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LIGHT-DRIVEN SODIUM TRANSPORT IN SUB-BACTERIAL PARTICLES OF *HALOBACTERIUM HALOBIUM*

MICHAEL EISENBACH, SHULAMIT COOPER, HAIM GARTY, ROSE M. JOHNSTONE *, HAGAI ROTTENBERG ** and S. ROY CAPLAN

Department of Membrane Research, Weizmann Institute of Science, Rehovot (Israel)

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Summary

Light-induced Na^+ efflux was observed in sub-bacterial particles of *Halobacterium halobium* loaded and suspended in 4 M NaCl solution. The Na^+ efflux was not ATP driven, since ATPase inhibitors were without effect or even enhanced efflux at low light intensity. Uncouplers, on the other hand, inhibited Na^+ efflux, the inhibition being complete at low light intensity. The Na^+ efflux was accompanied by proton influx. Both processes were dependent on light intensity, unaffected or enhanced by ATPase inhibitors and similarly affected by uncouplers. Proton influx was not observed in particles loaded with 4 M KCl instead of 4 M NaCl. Na^+ transport in the dark could be induced by artificial formation of a pH difference across the membrane; changing the sign of the pH difference reversed the direction of the Na^+ transport. Proton influx in the dark followed the artificial formation of a sodium gradient ($[Na^+]_{in} > [Na^+]_{out}$). These results may be explained by a Na^+/H^+ antiport mechanism. The fluxes of Na^+ and H^+ were of comparable magnitude, but the initial rate of Cl^- efflux in the same experiment was one-third of the initial rate of Na^+ efflux. Consequently Cl^- is not regarded as a participant in the Na^+ efflux mechanism.

Introduction

Extensive studies on transport phenomena in *Halobacterium halobium* have been carried out in various laboratories. Proton transport was found in the

* Present address: Department of Biochemistry, McGill University, Montreal, Canada.
** Present address: Department of Biochemistry, Tel-Aviv University, Ramat Aviv, Israel.
Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *P*-trifluoromethoxyphenylhydrazone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, morpholinopropane sulfonic acid; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); SF6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidene malononitrile; $TPMP^+Br^-$, triphenylmethylphosphonium bromide.

intact bacteria [1—3] as well as in sub-bacterial particles (cell envelopes) [4—6]. Potassium and rubidium transport were found in intact bacteria [7] as well as in sub-bacterial particles [5], and amino acid transport was investigated in intact bacteria [8] and in sub-bacterial particles [4,9,10].

It is a well-known fact that the internal concentration of K^+ in intact bacteria is higher than that of Na^+, irrespective of the K^+ content of the external or growth medium [11,12]. The phenomenon is especially prominent in *Halobacteria*, where the internal concentration of K^+ is near 4 M when external K^+ is 5 mM, internal Na^+ is 0.5—2 M, and external Na^+ is 4 M [13]. This suggests that these bacteria must possess some mechanism which expels Na^+ from the cell and which draws K^+ into the cell. According to Garty and Caplan [7], K^+ influx is a slow passive movement, driven entirely by the electrical potential gradient across the membrane. Very recently Lanyi et al. [10] published their observations on Na^+ transport in *H. halobium*, and suggested an electrogenic Na^+/H^+ exchange mechanism. In the present paper we present our own results on Na^+, H^+, and Cl^- transport. The data are consistent with the operation of a specific Na^+/H^+ antiport mechanism. Most of the reported experiments were carried out with sub-bacterial particles. These particles, as first described by MacDonald and Lanyi [4], are closed envelopes and their internal and external contents can be changed at will. Moreover, the absence of cytoplasmic and metabolic events means that transport processes can be measured free from such complications. Nonetheless a few experiments are reported with the intact cells.

Materials and Methods

Preparations. H. halobium M-1 strain was grown as described by Danon and Stoeckenius [14]. The growing medium was as described by Onishi et al. [15], but the amino acids were replaced by 7.5 g/ml vitamine-free casamino acids (DIFCO Laboratories). Sub-bacterial particles were prepared by sonication according to MacDonald and Lanyi [4] with some modifications. A variety of buffers were used in the washing and sonicating solutions (HEPES, PIPES, MES, and MOPS) and not necessarily Tris · HCl buffer as in ref. 4. In each preparation the same buffer was used throughout. Sonication was performed with six times 20-s periods of sonication at intensity 7 using the microtip of a Branson B-12 sonifier. The final washing and suspending solution contained 1 mM buffer. The particles maintained full activity for several weeks at 4°C; nevertheless in all the experiments they were less than 2 weeks old. Particles containing internal Na^+ were prepared in solutions containing 4 M NaCl, whereas K^+-containing vesicles were prepared in solutions in which K^+ was substituted for all the Na^+ in the appropriate solutions.

