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ESR studies of light-dependent volume changes in cell envelope vesicles from *Halobacterium halobium*

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Volume changes in illuminated cell envelope vesicles, prepared from various *Halobacterium halobium* strains, were measured with an ESR method. We demonstrated light-dependent swelling of vesicles which contained halorhodopsin (an inward-directed light-driven chloride pump), and shrinking of vesicles which contained bacteriorhodopsin (an outward-directed light-driven proton pump coupled to a proton/sodium antiporter). The swelling of the halorhodopsin vesicles was not inhibited by uncouplers or gramicidin, but the shrinking of the bacteriorhodopsin-vesicles was abolished by these ionophores. These findings confirm earlier models for ion circulation in these systems. Vesicles from strains which contained both pigments showed relatively small net volume changes upon illumination. A scheme of ionic transport in *H. halobium* cells is suggested, in which the inward movement of K^+ exceeds the outward movement of Na^+, and the difference equals the Cl^- uptake, so as to provide the net gain of KCl necessary for volume increases during cell growth.

Introduction

The cytoplasmic membrane of many *Halobacterium halobium* strains contains two different retinal proteins, which function as light-driven electrogenic transport systems: bacteriorhodopsin, an outward-directed proton pump [1], and halorhodopsin, an inward-directed chloride pump [2]. In wild-type strains bacteriorhodopsin is present in many-fold excess over halorhodopsin, and the protonmotive force, generated mostly by the proton pump upon illumination, is coupled so sodium ion extrusion via an electrogenic proton/sodium antiport system [3–5]. Uncoupler-sensitive rapid Na^+ efflux from illuminated cell envelope vesicles and whole cells via this mechanism was demonstrated directly with ^{22}Na measurements [3,4], atomic absorption determinations [5], and inferred from sodium-dependent amino-acid transport [6] as well as volume decreases monitored with light-scattering [7,8]. This is not the case with cells which contain only halorhodopsin. Cell envelope vesicles prepared from bacteriorhodopsin-deficient strains take up Na^+ upon illumination, but by a passive mechanism in response to the membrane potential created by chloride import through halorhodopsin. This Na^+ uptake was determined from volume increase followed with the light-scattering method [8], and found to be enhanced by gramicidin rather than inhibited, as expected for passive transport. Since all of the transport measurements used are subject to inaccuracies, and the light-scattering measurements can be used to calculate only relative volume changes, we sought to confirm the

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Abbreviations used: TEMPONE, 4-oxo-2,2,6,6-tetramethylpiperidine-*N*-oxyl; TEMPAMINE, 4-amino-2,2,6,6-tetramethylpiperidine-*N*-oxyl.

proposed models for ionic transport in *H. halobium* by repeating and quantitating the volume change measurements by an independent method. In the course of this work we examined also vesicles from strains which contain both pigments, since these two systems drive Na^+ transport in opposite directions, and we expected that the fluxes might cancel each other.

Uncharged spin probes, such as 4-oxo-2,2,6,6,-tetramethylpiperidine-*N*-oxyl (TEMPONE), partition freely between the two aqueous compartments, external and internal, of a vesicle system. The ESR signal can be used to detrmine the size of the internal compartment by quenching the signal from the external compartment with a membrane-impermeable paramagnetic line-broadening agent, since the magnitude of the residual signal is directly related to the volume of the vesicles [9–11]. In this study we used this ESR method to quantitate the volume of *H. halobium* vesicles and their changes.

Materials and Methods

H. halobium was grown and cell envelope vesicles were prepared as previously described [12]. Vesicles largely free of halo-opsin, but containing bacterio-opsin, were prepared from strain JW-5, a retinal-deficient strain. In vitro reconstitution of these vesicles with retinal [8] produced membranes with virtually only bacteriorhodopsin activity (bacteriorhodopsin vesicles). Vesicles free of bacteriorhodopsin, but containing halorhodopsin, were prepared directly from strains L-33 or OD-2, which are genetically defective in bacteriorhodopsin. Vesicles containing both retinal pigments were prepared from strain R-1. Vesicles from strain S-9 apparently contain bacteriorhodopsin, but little halorhodopsin.

