Rhodopsin Biosynthesis *in Vitro*†

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ABSTRACT: An *in vitro* system for the biochemical and autoradiographic study of rhodopsin biosynthesis has been developed. Using the isolated frog retina, the incorporation of radioactive leucine into rod outer segment rhodopsin was measured under a variety of experimental conditions. Incorporation of leucine into rhodopsin was shown to increase linearly with time and with increasing isotope concentration, and was sensitive to puromycin and oligomycin, but not chloramphenicol. When measured at various temperatures from 0 to 34°, maximal incorporation occurred at 26°. Reduced incorporation occurred when the retinas were incubated in phosphate–Ringer medium containing 15 mM phosphate when compared to bicarbonate–Ringer containing 1.25 mM phosphate. Incorporation was also reduced by concentrations of glucose greater than 20 mM and if casamino acids were added or if glycerol replaced glucose. Incubation was normally carried out in the dark using dark adapted, pigment epithelium-free retinas. If the retinas were partially bleached prior to incubation and incubated in the dark, no differences in incorporation of leucine were seen when compared to the fully dark-adapted retinas. However, the bleached rhodopsin did not regenerate during incubation. In addition, no difference was observed when retinas incubated under a constant illumination of 7 ft-candles were compared to those incubated in the dark. Finally, if the pigment epithelium was left attached during incubation, incorporation was significantly reduced.

The vertebrate retina contains specialized cells which are responsible for receiving light energy and translating it into neurochemical impulses. These cells, the photoreceptors, are composed of two nearly separate segments, each performing different functions in the cell. The inner segment is responsible for biosynthesis, metabolism, and maintenance of the cell, and contains the cell nucleus, the Golgi apparatus, endoplasmic reticulum, and a dense group of mitochondria which are located at its distal end. The cylindrical outer segment contains the light-trapping organelles, a stack of hundreds of lamellar membrane disks containing the visual pigment. These disks are separate in the rod outer segment, but continuous in the cone.

The rod outer segment (ROS)[1] undergoes continuous renewal by assembling new disks at the base of the outer segment, a process which displaces the old disks toward the pigment epithelium (Young, 1967; Young and Droz, 1968; Young and Bok, 1969). To maintain the rod outer segment at a uniform length, the outermost disks are continuously shed into the pigment epithelium (Young and Bok, 1969). Autoradiographic and biochemical experiments using labeled amino acids injected *in vivo* have shown that protein for new disks is synthesized in the inner segment and subsequently transported to the outer segment where disk assembly occurs (Young and Bok, 1969; Hall *et al.*, 1969).

Since each frog photoreceptor cell makes about 36 new disks per day (Young and Droz, 1968), this cell provides a model system for studying membrane biosynthesis. The disk membrane itself has a comparatively simple composition, being primarily protein and phospholipid. The major disk protein is rhodopsin, a glycoprotein containing a hexasaccharide in addition to its retinaldehyde chromophore and exhibiting a characteristic absorbance in the visible range.

To study the sequence of events in disk membrane synthesis requires a system in which components can be accurately monitored in a way that is readily amenable to experimental control. The isolated retina seems ideal for this purpose, as pointed out by Ames (1965) and O'Brien *et al.* (1972) in their arguments for the use of an *in vitro* system to study the metabolism and electrophysiology of the retina. An *in vitro* system was used by Santamaria *et al.* (1971) to follow respiration, metabolic characteristics, and the S-potential in the isolated, pigment epithelium-free, fish retina. The uptake of glycine (Bruun and Ehinger, 1972) and γ-aminobutyric acid (Starr and Voaden, 1972) by the isolated retina was studied using the radioactive forms of these possible neurotransmitters. The elegant *in vitro* system of O'Brien *et al.* (1972) demonstrated the incorporation of [^{14}C]leucine into visual pigment in the bovine retina. [^{14}C]Leucine incorporation into protein was linear for at least 6 hr, and the system was shown to be sensitive to puromycin. The [^{14}C]leucine was first incorporated into proteins synthesized on the microsomes, and then appeared in visual pigment purified from isolated rod outer segments. These authors have shown recently that [^{3}H]glucosamine, a component of the glycoprotein rhodopsin, is also incorporated into bovine rod outer segments (personal communication).

We have adapted this system to the frog retina and examined rhodopsin synthesis under a variety of metabolic parameters. The results of O'Brien *et al.* have been confirmed and extended in the present study. In addition, we have demonstrated the adaptability of this system to autoradiography, which enables one to "visualize" the results of parallel biochemical experiments. Finally, we have shown that this system can be sensitively controlled using temperature, inhibitors,

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[1] Abbreviations used are: RBG, Krebs–Ringer bicarbonate incubation medium; wash buffer, 0.067 M sodium phosphate–4 mM $MgCl_2$ (pH 7.0); ROS, rod outer segment; RR, retinal residues; CVP, crude visual pigment; OSI, outer segment insoluble; RRS, retinal residue soluble; RRI, retinal residue insoluble; ft^3/hr, standard cubic feet per hour.

and pulse-chase techniques, thus providing an ideal model for studies on the biosynthesis of a biological membrane.

Experimental Section

Materials. *Rana pipiens* weighing about 35 g are obtained from a local supplier. Gasses are obtained from Matheson Gas, Cucamonga, Calif. L-[U-^{14}C]Leucine (0.29 Ci/mmol) and L-[4,5-^{3}H]leucine (31.8 Ci/mmol) are from New England Nuclear, Boston, Mass. Chemicals are obtained from the following sources: potassium penicillin G and streptomycin sulfate: Calbiochem, La Jolla, Calif.; puromycin: Nutritional Biochemicals, Cleveland, Ohio; chloramphenicol and oligomycin (15% A, 85% B): Sigma Chemical Co., St. Louis, Mo.; Soluene and Permafluor: Packard Instruments, Downers Grove, Ill.; toluene, scintillation grade, and Bio-Solv (BBS-3): Beckman Instruments, Fullerton, Calif.; agarose (Bio-Gel A 1.5m, 100–200 mesh): Bio-Rad, Richmond, Calif.; calcium phosphate ($Ca_{10}(OH)_2(PO_4)_6$), reagent grade: J. T. Baker Chemical Co., Phillipsburg, N. J.; Celite: Van Waters and Rogers. Ammonyx-LO (lauryldimethylamine oxide) is a gift from the Onyx Chemical Co., Jersey City, N. J.