In experiments with intact bacteria, the cell suspension was prepared for experiments as described previously [3].

Methods. Protein was determined by the Lowry et al. method [16] using bovine serum albumin as standard. When the protein content of intact bacteria was examined, the protein was first dissolved in 1 M NaOH. In sub-bacterial particles this pre-treatment was unnecessary.

The bacteriorhodopsin content of the vesicles was determined by suspending them in pure water with or without the addition of Triton X-100 to the sus-

pension, and reading the absorbance at 570 nm, using an extinction coefficient of 63 000 $M^{-1} \cdot cm^{-1}$ [17]. Both methods yielded identical results.

Two methods were applied for the determination of the internal water space of the particles: a centrifugation method using ^3H_2O and [^{14}C]sucrose [18] and a filtration method. The first method was carried out as described previously by Bakker et al. [3]. The filtration method was performed as follows. The internal water space was measured directly by slowly permeating non-metabolized molecules, sorbitol and 2-deoxyribose. The particles were loaded with either [^{14}C]sorbitol or deoxy[^{14}C]ribose by incubation at 4°C until an equilibrium was reached (approx. 24 h), as verified by repetitive measurements at different time intervals. The particle suspension was then filtered, washed, and counted.

Na^+ transport in sub-bacterial particles was measured as follows. To a suspension of particles (5—20 mg protein/ml) in 4 M NaCl and 1 mM buffer (pH 7), either 2% (v/v) ^{22}NaCl (1 mCi/ml) or 10% (v/v) ^{22}NaCl (0.2 mCi/ml) were added, and the suspension was left at 4°C for 3—4 days for loading. After this period the external and internal specific activities were similar. Equal volumes of the suspension were distributed into cavities in a thermostated glass vessel, and the required reagents added and incubated with the suspension if necessary. The measurement was started upon turning on the light. During the experimental period the suspension was stirred with a magnetic stirrer. Samples (20 μl) were taken at intervals, transferred into 10 ml of cold suspending medium contained in a filtration device, and washed seven times with 3-ml aliquots of cold suspension medium. Cellulose nitrate Millipore filters of 0.45 μm pore size were used. The filter carrying the washed vesicles was transferred into a vial and counted in a Packard γ-counter (model No. 578). Other samples (5—10 μl) were transferred without filtration to a vial to determine the external specific activity of $^{22}Na^+$. $^{22}Na^+$ influx was measured as follows. One volume of a suspension of vesicles was added to two volumes of a mixture of 4 M NaCl, 0.5 mM PIPES, 0.5 mM MES (pH 6.8), and tracer $^{22}Na^+$. The resulting suspension was mixed rapidly and exposed to light for the desired period (ranging from 0 to 15 min). At the end of the illumination period, a 100-fold volume of a cold solution of 4 M NaCl was added and the content of the test tube was filtered on a Millipore and washed as described above. Cl^- efflux and influx in sub-bacterial particles were measured using the same procedures as for Na^+; 10% (v/v) $Na^{36}Cl$ (85 μCi/ml) were added and the samples were counted by a Packard 1230 Tri-carb scintillation spectrophotometer.

The pH measurements were carried out in a thermostated glass vessel using a Radiometer (Copenhagen) pH meter (type 64) connected to a high speed recorder (Varian A-25 with a response time of 0.5 s) and a combined pH electrode (Radiometer type GK2321^c). The source of light for both the pH and the transport measurements was a slide projector provided with an iodine quartz (24 V, 150 W) lamp. The variations in the light intensities were achieved by illuminating through neutral filters. The light intensity was measured by aYSI-Kettering radiometer (model 65A). Absorbance measurements were carried out with a Gilford 2400-S spectrophotometer. The electron microscope micrographs were obtained using the procedure described by Stoeckenius and Rowen [19].