ESR experiments were performed on a Varian E109E instrument, operating at a frequency of 9.53 GHz at room temperature. To avoid problems associated with a slow light-dependent decrease of the spin probe signal in the vesicles, volumes were determined in the dark after defined periods of illumination. Illumination of vesicles was in 0.6 ml test-tubes, at a protein concentration of 10 mg/ml, unless otherwise mentioned, with vigorous magnetic stirring to ensure uniform delivery of light. The light source was a 1000 W He arc lamp (Oriel Corp.), which provided sufficient illumination for saturating light responses, and was equipped with neutral density and 530 nm low-pass filters. The temperature of the samples was maintained at 22°C with a large water bath. At appropriate intervals, as indicated in the figures, 45 µl aliquots of the vesicle suspensions were withdrawn, 0.5 µl TEMPONE spin label and 5 µl paramagnetic broadening agent (sodium ferricyanide, 1 M) were added, and the samples were placed in 75 µl capacity glass capillaries for determination of the ESR signal intensity. In a similar way, light-induced pH differences between vesicle interior and exterior were determined with the spin probe 4-amino-2,2,6,6-tetramethyl piperidine-*N*-oxyl (TEMPAMINE), but on the basis of the partitioning of the probe in the two compartment according to the pH gradient [13].

The halorhodopsin content of OD-2 vesicles was determined by illuminating a suspension of these vesicles with yellow light in 4 M NaCl, pH adjusted to 9.5, as described before [14]. This sample, in which the halorhodopsin Schiff-base is deprotonated to give a 410 nm absorbing species [14], was used as a reference against a similar but unbleached sample. The halorhodopsin content was calculated from the difference spectrum obtained in this way, using 50000 $M^{-1} \cdot cm^{-1}$ as extinction coefficient at 580 nm [15].

Results

Reversibility of osmotic volume changes in vesicles

In order to test the ability of the cell envelope vesicles to swell and shrink as ideal osmometers in response to changing osmolarity, and to assess the accuracy and reliability of the ESR method for the halobacterial system, the vesicles were exposed to various NaCl concentrations expected to produce predictable osmotic volume changes. The vesicles were treated in one of two different ways: (1) they were equilibrated for several hours with 2 M NaCl and then exposed to higher NaCl concentrations for expected shrinking, and (2) they were equilibrated first with 2 M NaCl, then centrifuged and reequilibrated with 4 M NaCl (pre-shrinking), after which they were exposed to lower NaCl concentrations, for expected swelling. To avoid undue

volume decreases of the vesicle suspensions when the salt concentrations were adjusted, the added salt solutions were 5 M in NaCl for (1) and 1 M for (2). The volumes of L-33 vesicles exposed to different NaCl concentrations under these conditions are shown in Fig. 1. (left side) contains ESR traces at various osmolarities to demonstrate that the raw data obtained with single samples is accurate enough to allow volume determinations without statistical analysis. The ESR line heights obtained at the different NaCl concentrations are shown in Fig. 1 (right side). It is apparent that the ESR parameter (and thus the volumes) varied smoothly with the reciprocal of the molarity of the sodium chloride. At high osmotic strength a nearly ideal (linear) relationship is obtained, which extrapolates to zero volume at infinite osmotic strength, as expected for an ideal osmometer. Also as expected, the vesicles equilibrated with 2 M NaCl had higher volumes than those equilibrated with 4 M NaCl, but shrunk more at high salt concentrations. Reduction of the final salt concentration to below 2 M NaCl caused net volume losses, corresponding to the deviation from linearity in Fig. 1. This non-ideal behavior is probably due to lysis and/or the disintegration of the vesicles at suboptimal salt concentration, a phenomenon previously described for the membranes of the halobacteria [16]. Similar osmotic responses to those in Fig. 1 were obtained with vesicle preparations from the other strains used in this study.

