Incorporation of Leucine into Rhodopsin in Isolated Bovine Retina*

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ABSTRACT: Regeneration of vertebrate photoreceptor rod outer segments has been studied *in vivo* in the past. An *in vitro* system is desirable for further detailed studies. A system has been developed in which whole bovine retinas are incubated with radioactive leucine which is incorporated into the microsomal fraction very rapidly. At a later time, the rod outer segments become labeled. One of the proteins labeled during the process is rhodopsin which is isolated and purified by column chromatography on calcium phosphate–Celite. The spectral ratios A_{278}/A_{498} and A_{400}/A_{498} are 1.59 and 0.19, respectively. Rechromatography on agarose does not alter the spectral ratios of the rhodopsin but does resolve minor radioactive contaminants. Exposure to light causes bleaching of the purified rhodopsin and the resulting radioactive opsin is eluted from agarose ahead of rhodopsin with no change in specific activity. The labeling of purified rhodopsin as a function of time parallels the labeling of outer segments and follows a pattern predicted from the current understanding of the process of outer segment renewal.

Vertebrate photoreceptor cells show a high degree of differentiation both metabolically and structurally. Most of the metabolic and genetic functions are located in the inner segments of the cells while the visual pigments are components of lamellar membranous structures (discs) which completely fill the outer segments (Young, 1969a). Autoradiographic studies with rats and mice (Droz, 1963) showed a displacement of newly formed protein from the inner to the outer segments suggesting a continual process of outer segment renewal. This process was then studied in detail in rats, mice, and frogs (Young, 1967, 1968; Young and Droz, 1968; Young and Bok, 1969) where the rod outer segment was found to undergo continuous renewal by the addition of new discs at the base of the outer segment concommitant with removal of the oldest discs at the apical end of the outer segment and subsequent engulfment and digestion of the shed discs by the cells of the retinal pigment epithelium. The outer segment of the cone photoreceptor does not appear to undergo the same type of renewal process. Rather, newly formed protein seems to migrate to all parts of the outer segment, not simply to the basal discs (Young, 1969b).

Hall *et al.* (1968, 1969) demonstrated that the major component of frog rod outer segments labeled with radioactive amino acids during the course of this renewal process was rhodopsin. Subsequently two other laboratories presented similar results (Matsubara *et al.*, 1968; Bargoot *et al.*, 1969).

In order to study the renewal process in greater detail it has become necessary to develop an *in vitro* system which is amenable to experimental control while still retaining the essential features of the renewal process. An *in vitro* experimental system would permit inhibition of nucleic acid or protein synthesis without the ambiguity arising from simultaneous toxicity to whole animals. More immediate environmental controls would be possible and pulse–chase experiments could be readily carried out. Perhaps the greatest advantage would lie in the ability to present directly to the retina a variety of radioactive precursors without sustaining the enormous losses inherent in working with intact animals. This report describes such a system in which radioactive precursors are incorporated into retinal proteins, one of which has been extensively purified and shown to be rhodopsin.

Experimental Procedures

Preparation and Incubation of Retinas. Eyes are removed from freshly slaughtered cattle and placed immediately in the dark on ice for shipment to the laboratory. The maximum elapsed time before incubation is 4 hr. All subsequent operations are carried out in dim red light provided by two 25-W bulbs and filtered through a No. 70 Wratten filter (Eastman Kodak, Rochester, N. Y.). After removal of the cornea, ciliary body, and vitreous, the eye cup is filled with Krebs–Ringer bicarbonate buffer (Krebs and Henseleit, 1932) and the retina is gently removed. The retina is transferred to a petri dish of fresh buffer and any adhering vitreous or pigment epithelium is removed.

Incubations are carried out in 50-ml ehrlenmeyer flasks containing 2 retinas suspended in 10 ml of Krebs–Ringer bicarbonate buffer with the following additions: 20 mM glucose–0.1 mg of Casamino acids (Difco Laboratories, Detroit, Mich.)–350 units of penicillin G–potassium salt (Sigma Chemical Co., St. Louis, Mo.)–350 μg of streptomycin sulfate, USP (E. R. Squibb & Sons, New York, N. Y.)–5 μC of uniformly labeled L-[^{14}C]leucine (255 mCi/mmole, New England Nuclear, Boston, Mass.). Flasks are maintained at 37° with a water bath and each is continually gassed at a rate of 0.5 l./min with 95% oxygen–5% carbon dioxide previously saturated with water vapor by bubbling through distilled water at 37°. Without presaturation serious volume losses occur through evaporation. Each flask is fitted with a stopper perforated by two No. 15 hypodermic needles for inlet and venting of the gas mixture. The gas cannot be bubbled through the buffer solution because the resulting agitation is sufficient to break off the outer segments.

Incubations are terminated by chilling the flasks to 0° in a mixture of ice and water. The retinas are then isolated by centrifugation for 10 min at 12,000*g* and washed twice by

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resuspension in fresh buffer followed by recentrifugation. At this point the retinas can be frozen.

