Illumination of Bovine Photoreceptor Membranes Causes Phosphorylation of both Bleached and Unbleached Rhodopsin Molecules†

Bea Rapley Aton*
Department of Biochemistry, University of Virginia Medical School, Charlottesville, Virginia 22908
Received May 30, 1985

ABSTRACT: Bovine rod outer segments were given a series of flashes, each bleaching from 0.1% to 0.4% of the rhodopsin present. 9-*cis*-Retinal was then added, regenerating the bleaching pigment to isorhodopsin. The phosphorylated pigment species having either four and five or six and eight phosphates were isolated by chromatofocusing. The amounts of rhodopsin and isorhodopsin present in the phosphorylated species were determined spectrally. The species with four and five phosphates per rhodopsin were approximately 50% rhodopsin–50% isorhodopsin. The more highly phosphorylated species were almost entirely isorhodopsin. Presumably, the phosphorylated rhodopsin was phosphorylated without having been bleached. At a 4% bleach level, approximately 0.5 rhodopsin was phosphorylated with four to five phosphates for each rhodopsin that was bleached and phosphorylated.

Rhodopsin, the light-sensitive protein in the rod outer segment (ROS),[1] is phosphorylated by an intrinsic kinase after exposure to light (Kuhn & Dreyer, 1972; Bownds et al., 1972; Frank et al., 1973). Although the physiological significance of rhodopsin phosphorylation is not yet understood, it is thought to be involved in the regulation of the light-activated cGMP phosphodiesterase. When ATP is included in phosphodiesterase assays of ROS, the activity that is initiated by illumination is rapidly terminated (Liebman & Pugh, 1979). This is possibly mediated by the 48-kDa protein in the ROS which competes with the GTP binding protein for binding sites on phosphorylated bleached disks (Kuhn et al., 1984). However, the 48-kDa protein has been reported to quench cGMP phosphodiesterase activation (Zuckerman et al., 1985) independent of the phosphorylation of rhodopsin. While rhodopsin phosphorylation may or may not be involved in the rapid shut-off of cGMP phosphodiesterase activity, it appears to modulate the phosphodiesterase activity expressed when phosphorylated disks are recombined with the soluble proteins of the ROS (Aton & Litman, 1984; Arshavsky et al., 1985; see also Miller & Dratz (1983)]. Because the time scale of dephosphorylation is similar to that of dark adaptation, rhodopsin phosphorylation has also been implicated in this process (Kuhn, 1974).

Rhodopsin with at most 9 *P*/*R* has been isolated from bovine ROS bleached under bright lights for 30 min or more in the presence of ATP (Wilden & Kuhn, 1982; Aton et al., 1984). Under some low-bleach conditions, phosphate/rhodopsin bleached ratios have been much higher. Miller et al. (1977) report a phosphorylation level of 20–30 phosphates per rhodopsin bleached at a 1% bleach and 15–20 *P*/*R* at a 5% bleach but only 3–4 *P*/*R* for bleaches above 20% in frog. Sitaramayya & Liebman (1983) report 19 phosphates/rhodopsin bleached at 1.5×10^{-3}% bleach but only 0.32 at 1.7% bleach level in cattle ROS. Taken together, these previous results suggest that at low bleach levels either opsin kinase is able to phosphorylate many more sites on a bleached rhodopsin molecule or rhodopsins other than those bleached are phosphorylated as proposed by Miller et al. (1977). I have attempted to determine whether unbleached rhodopsin molecules are phosphorylated after a series of low, 0.1–0.4%, bleaches.