Media. Modified Krebs–Ringer bicarbonate (RBG) is 120 mM NaCl, 5.1 mM KCl, 2.75 mM $CaCl_2$, 1.25 mM $MgSO_4$, 1.25 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM D-glucose, 6.34 units/ml of penicillin G, and 4.6 mequiv/ml of streptomycin sulfate. The final medium is 302 mosm. All media are presaturated with O_2–CO_2 (95:5), bringing the pH to 7.3. Unless otherwise noted, incubation medium for four retinas consists of 10 ml of RBG and 5 μCi of [^{3}H]- or [^{14}C]leucine. Buffer used for washing retinal fractions is 0.067 M sodium phosphate–4 mM $MgCl_2$ (pH 7.0). When carrier L-leucine is present, its concentration is 10 μg/ml.

Preparation of Retinas. Frogs are dark adapted overnight and killed by decapitation. After the front of the eye and the lens are removed, a few drops of cold RBG are placed in the eye cup. The retina is carefully removed and placed in a pool of RBG. The pigment epithelium is gently peeled off, and the retinas are placed in petri dishes containing ice-cold RBG. Retinas which are torn or have more than traces of pigment epithelium are discarded. Incubation begins 15–30 min after the first frog is killed.

Incubation of Retinas. A modification of the method of O'Brien *et al.* (1972) is used. Four retinas are placed in a 50-ml erlenmeyer flask containing 10 ml of incubation medium. Each flask is gassed with O_2–CO_2 (95:5) at 1 ft^3/hr. The flasks are incubated in a reciprocal shaker at 40 cycles/min with the water temperature regulated at 20 or 25° by a Lauda K2/RD circulator. As many as eight flasks are run in each experiment. Incubation is stopped by diluting the media with cold wash buffer containing carrier leucine and precipitating the retinas by centrifugation at 4° for 20 min at 27,000*g*. In experiments where it is critical to stop incubation immediately, the retinas are poured over crushed frozen wash buffer. Pulse-chase experiments are performed by decanting the pulse incubation medium and rinsing the retinas in RBG, then transferring the retinas into a chase flask containing fresh nonradioactive medium and continuing incubation under the same conditions as during the pulse. Preincubation with inhibitors is carried out as above, with the time of isotope addition taken as zero time.

Retina Fractionation. The retinas are fractionated according to the procedure outlined in Figure 1. The precipitated retinas are homogenized in 30 ml of wash buffer plus leucine, centrifuged, and washed a further four times, using a Vortex mixer to resuspend the pellet in 30 ml of wash buffer. Rod outer segments (ROS) are isolated by a modification of the method of Heller (1968). The washed retinas are rehomogenized in 12 ml of 37% sucrose in wash buffer and centrifuged for 1 hr at 27,000*g* to float the ROS. The ROS are poured off, diluted with wash buffer, and precipitated by centrifugation. The ROS pellet and the retinal residue (RR) which remains after flotation of the ROS are each washed three times with wash buffer.

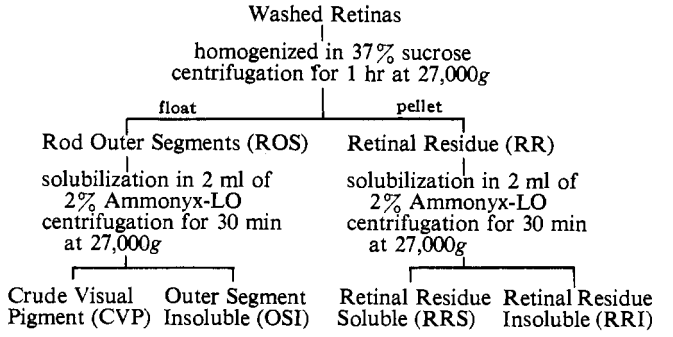

FIGURE 1: Summary of the procedure followed in fractionating the retina after *in vitro* incubation. Details as described in the Experimental Section.

Solubilization of ROS and Retinal Residue. The final washed pellets (ROS and RR) are solubilized overnight at 4° with either 2 ml of 2% Ammonyx-LO in 0.067 M sodium phosphate (pH 7.0) or 1 ml of Emulphogene column buffer (Shichi *et al.*, 1969). The two resulting extracts, crude visual pigment (CVP) and retinal residue soluble (RRS), are clarified by centrifugation for 30 min at 27,000*g* (Figure 1). The two small pellets of insoluble material remaining after the extraction of the rod outer segment and retinal residue fractions with detergent are designated outer segment insoluble (OSI) and retinal residue insoluble (RRI). These fractions are washed with sodium phosphate buffer and deionized water, transferred quantitatively to a scintillation vial and solubilized in 1 ml of Soluene for counting. The absorbances of the crude visual pigment and retinal residue soluble extracts are read at 280 and 500 nm on a Gilford 240 spectrophotometer, and 1-ml aliquots are counted in a Packard Tri-Carb liquid scintillation spectrophotometer with 10 ml of BBS–toluene (768 ml of toluene, 32 ml of Permafluor, 160 ml of BBS-3, and 14.4 ml of saturated citric acid). Counting efficiency is determined to be 80% for ^{14}C and 37% for ^{3}H by use of the external standard method.