Volume changes during illumination of halorhodopsin vesicles

Light-induced, chloride-dependent swelling of halorhodopsin vesicles could be demonstrated by equilibrating them with chloride-free salt solutions and monitoring their volumes after adding increasing amounts of chloride salt and illumination, as described for the earlier light-scattering studies.

After overnight dialysis against 3 M potassium phosphate the vesicles had very small volumes (about 1% of their initial volumes in NaCl) prior to the illumination. This result suggests that the membranes are much less permeable to phosphate than to chloride. Indeed, phosphate appears to be quite impermeant, since we could measure a light-induced pH difference caused by passive proton uptake, driven by chloride transport, with TEMPAMINE, and it was of similar magnitude in phosphate-equilibrated vesicles as in control vesicles in NaCl (result not shown). If appreciable amounts of phosphate had entered the vesicles, buffering by internal phosphate would have substantially diminished any pH difference from proton uptake.

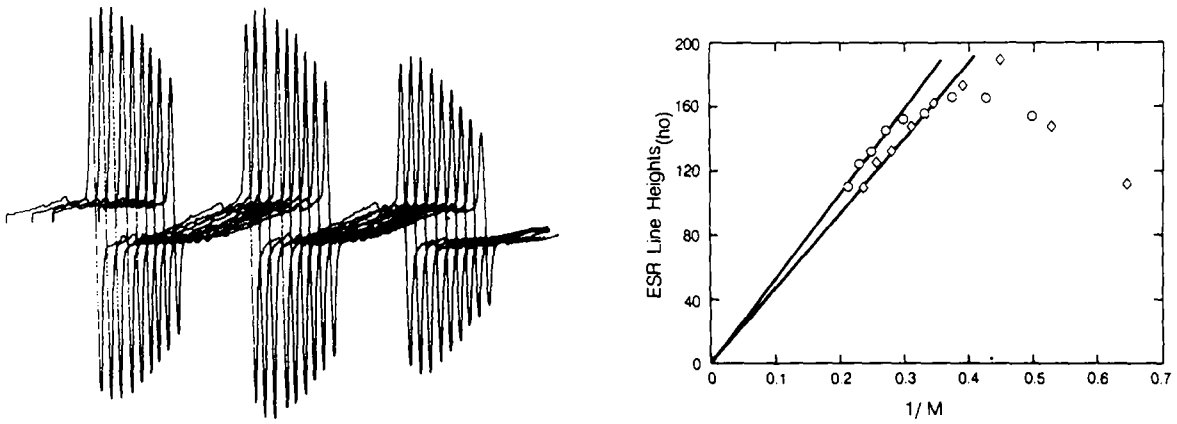

Fig. 1. Left side: ESR spectra used for the volume estimations of 4 M NaCl vesicles, as described in the text. The sharp lines arise from that fraction of the probe which is in the vesicle interior, while the underlying broad spectrum is due to probe in the exterior medium. Conditions: TEMPONE, 1 mM; $Na_3Fe(CN)_6$, 100 mM; vesicle protein 10 mg/ml. For clarity, successive spectra are displaced relative to one another by shifting the magnetic field by 1 G increments. Right side: Osmotic volume changes of L-33 cell envelope vesicles treated with hyper- and hypo-osmotic solutions of NaCl. The vesicles were initially equilibrated with 2 (◇) or with 4 (○) M NaCl.

In the presence of chloride, illumination caused swelling of the vesicles, as shown in Fig. 2. The volumes reached at the end of 45 mins of illumination were dependent on the chloride concentrations, and approached values above 1 μl/mg protein, or more than half the volumes in 4 M NaCl. In contrast, barely measurable rates of swelling were observed in the dark, or in the light, but without added chloride. Bromide allowed similar light-dependent volume increase to chloride. In the dark with 1.5 M chloride present, swelling occurred at rates about 3% of that in the light, and the volumes reached after 5 h reached only 0.14 μl/mg protein. The initial rates of the swelling were roughly unchanged between 0.2 and 1.5 M chloride (Fig. 2). In another series of experiments (not shown) lower chloride concentrations were tested, and half-maximal initial rates of swelling were seen at 50 mM chloride. An apparent affinity constant of this magnitude is consistent with the K_m for chloride transport determined by other means [8]. The light-induced swelling was essentially the same when the chloride was present largely on the vesicle exterior (illumination immediately after chloride addition, the half-life of a chloride gradient being over 2 h in these vesicles under these conditions), or both the interior and the exterior (illumination after several hours of incubation with chloride). The rate and extent of swelling were unaffected when potassium was replaced by sodium in the buffer (not shown).

