Light-Induced Permeability Changes in Sonicated Bovine Disks: Arsenazo III and Flow System Measurements†

H. Gilbert Smith, Jr.,‡ and Paul J. Bauer*

ABSTRACT: Light-induced permeability changes in bovine rod outer segments have been studied by two independent methods: (1) by use of the metallochromic indicator arsenazo III to measure light-induced calcium concentration changes in suspensions of disks loaded with calcium by sonication and (2) by use of a flow system to measure the efflux of radioactive calcium and other substances from immobilized sonicated disks [Smith, H. G., Fager, R. S., & Litman, B. J. (1977) *Biochemistry 16*, 1399]. Similar light-induced releases of internal calcium were reproducibly found with both types of experiments. Only part of the trapped calcium was released upon light exposure; therefore, the light-induced increase in permeability responsible for the release is only *transient*. The magnitude of the release increases with both the amount of internal calcium and with the amount of rhodopsin bleached. Under most conditions the amount of calcium released per *bleached* rhodopsin increases as the percent bleaching decreases. At 37 °C and pH 8.0, releases greater than one calcium per bleached rhodopsin were normally found except at high levels of bleaching. The magnitude of the release of substances other than calcium varied considerably from one substance to another; however, definite light-induced releases were found for ^{86}Rb, ^{22}Na, [^{32}P]phosphate, [^{3}H]glucose, and [^{3}H]sucrose in addition to ^{45}Ca. Experiments with trapped [^{3}H]inulin indicate that lysis of the sonicated disk vesicles makes almost no contribution to the light-induced releases discussed here.

The absorption of light by the chromophore of rhodopsin, the visual pigment located primarily in the disk membranes of rod outer segments, leads by a still unknown mechanism to a decrease in the sodium permeability of the separate plasma membrane. This decrease in sodium permeability causes a hyperpolarization of the plasma membrane and thereby initiates the electrical events of vision. Baylor & Fuortes (1970) proposed that some internal transmitter substance links the photochemical events of the disk membrane with the electrical events of the plasma membrane. Hagins and Yoshikami have refined this hypothesis into a persuasive model in which, upon light exposure, the disks release calcium which diffuses to the plasma membrane to reduce the sodium permeability by blocking sodium channels (Hagins, 1972; Yoshikami & Hagins, 1973; Hagins & Yoshikami, 1974). Other workers have suggested that calcium may control cyclic nucleotide levels in rod outer segments (Cohen et al., 1978).

The uptake and release of calcium by photoreceptor membranes have been studied by using a variety of methods and membrane preparations, but a unified picture of the effects of light on the calcium content of disks has not emerged [see Smith et al. (1977) for references]. Smith et al. (1977) found a light-induced release of about one calcium per bleached rhodopsin at pH 7.4 and 25 °C by using a flow system which measures the efflux of radioactive calcium from sonicated disks bound to glass particles. In order to more comprehensively study the characteristics of this release, we have extended similar flow system measurements to a greater range of conditions and have included other substances in addition to calcium. We have also employed the calcium ionophore A23187 to further characterize the release mechanism. To eliminate the possibility that binding to glass particles may influence the characteristics of the release, we have also measured light-induced calcium concentration changes in suspensions of sonicated disks by using the metallochromic indicator arsenazo III (Scarpa et al., 1978). We have reproducibly found similar light-induced releases of calcium with both types of experiment. Furthermore, we have investigated the specificity of the light-induced permeability changes by using the flow system to examine light-induced releases of various other substances in the presence and absence of calcium.

Materials and Methods

General Procedures. Disks were isolated from bovine rod outer segments by flotation on 5% Ficoll (Smith et al., 1975, 1977). During all procedures the samples were kept under argon or nitrogen to reduce oxidation. Rhodopsin concentrations were determined from the change in absorbance at 500 nm (ΔA_{500}) upon bleaching a solubilized disk sample in the presence of hydroxylamine. The molar extinction coefficient used was 40 000 M^{-1} cm^{-1}; the molecular weight used was 40 000. For both the arsenazo III and flow system experiments, calcium was trapped by sonicating a suspension of disks for 30 s at 4 °C with the microprobe of a Branson B-12 sonifier. The resulting disk vesicles were stored overnight under argon in a refrigerator to allow time for resealing. A separate sonication was done for each day's experiments. The suspension of disks typically contained 1–1.5 mg/mL rhodopsin, 0.1 M imidazole–chloride buffer, pH 7.4 or 8.0, and 15 mM $CaCl_2$. For the flow system experiments the disk suspension also included 50 μCi/mL ^{45}Ca. In some of the flow system experiments the suspension contained other isotopes either in place of or in addition to calcium. The specific activity of each isotope was usually $\sim 5.5 \times 10^6$ cpm/μmol.

Arsenazo III Experiments. In these experiments the released calcium was measured by using the metallochromic indicator arsenazo III, which has a maximal sensitivity for calcium at 652 nm. The arsenazo III was purified by the method of Kendrick (1976).

Before adding arsenazo III to the calcium-loaded disks, the external calcium was removed by treatment with the ion-exchange resin Chelex 100 (Kaupp & Junge, 1977). A 0.4-mL aliquot of sonicated disks containing 0.6 mg of rhodopsin was

†From the Institute for Neurobiology, KFA, D-5170 Jülich, West Germany. *Received January 10, 1979.* A preliminary report was given in a poster at the meeting "Visual Sensitivity and Adaptation" in Guildford, England, Sept 1978.

‡Supported by a postdoctoral grant of the Deutsche Forschungsgemeinschaft (SFB 160). Present address: Department of Physiology and Biophysics, Washington University, St. Louis, MO 63110.