Tissue Fractionation. Subcellular particles are isolated by the following modification of standard procedures. Four retinas are homogenized in a conical all-glass homogenizer with 20 ml of a buffer solution containing 0.35 M sucrose–25 mM KCl–4 mM $MgCl_2$–50 mM Tris-HCl, pH 7.6. The homogenate is centrifuged for 30 min at 27,000*g*. A crude microsomal fraction is obtained by centrifugation of the resulting supernatant for 30 min at 150,000*g*. The remainder of the homogenate sedimenting at 27,000*g* is suspended in 6 ml of 1.10 M sucrose in 66 mM sodium phosphate, pH 7.0, and centrifuged for 45 min at 27,000*g*. The supernatant containing the floating rod outer segments is diluted with three volumes of 66 mM sodium phosphate, pH 7.0, and centrifuged for 10 min at 12,000*g* to yield a crude rod outer segment preparation. The 27,000*g* pellet is then suspended in 10 ml of the original homogenizing medium and centrifuged for 5 min at 700*g*. The resulting supernatant is centrifuged for 10 min at 15,000*g* to yield a crude mitochondrial preparation. The use of the terms mitochondria and microsomes indicates that the cell fractions isolated are analogous to well-defined cellular elements only in terms of the centrifugal methods used for their isolation. Protein is determined by the method of Lowry *et al.* (1951). Samples are prepared for protein determinations by suspending the appropriate fraction in 1–2 ml of 0.5% sodium deoxycholate and precipitating the protein by the addition of 10 volumes of alcohol–ether (2:1, v/v). The precipitate is washed with alcohol–ether, then with ether and is finally dissolved in 0.1 N NaOH. Aliquots (0.1 ml) are also added to 1 ml of NCS solubilizer (Amersham-Searle, Des Plaines, Ill.) and counted in a scintillation spectrometer after adding 10 ml of a toluene solution of 0.4% 2,5-diphenyloxazole and 0.005% 1,4-bis[2-(5-phenyloxazolyl)]benzene.

Preparation of Rod Outer Segments. When no other subcellular fractions are required, rod outer segments are purified by a slight modification of the procedure of Heller (1968a). Four retinas are homogenized in a conical all-glass homogenizer with 10 ml of a buffer containing 66 mM sodium phosphate, pH 7.0, and 4 mM $MgCl_2$. The homogenate is centrifuged for 30 min at 27,000*g*. The resulting pellet is suspended in 10 ml of the same buffer containing 1.17 M sucrose and centrifuged for 45 min at 27,000*g*. The supernatant with the floating rod outer segments is diluted with 20 ml of buffer and centrifuged for 20 min at 27,000*g*. The crude rod outer segment pellet is suspened in 5 ml of buffer containing 1.08 M sucrose and centrifuged for 1 hr at 39,000*g*. A small pellet is discarded and the rod outer segments are sedimented by diluting the supernatant with 10 ml of buffer and centrifuging for 20 min at 27,000*g*. Finally, the rod outer segment preparation is washed 4 times by suspension in 30 ml of buffer and centrifugation for 10 min at 12,000*g*.

Extraction and Purification of Rhodopsin. Rod outer segments from 4 retinas are suspended in 1 ml of a solution containing 0.05 M Tris-HCl, pH 8.5, and 1% (v/v) of the nonionic detergent Emulphogene BC-720 (General Aniline and Film Corp., New York, N. Y.). After 15–30 min at room temperature, the suspension is frozen overnight. After thawing, an insoluble white or light pink residue is removed by centrifugation for 15 min at 27,000*g*. The spectral ratio A_{278}/A_{498} of the resulting clear solution is 4.8 to 5 with A_{498} ranging from 0.5 to 1.9 but averaging 1.1. There appears to be some seasonal variation as well as daily variation in the light exposure of animals. The lowest values of A_{498} occur in the summer months. However, within a single experiment the yields fall within a few per cent of each other.

Each 1 ml of extract is purified by passage over a column of 0.83 g of calcium phosphate ($Ca_{10}(OH)_2(PO_4)_6$, Fisher) and 1.17 g of Celite (Johns-Manville) prepared in Emulphogene–Tris buffer as described by Shichi *et al.* (1969) and run at room temperature. Air pressure (3–10 lb/in.^{2}) is applied to achieve a flow rate of about 0.5 ml/min. Recovery of rhodopsin as measured by 498-nm absorbance is in the range of 85–95%. Further purification is achieved by chromatography of the eluate from calcium phosphate–Celite on a 1.5 × 168 cm column of agarose (Bio-Gel A, 1.5 m, 100–200 mesh, Bio-Rad, Richmond, Calif.) as described by Heller (1968a). The flow rate is adjusted to 12 ml/hr with a metering pump and 3-ml fractions are collected. Samples of 1–5 ml are applied to the column with sucrose added to the sample to a final concentration of 20%. The column, equilibrated with Emulphogene–Tris buffer, is run at 4° in a dark cold room. Recovery of rhodopsin, measured as 498-nm absorbance, ranges from 91 to 100%.