Also shown in Fig. 2 is the shrinking of an illuminated vesicle suspension during subsequent incubation in the dark. The shrinking rate was quite slow, but the vesicle volume returned to nearly its original small value after more than 6 h of incubation. This finding strongly suggests that the illumination caused the vesicles to accumulate chloride at an internal concentration in considerable excess over that in the exterior medium, and it is the slow loss of this chloride which resulted in the return of the volume. Thus, during the illumination the chloride uptake must have proceeded against a concentration gradient (interior > exterior), as expected for an active chloride-transport system.

Vesicle swelling could be demonstrated also in vesicles much nearer to their original volume, e.g., when preshrunk with 5 M NaCl rather than with phosphate salts. As shown in Fig. 3, illumination of vesicles prepared from strain OD-2, which con-

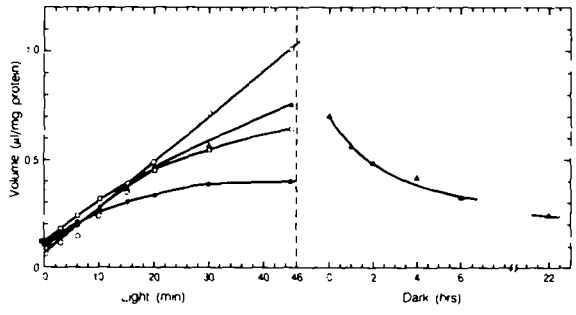

Fig. 2. Light-dependent swelling of L-33 cell envelope vesicles equilibrated with 3 M potassium phosphate, pH 7.0, as a function of added KCl concentration. ●, 0.2 M chloride; □, 0.375 M chloride; ▲, 0.75 M chloride; ○, 1.5 M chloride. No swelling was seen without added KCl. Conditions as in Fig. 1.

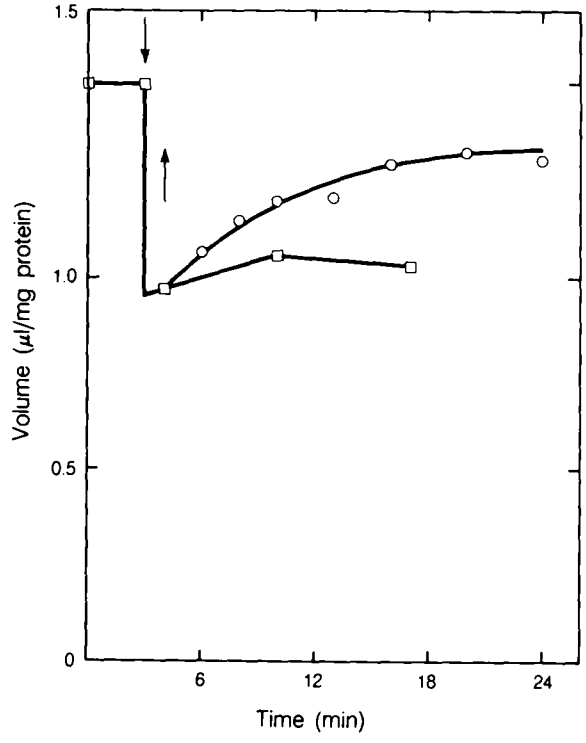

Fig. 3. Light-dependent swelling of OD-2 vesicles suspended in NaCl solution. Before illumination, the envelope vesicles were shrunken osmotically by adding sufficient amounts of 1 M NaCl to lower the osmolarity to 3 M, equilibrating the vesicles, and adding 5 M NaCl to raise the final osmolarity to 4 M (downward arrow). The beginning of illumination is indicated by the upward arrow. ○, illumination; □, dark. Conditions as in Fig. 1, but vesicle protein 20 mg/ml.

tains more halorhodopsin than L-33, resulted in swelling until the volumes approached values near those measured before the hypertonic preshrinking. In these experiments the time required for swelling was shorter than in phosphate: easily measurable volume increase occurred in two mins of illumination.

