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KINETIC STUDIES OF PHOTOTRANSIENTS IN BACTERIORHODOPSIN

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SUMMARY

Aqueous suspensions of bacteriorhodopsin in purple membrane fragments from *Halobacterium halobium* have been subjected to microsecond flash photometry utilizing both unpolarized and polarized light. Depletion of the ground state chromophore centered at 570 nm is accompanied by the formation of transients absorbing maximally at 410 nm and 660 nm with rise times of about 0.4 and 6 ms, respectively. Decay of both transients and reformation of the ground state chromophore occurs with identical first-order kinetics with a half life of about 6 ms. All three chromophores are polarized with dichroic ratios which remain constant throughout the transient lifetimes, indicating that Brownian rotation of the chromophore within the membrane is considerably restricted. Whereas agents which induce permeability of membranes to protons (2,4-dinitrophenol, carbonylcyanide-*m*-chlorophenylhydrazone) and non-specific univalent cations (gramicidin) or inhibit ATPase (ouabain) had no influence, the K^+-specific ionophore valinomycin in the presence of K^+ inhibited and quenched the formation of the 660 nm transient with concomitant increase in lifetime of the 410 nm transient and delay in recovery of the 570 nm chromophore. High concentrations of Na^+ produced an effect similar to that of valinomycin. The relationship of these data to the mechanism of the proton pump in the intact bacterium is discussed, with the conclusion that the 410 nm transient performs a key role.

INTRODUCTION

When grown under low oxygen tension in the light, *Halobacterium halobium* produces in its cell membrane a single rhodopsin-like protein, bacteriorhodopsin, possessing a broad absorption band with a maximum at about 570 nm [1]. Light energy absorbed by this chromophore is utilized by the organism to pump protons transversely across the membrane (to the outside), and the electrochemical energy associated with the resulting electrical and pH gradients is utilized in the phosphorylation of ADP to ATP [2–5]. When harvested and resuspended in aqueous solution with low salt concentration the cell membrane is fragmented. One of these fragments the so-called "purple membrane", can be isolated and has been shown to contain the

characteristic colour of the anaerobic bacterium and to be the site of the light-driven proton pump [1, 4]. Preliminary experiments undertaken in this laboratory with aqueous suspensions of purple membrane at room temperature using the modulation excitation technique identified two major transient species absorbing at 410 nm and 660 nm, which were concluded to be associated with the proton pump in *H. halobium* [6]. In the present work we confirm the existence of the transients at essentially physiological conditions using conventional microsecond flash photometry, and make a more detailed study of the kinetics. Additionally, we have used a procedure of producing transients with plane-polarized light pulses ("polarization flash photometry"). The purpose of this is to relate the dichroism of these chromophores to the resistance to rotation of the transient species which they represent. The influence of agents which act by inducing permeability in membranes towards protons (2,4-dinitrophenol, carbonylcyanide-*m*-chlorophenylhydrazone (CCCP)) and alkali metal ions (valinomycin, gramicidin), as well as the influence of an ATPase inhibitor (ouabain) on transient kinetics was also investigated.

MATERIALS AND METHODS

For all experiments bacteriorhodopsin in purple membrane fragments from *H. halobium* R_1 or M_1 strains [4] was used. The cells were concentrated at $8000 \times g$ and resuspended in basal salt containing DNAase. The mixture was then dialysed against water overnight and subsequently centrifuged at $50\,000 \times g$. The purple pellet thus obtained was resuspended in water and the centrifugation and resuspension repeated three times. Finally, the pellet was fractionated on a 25–50 % sucrose gradient and the purple band repeatedly washed with water, centrifuged, and finally suspended in 30 mM KCl in the absence of buffer at neutral pH. Inhibitory agents valinomycin (Sigma), ouabain (Sigma), and gramicidin (kindly supplied by Professor M. Avron) were added as concentrated enthanolic solutions such that the final concentration of ethanol in the solution to be photolyzed never exceeded 1 % v/v. Dinitrophenol (BDH) and CCCP (Sigma) were added as concentrated aqueous solutions.