Rhodopsin and other proteins are detected by measuring the A_{278} and A_{498} of column fractions in a Beckman DU spectrophotometer. Absorption spectra are recorded in a Cary Model 14 recording spectrophotometer against a blank consisting of the effluent from the column prior to application of the sample.

Radioactivity in column fractions is measured in a Packard liquid scintillation spectrometer. Aliquots are all brought to 1 ml with Emulphogene–Tris buffer and are added to 10 ml of a solution consisting of 1.5 l. of toluene, 500 ml of Triton X-100 (Packard Instrument Co., Downers Grove, Ill.), 11 g of 2,5-diphenyloxazole, and 0.25 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene.

Samples are concentrated for rechromatography with dry Sephadex G-25 (Pharmacia, Piscataway, N. J.). One gram of Sephadex absorbs about 2.2 ml of water in 30 min at 0°. The protein solution is then recovered by centrifugation through a glass fiber filter in a centrifugal filter holder (Gelman, Ann Arbor, Mich.). Recoveries of rhodopsin, measured as 498-nm absorbance, are in the range of 70–90%.

Results

Characteristics of the Incubation System. Isolated bovine retinas incorporated [^{14}C]leucine into total protein at a linear rate for at least 6 hr at 37°, following a short lag period. This can be seen (up to 4 hr) in Figure 1. Table I summarizes the effects of several experimental variables. There is no incorporation at 0° and the system is sensitive to puromycin, perhaps even more than indicated in Table I since the incubation time was relatively short and no attempt was made to preincubate with puromycin before adding the label. Optimal incorporation requires the presence of glucose as an energy source. In the absence of glucose the retinas begin to fragment during the incubations. The same effect can be seen on the omission of amino acids. Homogenizing or freezing seriously reduces the protein synthetic capacity of the retinas as does storage of the eye at 4° overnight. However, storage of the isolated retina in the incubation buffer at 4° overnight preserves most of the protein synthesizing activity. It should be noted that these results refer to incorporation into gross protein and not into rhodopsin specifically. The sensitivity of rhodopsin synthesis could be somewhat different.

Labeling of Subcellular Fractions. When the labeling of crude subcellular fractions is measured as a function of time the results seen in Figure 1 are obtained. While the labeling of the total protein of the retinal homogenate is essentially linear after a brief lag, the microsomes are labeled very rapidly and

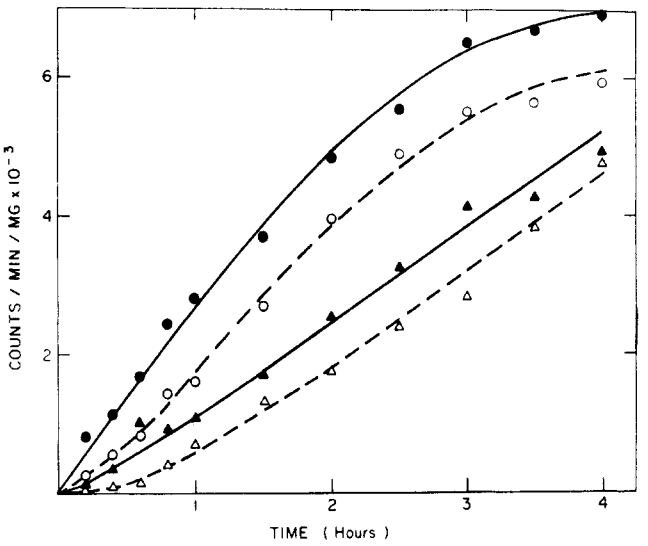

FIGURE 1: Labeling of subcellular fractions of bovine retina with [^{14}C]leucine. Incubation and tissue fractionation are described in Experimental Procedures. Specific activity of each fraction is expressed as counts/min per mg of protein. Symbols are: (●) microsomes; (○) mitochondria; (▲) whole homogenate; and (△) rod outer segments.

the rod outer segments show a substantial lag before label accumulates. This relationship in the labeling of microsomes and outer segments parallels the *in vivo* experimental observations of Young and Droz (1968) and Hall *et al.* (1969). Presumably, the lag in the labeling of outer segments corresponds to the time required for rhodopsin, newly synthesized by the microsomal fraction of the rod, to migrate to the outer segment and become incorporated into the basal disc.

Pulse Labeling of Outer Segments. An experiment was designed to test the precursor–product relationship of the radioactive protein in the microsomes and in the outer segments. Two sets of retinas were incubated as described under Experimental Procedures. At the end of 1 hr, one set was washed and homogenized and a sample was removed for specific activity determination. The remainder of the homogenate was carried through the tissue fractionation procedure and the specific activity of the fractions was determined. The second set of retinas was washed free of unincorporated [^{14}C]leucine by gentle suspension in several changes of Krebs–Ringer bicarbonate buffer. The washed retinas were then further incubated for 2 hr in fresh medium containing unlabeled leucine. At the end of the second incubation these retinas were washed and homogenized and processed in the same way as the first set.

This experiment measures the label in microsomes and outer segments at the end of the 1-hr pulse and follows the movement of that label, if any, during the 2-hr chase with unlabeled leucine (provided by the Casamino acid mixture). If there were a large intracellular pool of radioactive leucine, the labeling of all fractions should continue to increase as previously indicated in Figure 1. If not, the specific activity of the total protein should remain essentially constant. The results in Table II indicate that the latter alternative is the case.