Column Purification of Rhodopsin. Crude rhodopsin extracts are purified on columns of agarose or calcium phosphate–Celite. Agarose columns (0.9 × 95 cm) are equilibrated and eluted with 1% Ammonyx-LO in 0.067 M sodium phosphate buffer (pH 7.0) at 2 ml/hr. Calcium phosphate–Celite columns (1 × 10 cm) are run according to the method of Shichi *et al.* (1969). All columns are run at 4° in the dark. The absorbance of column fractions at 280 and 500 nm is read and 0.5- or 1.0-ml aliquots are counted for radioactivity in BBS–toluene. The specific activities of both column fractions and crude visual pigment extracts are calculated as dpm/ml per unit absorbance and are expressed as dpm/ml per A_{280} or dpm/ml per A_{500}. This normalizes the data and compensates for variations in the size of the retinas in separate incubation flasks, and can readily be converted to dpm/nmol using the molar extinction coefficient for rhodopsin$_{500}$. We have not made this conversion due to the controversy surrounding the value of ϵ for rhodopsin.

Autoradiography. Retinas for autoradiography are labeled

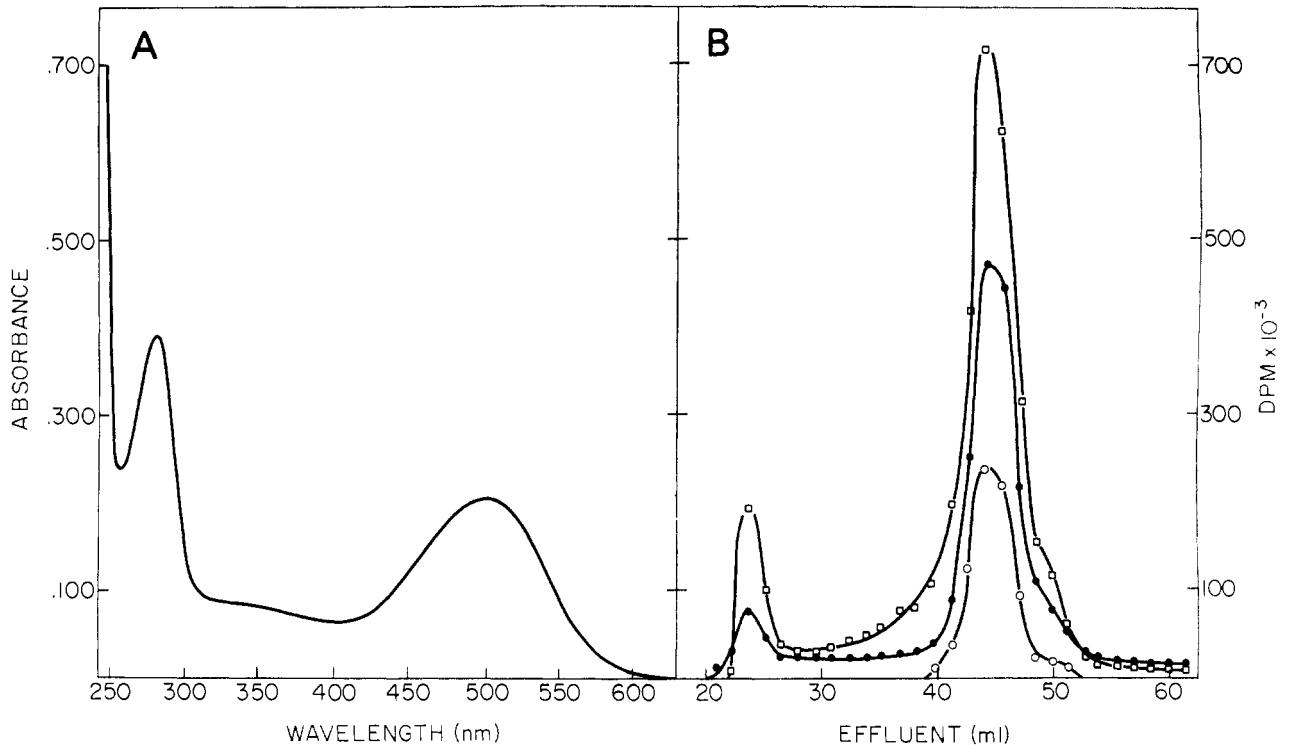

FIGURE 2: Spectral and chromatographic characteristics of rhodopsin isolated after incubation *in vitro*. (A) Absorption spectrum of rhodopsin purified by calcium phosphate–Celite chromatography as described in Experimental Section. (B) Agarose column elution profile of [^{3}H]-leucine-labeled rhodopsin isolated from 16 retinas pulsed 1 hr in 10 ml of RBG medium containing 80 μCi/ml of [^{3}H]leucine, then chased for 4 hr in 10 ml of RBG medium containing no isotope. Incubation temperature was 20° and gas flow 1.1 ft^3/hr. Rod outer segments isolated as described in the Experimental Section, solubilized in 1 ml of 1% Ammonyx-LO in 0.067 M sodium phosphate, and chromatographed on agarose as described in the Experimental Section. (○) A_{500nm}; (●) A_{280nm}; (□) dpm × 10^{-3}/ml.

in the same manner as those used for biochemicals studies, except that the [^{3}H]leucine concentration is 80 μCi/ml. Tissue preparation for autoradiography is done according to the method of Young and Bok (1969).

Results

Morphology of the Isolated Retina. Electron micrographs taken after seven hours of incubation revealed few morphological abnormalities. The outer segments, while devoid of attached pigment epithelium, were intact and very little membrane vesiculation had occurred. The interstitial matrix between the ROS appears to have been washed away during the incubation. The condition of the other retinal layers was generally indistinguishable from that of retinal sections prepared from a freshly enucleated eye.