A conventional flash photolysis system was used. The air-filled flash tubes operated at 20 kV and gave a flash profile with a half-width of about 5 μs and a total light energy of about 400 J. The continuous monitoring source was an Osram 100 W tungsten-quartz-iodine lamp operated from a stabilized 0–12 V d.c. power supply. The light transmitted by the sample contained in Pyrex cells (path lengths ranging from 2–8.2 cm) was detected by an EMI 9558 photomultiplier after passage through a Bausch and Lomb 500-mm monochrometer, and recorded with a Tektronix 564 B oscilloscope equipped with camera attachment. The earliest events which could be precisely measured were limited to those occurring after about 50 μs by the fatigue characteristics of the photomultiplier. For most studies, Corning 3486 cutoff filters (0 % T below 510 nm, 50 % T at 530 nm) were interposed between the flash tubes and the sample. Under these conditions excitation of the 570 nm chromophore was isolated. In studies aimed at investigating the growing in of the 570 nm depletion, photomultiplier fatigue was minimized by interposing a Schott GG4 400 nm cut-off between cell and monochrometer and flashing through Corning 9863 filters. These, together with the Pyrex sample cell, exposed the sample to radiation from 290–400 nm

and minimized the scattered light from the flash reaching the photomultiplier.

In polarization studies Polaroid type HNP'B ultraviolet polarizing filters were used. One polarizer was placed between the flash lamps and the cell, the other between the monitor lamp and the cell. The possibility of short term reversible bleaching of the polarizers was resolved by observing the transients from a sample flashed through a pair of crossed polarizers. Under these conditions the yield of transients at 410 nm and 570 nm was 3.5 % of that observed with parallel polarizers between the flash and sample.

During the course of the present study no attempt was made to maintain a constant temperature in the sample being flash-photolyzed. Ambient room temperature during the course of the work was 15–20 °C.

Purple membrane concentrations were calculated from optical density measurements at 570 nm assuming an extinction coefficient at this wavelength $\varepsilon_{570} = 5.4 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ [1].

RESULTS AND DISCUSSION

Flash photometry at room temperature of aqueous suspensions of purple membrane fragments produces temporary bleaching of the major chromophore of bacteriorhodopsin centered at 570 nm together with formation of new absorption bands with maxima at 410 nm and 660 nm (Fig. 1). The appearance and decay profiles at these maxima are shown in Fig. 2. (No permanent bleaching of the 570 nm chromophore was observed as a consequence of flash photolysis during the course of this work.) In the absence of additives decay of the chromophores centered at 410 nm and 660 nm (hereafter referred to as 410-transient and 660-transient) and recovery of the 570 nm ground state chromophore followed good first-order kinetics (Fig. 3) although some variation in half-life was noted from sample to sample. (It will be

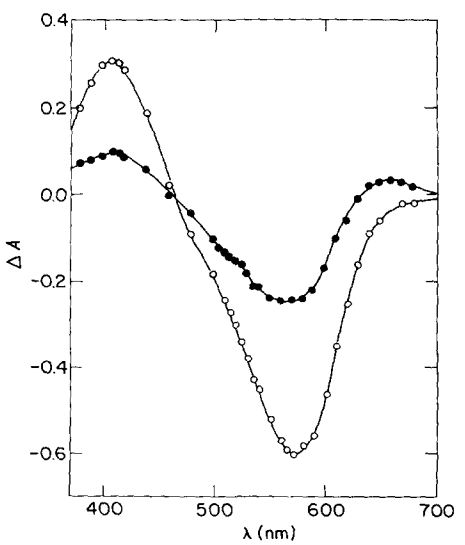

Fig. 1. Transient spectrum of flash-photolyzed aqueous suspension of purple membrane at ro temperature after 0.5 ms (○—○) and 10 ms (●—●).