0006-2960/79/0418-5067$01.00/0

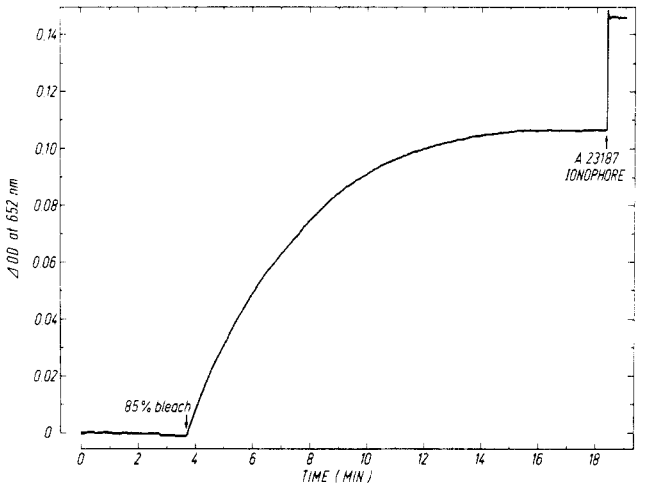

FIGURE 1: Calcium release measured in a typical arsenazo III experiment. 85% of the rhodopsin in the sample was bleached by a 6-s light exposure at the indicated time. 1.85 calcium molecules were released for each rhodopsin bleached, and an additional 0.75 calcium/rhodopsin was released by the treatment with ionophore, A23187. The sonicated suspension contained disks with 1.5 mg/mL rhodopsin, 15 mM $CaCl_2$, and 0.1 M imidazole–chloride buffer, pH 8.0. After dilution and treatment with Chelex 100, the suspension contained 43 μM arsenazo III, 0.29 mg/mL rhodopsin, 0.1 M KCl, and 0.1 M imidazole–chloride buffer, pH 8.0. During the absorbance measurements the temperature was 37 °C.

mixed with 1.6 mL of a 60 mg/mL suspension of Chelex 100 in the desired buffer. The mixture was gently shaken for 2 min and then filtered through a 5-μm pore Nuclepore filter to remove the Chelex 100 particles. This treatment reduced the external calcium concentration to ~25 μM. The buffer used was 0.1 M imidazole–chloride, pH 7.4 or 8.0. For some experiments 0.1 M KCl was added to give conditions closer to those expected in intact rod outer segments.

The filtered disk vesicles were added to 0.1 mL of a 0.75 mM arsenazo III solution, and 0.9-mL aliquots of this suspension were placed in thermostated cuvettes in the sample and reference compartments of a Cary 17 spectrophotometer. The absorbance difference was recorded at 652 nm vs. time. After a 10-min wait for temperature equilibration, a constant base line was reached and the sample was then bleached with incandescent light filtered through an orange cutoff filter (Schott OG 530). Absorption measurements showed that no bleaching of the reference occurred during the experiment. After bleaching, the absorbance difference at 652 nm increased and reached a plateau after 10–15 min (Figure 1). Then, 5 μL of a 0.2 mM ethanolic solution of the calcium ionophore, A23187, was added to the sample, resulting in an instantaneous OD increase. Next, the same amount of A23187 was added to the reference and again the OD change was measured.

To calculate quantitative calcium concentrations, we determined the amount of calcium-bound arsenazo III after each experiment. We measured the actual OD_{652} of both sample and reference vs. air, the maximal OD_{652} of each after adding 10 μL of 0.1 M $CaCl_2$ to bring arsenazo III fully into the calcium-bound form (OD_{652}^{max}), and the minimal OD_{652} after a desaturating addition of 10 μL of 0.4 M EDTA (OD_{652}^{min}).

The fraction of arsenazo III to which calcium is bound, α, is given by

$$\alpha = (OD_{652} - OD_{652}^{min})/(OD_{652}^{max} - OD_{652}^{min}) \quad (1)$$

The OD_{652} before addition of ionophore can be readily calculated for both sample and reference by subtracting the OD_{652} changes caused by ionophore addition from the values which were measured vs. air after ionophore addition. One then has all of the information necessary to calculate α for both sample and reference before and after addition of ionophore and from this to calculate the calcium concentrations by using the calcium binding constant and the extinction difference at 652 nm between calcium-free and saturated arsenazo III. These have been carefully determined for the various experimental conditions. In the experiments reported here, the pH was kept constant by buffering with 0.1 M imidazole–chloride, and our procedures eliminated any significant contributions from cations other than calcium. Therefore, the OD changes at 652 nm which we measure directly reflect changes in external calcium concentrations. The calcium binding constants and the extinction coefficient differences at 652 nm obtained in 0.1 M imidazole–chloride, pH 8.0 and 37 °C, were 3.80×10^5 M^{-1} and 28 500 M^{-1} cm^{-1} in the absence of KCl and 7.34×10^4 M^{-1} and 26 500 M^{-1} cm^{-1} in the presence of 0.1 M KCl.

In addition to the measurements described above, the OD_{652} of the reference vs. air was periodically measured during the experiment to monitor absorbance increases due to passive leakage. One can use these data to convert the measured relative OD changes into absolute OD for both sample and reference. One can then calculate α and calcium concentrations at any time during the experiment.

Finally, after each experiment we determined the percent bleaching of rhodopsin by adding to both sample and reference 0.1 mL of a solution containing 7% Emulphogene BC-720 and 0.5 M NH_2OH. We then measured the difference in absorbance at 500 nm (ΔA_{500}) before and after fully bleaching both sample and reference. The ΔA_{500} of the reference determines the rhodopsin concentration, and the ratio of the ΔA_{500} of the sample to that of the reference gives the fraction not bleached.