Materials. PIPES, Tris, HEPES, MES, MOPS, DNAase, FCCP, NADH, and menadione were obtained from Sigma Chemical Co.; DCCD from Fluka; $TPMP^+Br^-$ from K and K Laboratories; SF6847 was a gift from Dr. Y. Nishizawa, Sumimoto Chemical Industry, Osaka (Japan). [^{14}C]Sucrose, [^{14}C]-sorbitol, deoxy[^{14}C]ribose, ^3H_2O, ^{22}NaCl, and $Na^{36}Cl$ were obtained from Amersham Radiochemical Centre.

Results

Characterization of sub-bacterial particles from H. halobium

Fig. 1 is a comparison between the electron micrographs of intact purple bacteria (Fig. 1A) and of the particles prepared from them (Fig. 1B). It may be seen that the cytoplasmic content of the bacteria has been lost during the sonication. The average diameter of a particle, measured from this figure, is 0.5 μm. The amount of bacteriorhodopsin in the membrane was 3—8 nmol per mg protein (compared to 2.5 nmol as reported in ref. 4), and varied from batch to batch. The internal volume of the particles, measured using both methods described in Materials and Methods, gave values of 2.7 ± 0.3 (S.E.) μl/mg protein in accordance with the earlier report of 3.0 μl/mg [4].

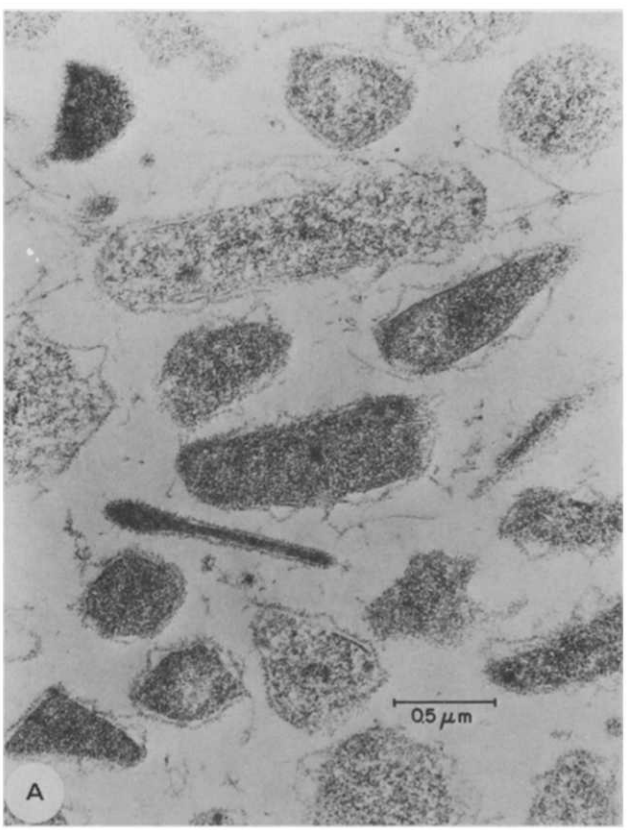


Fig. 1. For legend see opposite page.

The degree of integrity and the sidedness of the particles was determined by measuring the NADH-menadione reductase as first suggested by Lanyi [4,20]. Fig. 2 shows the activity of NADH-menadione reductase, an enzyme bound to the internal side of the membrane. Upon addition of the substrates (to which the intact membrane is impermeable) there is no change in NADH oxidation unless Triton is added. The low activity in the absence of detergent (curve I) and the high activity in the presence of Triton X-100 (curve II) provides evidence for both the intactness and the inside-in polarity of these particles. The fraction of intact inside-in particles was calculated from Fig. 2 to be 85%; in different batches the intactness varied from 80 to 100%. It should be emphasized that the kinetics reflected by curve II are those of the reaction only and not of the solubilization of the particles by Triton. The latter kinetics, which is reflected by curve III, are completed before the addition of the substrates.