Furthermore, if the *in vitro* system described here is indeed capable of outer segment renewal, then during the 2-hr chase there should be a migration of newly formed, labeled protein from the endoplasmic reticulum of the rod inner segment to the disc membranes of the outer segment (Young and Droz, 1968; Hall *et al.*, 1969). This migration would appear as a decrease in the specific activity of the microsomal fraction and a concommitant increase in the specific activity of the rod outer segments. Table II shows that the predicted changes in specific activity were found. Although not conclusive in itself, this experiment is strongly suggestive of protein migration. The label in the outer segments more than doubled during the chase. The decrease in microsomal specific activity was much less dramatic because this fraction is derived from all cell types in the retina, not simply the rod cells, and probably is comprised of many types of membrane fragments in addition to endoplasmic reticulum.

Although the labeling patterns indicate a migration of preformed polypeptide from inner to outer segments in the manner observed *in vivo* (Young and Droz, 1968; Hall *et al.*, 1969), this *in vitro* system cannot be said to be capable of outer segment renewal unless it can be demonstrated that some of the newly formed outer segment protein is rhodopsin. For this reason it became necessary to isolate and purify rhodopsin from retinas incubated with radioactive leucine.

Purification of Rhodopsin. In view of the possibility that a contaminating radioactive protein could cochromatograph with rhodopsin, special pains were taken to purify rhodopsin as extensively as possible. In order to accomplish this, two procedures were coupled, each one having been used indepen-

TABLE I: [^{14}C]Leucine Incorporation into Total Retinal Protein.

Variables	Per cent of total[a]
Complete system[b]	100
0°	5
0°, 0 time	0.03
+ Puromycin, 20 μg/ml	17
− Glucose	48
Retinas cut in 10 pieces each	103
Retinas homogenized in incubation buffer	21
Retinas frozen and thawed	20
Retinas frozen, thawed, and homogenized	6
Retinas stored 16 hr, 4°, in incubation buffer	78
Eyes stored 16 hr, 4°	32

[a] Percentage refers to counts per min per mg of protein in the retinal homogenate. [b] Incubation system is as described in Experimental Procedures, 1 hr, 37°.

TABLE II: Specific Activity of Cell Fractions after Pulse Labeling with [^{14}C]Leucine.[a]

	Counts/min per mg of protein		
Retinal cell fraction	1-hr pulse	1-hr pulse and 2-hr chase	Difference
Total protein	1320	1270	−50
Microsomes	2682	2300	−382
Rod outer segments	470	1020	+550

[a] Incubation system as described in Experimental Procedures; experimental details as described in Results.

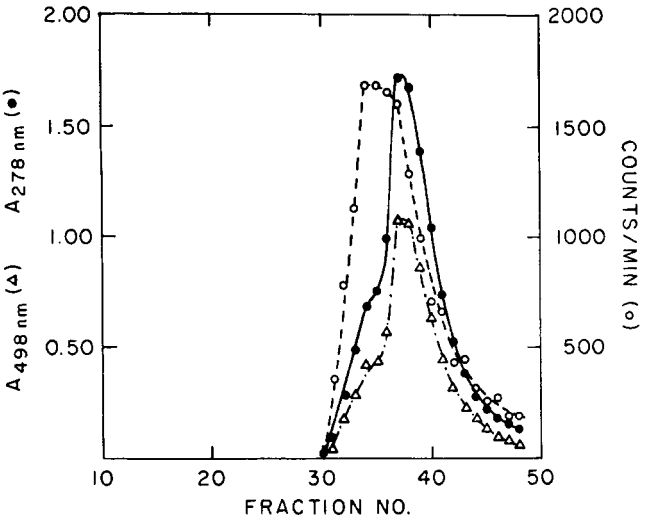

FIGURE 2: Calcium phosphate–Celite chromatography of radioactive rhodopsin. Rod outer segments from 24 bovine retinas incubated with [^{14}C]leucine for 4 hr were extracted with 6 ml of 1% Emulphogene in 0.05 M Tris-HCl, pH 8.5. The extract was passed over a column of 5 g of calcium phosphate and 7 g of Celite under 10 lb/in^2 air pressure. Fractions were 1.6 ml of which 0.1 ml was used for radioactivity measurements. Total counts are plotted along with absorbance at 278 nm and 498 nm.