The Flow System Experiment. The procedures used were basically those of Smith et al. (1977). An aliquot of disks which had been sonicated with ^{45}Ca or other radioactive substances was loaded onto a small bed (100–200 μL) of 5–10-μm diameter controlled pore glass particles (Corning, CPG-250) which was supported by a Nucleopore filter (0.4-μm pore diameter) in a 13-mm Millipore filter holder fitted with a clear top. More than 90% of the disk membranes were retained by the glass particles in typical experiments with 0.1 mg of rhodopsin and 30 mg of the glass particles.

The filter holder with glass particles and adhering disk membranes was mounted in a thermostated jacket and connected to a peristaltic pump and a fraction collector. After a 20-min prewash at room temperature with a buffer flow rate of 2 mL/min to remove external calcium, the temperature of the thermostating liquid was adjusted and the flow rate of the washing buffer was reduced to 1 mL/min. Fractions of the effluent from the filter holder were then collected every 0.6 min (Figure 2). After 20 fractions had been collected to establish the base line, the disks were bleached by light from a microscope lamp. Buffer flow and fraction collection were continuous.

The amount of rhodopsin bleached was determined by measuring the ΔA_{500} of separate nonradioactive samples that were treated as in the normal experiment and then eluted from the glass particles and filter with 2 mL of 5% Emulphogene, 0.1 M NH_2OH, and 0.1 M imidazole–chloride, pH 7.3. The total retention of disk vesicles by the glass beads was similarly measured in a sample that was not bleached.

The fractions were counted in a three-channel Berthold liquid scintillation counter using Instagel scintillation fluid (Packard). The specific activity was determined by counting a small aliquot of the suspension of sonicated disks used for each experiment. In the double-label experiments the activity

Table I: Magnitude of the Light-Induced Release Measured in Flow System Experiments[a]

conditions[b] (wash buffer)	data for 25% bleaches: light-induced Ca release per bleached rho	data for 25% bleaches: trapped Ca per total rho[c]	data for 25% bleaches: no. of expt	data for 95% bleaches: light-induced Ca release per bleached rho	data for 95% bleaches: trapped Ca per total rho[c]	data for 95% bleaches: no. of expt	ratio,[d] 25%/95%
pH 8.0, 37 °C,	2.31 ± 0.81	1.23 ± 0.35	7	0.81 ± 0.20	3.37 ± 2.50	9	2.9
+ 0.1 M KCl	1.68 ± 0.51	1.08 ± 0.47	6	0.66 ± 0.37	1.51 ± 0.39	13	2.5
pH 8.0, 25 °C,	1.52 ± 0.46	1.90 ± 0.19	4	0.55 ± 0.12	1.95 ± 0.02	2	2.8
+ 0.1 M KCl	1.75 ± 0.42	1.55 ± 0.32	5	0.97 ± 0.11	2.66 ± 0.24	3	1.8
pH 7.4, 37 °C,	1.49 ± 0.42	1.16 ± 0.24	4	0.86 ± 0.15	1.93 ± 0.62	12	1.7
+ 0.1 M KCl	1.23 ± 0.19	1.30 ± 0.28	4	0.88 ± 0.58	1.63 ± 0.51	3	1.4
pH 7.4, 25 °C,	0.64 ± 0.28	1.71 ± 0.14	6	0.37 ± 0.18	4.55 ± 2.70	5	1.7
+ 0.1 M KCl	0.53 ± 0.17	1.23 ± 0.41	7				

[a] The reproducibility of the data is indicated by the standard deviation ($n > 2$) or range ($n = 2$) of the data. [b] The wash buffer also contained 0.1 M KCl for all of these experiments. [c] The amount of trapped calcium within the sonicated disks at the time of bleaching is estimated by the sum of the released calcium and the calcium which remained with the disks at the end of the experiment. [d] Ratio of light-induced release per bleached rhodopsin at 25% bleaching to that at 95% bleaching.

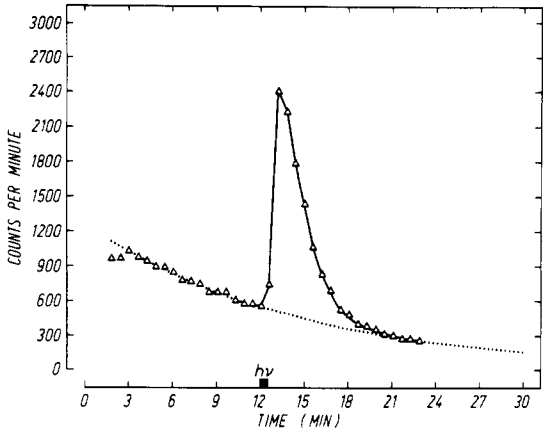

FIGURE 2: ^{45}Ca release measured in a typical flow system experiment. Approximately 95% of the rhodopsin was bleached by a 0.6-min light exposure during the indicated time period. The light-induced release was 0.78 calcium for each rhodopsin bleached, and an additional 1.3 calcium/rhodopsin remained at the end of the experiment with the disk membranes adhering to the glass beads. The sonicated suspension contained disks with 1.0 mg/mL rhodopsin, 15 mM $^{45}CaCl_2$, and 0.1 M imidazole–chloride, pH 8.0. The wash buffer contained 0.1 M KCl and 0.1 M imidazole–chloride, pH 8.0. The temperature during fraction collection was 37 °C. The ordinate gives the amount of isotope collected in each fraction, with 1000 cpm representing 0.24 nmol of calcium. The dotted line is the estimated release base line.