These results were qualitatively verified by using a fragiligraph [21]. The suspension was injected into a fragiligraph and dialyzed against pure water. A decrease in transmission was observed within 10 min at which time a new pla-

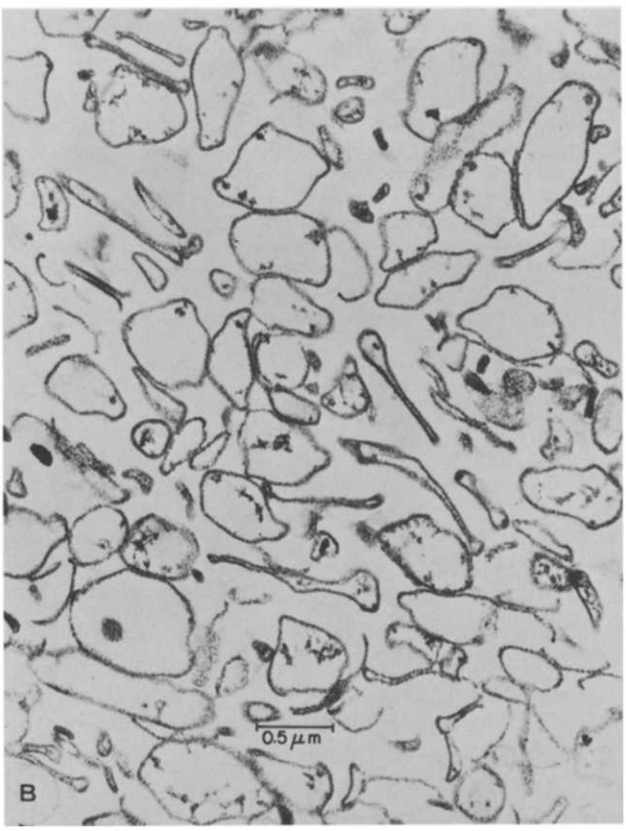
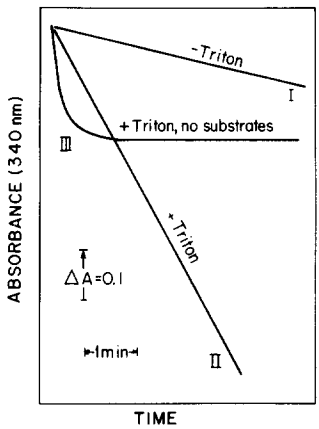


Fig. 1. Section through pellets of intact *H. halobium* cells (A) and of sub-bacterial particles (B). The intact purple bacteria were collected after 4 days in the light in anaerobic conditions and treated as described under Materials and Methods. Another portion of the same batch was used for preparation of the sub-bacterial particles.



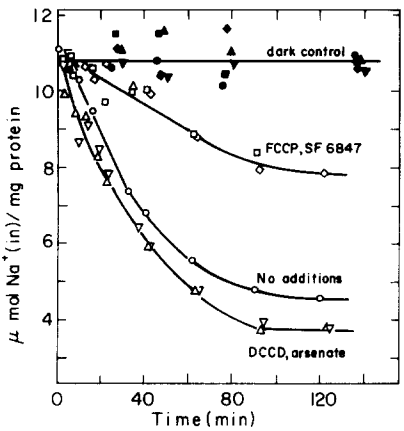


Fig. 2. The effect of Triton X-100 on the activity of NADH-menadione reductase. The suspension of the vesicles contained 0.5 mg protein/ml in 4 M NaCl, 0.5 mM MES, and 0.5 mM PIPES (pH 6.5). The reaction was followed at 340 nm at 25°C. I. Menadione (0.5 mM) was added to the suspension of the sub-bacterial particles and the reaction was initiated by addition of NADH (0.5 mM). II. As in I, but the suspension was incubated with 0.01% (v/v) Triton X-100 for 15—30 min before initiating the experiment by the addition of menadione and NADH. III. The experiment was initiated by addition of 0.01% (v/v) Triton X-100 to the suspension of vesicles. NADH and menadione were absent.

Fig. 3. The effects of uncouplers and ATPase inhibitors on the Na^+ efflux. Sub-bacterial particles (8 mg/ml) in a solution of 4 M NaCl plus 1 mM MOPS (pH 7.2) were loaded with $^{22}NaCl$ as described under Materials and Methods. Before the experiment the suspension was distributed between two thermostated glass vessels (21.5°C). The reagents were added to different cavities in the same vessel; a vessel in the light (open symbols) and a vessel in the dark (closed symbols). The reagents (except for arsenate) were dissolved in alcoholic solution, the final alcohol concentration in all the cavities (including the control and arsenate) being 0.1% (v/v). The experiment was carried out as described under Materials and Methods. It was initiated by illuminating one of the vessels (I = 450 W/m^2) while the other remained in the dark as a control. Specific activity, 4000 cpm/μmol Na^+(in). ○, 0.1% alcohol only; □, 20 μM FCCP; ◇, 10 μM SF6847; △, 1 mM arsenate; ▽, 0.1 mM DCCD (added 60 min before the initiation of the experiment).

teau was reached. This supports the idea that the particles were initially intact and that 10 min were required to rupture them.