Materials and Methods

ROS were isolated from frozen dark-adapted cattle retina (Lawson Co.) following the procedure of McDowell & Kuhn (1977). Approximately 250 nmol of rhodopsin starting material was used for each determination. The ROS in isolation buffer were mixed with assay buffer and ATP. The final concentrations were 20 μM rhodosin, 3.6 mM ATP, 0.1 mM GTP, 1.8 mM $MgCl_2$, 4.4 mM dithiothreitol, and 135 mM sodium phosphate buffer, pH 7.0. The ROS suspension was then placed in a water bath at 30 °C and allowed approximately 1 min to warm. The sample beaker was approximately 30 cm below a Sunpak 611 flash attachment with a Wratten 8 gelatin filter and a 0.7 neutral density filter. The flash was fired every 6 s for 4.6 min. the power ratio settings on the flash attachment were 1/64, 1/32, and 1/16, bleaching 4.2 ± 1.3%, 8.4 ± 2.6%, and 16.9 ± 5.2% of the rhodopsin, respectively. The sample was stirred continuously during the bleaching. In each experiment, a 0% bleach sample was treated in an identical manner, excluding only the bleaching. Ten minutes after the sample had been illuminated, at least a 3-fold excess of 9-*cis*-retinal was added. The sample was incubated for approximately 1.5 h at 30 °C. The ROS were pelleted and washed 1 time in 0.1 M sodium phosphate buffer, pH 7, before being solubilized in 5 mL of 70 mM OG, 5 mM EDTA, 5.5 mM $MgCl_2$, and 50 mM Tris–acetate, pH 7, solution. The rhodopsin was then isolated by affinity chromatography on Con A (Litman, 1982); 5 mM EDTA and 5.5 mM $MgCl_2$ were included in the buffers. The rhodopsin (aproximately 200 nmol) was chromatofocused on PBE 94 with a pH 6.6–3.9 gradient of polybuffer 74 diluted in a ratio of 1/12 with water, 50 mM OG, pH 3.9 (Aton et al., 1984).

† This work was supported by grants from the National Eye Institute (EY04430 to B.R.A. and EY00548 to B. J. Litman).

* Address correspondence to this author at the Chemistry Department, Southern Methodist University, Dallas, TX 75275. Part of this work was also done at this address.

[1] Abbreviations: ROS, rod outer segment(s); *P*/*R*, moles of phosphate per mole of rhodopsin; OG, octyl β-D-glucoside; Tris–acetate, equimolar amounts of tris(hydroxymethyl)aminomethane and sodium acetate; Con A, concanavalin A–Sepharose 4B; EDTA, ethylenediaminetetraacetic acid; TA buffer, 50 mM Tris–acetate, 30 mM OG, 0.5 mM EDTA, and 0.55 mM $MgCl_2$, pH 7.0; kDa, kilodalton(s); cGMP, guanosine cyclic 3′,5′-phosphate; IEF, isoelectric focusing.

0006-2960/86/0425-0677$01.50/0 © 1986 American Chemical Society

The flow rate was 7 mL/h, and fractions were collected every 18 min. After 60–65 fractions had been collected, a 0.5 M NaCl, 25 mM histidine, and 50 mM OG salt wash was started. The absorbance of the column eluant was monitored at 280 nm. The pH and the absorbance at 500 nm of each of the fractions were measured. Fractions of interest were combined to constitute three separate fraction pools. The approximate pH ranges are from pH 5.44 to pH 4.72 and from pH 4.72 to pH 4.0 for fraction pools 1 and 2, respectively; the fractions with absorbance at 500 nm in the salt wash constitute fraction pool 3. These fraction pools were then adjusted to pH 7 with 0.05 M NaOH, and each was put over an approximately 0.75-mL bed volume Con A column. Each column was then washed with 2 mL of TA buffer, and the rhodopsin and isorhodopsin were removed with 5 mL of 0.1 M methyl α-D-mannoside in TA buffer. Each sample was then concentrated to approximately 1 mL by using an Amicon stirred cell with a PM10 membrane. Forty microliters of hydroxylamine (1 M, pH 7, prepared fresh weekly) was added to 400 μL of each cantle, and ΔA, the difference in absorbance before and after bleaching, from 550 to 450 nm for each was determined.