Nature of in Vitro Rhodopsin. The rhodopsin isolated from retinas after incubation *in vitro* is indistinguishable from that prepared from freshly dissected eyes. The absorption spectrum of rhodopsin purified on a calcium phosphate–Celite column is shown in Figure 2A. Figure 2B shows the elution profile of [^{3}H]leucine-labeled rhodopsin from an agarose column. The high specific activity (3×10^6 dpm/ml per A_{500}) of the rhodopsin in this experiment resulted from using a dose of [^{3}H]-leucine suitable for autoradiography (80 μCi/ml).

Fractionation of the Retina. Ammonyx-LO detergent was used because of its seemingly high specificity for visual pigment (Ebrey, 1971). The ratio of A_{280}/A_{500} in the crude visual pigment extract is about 3.6, and after column purification on calcium phosphate–Celite is about 1.8.

As detailed in Methods and outlined in Figure 1, we have fractionated the retinas in a purely operational way, making no attempt to isolate subcellular particles such as mitochondria or microsomes. This was done primarily because the photoreceptor subcellular particles would be contaminated by those from cells in other retinal layers, and thus would not truly reflect metabolism occurring in the photoreceptor inner segment.

The four fractions obtained with our procedure (Figure 1) require some explanation. The crude visual pigment fraction is obtained from the detergent-solubilized outer segments and contains 95% of the rhodopsin of the retina as determined by difference spectra after total bleaching. The outer segment insoluble fraction is composed of contaminating material from the inner segments, such as mitochondria, and other traces of retinal debris which contaminate the ROS float.[2] The retinal residue soluble fraction is obtained by detergent extraction of the retinal residue (retina minus outer segments) and contains a small amount of rhodopsin (5%) as well as other soluble material from the retinal cells. The retinal residue insoluble fraction contains the detergent-insoluble material from the retina minus the outer segments, plus traces of pigment epithelium. The incorporation into these four fractions can be summed to indicate the total incorporation into the retina, a useful figure when comparing the effects of various inhibitors or when monitoring overall retinal metabolism.

Biosynthesis of Rhodopsin. Frog retinas incubated *in vitro*

[2] The purity of the ROS preparation as isolated by sucrose flotation was found to be 90% based on electron micrographs of ROS pellets and assays for various mitochondrial enzymes present in the ROS fraction (Heller *et al.*, 1971; Ostwald, 1972).

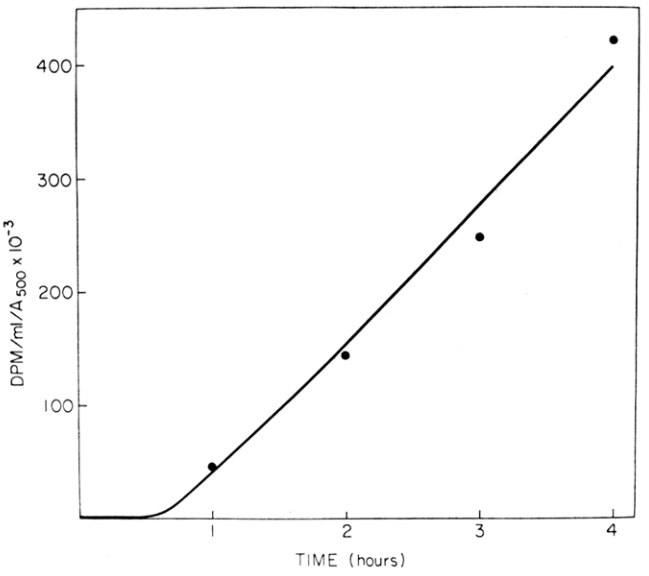

FIGURE 3: Time course of incorporation of [^{3}H]leucine into rhodopsin. Each time point was obtained from four retinas incubated in 10 ml of RBG medium containing 2.5 μCi/ml of [^{3}H]leucine. Incubation temperature was 19.5° and gas flow 1.0 ft^3/hr. As described in the Experimental Section, ROS were isolated, solubilized in 1.0 ml of 1% Emulphogene in 5 mM sodium phosphate buffer (pH 7.0), and chromatographed on a calcium phosphate–Celite column. Specific activity is expressed as dpm/ml per A_{500} in the purified rhodopsin.

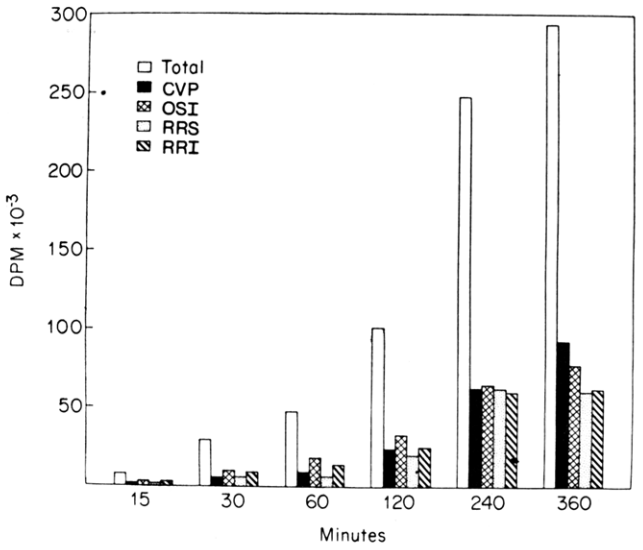

FIGURE 4: Histogram showing the time course of incorporation of [^{14}C]leucine into the various retinal fractions. Each time point was obtained from four retinas incubated in 10 ml of RBG medium containing 0.4 μCi/ml of [^{14}C]leucine. Incubation temperature was 19.5° and gas flow 1.0 ft^3/hr. Retinas fractionated as outlined in Figure 1 and detailed in the Experimental Section.

incorporate radioactive leucine into rhodopsin in a linear fashion for at least 4 hr, as shown in Figure 3. As with the bovine system (O'Brien *et al.*, 1972), there is a lag period of about 35 min, reflecting the time needed for uptake into the cell, biosynthesis, transport to the outer segment, and disk assembly. This time course of incorporation is similar to that found *in vivo* (Hall *et al.*, 1969).