Transport measurements

Fig. 3 describes a typical determination of Na^+ efflux from sub-bacterial particles and the effects of uncouplers and energy transfer inhibitors on the efflux. Turning on the light induced a loss of $^{22}Na^+$ from the particles at an initial rate of 7.3 μmol $Na^+ \cdot h^{-1} \cdot (mg\ protein)^{-1}$ in the absence of any reagent. The rate gradually decreased and the process levelled off after 90 min. Removal of the light source after a period of illumination resulted in a reversed flow of $^{22}Na^+$, i.e. a slow uptake of $^{22}Na^+$ by the particles (not shown) in accordance with the previous report of Lanyi et al. [10]. No flux of $^{22}Na^+$ could be detected in the control experiment carried out in the dark (Fig. 3). It should be emphasized that upon illumination (after loading) only efflux of Na^+ was observed, even when the external specific activity of $^{22}Na^+$ was higher than the internal one. This observation together with the fact that Na^+ was moving against its electrochemical potential gradient (ref. 22 and Garty, H., Cooper, S. and Eisenbach, M., unpublished observations) indicates that the movement of Na^+ is not a process

of passive diffusion. We therefore examined whether the light-dependent Na^+ efflux is mediated by ATP. It is shown in Fig. 3 that while the uncouplers (FCCP and SF6847) decreased the initial rate of light-induced efflux by about 70%, the ATPase inhibitors (DCCD and arsenate) enhanced the rate by about 25%. Qualitatively similar results were observed with intact bacteria. If ATP were the energy source for efflux, both classes of inhibitors would reduce the flux. These observations argue against the possibility that Na^+ efflux requires ATP. To substantiate this conclusion we prepared particles containing internal ADP, P_i, and Mg^{2+} (all at 5 mM), and incubated them in a medium containing the same concentration of these substances. If ATP is produced by illumination and then used for Na^+ efflux, the above treatment would be expected to enhance Na^+ efflux. In fact, there was no difference (within experimental variation) in Na^+ loss with or without the above treatment, nor was there any difference in response to arsenate or DCCD after exposure to ADP, P_i, and Mg^{2+}.

To determine whether light-dependent $^{22}Na^+$ fluxes correctly reflect the behaviour of net Na^+ fluxes would require a measurement of the net flux by following efflux and influx of $^{22}Na^+$ simultaneously. Because Na^+ is eliminated from the particle with great efficacy as soon as illumination begins, it was impossible to follow $^{22}Na^+$ uptake accurately by a conventional experiment, namely by adding $^{22}Na^+$ to a particle suspension and following the appearance of $^{22}Na^+$ in the particles (similar difficulties were found with intact bacteria). We circumvented this problem by doing the $^{22}Na^+$ influx measurement in the manner described under Materials and Methods, both under illumination and in the dark (as a control). The results are shown in Fig. 4. It may be seen that the kinetics of the $^{22}Na^+$ uptake consisted of two phases: a rapid phase followed by a slow phase. It seems reasonable to suppose that the rapid phase represents the adsorption of $^{22}Na^+$ by the membrane, while the slow process represents the penetration of $^{22}Na^+$ into the particle. The significant observation is that, within the limits of experimental error, $^{22}Na^+$ uptake is the same with and without illumination and represents an equilibration between internal and external $^{22}Na^+$. Since there is no light-dependent $^{22}Na^+$ uptake, the observed light-dependent $^{22}Na^+$ efflux represents a net loss of Na^+.