The amount of rhodopsin and isorhodopsin in each fraction pool was calculated by using the ΔA's at 540, 530, 470, and 460 nm and the equations:

$$K_I = 2.17\Delta A_{460} - 2.64\Delta A_{540} \quad (1)$$

$$K_I = 2.45\Delta A_{470} - 2.78\Delta A_{530} \quad (2)$$

$$K_R = 3.29\Delta A_{540} - 1.22\Delta A_{460} \quad (3)$$

$$K_R = 3.20\Delta A_{530} - 1.60\Delta A_{470} \quad (4)$$

K_I is the absorbance at 486 nm due to the presence of isorhodopsin. K_R is the absorbance at 498 nm due to the presence of rhodopsin. The coefficients of eq 1–4 were determined by using phosphorylated rhodopsin and phosphorylated isorhodopsin. ROS were incubated with ATP in the light for 30 min. Hydroxylamine, 0.1 M final concentration, was added. The ROS were pelleted and washed 1 time in 0.1 M sodium phosphate buffer. A 3-fold excess of either 9-*cis*- or 11-*cis*-retinal was added. These samples were then treated the same as the low-bleach samples. The ΔA's at 540, 530, 498, 486, 470, and 460 nm from the fraction pools were used to determine the coefficients (Hubbard, 1956).

The fraction pools were isoelectrically focused on 0.5-mm-thick acrylamide slab gels in the dark using a gradient ranging approximately from pH 6.5 to pH 3.7. The gel contained 5% (w/v) acrylamide, 0.15% (w/v) *N*,*N*′-methylenebis(acrylamide), 45 mM OG, 1.88% (v/v) pH 5–7 ampholytes, and 0.62% (v/v) pH 3.5–5 ampholytes (LKB). The cathode buffer was 0.5 M NaOH; the anode buffer was 0.5 M acetic acid. Before the sample was loaded, the ionic strength of the TA buffer in the fraction pools was reduced to below 10 mM by diluting and concentrating with a Centricon-10 microconcentrator; 10–20 mg of protein was loaded in each lane on the gel by pipetting onto application paper located approximately 2 cm from the anode strip. The gel was run horizontally at 25 W, 6 °C, for 2 h. The gel was stained with Coomassie Brilliant Blue R250 and destained as described by LKB instructions for high-performance analytical electrofocusing (Instruction 1818-P).

Results and Discussion

A typical profile from a chromatofocusing column run is shown in Figure 1. There is considerable variation in the pH at which each phosphorylated rhodopsin species elutes from the column. To ensure that the same phosphorylated species were combined to make up the same fraction pools in each column run, the following three characteristics were used to decide which fractions to combine to make up the separate fraction pools: (1) the scan of the 280-nm absorbance from the column run; (2) the 500-nm absorbance of each fraction; and (3) the pH of the fraction.