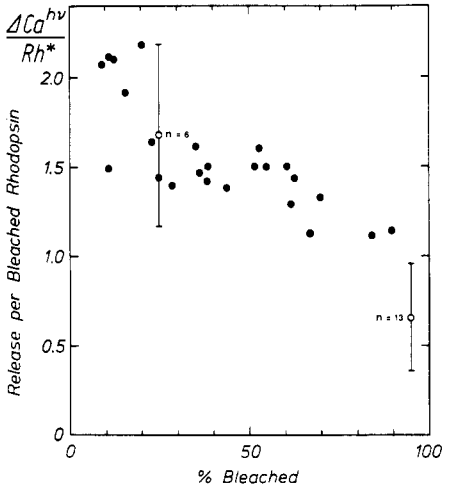

FIGURE 3: Dependence of the calcium release per bleached rhodopsin upon the percent rhodopsin bleached. The solid points are taken from four sets of arsenazo III experiments. Each set of data is normalized to the mean value of 1.5 calcium/bleached rhodopsin at 50% bleaching to reduce the scatter between sets of experiments which is due to variations of the internal calcium concentration of the disk vesicles (see text). The actual data gave between about 0.9 and 2.0 calcium released/bleached rhodopsin at this level of bleaching. The open points show the mean ± standard deviation for flow system experiments at 25 and 95% bleaching (Table I). The suspension of sonicated disks in the arsenazo III experiments contained 0.1 M KCl in addition to arsenazo III and pH 8.0 imidazole–chloride buffer. The wash buffer for the flow system experiments contained 0.1 M KCl and 0.1 M imidazole, pH 8.0.

of each isotope was calculated from the counts per minute obtained by using an appropriate two channels of the counter and the relative counting efficiencies for each isotope in those channels. The latter were determined by using standards for each isotope prepared the same as the experimental samples.

To calculate the magnitude of the light-induced release in each experiment, we extrapolated the base line before bleaching (the dark release) by fitting the counts per minute of the 10 fractions immediately before bleaching to a first-order exponential (see dotted line in Figure 2). The area enclosed by the peak over this base line is taken as the amount of light-induced isotope release.

Results

The Light-Induced Calcium Release. The total light-induced release depends upon the amount of rhodopsin bleached, the amount of calcium trapped within the disk vesicles, and other experimental conditions. Typically, both flow system and arsenazo III measurements show 0.7–1.0 calcium released for each rhodopsin in the sample at high levels of bleaching under the conditions of Figure 3 (pH 8.0, 0.1 M KCl, and 37 °C). The values obtained with the flow system tended to be somewhat lower than those obtained in the arsenazo III experiments under these conditions.

Both arsenazo III and flow system experiments show that the magnitude of the light-induced calcium release increases with the amount of rhodopsin bleached. The amount of calcium released, however, is not quite proportional to the percent bleaching. Under all conditions studied, flow system experiments show that the magnitude of the release per *bleached* rhodopsin increases as the percent bleaching decreases (Table I). Arsenazo III experiments also show this dependence in the presence of 0.1 M KCl (Figure 3); however, in the absence of KCl such a dependence is not seen because of calcium binding to the disk membranes. Such binding will affect the data obtained with arsenazo III experiments but not those obtained with flow system experiments where the external solution is kept calcium free by continual replacement. Several workers have reported high-affinity binding sites that are strongly dependent on the potassium concentration (Hem-

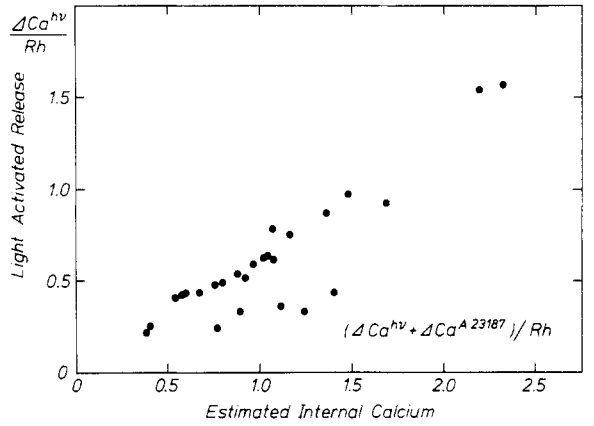

FIGURE 4: Dependence of the light-induced calcium release upon the amount of internal calcium. In these arsenazo III experiments, the amount of internal calcium was estimated by the sum of the light-induced release and the release from the sample produced by treating with the ionophore, A23187. All suspensions of sonicated disks contained 0.1 M KCl in addition to arsenazo III and pH 8.0 imidazole–chloride buffer. 80 ± 5% of the rhodopsin was bleached in each of these experiments. The amount of light-induced calcium release and the estimated internal calcium were normalized to the total amount of rhodopsin in the sample.

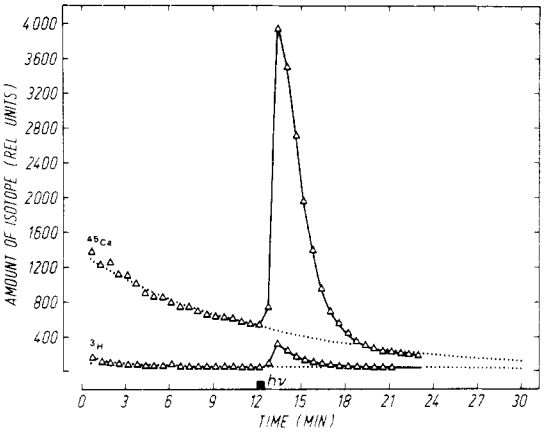

FIGURE 5: Simultaneous light-induced release of ^{45}Ca and [^{3}H]inulin. In this experiment the suspension of disks that was sonicated contained 15 mM $^{45}CaCl_2$, 5 mM [^{3}H]inulin, and 0.1 M imidazole–chloride, pH 8.0. The amount of isotope collected in each fraction is expressed as counts per minute after a small adjustment for the difference in specific activity between the two isotopes. A value of 1000 represents 0.182 nmol of either calcium or inulin. 0.91 calcium and 0.08 inulin were released for each rhodopsin bleached with ~95% of the rhodopsin being bleached. At the end of the experiment, 0.80 calcium and 0.21 inulin remained per rhodopsin.