In general, the time course of incorporation of radioactivity into purified rhodopsin (Figure 3) parallels that into the whole retina (Figure 4). During this time, the percentage of counts in the crude visual pigment which are isolated as purified rhodopsin increases from 15% at 1 hr to 65% at 4 hr. The remaining counts in the crude visual pigment fraction are eluted from an agarose column in either the void volume fraction (mol wt >200,000) or in the small molecules fraction. A small amount of labeled protein is eluted from the column just prior to rhodopsin. This probably represents some rhodopsin which is bleached during the preparation of the ROS (Heller, 1968).

Autoradiography. Figure 5 shows an autoradiogram prepared from a retina incubated with [^{3}H]leucine *in vitro*. As with those autoradiograms prepared after the *in vivo* injection of [^{3}H]leucine (Young, 1967; Young and Droz, 1968; Hall *et al.*, 1969), a band of radioactivity is seen at the base of the outer segment, reflecting the newly synthesized rhodopsin molecules which have been incorporated into the basal disks. The myoid and ellipsoid areas of the inner segment, the sites of biosynthesis and transport, respectively, show a less prominent reaction, reflecting a 6-hr "chase" in medium containing no radioactive leucine.

Metabolic Characteristics of the in Vitro System. We have tried to develop an *in vitro* system which is both optimal for rhodopsin biosynthesis and amenable to experimental variation. Some characteristics of the system are shown in Table I. Leucine incorporation into visual pigment at 0° for 2 hr is negligible, and extending the time at 0° to four hours yields only 2.5% of the control. Negligible nonspecific binding was found to occur.

Reduced retinal metabolism in phosphate buffer was first

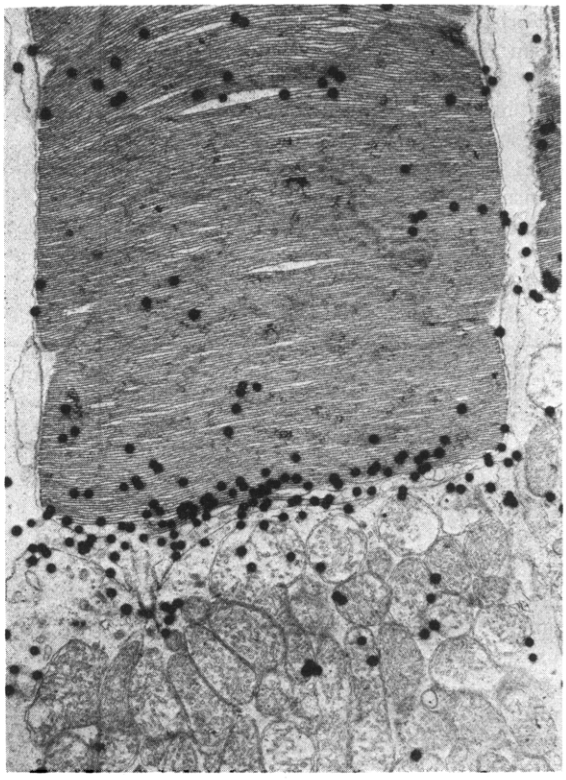

FIGURE 5: Electron microscope autoradiogram prepared from a retina incubated 1 hr in RBG medium containing 80 μCi/ml of [^{3}H]leucine, then 6 hr in RBG medium without [^{3}H]leucine. Incubation temperature was 19.5° and gas flow 1.0 ft^3/hr. The retina was fixed and sections prepared for autoradiography as described in the Experimental Section. Magnification = 8000×.

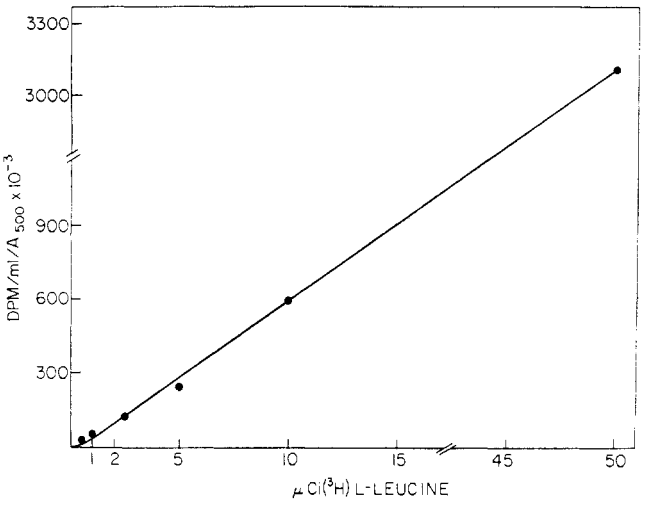

FIGURE 6: Incorporation of [^{3}H]leucine into the CVP fraction as a function of isotope concentration. Abscissa indicates amount of [^{3}H]leucine in each incubation flask containing four retinas in 10 ml of RBG medium. Incubation was for 2 hr at 19.5° and gas flow of 1.0 ft^3/hr. CVP fraction was prepared as outlined in Figure 1 and detailed in the Experimental Section.

reported by Laser (1935), apparently owing to the inhibition of oxygen uptake in this buffer (Dickens and Greville, 1935). Other authors have confirmed this effect in the isolated pig, rabbit, and rat retina (Riley and Voaden, 1970) and in the isolated fish retina (Santamaria *et al.*, 1971). Our results with phosphate–Ringer, where leucine incorporation into visual pigment was 26% of the value in bicarbonate–Ringer (Table I), support this "phosphate effect." A slight inhibition (20%) is seen in both buffers if the ionic strength is lowered (200 mosm *vs.* 300 mosm), although electron micrographs show no apparent difference in the appearance of the retina incubation at each osmolarity.

Table I also shows the results of varying aeration conditions. A significant inhibition occurs if O_2–CO_2 is replaced by N_2 or no aeration. Pure O_2 gave widely varying results in repeated experiments, possible due to an instability in pH caused by lack of CO_2.