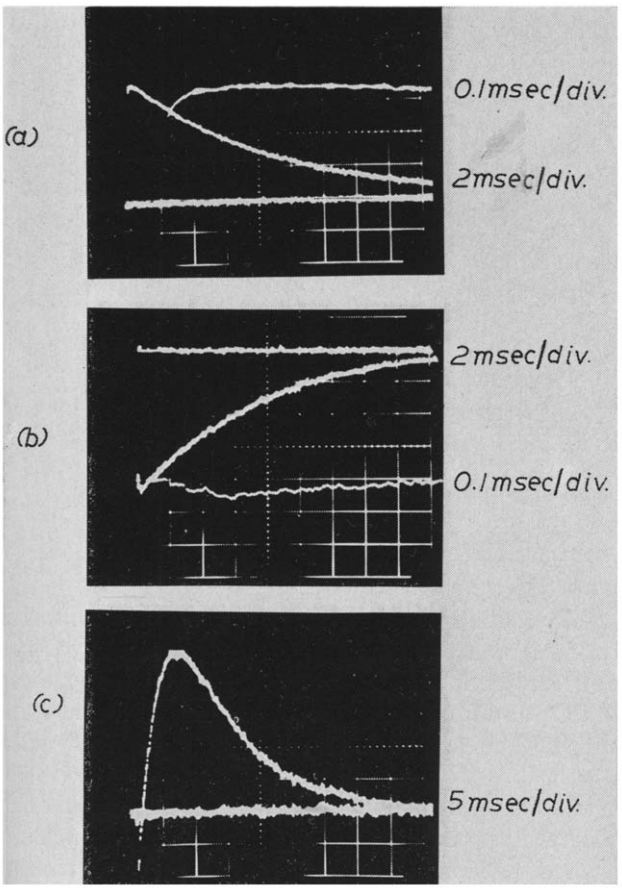

Fig. 2. Appearance and decay profiles of transients in flash-photolyzed aqueous suspensions of purple membrane at room temperature. Percent transmission increases on going from top to bottom. (a), appearance (time base 0.1 ms/div) and decay (2 ms/div) at 410 nm; (b), appearance (0.1 ms/div) and decay (2 ms/div) of depletion at 570 nm; (c), appearance and decay at 660 nm (5 ms/div).

shown elsewhere [7] that these variations are attributable primarily to the influence of variations in room temperature on the unthermostated samples. In particular, the 660-transient yield is temperature-sensitive, becoming undetectable below 0 °C.) In general, the half-lives of all three chromophores were essentially identical for an individual sample (mean value 6 ms). The relative changes in optical density (as compared with bacteriorhodopsin in its ground state) at times of maximum development of the 410- and 660-transients and the 570-depletion were 0.5 : 0.08 : 1, respectively. Grow-in times of the 410-transient and 570-depletion were also essentially the same (0.5 ms), while that for the 660-transient was an order of magnitude larger (6 ms).

Deaeration by argon bubbling had no significant effect on transient characteristics. However, rigorous freeze-thaw degassing reduced the grow-in times at 410 nm and 570 nm to below that resolvable (0.1 ms). The grow-in phenomenon did not reappear immediately after opening the cell or bubbling O_2, but did return on

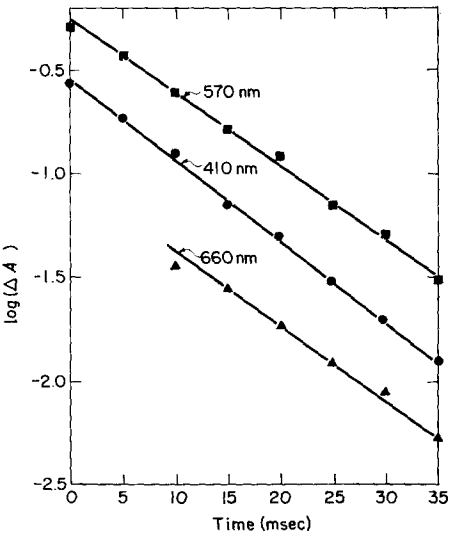

Fig. 3. First-order plots of transient decay data of Fig. 2.

standing in air overnight. Evidently the situation of the bacteriorhodopsin within th purple membrane is such that considerable time is required for O_2 or possibly CO_2 t penetrate to the site responsible for producing light transients (likewise significar time must elapse for the O_2 or CO_2 to diffuse away from this site as indicated by th ineffectiveness of argon-bubbling). The reasons for these phenomena are at prese unclear.