minki, 1975; Hendricks et al., 1977; Ostrovsky, 1978; Schnetkamp, 1979). In flow system experiments the increase in the magnitude of the release per bleached rhodopsin at lower bleaches is less pronounced at pH 7.4 than at pH 8.0 (Table I). In both the flow system and arsenazo III experiments the total magnitude of the release increases with increasing pH.

After the light-induced calcium release had subsided, an additional calcium release was observed with both methods upon addition of the calcium ionophore, A23187, indicating that light exposure had released only part of the internal calcium. This ionophore treatment was routinely carried out in the arsenazo III experiments. In these experiments the sum of the light-induced and ionophore-induced calcium releases from the sample was always about the same as the ionophore-induced calcium release from the unbleached reference and can thus be used as a measure of the total amount of internal calcium in the disk vesicles at the time of bleaching. By this measure, the internal calcium of the disk vesicles was always reasonably constant for different experiments using vesicles from the same sonication. There was a larger ionophore-induced release from the sample in experiments where only a small percentage of the rhodopsin had been bleached than in experiments where most of the rhodopsin had been bleached.

The amount of internal calcium in the disk vesicles varied somewhat between different sonications because of differences in the amount of calcium trapped and because of differences in the permeability of the vesicles to calcium in the dark. As shown in Figure 4, the light-induced calcium release increases with increasing internal calcium under conditions where the buffer contains 0.1 M KCl and where the amount of bleached rhodopsin is constant. Similar results were obtained without KCl and, also, in flow system experiments, but the dependence was less pronounced. In flow system experiments, the internal calcium of the disk vesicles was estimated from the counts per minute remaining with the membranes adhering to the glass beads at the end of the experiments (Table I).

Treating disk vesicles with ionophore before bleaching results in an exhaustive calcium release, and no further release is observed upon subsequent bleaching. In the arsenazo III experiments a light-scattering artifact gave a small, rapid OD decrease which was only ~1% of the OD increase observed after a full bleach in a normal experiment. This was also seen in control experiments that contained no arsenazo III and in experiments using sonicated disks that were prepared without calcium.

Specificity Studies. The specificity of the light-induced release was studied in flow system experiments where the disks were sonicated with radioactive substances other than calcium and in double-label experiments where the sonication suspension contained both ^{45}Ca and another radioactive substance. The latter type of experiment permits the simultaneous measurement of the release of both ^{45}Ca and the second substance, as shown in Figure 5. In these experiments definite light-induced releases have been seen with ^{86}Rb, ^{22}Na, [^{32}P]phosphate, [^{3}H]glucose, and [^{3}H]sucrose in addition to ^{45}Ca (Figure 6). In experiments with [^{3}H]inulin, only very small light-induced releases were measured (Figure 5).

Both single- and double-label experiments with ^{86}Rb gave extremely low Rb releases (less than 0.1 mol of Rb released per mol of rhodopsin) even though most of the experiments at 25 °C had more than 0.5 mol of Rb per mol of rhodopsin remaining at the end of the experiment. The double-label experiments showed that the calcium release mechanism was functioning even under conditions where essentially no Rb release was observed.

In contrast to ^{86}Rb, all experiments with ^{22}Na showed a definite light-induced release which in some instances was almost as high as that normally seen for calcium.

Single-label experiments with [^{32}P]phosphate showed a definite light-activated release plus a particularly high retention at the end of the experiment. This indicates that the sonicated membranes either bind phosphate or are less permeable in the dark to phosphate than to the cations and sugars tested.

In double-label experiments there was a small but definite light-induced release of [^{3}H]glucose. The simultaneous calcium release was found to be smaller than that obtained in single-label experiments under the same conditions.

Both single- and double-label experiments with [^{3}H]sucrose gave definite light-induced releases. The simultaneous calcium release was found to be about the same as in single-label calcium experiments. A particularly large release of almost 2 sucrose/rhodopsin was measured in experiments where the