Various glucose concentrations from 0 to 50 mM were tested, and 10 mM was found to be optimal for a two hour incubation. Significant inhibition occurs if either no glucose, or a concentration greater than 20 mM, is used. Contrary to the stimulation found in the bovine system when glycerol replaces glucose (O'Brien *et al.*, 1972), the frog system shows a slight inhibition in the presence of either 0.06 or 0.12 M glycerol. If either casamino acids (10 μg/ml), or a complete amino acid mixture minus leucine, are added, incorporation is significantly inhibited.

In an attempt to reduce possible endogenous substrate, we preincubated the retinas for up to one hour before the addition of radioactive leucine, but noticed no significant differences in incorporation. This observation is of some value if one wishes to preincubate with inhibitors.

Effect of [^{3}H]Leucine Concentration. Figure 6 shows the effect of increasing the concentration of [^{3}H]leucine in the incubation medium. Incorporation of [^{3}H]leucine is linear through 50 μCi/10 ml although 5 μCi/10 ml yields an adequate number of counts in all retinal fractions. This linearity is important when the higher concentrations needed for autoradiography are used. The isotope used in this experiment was of a very high specific activity (31.8 Ci/mmol), so that even at 50 μCi/10 ml, only 0.2 μg of leucine was present.

Effect of Light on Leucine Incorporation. As mentioned in Methods, the retinas used for these experiments are removed from frogs which have been completely dark adapted (15 hr). This facilitates the complete removal of the pigment epithelium, yielding more homogeneous retinal fractions. In a control experiment, retinas from light-adapted frogs were used, and no signficant difference in incorporation was observed. Since the light-adapted retinas were about 50% bleached, the specific activities were compared as dpm/ml per A_{280}. A similar observation was made by Starr and Voaden (1972) using the rat retina. Since the incubation of these light-adapted retinas was carried out in the dark, they were examined for regeneration of rhodopsin during the course of incubation. No regeneration was observed, which is in agreement with the results reported by Ames (1965).

To investigate the effect of continuous light exposure on incorporation, dark-adapted retinas were placed in cold RBG and exposed to 7 ft-candles of illumination. After the retinas were completely bleached (30 min) they were placed in an incubation flask, and the incorporation of [^{3}H]leucine into opsin was measured after 1, 2, and 4 hr of incubation under a continuous illumination of 7 ft-candles. A group of dark-adapted control retinas were run in parallel, receiving no illumination. The outer segments isolated from both the retinas incubated in the light and in the dark were extracted with detergent and the extracts were purified by column chromatography. In the case of the bleached retinas, radioactivity was eluted as opsin rather than rhodopsin, so the specific activities were compared as dpm/ml per A_{280} rather than as dpm/ml per A_{500}. No significant differences in incorporation were observed between the light-exposed or dark-adapted retinas at any of the time intervals studied. This is in agreement with the results of Starr and Voaden (1972). When viewed by electron microscopy, the morphology of the light-exposed retinas was indistinguishable from the morphology of those kept in the dark. Very little fragmentation or vesiculation of the photoreceptors had occurred.

Effect of the Presence of the Pigment Epithelium. When the pigment epithelium layer was left attached to the retinas

TABLE I: Leucine Incorporation into Crude Visual Pigment.[a]

Conditions	%[b]
Complete system	100
0°, zero time	1.0
0°, 2 hr	1.5
No aeration	74
No glucose	73
Pigment epithelium attached	51
Plus casamino acids	48
N_2 replace O_2–CO_2	41
Phosphate–Ringer	26
Retinas homogenized prior to incubation	22

[a] Incubation conditions (except as noted under Conditions): four retinas in 10 ml of RBG medium with 0.5 μCi/ml of [^{3}H]leucine, temperature at 19.5°, gas flow at 1.0 ft^3/hr, and an incubation time of 2 hr. [b] Per cent as dpm/ml per A_{500} in crude visual pigment fraction.

TABLE II: Effect of Inhibitors on Leucine Incorporation into Crude Visual Pigment and Purified Rhodopsin.

Inhibitor	Preincubation (min)	Per Cent[a] Crude Visual Pigment	Per Cent[a] Purified Rhodopsin
Control	None[b,c]	100	100
Puromycin			
10.0 μg/ml	None[b]	48	
50.0 μg/ml	None[c]	8	12
50.0 μg/ml	30[c]	11	15
50.0 μg/ml	60[c]	9	15
100.0 μg/ml	60[c]	12	17
Oligomycin			
1.8 μg/ml	None[b]	79	
9.8 μg/ml	None[b]	42	
18.0 μg/ml	None[c]	30	24
18.0 μg/ml	60[c]	17	12
Chloramphenicol			
10.0 μg/ml	None[b]	104	
50.0 μg/ml	None[b]	96	

[a] Per cent as (dpm/ml per A_{500} in inhibited)/(dpm/ml per A_{500} in noninhibited control). [b] Incubation conditions: four retinas in 10 ml of RBG medium with 1.0 μCi/ml of [^{3}H]-leucine, temperature at 20°, gas flow at 1.1 ft^3/hr, and an incubation time of 2 hr, 45 min. [c] Incubation conditions: same as footnote *b* except temperature at 25° and incubation time of 4 hr.