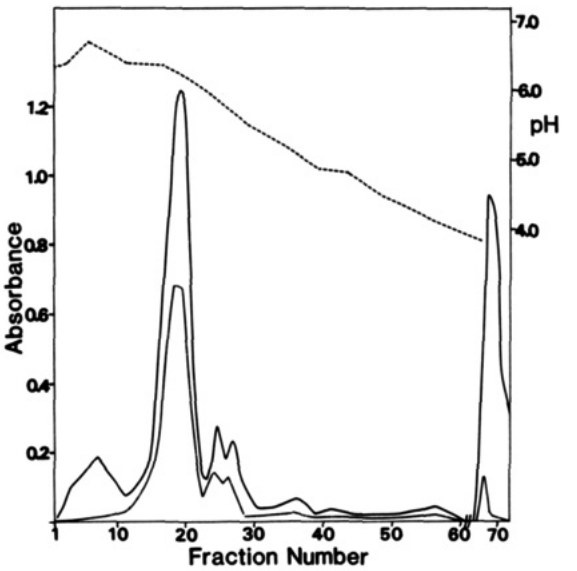

FIGURE 1: Profile of an 8.4% bleached sample elution from a chromatofocusing column. An 8.4% bleached sample regenerated with 9-*cis*-retinal was chromatofocused as described in the text. Approximately 8.4 mg of rhodopsin was loaded onto the PBE column. The absorbance of the column eluant was monitored at 280 nm (—). The absorbance at 500 nm (···) and the pH (- - -) of each fraction were determined. The large absorbance at 280 nm in the last fractions was a result of the difference of index of refractions of the salt wash and the polybuffer gradient solutions. Fractions pooled were 32–46 for fraction pool 1, 47–61 for fraction pool 2, and 68 and 69 for fraction pool 3.

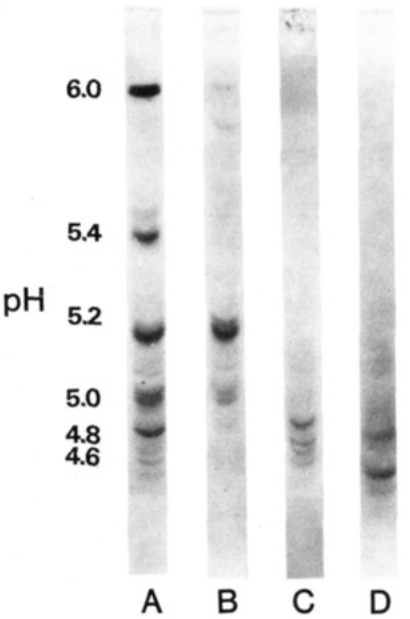

FIGURE 2: Isoelectric focusing of the fraction pools. Lane A is the isoelectric focusing pattern of a sample of phosphorylated ROS >95% bleached, regenerated with 11-*cis*-retinal and is used as a standard for establishing the pH gradient in the gel. Lanes B, C, and D are the patterns for fraction pools 1, 2, and 3, respectively, of an 8.4% bleaching sample.

The species composition of each sample pool was examined by isoelectric focusing (see Figure 2). Fraction pool 1 was composed of the species that focused with the isoelectric points of rhodopsin with 4 and 5 *P*/*R*; fraction pool 2 was the species

Table I: Distribution of Rhodopsin and Isorhodopsin between the Chromatofocusing Cantles

fraction pool	% bleach[a]	nmol of rhodopsin[b]	nmol of isorhodopsin[c]	% rhodopsin[d]
1	0	0.72 ± 0.14		
2		0.15 ± 0.06		
3		0.10 ± 0.03		
1	4.2 ± 1.3	1.78 ± 0.59	0.62 ± 0.30	63 ± 24
2		0.25 ± 0.17	1.02 ± 0.22	9 ± 13
3		0.08 ± 0.03	0.78 ± 0.56	0 ± 3
1	8.4 ± 2.6	1.40 ± 0.64	0.85 ± 0.50	44 ± 23
2		0.45 ± 0.26	2.95 ± 0.49	9 ± 10
3		0.12 ± 0.09	3.30 ± 1.22	1 ± 3
1	16.9 ± 5.2	3.75 ± 1.55	1.70 ± 1.35	64 ± 23
2		0.98 ± 1.16	4.72 ± 0.59	15 ± 14
3		0.08 ± 0.11	6.30 ± 4.05	0 ± 3

[a] The percent bleach was determined by taking an aliquot of the ROS suspension after the controlled bleach, before the 9-cis addition, and comparing the ΔA_{500} of this aliquot to that of an aliquot taken from the 0% bleach sample. [b] The nanomoles of rhodopsin was determined from eq 3 and 4 by using an extinction coefficient of 40 000. Each entry is an average of at least three determinations. [c] The nanomoles of isorhodopsin was determined with eq 1 and 2 by using an extinction coefficient of 43 000. [d] The percent rhodopsin was calculated by subtracting the nanomoles of rhodopsin in the 0% bleach fraction pool from the nanomoles of rhodopsin in the corresponding fraction pool for the controlled bleach samples and determining the percent rhodopsin in the total (rhodopsin plus isorhodopsin) for that fraction pool. Each experiment is calculated separately, the values are averaged, and the standard deviation is determined for the average.

that focused with the isoelectric points of rhodopsin with 6 and 8 P/R (Aton et al., 1984). The protein in fraction pool 3 focused with the species that have an average P/R from 7 to 9 (unpublished results).