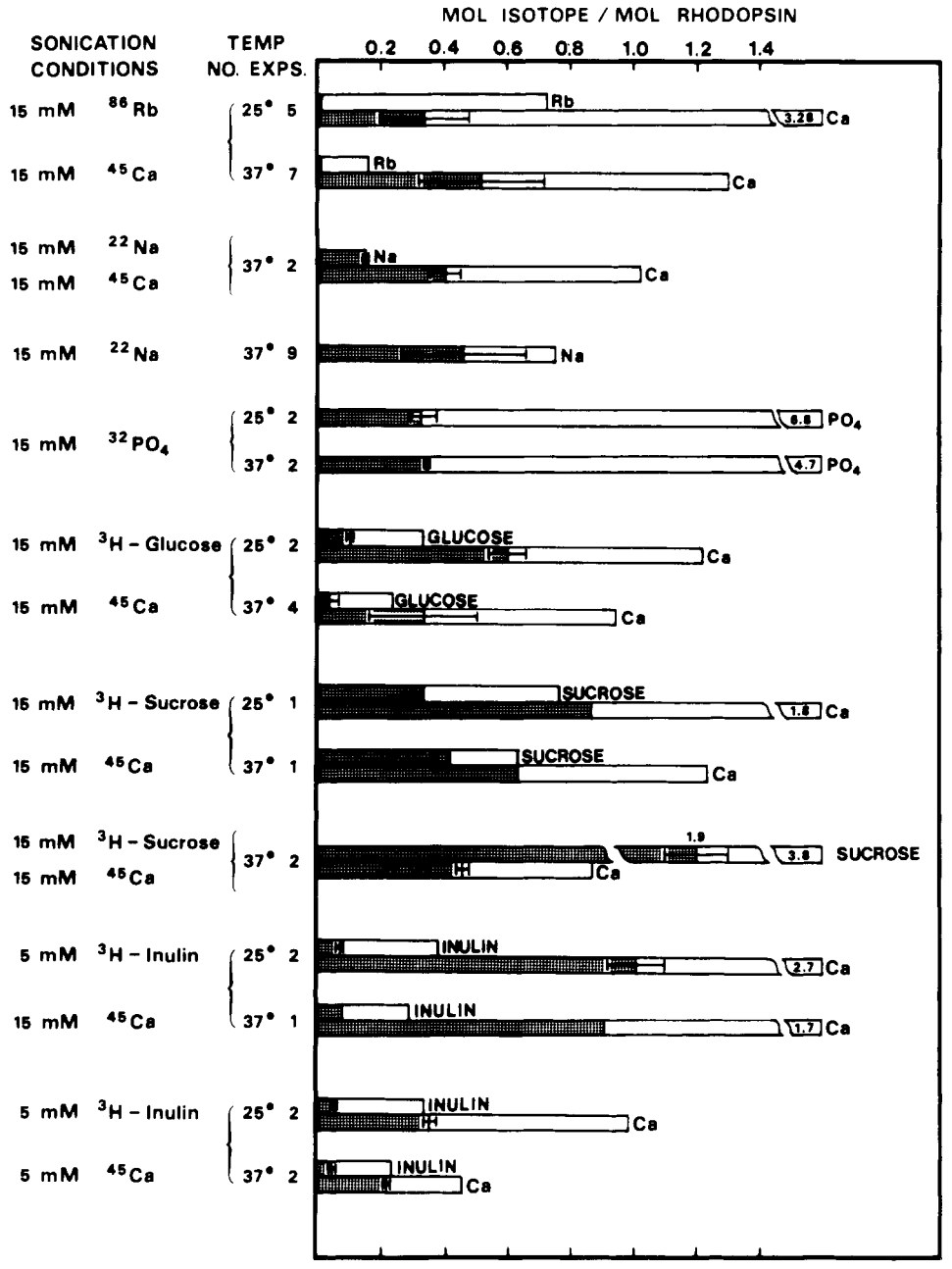

FIGURE 6: Light-induced release and total trapping of various substances in single- and double-label experiments. The shaded portion of each bar represents the mean magnitude of the light-activated release, normalized to the amount of rhodopsin in the sample. The inset error bars give the standard deviation ($n > 2$) or range ($n = 2$) of the data to indicate their reproducibility. The total height of each bar represents the mean amount of isotope trapped as estimated by the sum of the calcium remaining at the end of the experiment plus the light-induced release. All sonications contained 0.1 M imidazole–chloride buffer at pH 7.4 or 8.0 in addition to the indicated isotopes. The counterion for the cations was Cl, and for phosphate, K and Na. The wash buffer was 0.1 M imidazole–chloride, pH 7.4, for the Rb and Na experiments and 0.1 M imidazole–chloride with 0.1 M KCl, pH 8.0, for the other experiments. Approximately 95% of rhodopsin was bleached in each experiment. The small numbers in the broken bars indicate the magnitude of the trapping and/or release for experiments with data greater than the indicated abscissa.

disks were sonicated in 0.1 M [^{3}H]sucrose and 15 mM ^{45}Ca instead of the 15 mM concentration of both substances that was used for the other experiments. Here the calcium release was smaller than in the earlier experiments. This 6.7-fold increase in the sucrose concentration gave an average 4.5-fold increase in the sucrose release while reducing the calcium release by ~30%. Under these same conditions, reducing the percent bleaching from 95 to 25% caused the sucrose and calcium releases to be reduced by 24 and 23%, respectively.

In both double- and single-label experiments, very small but statistically significant releases of [^{3}H]inulin (M_r ~5000) were observed (Figure 5). Because of its limited solubility, the highest possible inulin concentration was 5 mM. Double-label experiments including [^{3}H]inulin were done with both 15 and 5 mM calcium. Going from 15 to 5 mM calcium reduced the magnitude of the calcium release by ~70%. Even with 5 mM calcium the magnitude of the calcium release was at least 4 times greater than that of the inulin release.

Discussion

The results clearly demonstrate that the permeability of the disk membranes for calcium as well as for some other substances increases after light absorption. The magnitude of this light-induced release increases with the amount of a substance trapped within the disk vesicles and with the amount of rhodopsin bleached. The magnitude of the measured light-induced

calcium release is similar in both the arsenazo III and flow system experiments. The arsenazo III experiments measure increases in external calcium resulting from net fluxes across the membrane less any binding to the membrane. The flow system experiments measure the rate of release as a function of time and, because the external calcium concentration is kept low, reflect unidirectional fluxes across the membrane.