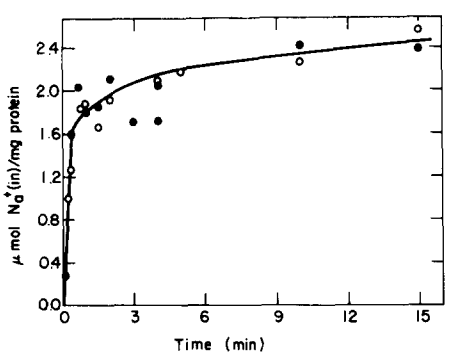


Fig. 4. Undirectional Na^+ influx in short periods of time. Samples of 7-μl vesicles (13 mg/ml) were treated and examined at room temperature as described in the text. Specific activity, 3500 cpm/μmol Na^+. ○, light; ●, dark control.

TABLE I

THE EFFECTS OF LIGHT INTENSITY, UNCOUPLERS AND ATPase INHIBITORS ON THE INITIAL RATE OF THE Na^+ EFFLUX

Sub-bacterial particles, preloaded with $^{22}Na^+$, were suspended in 4 M NaCl solution including 1 mM MOPS (pH 7.2) to approx. 5 mg protein/ml. The suspension was distributed among several cavities in a thermostated glass vessel, and to each cavity a different reagent was added. The reagents (except for arsenate) were dissolved in absolute ethanol, and their concentrations adjusted so that the desired final concentration in the suspension could be reached by an 0.1% (by volume) addition of the alcoholic solution. Arsenate was dissolved in water. The arsenate and the control experiments also included 0.1% ethanol. DCCD was added at least 1 h before the initiation of the experiment and was incubated with the vesicles at room temperature. Samples for filtration (including dark-control samples) were taken from the moment the light was switched on. The vessel was illuminated by a slide projector and the light intensity changed by neutral filters. The temperature was 21.5°C. Since in all the experiments which are shown in the table the external and internal specific activities were identical, no flux of $^{22}Na^+$ was observed in the dark control.

Reagent added	Initial rate of Na^+ efflux (μmol $Na^+ \cdot h^{-1} \cdot$ (mg protein)$^{-1}$)		
	Light intensity (W/m^2)		
	37	120	800
No addition	0	0.8	7.3
10 μM SF6847	0	0	1.2
10 μM FCCP	0	0	4.6
10 μM DCCD	0	1.8	7.3
10 μM oligomycin	—	—	7.3
14 mM arsenate	—	—	7.3

In agreement with Lanyi et al. [10] and based on the present data, an attractive model for the Na^+ efflux mechanism would be a Na^+/H^+ antiport system as suggested by West and Mitchel [23] for *Escherichia coli*. In such a scheme, Na^+ efflux would be dependent on the electrochemical potential difference $\Delta\tilde{\mu}_{H^+}$ (or ΔpH, if the process were electroneutral).

The reports of Lanyi and his associates [4,22] suggest that in particles from *H. halobium* $\Delta\tilde{\mu}_{H^+}$ depends on light intensity. If Na^+ efflux depends on $\Delta\tilde{\mu}_{H^+}$, light intensity would affect Na^+ efflux. The results of these experiments are shown in Table I. The data include experiments with uncouplers and ATPase inhibitors. It is apparent that increased light intensity increased the efflux of Na^+ at early times. It is also evident that at very low light intensity there was no Na^+ loss. At medium light intensities (100—200 W/m^2) the uncouplers abolished Na^+ efflux completely while the ATPase inhibitors doubled the rate of Na^+ loss. In contrast, at high light intensity, the uncouplers caused only partial inhibition (16 and 63% of the original activity with SF6847 and FCCP, respectively) and the ATPase inhibitors had no effect (note in Fig. 3, at a light intensity of 450 W/m^2, the ATPase inhibitors enhanced the efflux by 25%). The average initial rate of the Na^+ loss in the control experiment was 7 μmol $Na^+ \cdot h^{-1} \cdot$ (mg protein)$^{-1}$ and the variation from batch to batch was less than ± 4 μmol $Na^+ \cdot h^{-1} \cdot$ (mg protein)$^{-1}$. The fact that SF6847 inhibits the efflux at high light intensities to a greater extent than FCCP is explained by the higher efficiency of SF6847 as an uncoupler as was found in mitochondria [24] and in chloroplasts (Avron, M., personal communication). In Table I the data given,