The quantities of rhodopsin and isorhodopsin recovered in each fraction pool are given in Table I. Fraction pool 1 contained a large percentage of rhodopsin for all bleaching levels examined. In one experiment, as much as 88% and, in another, as little as 23% of the protein was determined as having the 11-cis chromophore. The pigment in fraction pool 2 was made up of approximately 10% rhodopsin and in fraction pool 3 was less than 2% rhodopsin. The error in determining the fractions of rhodopsin and isorhodopsin in each fraction pool was less than 10%. However, as indicated, there were large variations from experiment to experiment. I feel that this was probably due to variability in the ROS since I obtained relatively high or low ratios for each bleach level in a particular experiment. I was not able to analyze the rhodopsin fractions with 2 P/R because they were not always well separated from 0 P/R fractions under the gradient conditions used.

To rule out the possibility that residual 11-*cis*-retinal in the ROS was preferentially regenerating with the opsin species in fraction pool 1, the 9-*cis*-retinal was added immediately after a 5% bleach instead of waiting 10 min. In this experiment, fraction pool 1 contained 45%, fraction pool 2, 0%, and fraction pool 3, 8% rhodopsin. These values are in the range of values I get for the longer incubation times. Furthermore, the moles of free 11-*cis*-retinal or retinal precursor in the membrane would have to be from 3% to 8% of the moles of rhodopsin in the ROS. In isolated frog ROS, free 11-*cis*-retinol has been determined to be approximately 2.5% (Bridges, 1976). Furthermore, the pigment of unbleached ROS incubated with a 3-fold excess of 9-*cis*-retinal at room temperature for 48 h was >95% rhodopsin. There was no exchange with the free retinal.

A related alternative possibly explaining my data is reisomerization and regeneration to rhodopsin during the flash sequence. Two identical ROS samples were prepared for bleaching and phosphorylating. To one sample was added hydroxylamine, final concentration 0.1 M, before bleaching. To the other was added an equivalent quantity of water. An aliquot of each was saved to determine the ΔA_{500} values before bleaching. The samples were then given the series of flashes that bleach approximately 16% of the rhodopsin. The ratios of the ΔA_{500} values after and before bleaching for the ROS sample without hydroxylamine and the sample with hydroxylamine were 0.855 and 0.845 ± 0.005, respectively. I estimate that the difference in this ratio would be at least 0.040 in order to account for the amount of rhodopsin isolated in the phosphorylated species if the phosphorylated rhodopsin had been generated by the reisomerization mechanism. This estimate of the difference also requires two assumptions; otherwise, the estimated difference would be even larger. First, the opsin with 4 and 5 P/R must have a higher affinity for 11-*cis*-retinal than any of the other forms of opsin, phosphorylated or unphosphorylated. Second, the *all-trans*-retinal must be isomerized to 11-cis and not 9-cis; the 9-cis form would contribute to the difference in the ratio without contributing to the phosphorylated rhodopsin. Therefore, I feel that reisomerization and regeneration contribute little if any to the effect measured.