We have found that raising the temperature from 25 to 37 °C and the pH from 7.4 to 8.0 increases the magnitude of the light-induced calcium release significantly over that previously reported (Smith et al., 1977; Mason et al., 1974); however, under the conditions which most closely approximate those of the earlier studies, pH 7.4 and 25 °C, we find in flow system experiments at both 25 and 95% bleaching lower releases than previously reported. This is most probably due to the fact that in the experiments reported in this paper lower levels of internal calcium were obtained which, therefore, produced a smaller calcium release. These lower levels of trapping could be due to the slightly different sonication conditions used or to differences in the permeability of the resulting vesicles to calcium in the dark. This may also explain why we found that the magnitude of the release per bleached rhodopsin varies within the percent bleaching whereas the earlier workers did not see such an effect. The reduction in the amount of calcium released per bleached rhodopsin which we see at the higher bleaching levels is at least partly caused by depletion of the internal calcium. Such a depletion effect is more significant in our current experiments than in the earlier studies where the observed releases removed a smaller fraction of the internal calcium.

Hagins & Yoshikami (1978) have provided evidence that upon light exposure several hundred transmitter molecules must be released into the rod outer segment cytoplasm in less than a second. Although the magnitude and rate of the calcium release which we measure from isolated disks are smaller than this, a direct comparison is not possible because of the relatively high bleaching levels used in our experiment. The release stoichiometry which we measure does increase as the bleaching level decreases, but we hestitate to extrapolate this trend to physiological levels of bleaching. Although our results are perhaps more consistent with a regulatory role than with an excitatory role for calcium, firm conclusions regarding the role of calcium in intact rod outer segments under physiological light levels are not warranted at this time.

We have interpreted our results in terms of calcium being transported across the membrane, an interpretation than is supported by the following evidence. In both arsenazo III and flow system experiments the disk vesicles can be treated with the calcium chelating resin, Chelex 100, without abolishing the release, and in the flow system the release is also obtained even after a lengthy wash with calcium-free buffer. If the released calcium came from the outside surface of the vesicles, both treatments would be expected to eliminate the release. Secondly, in both systems no light-induced release is seen from sonicated disks when the internal calcium is depleted before bleaching by treatment with the ionophore, A23187, indicating that the light-release mechanism and the ionophore draw upon the same internal calcium pool. Finally, in the flow system it has been reported that disks which had been sonicated in the absence of calcium, allowed to reseal, and later incubated with radioactive calcium gave no light-induced release (Smith et al., 1977). Similarly, intact disks which had been incubated with ^{45}Ca gave no light-induced release. The effect of light is, therefore, apparently to increase the permeability of the disk membrane for calcium and probably for the other substances as well. This confirms previous measurements of transmembrane transport by rhodopsin (O'Brien et al., 1977; Darszon et al., 1977; Hubbel et al., 1977) and is consistent with evidence that rhodopsin is a transmembrane protein (Fung & Hubbel, 1978). Our results are in apparent disagreement with those of Kaupp et al. (1979), who found a rapid light-induced release of calcium only from rod outer segments that had been treated with ionophore.

We found in both arsenazo III and flow system experiments that light usually causes only part of the trapped calcium to be released. This is shown by the strong dependence of the magnitude of the release upon the percent bleaching; small bleaches release only part of the calcium that can be released in experiments with larger bleaches. Also, the ionophore-induced release from the sample after bleaching in the arsenazo III experiments and the calcium remaining with the vesicles at the end of the flow system experiments show that even when most of the rhodopsin in a sample is bleached, a substantial amount of calcium remains trapped. These findings strongly indicate that the permeability increase must be transient.

The specificity studies show that the permeability change is relatively nonspecific but do not permit any final conclusions about the selectivity of the release mechanism among the various substances. In those experiments where the concentration of the released substance was 15 mM during the sonication, all of the experiments gave larger releases of calcium than of the other substances; however, the amounts of the other substances trapped at the time of bleaching were also lower than the amount of trapped calcium. This may reflect a selectivity in the trapping of the various substances during sonication, perhaps due to preferential binding of calcium to membrane sites, or a selectivity in the permeability of the membrane in the dark. The relative rates of release in the dark can be roughly estimated from the prebleach base line in the flow system experiments. Such estimates suggest that the latter factor is of primary importance for sodium and rubidium and that the first factor is of primary importance for the other substances tested. The permeability increase seems definitely to favor calcium over some substances such as rubidium, but with other substances such as sucrose the internal concentrations may be the controlling factor determining the relative amounts of release. There is some evidence for competition between calcium and the other substances used in the double-label experiments. In most of these experiments the calcium release is smaller than that seen in single-label calcium experiments under similar conditions. Also, in the sucrose–calcium double-label experiments, raising the sucrose concentration from 15 to 100 mM reduces the magnitude of the calcium release while increasing the sucrose release. The implication of such competition for the release mechanism is unclear. In the double-label experiments with [^{3}H]inulin and ^{45}Ca, only a small percentage of the trapped inulin is released while the percentage of the trapped calcium released is comparable to that found in experiments with only calcium. This confirms that the light-induced permeability changes reported in this paper are not caused by lysis of the vesicle membranes.

Comparable specificity studies have been done by others using reconstituted membranes formed from purified rhodopsin and lipids. In vesicles formed from such artificial membranes, light-induced permeability changes have been found for Na, Ca, Mn, Co, Cs, Eu, glycerol, and glucose but not for sucrose, inulin, or Cl (O'Brien et al., 1977; Darszon et al., 1977; Hubbel et al., 1977). The fact that we do find a light-induced release of sucrose probably reflects basic differences between the disk membranes we have used and the various reconstituted systems

used by others. Another important difference is that these workers find the permeability changes to be *permanent* (all of the trapped substance is released) whereas we find the permeability change to be *transient*.