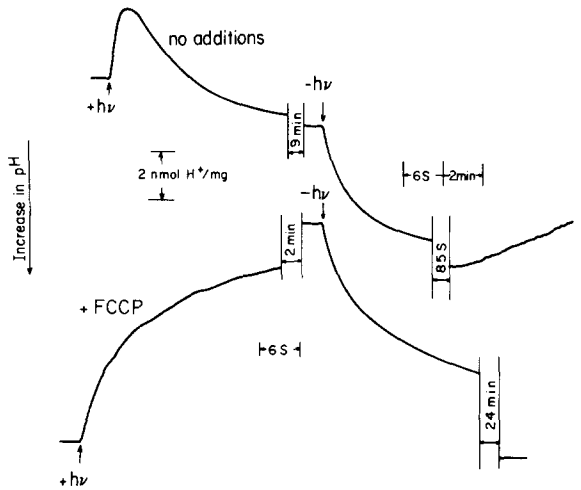


Fig. 5. Light-induced pH changes at high light intensity. Sub-bacterial particles were suspended in 4 M NaCl to a concentration of 2 mg/ml. The suspension was pre-illuminated and then re-illuminated through a 517 nm "cut-on" filter (in order to avoid electrode artifacts) at light intensity of 1250 W/m^2. The upper curve is the pH change during the second illumination in the absence of any added reagents. The lower curve is another experiment with the same batch of vesicles which was done in the presence of 10 μM FCCP. The initial pH in both experiments was 6.5. Temperature, 25°C.

although taken from a single experiment, are representative of 12 similar experiments with the same qualitative results.

Assuming the Na^+/H^+ antiport model, an influx of H^+ should accompany efflux of Na^+. Fig. 5 (upper curve) describes the pH changes observed upon illumination at a high light intensity (1250 W/m^2). It may be seen that on exposure to light, acidification of the medium is followed by a slower alkalinization. In contrast, if the light is turned off, the opposite sequence of events is

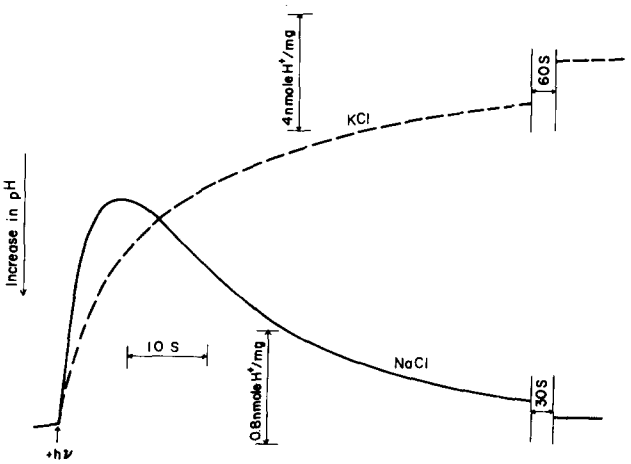


Fig. 6. A comparison between the light-induced pH changes in KCl-loaded and in NaCl-loaded sub-bacterial particles. The KCl- and NaCl-loaded vesicles were prepared simultaneously as described under Materials and Methods, and suspended respectively in either 4 M KCl or 4 M NaCl plus 1 mM MOPS (pH 7.2) to a concentration of 2.4 or 4.2 mg/ml, respectively. The experiment was carried out as described in Fig. 5. I = 400 W/m^2. Temperature, 25°C.

observed albeit at a slower rate: an initial alkalinization is followed by a very slow acidification. If the slow alkalinization observed in Fig. 5 during the illumination represents the Na^+/H^+ antiport system, which is obligatory for Na^+, it should be observed only in a medium containing Na^+. The data in Fig. 6 show light-induced changes in pH with KCl- and NaCl-containing particles. It is apparent that the second phase of pH change observed in Na^+-containing vesicles is absent in the K^+-containing vesicles. These results favour the Na^+/H^+ antiport model and suggest the following manner of operation.

(1) When the light is turned on, the proton pump extrudes H^+, and a $\Delta\tilde{\mu}_{H^+}$ is formed (the medium is acidified). At a certain stage protons reenter the vesicle in exchange for vesicular Na^+ resulting in an alkalinization of the medium and an extrusion of Na^+.

(2) When the light is turned off, the proton pump ceases to operate, and protons enter the vesicle with an alkalinization of the medium (because of the back diffusion of H^+ down its electrochemical potential gradient). With the entry of H^+, $\Delta\tilde{\mu}_{H^+}$ decreases. Since internal Na^+ is less than external ($[Na^+]_{in}$ = 3.3 M, $[Na^+]_{out}$ = 4 M after 10 min illumination (calculated from parallel experiments)), there is now a tendency for medium Na^+ to exchange with vesicular H^+ causing reacidification of the medium.