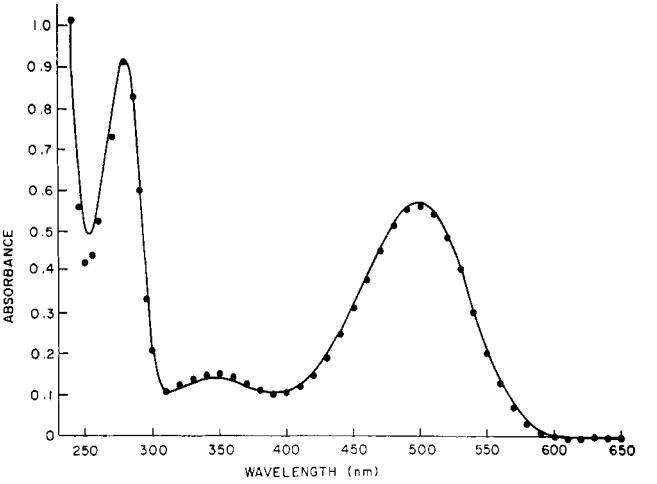

FIGURE 3: Absorption spectra of bovine rhodopsin purified as described in Experimental Procedures. The solid line represents a peak fraction eluted from a calcium phosphate–Celite column. The dots represent a peak fraction of rhodopsin eluted from an agarose column after prior purification on calcium phosphate–Celite, each point being three times the absorbance actually recorded, for ease of comparison.

dently to obtain rhodopsin of high purity. Chromatography of rhodopsin extracts on calcium phosphate–Celite columns (Shichi *et al.*, 1969) produces a high purity rhodopsin especially in the peak tubes (Figure 2) with a spectral ratio A_{278}/A_{498} around 1.6. This ratio rises slightly on the trailing shoulder of the peak indicating the presence of some additional 278-nm absorbing components. The spectrum of a peak fraction obtained by chromatography on calcium phosphate–Celite is shown in Figure 3. The spectral ratios A_{278}/A_{498} and A_{400}/A_{498} are 1.59 and 0.19, respectively, with an absorption minimum at 390 nm and absorption maxima at 498, 278, and 344–348 nm, essentially as reported by Shichi *et al.* (1969). Rechromatography of such fractions on agarose (Heller, 1968a) produces essentially a single protein peak (Figure 4) with only traces of 278-nm absorption in other regions of the elution profile. The A_{278}/A_{498} ratio sometimes rises slightly on the trailing shoulder of the rhodopsin peak. As can be seen in Figure 3, the spectral characteristics are unchanged by this additional purification procedure. However, there is a partial resolution of contaminating radioactive components, permitting a more accurate determination of specific activity. This will be discussed in further detail below. Chromatography of rhodopsin extracts directly on agarose without prior purification on calcium phosphate–Celite is unsatisfactory with A_{278}/A_{498} ratios of peak fractions exceeding 2.5. Considerable 278-nm absorbing material is found in the void volume and also overlapping the rhodopsin from both directions. Hall *et al.* (1969) obtained far greater purification of frog rhodopsin by chromatography of cetyltrimethylammonium bromide (CTAB)[1] extracts of frog rod outer segments on a similar agarose column. However, the A_{278}/A_{498} ratio of their original extract was only 3.2–3.5 whereas the Emulphogene extracts of bovine rod outer segments reported here exhibit a ratio of 4.8–5.0. The additional 278-nm absorbing material may be related to species differences since Millar *et al.* (1969) found spectral ratios of 5.55–8.80 with CTAB extracts of bovine rod outer segments. Shichi (1970) also observed a high spectral ratio of 3.7 when bovine rhodopsin was extracted with CTAB and chromatographed on a Sephadex G-200 column.

Purification of Radioactive Rhodopsin. Rhodopsin was extracted with Emulphogene from rod outer segments isolated from 24 retinas which had been incubated for 4 hr with [^{14}C]-leucine. The extract was passed over a column of calcium phosphate–Celite to produce the elution pattern shown in Figure 2. It is apparent that the radioactivity profile does not coincide with the A_{278} and A_{498} peak. A highly labeled protein, present in trace quantities, is eluted just before the bulk of the rhodopsin. This protein, possibly a precursor of rhodopsin, has a chromatographic mobility on agarose similar to opsin. When the highly labeled fractions preceding the bulk of the rhodopsin

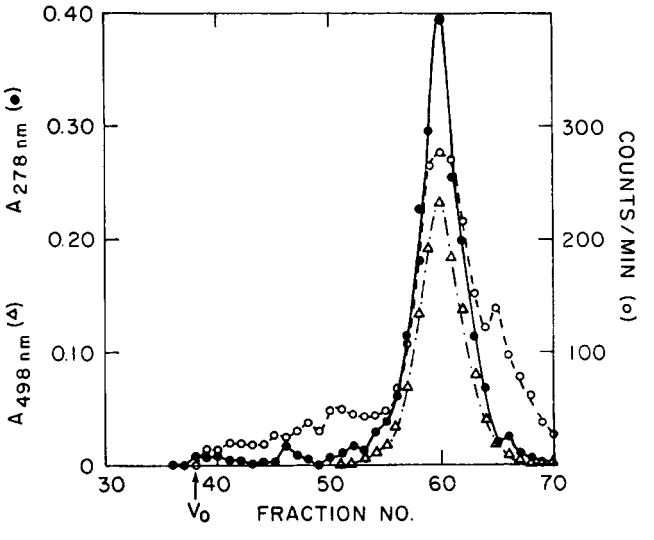

FIGURE 4: Agarose chromatography of peak rhodopsin fractions from calcium phosphate–Celite (see Figure 2) as described in Results. Agarose column is described in Experimental Procedures. Total counts are plotted along with absorbance at 278 nm and 498 nm.