Volume changes during illumination of bacteriorhodopsin-vesicles

Bacteriorhodopsin-containing vesicles suspended in 4 M NaCl showed dramatic light-induced volume decrease (Fig. 4). The initial volume was about 1.4 μl/mg protein, which decreased linearly to about 0.9 μl/mg after 20 min of illumination. The decrease in the ESR signal was not due to reduction or degradation of the TEMPONE, because the signal returned upon swelling the vesicles by suspending them in 2.7–3.7 M NaCl, proportionally to the extent of hypoosmolar change (Fig. 4, right side). Envelope vesicles prepared from strain S-9, which were expected to contain little halorhodopsin but large amounts of bacteriorhodopsin, showed only light-dependent shrinking. The shrinking was inhibited by uncouplers. Thus, S-9 vesicles behaved like bacteriorhodopsin vesicles. Maximal rates of shrinking were observed near neutral pH, i.e., the rates were greater at 7.2 than at either 6.0 or 8.2. No light-dependent shrinking was seen in 3.5 M KCl.

Light-scattering experiments have already suggested that bacteriorhodopsin vesicles show light-induced loss of volume [7,8]. Because this process is due to energized sodium export by a sodium/proton antiporter, a system sharply gated by protonmotive force, it was expected that the volume decrease would be similarly gated. This question was examined by following ESR signals from TEMPAMINE, because antiport activity is evident from a characteristic pattern of internal acidification which follows internal alkalinization, as described previously [3,8]. The addition of a small amount of uncoupler would produce a small decrease in the protonmotive force, with a much more than proportional decrease in the antiport activity. We found that small concentrations of uncoupler (20 μM) converted the characteristic biphasic pH change to simple internal alkalinization, which originates from bacteriorhodopsin without antiport activity (not shown). Such simple internal alkalinization was seen also in vesicles containing less than a threshold amount of bacteriorhodopsin, as expected. Adding 20 μM uncoupler to the vesicles abolished the light-induced shrinking.

Volume changes during illumination of vesicles containing both bacteriorhodopsin and halorhodopsin

Vesicles prepared from strain R-1 showed relatively little light-dependent volume change. When these vesicles, which contain both bacteriorhodopsin and halorhodopsin, were illuminated only a 14% volume decrease could be observed at pH 7 or 8 (data not shown).

Special problems of volume determinations in the halobacterial system

Although the ESR volume determinations are a powerful tool in the study of halobacterial bioenergetics, certain precautions must be exercised in this system. The most significant of these is that at the high salt concentrations used, the nature of the salt affects the distribution of TEMPONE between