In conclusion, our results clearly show that light induces a transient change in the permeability of the disk membrane and thereby causes a definite release of part of the trapped calcium from the interior of the disk vesicles. The amount of calcium released is determined primarily by the amount of rhodopsin bleached and by the amount of the calcium within the disk vesicles at the time of bleaching. Similar releases were obtained for substances other than calcium, indicating that the permeability change is relatively nonspecific.

Acknowledgments

We thank Dr. Henning Stieve and Dr. Hermann Kühn for their numerous suggestions and acknowledge the technical assistance of U. Kaysser.

References

Baylor, D. A., & Fuortes, M. G. F. (1970) *J. Physiol. (London) 207*, 77.
Cohen, A. I., Hall, I. A., & Ferrendelli, J. A. (1978) *J. Gen. Physiol. 71*, 595.
Darszon, A., Montal, M., & Zarco, J. (1977) *Biochem. Biophys. Res. Commun. 76*, 820.
Fung, B. K.-K., & Hubbel, W. L. (1978) *Biochemistry 17*, 4403.
Hagins, W. A. (1972) *Annu. Rev. Biophys. Bioeng. 1*, 131.
Hagins, W. A., & Yoshikami, S. (1974) *Exp. Eye Res. 18*, 299.
Hagins, W. A., & Yoshikami, S. (1977) in *Vertebrate Photoreception* (Barlow, H. B., & Fatt, P., Eds.) pp 97–139, Academic Press, New York.
Hemminki, K. (1975) *Vision Res. 15*, 69.
Hendriks, T., van Haard, P. M. M., Daemen, F. J. M., & Bonting, S. L. (1977) *Biochim. Biophys. Acta 467*, 175.
Hubbel, W., Fung, K.-K., Hong, K., & Chen, Y. S. (1977) in *Vertebrate Photoreception* (Barlow, H. B., & Fatt, P., Eds.) p 41 ff, Academic Press, New York.
Kaupp, U. B., & Junge, W. (1977) *FEBS Lett. 81*, 229.
Kaupp, U. B., Schnetkamp, P. P. M., & Junge, W. (1979) *Biochim. Biophys. Acta 552*, 390.
Kendrick, N. C. (1976) *Anal. Biochem. 76*, 487.
Mason, W. T., Fager, R. S., & Abrahamson, E. W. (1974) *Nature (London) 247*, 562.
O'Brien, D. F., Zumbulyadis, N., Michaels, F. M., & Ott, R. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5222.
Ostrovsky, M. A. (1978) in *Membrane Transport Processes* (Tosteson, D. C., Ovchinikov, Y. A., & Latorre, R., Eds.) Vol. 2, p 217 ff, Raven Press, New York.
Scarpa, A., Brinley, F. J., Tiffert, T., & Dubyak, G. R. (1978) *Ann. N.Y. Acad. Sci. 307*, 86.
Schnetkamp, P. P. M. (1979) *Biochim. Biophys. Acta* (in press).
Smith, H. G., Stubbs, G. W., & Litman, B. J. (1975) *Exp. Eye Res. 20*, 211.
Smith, H. G., Fager, R. S., & Litman, B. J. (1977) *Biochemistry 16*, 1399.
Yoshikami, S., & Hagins, W. A. (1973) in *Biochemistry and Physiology of Visual Pigments* (Langer, H., Ed.) p 245 ff, Springer-Verlag, New York.

Energy Transfer among the Chromophores in Phycocyanins Measured by Picosecond Kinetics†

T. Kobayashi, E. O. Degenkolb,‡ R. Bersohn,§ P. M. Rentzepis,* R. MacColl, and D. S. Berns

ABSTRACT: Energy-transfer processes in the algal light-harvesting proteins, the phycocyanins, have been studied by means of picosecond absorption spectroscopy. After excitation at 530 nm, the absorption at several wavelengths in the range 480–669 nm decayed with a short time constant (picosecond) and a long time constant (>1 ns). For *C*-phycocyanin, energy transfer from the β to the α subunits is interpreted as being a likely candidate for the short time constant; the long time constant probably is the excitation lifetime of the chromophore on the α subunits. The time constants for energy transfer in monomers, trimers, and hexamers of *C*-phycocyanin extracted from a blue-green alga, *Phormidium luridum*, were measured as ~85, ~56, and ~32 ps, respectively. The corresponding time constant in the cryptomonad phycocyanin 645 from *Chroomonas* species was found to be less than 8 ps.

Algae are assigned according to their color and morphology to 15 classes, e.g., cyanophyta (blue-green algae), rhodophyta (red algae), cryptophyta (cryptomonads), chlorophyta (green algae), etc. The first three classes are of special interest because in addition to chlorophylls and carotenoids, they contain an array of accessory pigments called phycobiliproteins whose function is to absorb visible light and transfer energy stepwise until it eventually reaches chlorophyll *a*. The accessory pigments, phycocyanins, allophycocyanins, and phycoerythrins, consist of tetrapyrrole chromophores covalently linked to a protein (Troxler, 1975; Bogorad, 1975). Direct observation of the energy-transfer process between two or more different kinds of phycobiliproteins on the time scale of several tens of hundreds of picoseconds has been studied recently by Porter et al. (1978) for intact algae and also for the isolated light-harvesting complex by Searle et al. (1978). Fluorescence picosecond spectroscopy was applied to the energy transfer between different kinds of phycobiliprotein in these studies.

† From Bell Laboratories, Murray Hill, New Jersey 07974 (T.K., E.O.D., R.B., and P.M.R.), and Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201 (R.M. and D.S.B.). *Received February 16, 1979.*
‡ Present address: GTE Laboratories, Consumer Products Division, Waltham, MA 02154.
§ Present address: Department of Chemistry, Columbia University, New York, NY 10027.

0006-2960/79/0418-5073$01.00/0