[1] Abbreviation used is: CTAB, cetyltrimethylammonium bromide.

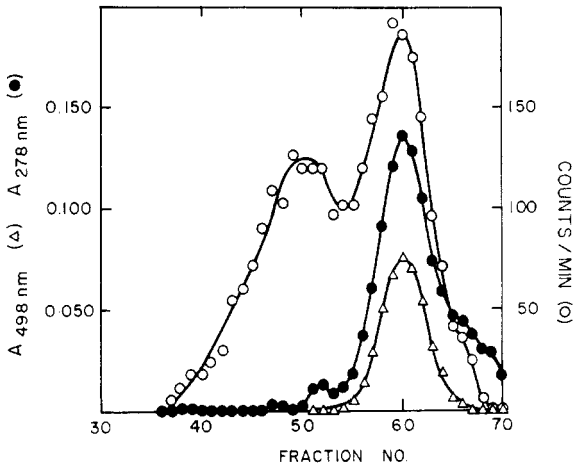

FIGURE 5: Agarose chromatography of highly labeled fractions preceding the bulk of rhodopsin from calcium phosphate–Celite (see Figure 2) as described in Results. The agarose chromatography system is described in Experimental Procedures. Total counts are plotted along with absorbance at 278 nm and 498 nm.

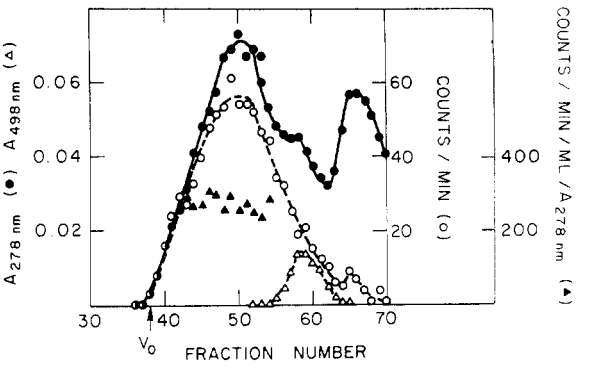

FIGURE 6: Agarose chromatography of bleached radioactive rhodopsin with added unlabeled rhodopsin marker. The five peak fractions shown in Figure 4 were pooled, concentrated, and bleached as described in Results. After addition of marker rhodopsin the mixture was chromatographed on agarose as described in Experimental Procedures. Total counts are plotted along with absorbance at 278 and 498 nm. Specific activity of opsin is plotted as far as the rhodopsin marker. Trailing 278 nm peak is derived from Sephadex G-25 used to concentrate sample.

(for example, numbers 34 and 35) are pooled, concentrated threefold with Sephadex G-25, and chromatographed on an agarose column, the elution profile depicted in Figure 5 is obtained. One of the two major radioactive components coincides with rhodopsin while the other is eluted from the column in a broad peak suggestive of the elution profile of opsin (Figure 6) which will be discussed below. Only traces of 278-nm absorbance can be detected in the opsin region. A small amount of 278-nm absorbing material derived from the Sephadex used for concentration can be seen on the trailing shoulder of rhodopsin. It occurs at no other place on the column.

The peak 498-nm absorbing fractions, numbers 37–39, when pooled and chromatographed on agarose, give rise to the elution pattern shown in Figure 4. These fractions contain only small amounts of the labeled opsin-like protein and a minor radioactive peak trailing rhodopsin. But approximately 70% of the radioactive protein coincides with rhodopsin, the contaminants having been largely removed. The radioactivity applied to the column is totally recovered, with 72% found in the fractions shown and the remaining 28% found in the region where small molecules are eluted, presumably as free leucine.

The two-step purification procedure described here is essential for the isolation of purified radioactive rhodopsin. The calcium phosphate–Celite column retains 85–90% of the radioactivity present in the crude rhodopsin extracts. If the extracts are chromatographed directly on agarose, the bulk of this radioactivity is eluted at or near the void volume with the remainder trailing back throughout the entire elution profile and masking the rhodopsin peak.

Light Sensitivity of Radioactive Rhodopsin. In order to eliminate the possibility of coincidental elution of rhodopsin and contaminating radioactive protein, an experiment was designed to take advantage of a property of rhodopsin first described by Heller (1968b). He observed that in CTAB solutions bleached rhodopsin was eluted from an agarose column just ahead of unbleached rhodopsin. In the present studies, this shift in elution position was found to be even more pronounced in Emulphogene or Triton solutions.