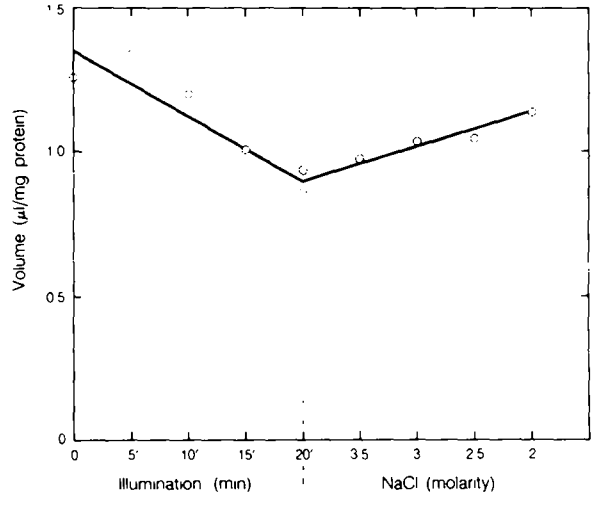

Fig. 4. Light-dependent shrinking of JW-5 vesicles reconstituted with retinal (these vesicles contain bacteriorhodopsin, but insignificant amounts of halorhodopsin). In order to reswell the vesicles after 20 min illumination, NaCl solutions were added in the dark at the concentrations indicated on the abscissa, which resulted in the following final osmolarities: for 3.5 M addition, 3.67 M; for 3 M addition, 3.34 M; for 2.5 M addition, 3.01 M; and for 2 M addition, 2.68 M. Conditions as in Fig. 1.

the compartments. Thus, the ESR probe tends to be excluded from the compartment with the phosphate, in favor of the compartment with chloride. If chloride is taken up by phosphate-equilibrated vesicles, this tendency will cause TEMPONE uptake, which would be mistakenly interpreted as swelling. The magnitude of such apparent swelling is insignificant relative to the changes seen in Fig. 2, however.

It would have been convenient to illuminate the vesicle suspensions, together with the TEMPONE and the external paramagnetic broadening agent, directly in the ESR instrument cavity in order to follow the changes continuously. However, careful comparisons of volume changes in L-33 vesicles outside the cavity, with and without the ESR probe plus quencher present during the illumination, revealed that the apparent swelling was somewhat less in the former case than in the latter. It seems likely that a slight loss of the ESR probe occurs under prolonged illumination under these conditions, and this accounts for the lesser increase of the ESR signal. To simplify matters, the illumination was carried out prior to addition of probe and quencher, followed by addition of these agents and measurement of the signal. Control experiments showed that volume changes in the dark during the manipulations required for this were insignificant.

Discussion

We have demonstrated that at NaCl concentrations higher than about 3 M, cell envelope vesicles prepared from *H. halobium* behave as perfect osmometers. The integrity of the membrane of these vesicles, and the suitability of this system for ion transport measurements, are thereby confirmed. Volume changes during illumination of vesicles from various strains, containing bacteriorhodopsin or halorhodopsin, previously inferred from-light-scattering, have been measured quantitatively. The ion movements in halobacterial cells, suggested by previous results and results reported here, are shown schematically in Fig. 5. The protons extruded by bacteriorhodopsin are recirculated by the sodium/proton antiporter, which provides a sustained flux of sodium ions toward the exterior during the illumination. The

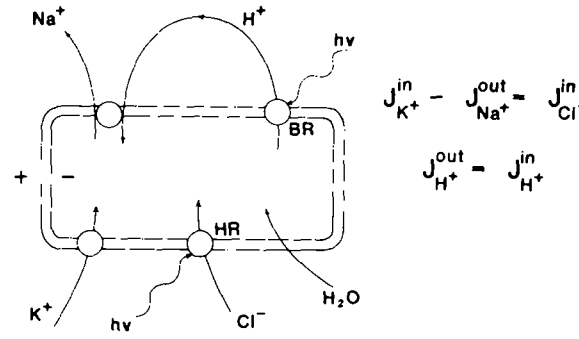

Fig. 5. Schematic diagram of the proposed volume regulation in *H. halobium* cells during illumination. Details are explained in the text. BR is bacteriorhodopsin, HR is halorhodopsin. J with the appropriate subscripts and superscripts refer to fluxes of potassium, sodium, protons and chloride, inward and outward. Unlike the fluxes of sodium, potassium and chloride, which are continuous during steady-state light-dependent growth, the net steady-state flux of protons is zero.

membrane potential (interior negative) generated is increased by the activity of the antiport [3], and will drive passive chloride efflux and potassium influx. Bacteriorhodopsin-containing vesicles show only this effect, and when suspended in NaCl (in the absence of KCl), show net salt and thus volume loss. The physiological anion, chloride, is apparently permeant enough in the vesicle membranes to allow for the massive volume changes we measured. Since the volume loss is driven by protonmotive force, the effect is expected to be inhibited by uncouplers. The results of the ESR volume measurements with bacteriorhodopsin vesicles in this study confirm this part of the model. The pH optimum for the process is somewhat above the pH optimum for the generation of membrane potential, probably reflecting the pH requirements of the antiporter.