Small concentrations of ethanol or methanol (< 1 M) had no effect on the lig response, but the presence of diethyl ether resulted in irreversible light bleaching of t 570 nm chromophore and increased the lifetimes of both the 410- and 660-transien In the presence of a sodium chloride concentration approaching that of basal s solution (3 M) the 660-transient was not observed while the lifetimes of the 41 transient and the 570-depletion were increased to 77 ms and 105 ms, respectively. T presence of potassium chloride (up to saturation), however, had no effect on yields lifetimes.

In previous work on light-induced transients reported from other laborator [2, 3, 5] only the 410-transient (variously reported as having a maximum at 412 1 or 415 nm) was observed. These studies utilized low temperature and/or orga solvents in the presence of high salt concentrations in order to extend the lifetime the transient. It is these conditions of high salt concentration and/or low temperat which probably led to the failure to observe the 660-transient previously found our laboratory [6] and confirmed in this present work. The possibility that the 6 transient may be an artifact arising from our procedure for isolating the purple m brane is excluded by our observation of both the 410- and 660-transients in fl photolysis experiments with suspensions of intact bacteria in basal salt solution [7

The formation and decay times of the 410-transient presented here are c sistent with the laser-flash spectra results of Stoeckenius and Lozier [8]. Howe their spectra obtained at between -90 to -196 °C do not reveal any species in 660 nm region with a millisecond lifetime. This clearly is a consequence of t

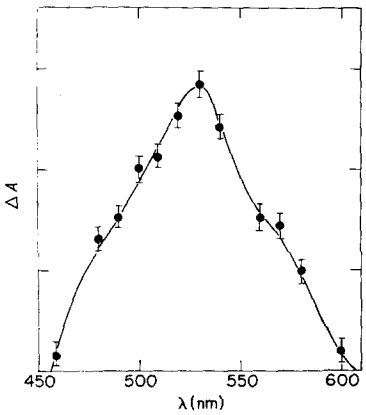

Fig. 4. Spectrum of grow-in of the 570 nm depletion band. The ordinate represents differences between optical densities at 0.1 ms and 0.4 ms.

working outside the physiological temperature range of *H. halobium* since we find [7] that the 660-transient yield drops with decreasing temperature and becomes insignificant even at 0 °C.

The grow-in times of the 410- and 660-transients clearly show that neither of these absorptions represent primary photolysis products. The relatively long grow-in time of the 570-depletion at first seems anomalous since excitation of the 570 nm chromophore must be the primary photochemical event. A clue to the explanation of this apparent anomaly comes from modulation excitation work [6] in which a partly-resolved short-lived (< 1 ms) absorption centered around 510 nm was observed. If this 510-transient is relatively broad and extends into the region of the 570 nm chromophore, then the presence of the former would detract from the 570-depletion and its decay would appear as an increase in the intensity of depletion in the 570 nm band. This speculation is supported in the present study by the data presented in Fig. 4, in which differences in optical density at 0.4 ms and 0.1 ms are plotted against wavelength. The data presented in this manner show the presence of an absorbing species with a maximum at about 530 nm and lifetime of less than 1 ms. The identity of the grow-in times of the 570-depletion and the 410-transient point to this 530-transient being the precursor of the 410-transient. A broad absorption with maximum at 550 nm and half-life of 40 μs observed by Stoeckenius and Lozier [8] at room temperature seems to be consistent with our 530-transient.

Flash photometry with plane-polarized light showed the transient chromophores at 410, 570, and 660 nm to be polarized, consistent with the modulation excitation work except that the two 410-transients possessing different phases could not be distinguished. Dichroic ratios, $R = A_{\parallel}/A_{\perp}$, presented in Table I (second column), were found to remain constant over the observable lifetime of transients (Fig. 5 shows superimposed decays of the 410- and 660-transients and reappearance of 570 nm chromophore with polarizers on the flash tubes and analysing light parallel and perpendicular*). The lower values reported here are a result of longer light paths

* In a brief publication, Chapman and coworkers have similarly noted time-independent dichroism in the 410-transient and the 570-depletion [9].