The five fractions containing the highest concentrations of rhodopsin, numbers 58–62, were pooled and concentrated fourfold with Sephadex G-25 to a volume of 2.5 ml. The rhodopsin was then bleached during a 30-min exposure to room light at 0°. The solution was returned to the dark and 0.7 ml of a solution of unlabeled rhodopsin was added as a marker. The marker rhodopsin was a peak fraction from a calcium phosphate–Celite column with an A_{498} of 0.779. The resulting solution was then applied to the agarose column. If the radioactive protein which had previously coincided with rhodopsin was, in fact, rhodopsin it would have been bleached during the exposure to light and should migrate with opsin on rechromatography. Figure 6 shows that the radioactive peak still coincides with the A_{278} peak of opsin, that both now elute at an earlier position from the column, and that neither coincides with the rhodopsin marker. The specific activity is constant across the peak and remains the same as the specific activity of the pooled fractions applied to the column; namely, 304 counts/min per ml per A_{278} in the pooled fractions and 301 counts/min per ml per A_{278} averaged across the peak to the point where the rhodopsin marker begins to influence the A_{278} measurement. Quenching as measured by both the external standard and channels ratio methods is constant across the peak. The recovery of radioactivity is 78% and that of A_{278} is about 80% during the concentration with Sephadex G-25 and rechromatography. The additional A_{278} component following the rhodopsin marker is derived from the Sephadex G-25 during the concentration step.

Control experiments were performed to eliminate several possible explanations of the above observations. First, radioactive rhodopsin was concentrated and rechromatographed without bleaching. The peak of radioactivity as well as of the rhodopsin emerged from the column at fraction 60, the normal elution position of rhodopsin. No radioactivity or 278-nm absorbing material could be detected in the opsin region. These observations indicate that the shift in the elution position observed in the bleaching experiment was not the result of the concentration and rechromatography. This reasoning applies equally to the radioactivity and to the 278-nm absorbing protein peak. Secondly, in a bleaching experiment with no added marker rhodopsin, both the radioactivity and the 278-nm absorbance shifted to an earlier elution position with constant specific activity across the entire peak. This control demonstrates that the added marker rhodopsin was not responsible for the shift in elution position of either the radioactivity or the opsin. As before, the recovery of radioactivity was 78% and that of A_{278} was 84% of the pooled fractions used for the experi-

ment, the losses occurring in the concentration step where 498-nm absorbance was followed prior to bleaching, with an 80% recovery.

These experiments demonstrate that the newly synthesized radioactive protein which coincides with rhodopsin on an agarose column is light sensitive and is therefore rhodopsin.

Time Course of Rhodopsin Labeling. Rhodopsin was isolated and purified as described in Experimental Procedures from three groups of four retinas incubated with [^{14}C]leucine for 1.5, 3, and 4.5 hr. The specific activity of rhodopsin was determined for each time point and is plotted in Figure 7. The labeling of rhodopsin parallels the labeling of the whole rod outer segments described above. But since outer segment preparations are rarely homogeneous and since the outer segments also contain soluble proteins that may turn over at a rate different from membrane proteins, the measurement of rhodopsin specific activity is a far more reliable indicator of outer segment membrane renewal.

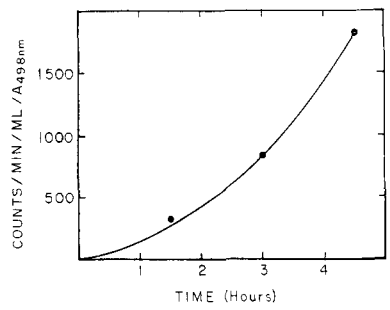

FIGURE 7: Specific activity of purified rhodopsin isolated from retinas incubated for the indicated times. Rhodopsin was isolated and purified as described in Experimental Procedures. Specific activity is expressed in terms of the radioactivity in 1 ml of eluates from agarose columns after prior purification on calcium phosphate–Celite.

Discussion

The experimental conditions as described are probably amenable to modification. In situtations where higher levels of amino acid incorporation are desired, the casein hydrolysate could be replaced by a mixture of amino acids lacking the one bearing the label. In addition, the glucose used as a carbon source can be replaced by 0.14 M glycerol with apparently less dilution of radioactive leucine, the incorporation being almost fivefold greater.[2]

The identity of radioactive rhodopsin rests on the chromatographic distinction between rhodopsin and opsin and the fact that light alters the properties of the radioactive protein from those of rhodopsin to those of opsin. The product formed on light exposure of the radioactive protein migrates at a new position on the agarose column, one which coincides with that of the bleach product of rhodopsin, namely opsin. In addition, most, if not all, of the radioactivity found in the rhodopsin peak on agarose is light sensitive. Consequently the specific activity of rhodopsin determined by the two-step chromatographic purification procedure described here is essentially error free.

It is of interest that the change in chromatographic elution position induced by light is far more dramatic in Emulphogene or Triton solutions than the relatively small shift observed by Heller (1968b) using CTAB as the solubilizing detergent. Heller attributed the shift to a change in hydrodynamic volume brought about by a configurational change in the protein. In the present studies there appears to be some aggregation with a broad peak resulting, reaching almost to the void volume.

Although the kinetics of labeling the outer segments and rhodopsin are those predicted by the model system described in rats, mice, and frogs by Young and Droz (1968) and by Hall *et al.* (1969), the experiments reported here give no direct evidence that the processes are identical. Since these authors described the renewal of outer segments on the basis of autoradiographic observations, the only true comparison of their *in vivo* systems with the *in vitro* system reported here would be an autoradiographic study with the *in vitro* system.