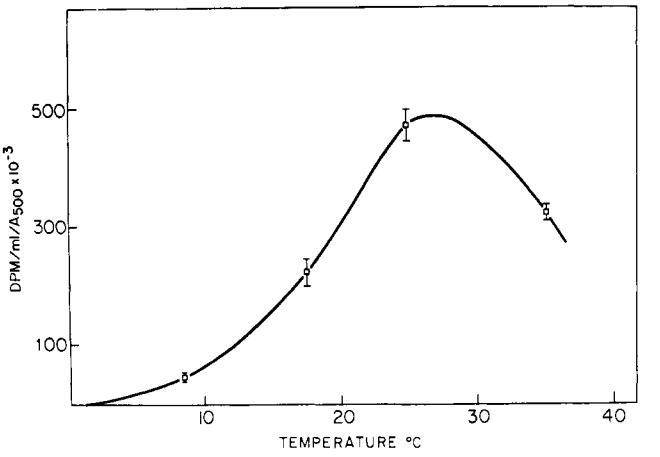

FIGURE 7: Incorporation of [^{3}H]leucine into the CVP fraction as a function of incubation temperature. Each point is the average of two separate flasks; each contains four retinas in 10 ml of RBG medium with 0.50 μCi/ml of [^{3}H]leucine. Gas flow was 1.0 ft^3/hr and incubation was for 3 hr. CVP fraction was prepared as in Figure 1 and detailed in the Experimental Section.

during incubation, incorporation of [^{3}H]leucine into the rod outer segments was only half that of the epithelium-free retinas. Since this cell layer mediates the transport of metabolites into the photoreceptors and is itself metabolically active (Glocklin and Potts, 1965), it provides a competitive barrier to uptake by the inner segment.

These experiments indicate the importance of using only epithelium-free retinas for *in vitro* studies. Complete dark adaptation and rejection of those retinas with more than a trace of pigment epithelium ensure that this criterion is met.

Effect of Temperature on Incorporation. To determine the effect of temperature on incorporation of [^{3}H]leucine, the retinas were incubated for 3 hr at 8.5, 17.5, 23, and 34°. Figure 7 shows the results of this experiment. Incorporation increased with increasing temperature until about 25°, at which point it decreased. We have not examined the reasons for the decreased incorporation above 25°, although some type of thermal inactivation seems plausible. In any case, the direct dependence of incorporation rate on temperature allows one to sensitively control retinal metabolism.

Effect of Inhibitors. Puromycin, oligomycin, and chloramphenicol were examined for their effect on rhodopsin biosynthesis, and the results are shown in Table II. Incubation was for 3 or 4 hr, and inhibitors were added either at zero time or after 30 or 60 min of preincubation. As in the bovine system (O'Brien *et al.*, 1972), [^{3}H]leucine incorporation into rhodopsin is sensitive to puromycin, with 50% inhibition occurring at a concentration of 10 μg/ml.

Chloramphenicol, an inhibitor of mitrochondrial protein synthesis, has no effect on the incorporation of [^{3}H]leucine into the crude visual pigment fraction over a range of concentrations used (10–50 μg/ml). The incubation time (3 hr) may have been too short for the effect of the chloramphenicol on the mitochondria to be translated into inhibition of leucine incorporation.

Oligomycin, an inhibitor of mitochondrial ATPase and thus an inhibitor of electron transport and energy production, has a significant effect on leucine incorporation into outer segment rhodopsin. This no doubt emphasizes the large amount of energy required for both biosynthesis and transport, and may explain why dense clusters of mitochondria are concentrated in the ellipsoid portion of the photoreceptor. At the higher concentration of oligomycin, the percentage of the total disintegrations per minute which is present in the outer segments is reduced with a concomitant rise in the other retinal fractions, indicating that transport of rhodopsin to the outer segment, rather than biosynthesis, seems to be affected.

Discussion

The rod photoreceptor cells of all vertebrates studied thus far continually renew the components of their outer segments (Young, 1973). New disk membranes are assembled at the base of the outer segment utilizing components synthesized in the inner segment of the cell. These components, primarily protein and phospholipid, are transported from their sites of synthesis in the inner segment, through the connecting cilium to the base of the outer segment, where disk assembly occurs. The major protein of the ROS disk membrane is the photosensitive pigment rhodopsin. This protein comprises 80–90% of the structural protein of washed ROS disk membranes (Hall *et al.*, 1969; Robinson *et al.*, 1972). In addition, it is responsible for the absorption of light energy, the first step in the sequence of events leading to the transmission of a neural impulse in the retina. Thus, rhodopsin plays both a vital structural and a functional role in the retina.

Since a single disk in the frog ROS contains approximately 2×10^6 molecules of rhodopsin, and each photoreceptor cell synthesizes a new disk every 40 min, an appreciable portion of

the synthetic machinery of this cell must be involved in the synthesis of rhodopsin and phospholipids for new disks. When one considers that a frog retina contains at least 2×10^6 rod cells, it becomes apparent that the retina is an excellent system for studying the biosynthesis of a membrane protein and its mode of assembly into disk membrane.

Our early studies on the biosynthesis of rhodopsin and its assembly into the disk membrane were carried out in the living frog. However, it proved impossible to study the early events occurring in the inner segment with this *in vivo* approach. Recently, O'Brien *et al.* (1972) published an *in vitro* system using bovine retinas in which rhodopsin biosynthesis continued for a period of hours. We have adapted this system to the frog retina. The metabolic parameters and kinetics of leucine incorporation into rhodopsin in the isolated frog retina are nearly identical with those in the bovine system. The frog retina, however, offers certain advantages over the bovine system in that fresh tissue is available immediately before incubation and the extent of dark adaptation is under laboratory control. In addition, a direct comparison can be made to previous *in vivo* experiments carried out using the frog, and parallel *in vivo* and *in vitro* experiments are possible. The system is metabolically active for at least 7 hr, enough time to allow significant incorporation into basal disks, as shown by both the autoradiographic and biochemical experiments reported here.

The incorporation of rhodopsin into newly synthesized basal disks follows a time dependent course similar to that previously shown *in vivo* (Hall *et al.*, 1969) and *in vitro* (O'Brien *et al.*, 1972). Prior to about 35 min, no labeled rhodopsin can be extracted from the outer segments. This lag presumably reflects the time required for the rhodopsin synthesized on the ribosomes of the inner segment to be transported to the outer segment and to be incorporated into a newly forming disk membrane. Thereafter, in a continuous pulse experiment, a stream of labeled rhodopsin molecules is transported to the outer segment for as long as the retinal preparation remains viable. Once incorporated into the disk membrane, rhodopsin undergoes no turnover (Hall *et al.*, 1969), so that with time, labeled rhodopsin comprises an ever increasing proportion of the total rhodopsin of the outer segment.