The active transport of chloride into the cells by halorhodopsin is also illustrated in Fig. 5. In halorhodopsin vesicles the cations taken up due to the membrane potential generated (interior negative) are first protons, but as the interior becomes acidified and the protonmotive force tends to zero [17], they are increasingly sodium ions (or potassium if present). Hence, this component of the model will generate light-dependent swelling. Because this transport system is not driven by protonmotive force, addition of uncoupler should have no effect on the volume change. Other ionophores

which increase permeability to cations, e.g., gramicidin or valinomycin, also should not be inhibitory. This pattern of volume change is indeed confirmed for halorhodopsin-vesicles in this study, as is the previously described specificity of the system for chloride [8]. It seems reasonable to expect that when both bacteriorhodopsin plus sodium/proton antiport and halorhodopsin are present in the same membrane, the net volume change would be given by the linear combination of the two separate effects [17]. The data with R-1 vesicles is consistent with this idea.

It has long been known that *H. halobium* cells accumulate potassium and extrude sodium ions during growth [18]. The predominant anion, chloride, on the other hand, is present in roughly equal concentration in the cell interior and exterior. The pathways of ion transport described above will account for this balance of ionic composition in the halobacteria. For growing cells the removal of sodium is facilitated by the sodium/proton antiporter, energized by protonmotive force generated by either respiration, ATP hydrolysis, or bacteriorhodopsin during illumination. The uptake of potassium ions, which replace the sodium, is accounted for, partly or fully, by passive transport driven by the membrane potential. But growing cells must increase their internal volume, and to the extent required by this increase the potassium uptake must exceed the sodium loss. The uptake of chloride equivalent to the amount of this excess cation gain should be accounted for by the activity of halorhodopsin in the light, but another system (driven by respiration) will have to operate in addition, in order to support growth in the dark. Such a chloride transport system has been observed (Wagner, G., personal communication). Since the halobacteria grow slowly (the generation time is 6–8 h), and the net volume increase of cells is likely to be under metabolic control, the behavior of vesicles could not be expected to quantitatively reflect the behavior of intact cells. However, driving each component of the scheme in Fig. 5 at its maximum rate in the vesicles, individually and together, allowed us to confirm the elements of the overall ion transport scheme described above.

An important advantage of the ESR method is that the volume changes can be quantitated and related to the ion fluxes during the illumination. We present here a calculation to estimate the chloride flux in the OD-2 vesicles at saturating light-intensities, in order to test the assumption that the chloride transport is directly related to the halorhodopsin photocycle. The halorhodopsin content of the vesicles used for the experiments in Fig. 3 was determined to be about 0.4 nmol/mg protein, which gives a concentration of 4 μM. The vesicles accumulated NaCl at an initial rate of 4.1 mM/min. Combining these numbers, we get 17 mol chloride transported per mol halorhodopsin per s, or a turnover time of about 60 ms. From flash experiments the photocycle of halorhodopsin in these vesicles was determined to have a half-life of about 10 ms [14], i.e., it takes about 30 ms for 90% of the halorhodopsin molecules to recover from the light-induced photochemical changes. Thus, it appears that all or nearly all of the halorhodopsin transports chloride, and with rather high efficiency per photocycle. It has been shown for bacteriorhodopsin that the overall photocycling rate is regulated by membrane potential [19,20]; it would seem plausible that a similar control mechanism exists for halorhodopsin. Substantial membrane potentials have been measured during illumination of halorhodopsin vesicles, and thus it is possible that under the conditions of the experiment the photocycle of halorhodopsin is slower. If so, the match between the photocycling rate and the transport rate is even closer.

Acknowledgements

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