Another possible explanation of the data might be chromophore exchange between phosphorylated and unphosphorylated species. After 9-*cis*-retinal addition to an 8.4% bleached sample, ROS samples were incubated for 10 and 21 h instead of 2 h. The percentages of rhodopsin in fraction pools 1, 2, and 3 were 45, 12, and 6%, respectively, in the 10-h sample and 79, 18, and 1% in the 21-h sample. Again, these values are within the ranges I see for 2-h incubations. Although unlikely, there is the possibility that some mechanism could exist that allows for exchange between the 4 and 5 P/R species and 0 P/R species and has a time course faster than 2 h. Because the 21-h sample was on the high end of the range determined, a further experiment was performed. An ROS sample was given the series of flashes resulting in a 17% bleach. No 9- or 11-*cis*-retinal was added. Fifteen minutes after bleaching the sample, the phosphorylation was stopped by diluting the ROS in 0.1 M sodium phosphate buffer, pH 7, pelleting the sample, and raising up the pellet in 0.1 M sodium phosphate buffer. The sample was then divided into two parts. One part was pelleted and solubilized in 100 mM OG in TA buffer. The other was incubated for 24 h before being pelleted and solubilized. The rhodopsin from both samples was then isolated on a Con A column. Under the conditions used, the opsin remains attached to the column materials. The P/R values were determined to be the same, approximately 0.6 P/R, for each sample. If there had been significant transfer to the 11-cis chromophore to phosphorylated opsin, then the P/R of the sample incubated for 24 h would have been higher.

Typically, I recovered 60–70% of the material as phosphorylated isorhodopsin that was estimated to have been bleached in the sample. This estimate was made by taking into account three factors: (1) 70–80% of the rhodopsin was phosphorylated in a total bleach experiment; (2) I recovered approximately 80% of the material loaded on a chromatofocusing column and (3) 80% of the material loaded on a Con A column. Furthermore, because the gradient fractions containing 2 P/R were unresolved from 0 P/R fractions, I was not able to include them in my total quantities. In high-bleach experiments, considerable amounts of rhodopsin were found in 2 P/R species. Assuming the same distribution of species for the low-bleach level as for the high-bleach levels, I can account for 80–100% of the material that was bleached.

In this discussion, I call the enzyme that phosphorylates rhodopsin that was absorbed a photon "opsin kinase" and the

enzyme that phosphorylates unbleached rhodopsin "rhodopsin kinase". These need not be separate enzymes, only separate activities. I have speculated that the phosphorylated species with isoelectric points of 5.2, 5.0, 4.85, and 4.7 isolated under these bleaching conditions have respectively 4, 5, 6, and 8 P/R as determined previously (Aton et al., 1984). The original P/R determinations were made on rhodopsin samples that had been >95% bleached. In Figure 2, lane A is an IEF pattern from ROS with >95% bleach, while lane B is a pattern from fraction pool 2 with an 8.4% bleach sample. In this gel, there are apparently no new phosphorylated species generated by rhodopsin kinase that are not seen in the sample which should be phosphorylated entirely by opsin kinase.

These data indicate that, under the bleaching conditions used, rhodopsin was phosphorylated that initially was not bleached. Although the high ratios of phosphate to rhodopsin determined for frog ROS at the 1–5% bleach levels have not been reported in cattle, the mechanism of phosphorylation of rhodopsin is apparently present in both frog and cattle. Since there are large differences at low bleach levels in the rates of hydrolysis of cGMP by the phosphodiestrase between cattle and frog (Liebman & Pugh, 1982), it is perhaps not unexpected to find such differences in the P/R vs. bleach level between frog and cattle.

A few observations can be made about how the rhodopsin was phosphorylated. The rhodopsin could be phosphorylated by transfer of phosphate from previously phosphorylated rhodopsin. In the samples incubated for 10 and 21 h, the amounts of isorhodopsin in both fraction pools 3, the most likely candidates for donor phosphates, were not depleted during the longer incubation times. Furthermore, several phosphates must be transferred to the same rhodopsin cooperatively. The rhodopsin could have been "bleached" by having its chromophore removed by a phosphorylated rhodopsin intermediate with a higher affinity for the 11-cis chromophore. However, the all-trans chromophore must be displaced by the 11-cis chromophore of an unbleached rhodopsin. Rhodopsins adjacent to a bleached molecule could become activated by the energetic bleached molecule and then available as substrate for the kinase. This mechanism would imply an initial plateau region in a plot of phosphates incorporated per rhodopsin bleached. Finally, rhodopsin could be phosphorylated by an activated kinase, in which case, the rhodopsin kinase, either the same or different from opsin kinase, phosphorylates primarily only four or five sites on the unbleached form.