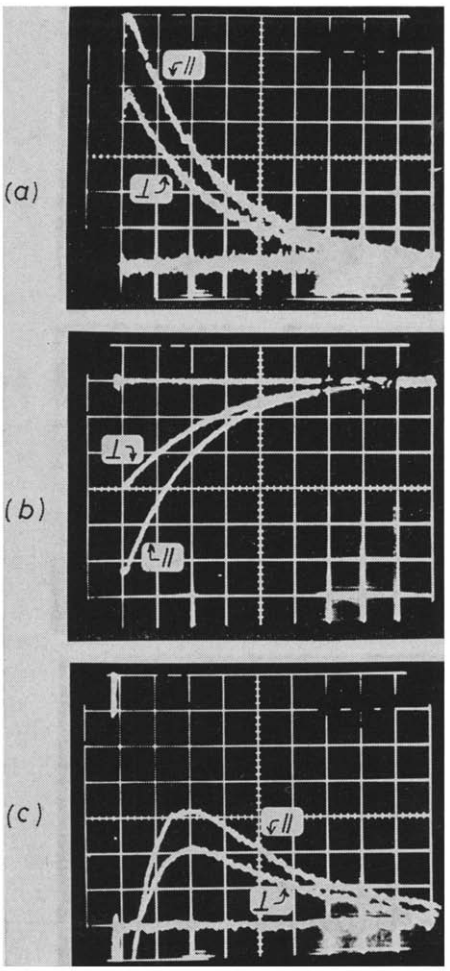

Fig. 5. Decay of transient produced with plane polarized light.

TABLE I

DICHROISM IN FLASH PHOTOLYSIS OF PURPLE MEMBRANE SUSPENSIONS, ALONE AND IN THE PRESENCE OF VALINOMYCIN[a]

λ(nm)	R[b] valinomycin absent	R[c] valinomycin present
410	1.4	1.5
570	1.5	1.6
660	1.4	–[d]

[a] PM and valinomycin concentrations, respectively, $6.5 \cdot 10^{-6}$ M and $2.0 \cdot 10^{-5}$ M.

[b] $R = A_{\parallel}/A_{\perp}$. Values constant ($\pm 10\,\%$) throughout detectable transient lifetime.

[c] Initial dichroic ratios. A small ($\sim 20\,\%$) decrease in R values was detectable at the end of th transient lifetime (> 30 ms).

[d] 660-transient undetectable.

used in our flash photometry configuration and the resultant increased scattering losses. The longer decay times reported in the previous work are a manifestation of a temperature effect which will be discussed in detail elsewhere [7]. Polarization flash photometry may be considered analogous to the well-studied fluorescence polarization technique [10, 22] in which in the absence of intermolecular electronic energy transfer, time dependent depolarization of emission (here absorption) of a transient chromophore is a measure of rate of Brownian rotation of the transient. Rate of rotation depends on both the dimensions of the species containing the chromophore and the viscosity of the medium. In the system presently under investigation rotation during the lifetime of the transient consists of two parts: rotation of the bacteriorhodopsin molecule within the purple membrane fragment, and overall rotation of the fragment suspended in the aqueous medium. Purple membrane fragments appear to consist of oval plates, about 0.5 μm in diameter and 50 Å thick with the protein molecule embedded in the membrane lipid having a dimension of about 30 Å [11, 12]. The rotational relaxation time, τ, of a prolate sphere may be expressed by Perrin's formula [13]:

$$\tau = \eta 2\pi r^3/3kT$$

where η is the viscosity and r the small axis. The rotational relaxation time for purple membrane fragments in water at room temperature ($\eta = 1.0$ centipoise) is therefore 63 ms. This exceeds the transient lifetime, and hence the estimates of purple membrane size are consistent with our inability to observe any decrease in dichroic ratio during transient lifetimes. If the estimates [11, 12] of the dimension of the bacteriorhodopsin molecule are also accepted, then since the relaxation lifetime exceeds that of the transient we are led to the conclusion that if rotation is limited by the viscosity of the lipid of the purple membrane, then this viscosity has a lower limit of $7 \cdot 10^3$ poise. This value is completely inconsistent with that determined for membrane lipids extracted from the related halophile, *H. cutirubrum*, by the fluorescent probe technique [14], and therefore suggests that rotation of the bacteriorhodopsin molecule is restricted by some form of binding (e.g. hydrogen bonds) or by its location within some directional cavity within the lipid structure. Formal covalent bonding to membrane lipid would seem to be ruled out by the observation [1] that detergents are able to separate the lipid from the protein molecule and expose the Schiff base to chemical attack.

The values observed for transient dichroic ratios are significantly below the theoretical maximum of 3.0 [10, 15]. This might have been taken to reflect rotation of the molecule, but the probable prime reason for this is polarization loss resulting from the considerable light scattering present in the purple membrane suspension. Cone [16] working with rhodopsin immobilized by glutaraldehyde has similarly observed dichroic ratios significantly below the theoretical maximum. It is interesting to note that although many similarities exist between bacteriorhodopsin and the visual pigment (amino acid composition, retinal prosthetic group attached by Schiff base linkage, molecular weight), the latter is characterized by a high degree of freedom to undergo Brownian rotation [16, 17]. Our present data show that quite the reverse is true for the chromophore in *H. halobium*. Recently, Chignell and Chignell have drawn the same conclusion based on a spin-label study of purple membrane [18].

In the absence of added potassium ions, valinomycin has only a small effect on

TABLE II

EFFECT OF VALINOMYCIN ON TRANSIENT YIELDS AND LIFETIMES IN FLASH-PHOTOLYZED AQUEOUS SUSPENSIONS OF PURPLE MEMBRANE (PM)[a]

Expt. No.[b]	PM, μM	valinomycin, μM	410-transient		660-transient	
			Yield[c]	$t_{\frac{1}{2}}$, ms[d]	Yield[c]	$t_{\frac{1}{2}}$, ms
2	3.7	0[e]	1.00	6.5	1.00	7.2
2	3.7	> 10[e, f]	1.05	10, 16	0.54	7.5
2	3.7	> 10[f]	–	48	0.00	–
13	3.3	0	1.00	4.0	1.00	5.0
13	3.3	0.01	1.00	4.4	0.96	4.0
13	3.3	0.1	1.03	5.0, 13	0.88	5.9
13	3.3	1.0	1.17	10, 17	0.23	$\approx$ 5.0
13	3.3	10	1.20	15, 21	0.01	–
29	22	0	1.00	5.6	1.00	6.3
29	22	0.1	1.00	5.2	1.00	6.3
29	22	0.4	1.05	5.9, 10	0.91	5.0
29	22	1.0	1.05	7.2, 17	0.76	5.6
29	22	5.0	1.15	12, 26	0.33	–
29	22	10	1.15	20, 36	0.03	–
29	22	20	1.15	16, 38	0.06	–
29	22	40	1.15	16, 38	–	–
29	22	40[g]	–	27, 87	0.00	–

[a] All solutions contain 30 mM KCl unless otherwise stated.

[b] Identifies the stock solution to which aliquots of valinomycin were added.

[c] Maximum optical density of transient relative to that of valinomycin-free stock solution.

[d] Valinomycin-containing solutions show more rapid initial decay. First-order plots may be separated into two portions with different apparent half-lives.

[e] KCl absent.

[f] Saturated valinomycin.

[g] On standing for 5 days under refrigeration.