During the course of a 4-hr incubation, the total radioactivity of the crude visual pigment fraction extracted from isolated ROS increases about sevenfold between 1 and 4 hr (Figure 4). However, at 4 hr, only about 65% of the total counts in the crude visual pigment can be isolated as purified rhodopsin. The remaining 35% could be derived from a number of sources. Bok and Young (1972) have shown that a diffuse labeling of frog ROS occurs within 15 minutes after injection of a labeled amino acid. This protein component might be isolated during the preparation of the ROS, and since it is turned over quite rapidly, its specific activity could reach a high value during a continuous pulse experiment. Thus, even though it might comprise only a small percentage of the total ROS protein, it could contribute significantly to the total radioactivity of the crude visual pigment fraction. Additionally, the ROS fraction isolated by the flotation procedure is at most 90% pure (Heller *et al.*, 1971; Ostwald, 1972). The main contaminants are mitochondria and ribosomes trapped when the outer segments break off below the connecting cilium. These organelles could also contribute significantly to the total radioactivity of the crude visual pigment fraction. Finally, some of the unincorporated leucine, as well as other low molecular weight substances, are not removed during the preparation of the ROS. These would also contribute to the total radioactivity of the crude visual pigment fraction, but are removed during the column procedures used for the purification of rhodopsin. The extent of radioactive contamination of the crude visual pigment fraction is substantially reduced in a pulse-chase experiment. When retinas were pulsed for 1 hr in the presence of [^{3}H]leucine, then chased for four hours in nonradioactive medium, labeled rhodopsin represented 82% of the total radioactivity in the ROS extract (Figure 3). This supports the suggestion that many of the contaminants are substances which are undergoing a rapid turnover.

Protein synthesis in this system is predictably inhibited by both puromycin and oligomycin, while chloramphenicol has no effect at the concentrations used. The degree of inhibition in column-purified rhodopsin is consistently reflected by the degree of inhibition in the crude visual pigment extract (Table II), making the crude visual pigment fraction an accurate indicator of the extent of rhodopsin biosynthesis. The inhibitory effect of oligomycin is increased by preincubation, reflecting its role as an inhibitor of oxidative phosphorylation. Protein synthesis continues until the preexisting stores of ATP are used up, a condition which is enhanced by preincubation. Additionally, rhodopsin transport to the ROS is probably inhibited in the presence of oligomycin. The effect of puromycin is also increased through preincubation, but, as seen in Table II, at a concentration of 50 μg/ml or more, a level of about 90% inhibition is reached, at which point higher concentrations of puromycin have no further effect. The effect that inhibiting rhodopsin biosynthesis has on disk membrane assembly is being examined at the present time.

In two different experiments, the effect of light and dark on rhodopsin synthesis was compared. In one experiment, partially bleached (about 50%) retinas from light-adapted frogs were incubated in the dark, along with dark-adapted control retinas. Although no regeneration of the bleached visual pigment occurred during the incubation, the newly synthesized product was rhodopsin, as judged by its elution from columns of agarose. Further, the rate and level of visual pigment synthesis was the same as in the control flask, which contained fully dark-adapted retinas. In the second experiment, retinas were completely bleached prior to incubation, then incubated under an illumination of 7 ft-candles and compared to fully dark-adapted retinas incubated in the dark. Only radioactive opsin could be isolated from the light-incubated retinas, but again no difference was noted when compared to the dark-incubated controls. Because the visual pigment was partially or fully bleached in these experiments, dpm/ml per A_{280} was used to compare specific activities. A question of some interest is whether vitamin A (retinal) is added to opsin to form rhodopsin before or after the protein is inserted into the growing disk membrane. These experiments do not answer this question, but do show that the absence of a photosensitive form of rhodopsin does not prevent the insertion of the protein into the growing disk membrane. They do not rule out the possibility that vitamin A is bound to opsin during transport to the outer segment and insertion into the membrane in a form which is not photosensitive. Similar experiments using radiolabeled vitamin A are in progress to answer this question.

Using amino acid autoradiography, we have previously shown that a protein, presumed to be rhodopsin, migrates from the ribosomes of the inner segment, through the Golgi, to the base of the outer segment, where it is incorporated into the newly forming disk membranes (Hall *et al.*, 1969). In an *in vitro* pulse-chase experiment, O'Brien *et al.* (1972) have provided indirect biochemical evidence that this migration of rhodopsin does occur. However, it has been impossible thus far to isolate

a precursor of rhodopsin from the inner segment prior to its incorporation into the outer segment disk membrane. Rhodopsin is a glycoprotein containing 11-*cis*-retinal as a prosthetic group, and would presumably exist in the inner segment first as a nascent protein, then a glycoprotein, and finally as the retinal-conjugated protein. In addition, at some point it is probably "packaged" into a phospholipid micelle as it is transported to the outer segment. Thus, the exact form in which rhodopsin exists during its transport to the outer segment is still unknown. However, the capacity of this *in vitro* system for synthesizing rhodopsin is such that the isolation of rhodopsin precursors should be possible. The fact that incorporation is linear with isotope concentration, supported by the excellent results obtained with the high dose necessary for autoradiography, shows that the system is not affected by high concentrations of isotope. In addition, the system is sensitive to temperature, operating at an increasing efficiency up to 26°. Thus, by lowering the temperature and using a high concentration of a high specific activity isotope, it might prove possible to accumulate sufficient amounts of labeled rhodopsin precursors in the inner segment to allow their isolation and identification, and to study their mode of assembly into the ROS disk membrane.

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