In the present system, the specific activity of the outer segments and rhodopsin rises sharply after a lag period in a fashion paralleling the kinetics of visual pigment incorporation into frog rod outer segment discs observed by Hall *et al.* (1969) *in vivo*. It is possible that the time lag observed in the *in vitro* labeling of rhodopsin is the result of the presence of a large pool of precursors which become labeled slowly relative to other proteins in the retina. However, it seems reasonable to assume that the renewal process does not differ radically in the two biological systems. Thus, it would appear that the experimental system described here is capable of sustaining *in vitro* at least some of the fundamental processes of outer segment renewal as currently understood. In any event, the existence of an *in vitro* biosynthetic system makes it possible to investigate the fine details of rhodopsin synthesis as well as the broader question of the renewal of rod outer segments.

The presence in outer segments of a highly labeled protein with a mobility on agarose similar to opsin raises the intriguing prospect that it could be a precursor to rhodopsin. However, the evidence is totally circumstantial. Many proteins could exhibit similar elution patterns, and no precursor–product relationship between this protein and rhodopsin has been definitely established. Moreover, a deficiency of retinal in some rod cells could also result in incomplete protein molecules being incorporated into outer segment discs. However, the evidence at hand points to a highly labeled protein present in quantities too small to be detected by 278-nm absorbance measurements, suggesting a precursor rather than some other component of outer segment membranes which might be expected to show some measurable 278-nm absorbance. In addition, preliminary experiments indicate that the labeling of rhodopsin becomes progressively greater with time relative to the opsin-like protein,[2] suggesting that the latter might actually be a precursor of rhodopsin. If it could be established that the highly labeled protein is opsin and, in time, gives rise to rhodopsin, there would be cause to suspect that newly formed opsin migrates to the outer segment prior to the addition of retinal. Additional experiments are needed to resolve this question.

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Noncovalent Association of Tyrocidine B*

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ABSTRACT: The noncovalent association of tyrocidine B was investigated by equilibrium ultracentrifugation. In solvents containing several per cent of acetic acid, the association is relatively simple and equilibrium is rapidly attained. The stoichiometry can be adequately represented as an equilibrium between monomer and two polymers of different sizes. In aqueous salt solution, however, the association is complex: the size of the associated units gradually increases after the solution is prepared. This increase can be reversed by warming the solutions. Preliminary investigations of the related polypeptides tyrocidine C, gramicidin S, and an acetylated derivative of gramicidin S suggest a complex relationship between the structure of the peptide and its association behavior.

The tyrocidines, cyclic decapeptides obtainable from cultures of *Bacillus brevis*, are useful molecules for study of the noncovalent association of polypeptides in aqueous solution. They are readily soluble and have high extinction coefficients in the ultraviolet spectrum. Near neutral pH they possess only a single electrostatic charge and thus do not have the large ratio of charge to mass common to most water-soluble small peptides. Their molecular weights and chemical structures are known (Paladini and Craig, 1954; King and Craig, 1955; Ruttenberg *et al.*, 1965). The tendency of tyrocidines to associate has been known since Pedersen and Synge (1948) observed that the diffusion coefficients of partially purified fractions of these peptides were consistent with molecular weights in the range 1900–5100. King and Craig (1955) noted that purified tyrocidine A passed through dialysis membranes unexpectedly slowly. Ruttenberg, King, and Craig (1966) investigated the association of tyrocidine B and several of its derivatives by thin film dialysis (Craig, 1964). They found extensive association of tyrocidine B, diiodotyrocidine B, *N*-succinyltyrocidine B, *O*-methyl-*N*-succinyltyrocidine B, and of a derivative of tyrocidine A in which the aromatic groups had been catalytically reduced. An open-ring derivative of tyrocidine B, prepared by reductive cleavage of the proline–tryptophan bond, did not associate strongly. They concluded that interactions between aromatic groups are not important, but that the cyclic structure of the peptide is necessary for the association. The experiments reported below were conducted in order to elucidate the strength and stoichiometry of the association and to investigate the effects of solvent and temperature upon it.

Experimental Section

Materials. Tyrocidine hydrochloride (Wallerstein Co., lot no. ON 13554) was separated into its components by countercurrent distribution to 3000 transfers in a system composed of chloroform–methanol–0.01 M HCl (2:2:1, v/v) (Ruttenberg, 1965). A detailed analysis of this separation was carried out (Williams and Craig, 1967), with the conclusion that the tyrocidine B fraction contains no more than 3% of the similar peptide, tyrocidine C; and that the tyrocidine C fraction contains a smaller proportion of tyrocidine A. Gramicidin S-A, prepared by countercurrent distribution (Craig *et al.*, 1949), was the gift of Dr. Michael Ruttenberg.

Acetylated gramicidin S-A was prepared by allowing the peptide to react with a limiting amount of acetic anhydride in a solvent of ethanol and triethylamine. Three products were separated from the reaction mixture by countercurrent distribution. Ninhydrin reactions showed that these products possessed zero, one, and two free amino groups per molecule. It was concluded that mono- and di-*N*-acetylgramicidin

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