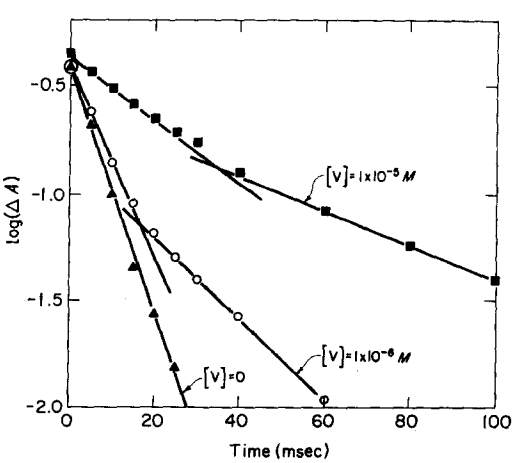

Fig. 6. Examples of first-order plots for decay of 410-transient in presence of valinomycin.

the major transients. In the presence of 30 mM K^+, the lifetime of the 410-transient increases progressively with increasing valinomycin concentration while the 660-transient intensity (but not its lifetime) is decreased (Table II). Concomitantly there is a slight, but significant, increase in the relative maximum intensity of the 410-transient. The data also show that sensitivity to valinomycin depends on the ratio of its concentration to that of bacteriorhodopsin. A non-specific metal cation ionophore (gramicidin) at similar concentrations produced no detectable effect, nor did similar concentrations of proton carriers (dinitrophenol, CCCP) nor the ATPase inhibitor ouabain. Decay kinetics at the higher valinomycin concentrations may be fitted to two first order plots (Fig. 6); at intermediate concentrations the initial portion has the same half life as that of purple membrane suspension in the absence of this agent. As in valinomycin-free solutions the reappearance kinetics of the 570 nm chromophore parallelled that for the decay of the 410-transient.

Valinomycin has been shown to profoundly influence the light-stimulated proton gradient produced in intact cells and the accompanying ATP synthesis [19]. Light-driven ATP synthesis or photophosphorylation is thought to be coupled energetically through the setting up of a transverse proton gradient across the cell wall as in the case of oxidative phosphorylation during respiration [20]. Valinomycin has the capacity to solubilize potassium ions in hydrophobic media and hence transport them across membranes. Halophilic bacteria are characterized by a very high internal potassium ion concentration [21], and permeability to these ions produced by valinomycin permits an outflux which affects the electrochemical potential gradient driving protons inward through the phosphorylation pathway. Penetration of purple membrane in the present work by valinomycin-K^+ complexes may be expected to produce crowding and thereby stiffening of the lipid environment of the protein chromophore. The observation that valinomycin retards the decay of the 410-species to the 570 nm ground state is consistent with involvement of either (i) lateral movement of a species (this could be hydrogen ions in some form) through the membrane, or (ii) internal motion (e.g. conformational change) within the protein chromophore. The present experiments do not discriminate between these two possibilities. Polarization data with valinomycin solutions (Table II, last column) yield dichroic ratios which are not significantly larger than those of valinomycin-free solutions. Since bacteriorhodopsin is already immobilized in the absence of valinomycin, any further membrane stiffening effect would have no influence on dichroism.

The similarity between the decay kinetics of the 410-transient and that of the regeneration of the 570 nm chromophore, both in the absence and presence of valinomycin, strongly points to the former being a last step in the deexcitation process leading back to the ground state represented by the 570 nm chromophore. The role of the 660-transient in the proton-pumping apparatus of *H. halobium*, however, is less clear. As pointed out previously [6] these two intermediates are linked kinetically, and we have now shown that they possess identical depolarization properties. The presence of valinomycin, however, clearly distinguishes the two intermediates. While the yield of the 660-transient is sensitive to valinomycin, its kinetics are not. In view of the small but distinct increase in 410-transient yield accompanying inhibition of the 660-transient, it is tempting to speculate that these two intermediates occupy parallel pathways, and blocking of the pathway to one by the presence of valinomycin leads to an increase in the yield of the other. The lack of correlation

between the recovery of the 570 nm chromophore and the decay of the 660-transien at low valinomycin concentration suggests that the 410-transient occupies the majo pathway in the spontaneous de-excitation of light-stimulated bacteriorhodopsin.

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