By dividing the total nanomoles of rhodopsin minus the correction from the unbleached sample by the total nanomoles of isorhodopsin collected in the fraction pools (Table I), I can estimate how many rhodopsin molecules were phosphorylated for each one that was bleached and phosphorylated. I get 0.47, 0.14, and 0.30 for the 4, 8, and 17% bleach levels, respectively, suggesting the same trend that is observed in frog ROS. Chromatofocusing profiles of phosphorylated rhodopsin reveal two forms of rhodopsin with 2 P/R as well as possible multiple forms of the highly phosphorylated species. The data presented here indicate that there are at least two mechanisms for the phosphorylation of rhodopsin and opsin. The hypothesis that the only role of phosphorylation is to build a large negative charge in the carboxy terminus of rhodopsin must be evaluated critically. Rhodopsin phosphorylation may be similar to other systems in which specific site phosphorylation is significant.

ACKNOWLEDGMENTS

I thank Dr. David Bridges for discussions on this work and especially Dr. Burton Litman for support and use of laboratory facilities. I gratefully acknowledge the generous supplies of 9-*cis*-retinal and 11-*cis*-retinal from Hoffmann-La Roche, Inc.

REFERENCES

Arshavsky, V. Y., Dizhoor, A. M., Shestakova, I. K., & Philippov, P. P. (1985) *FEBS Lett. 181*, 264–266.

Aton, B., & Litman, B. J. (1984) *Exp. Eye Res. 38*, 547–559.

Aton, B. R. Litman, B. J., & Jackson, M. L. (1984) *Biochemistry 23*, 1737–1741.

Bownds, D., Dawes, J., Miiller, J., & Stahlman, M. (1972) *Nature (London), New Biol. 237*, 125–127.

Bridges, C. D. B. (1976) *Nature (London) 259*, 247–248.

Frank, R. N., Cavanagh, H. D., & Kenyon, K. R. (1973) *J. Biol. Chem. 248*, 596–609.

Hubbard, R. (1956) *J. Gen. Physiol. 39*, 935–962.

Kuhn, H. (1974) *Nature (London) 250*, 588–590.

Kuhn, H., & Dreyer, W. J. (1972) *FEBS Lett. 20*, 1–6.

Kuhn, H., Hall, S. W., & Wilden, U. (1984) *FEBS Lett. 176*, 473–478.

Liebman, P. A., & Pugh, E. N., Jr. (1979) *Vision Res. 19*, 375–380.

Liebman, P. A., & Pugh, E. N., Jr. (1981) *Vision Res. 22*, 1475–1480.

Litman, B. J. (1982) *Methods Enzymol. 31*, 150–153.

McDowell, J. H., & Kuhn, H. (1977) *Biochemistry 16*, 4054–4060.

Miller, J. L., & Dratz, E. A. (1984) *Vision Res. 24*, 1509–1521.

Miller, J.A., Paulsen, R., & Bownds, M. D. (1977) *Biochemistry 16*, 2633–2639.

Sitaramayya, A., & Liebman, P. A. (1983) *J. Biol. Chem. 258*, 12106–12109.

Wilden, U., & Kuhn, H. (1982) *Biochemistry 21*, 3014–3022.

Zuckerman, R., Buzdygon, B., & Liebman, P. (1985) *Invest. Ophthalmol. Visual Sci. 26*, 45 (ARVO abstract).