Comparing the rate of the back-flow of Na^+ upon turning off the light [10] to the rate of the H^+ back-flow under the same conditions (upper "off" curve in Fig. 5) shows that both the influx of Na^+ and the acidification are much slower than the preceding initial rates in the light. The type of pH changes seen in Fig. 5 were observed also at lower light intensities, except that at lower light intensity the extent and the rate of the alkalinization that followed the acidification in the light decreased markedly. At light intensities below 40 W/m^2 the alkalinization phase vanished completely. Thus the alkalinization phase and Na^+ efflux are similarly dependent on the light intensity (cf. Table I). The conclusion that the alkalinization is associated with Na^+ efflux is supported by the observation that with FCCP no alkalinization occurs (lower curve, Fig. 5). In Fig. 3 and Table I, we showed data indicating that FCCP inhibits Na^+ efflux. In fact, FCCP also decreases the extent of the acidification, but because of the subsequent alkalinization in the absence of FCCP, this effect is not readily apparent by comparing the upper and lower curves in Fig. 5. The decreased acidification by FCCP can be observed only at low light intensities (approx. 40 W/m^2) where no alkalinization takes place to perturb the acidification process (Eisenbach, M. and Caplan, S.R., unpublished results). Moreover, Fig. 7 shows that an ATPase inhibitor had the same effect on alkalinization as on Na^+ efflux, namely DCCD increased both the net proton uptake probably by decreasing the leak (or inhibiting phosphorylation) (Fig. 7) and the net efflux of Na^+ (Fig. 3 and Table I).

If our assumption is correct that the light-induced ΔpH or $\Delta\tilde{\mu}_{H^+}$ is the force which drives Na^+ transport in the light, then an artifically induced ΔpH in the dark should also drive a sodium flux. Fig. 8 shows the effect of HCl or NaOH added externally to a suspension of the vesicles. Curve I describes an experiment which imitates the effect of light, i.e. inducing a ΔpH where pH(in) > pH(out). The addition of HCl decreased the external pH from 6.7 to 4.0 and resulted in a fast efflux of Na^+. Curve II shows the results of a control experi-

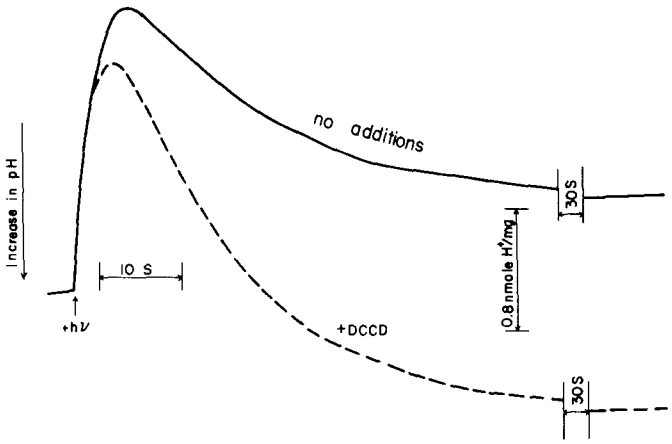


Fig. 7. The effect of DCCD on the light-induced pH changes. Sub-bacterial particles were suspended in 4 M NaCl plus 1 mM MOPS (pH 7.2) at a concentration of 4.2 mg/ml. The pH was followed under the same experimental conditions as in Fig. 6, either in the absence of DCCD (upper curve) or in the presence of 10 μM DCCD (lower curve) after 30 min incubation of the vesicles with the inhibitor.

ment in which no reagents were added. A ΔpH in the direction opposite to the light effect is induced in the experiment described by curve III. Addition of NaOH changed the external pH from 6.7 to 8.5, and a transient Na^+ influx was observed. This was followed by a slow decrease in the external pH and a slow efflux of Na^+. The lack of reversal (absence of Na^+ uptake) at low pH in Fig. 8 (curve I) may be due to the fact that the internal buffer capacity is higher at low pH (Garty, H. and Eisenbach, M., manuscript in preparation), or alterna-

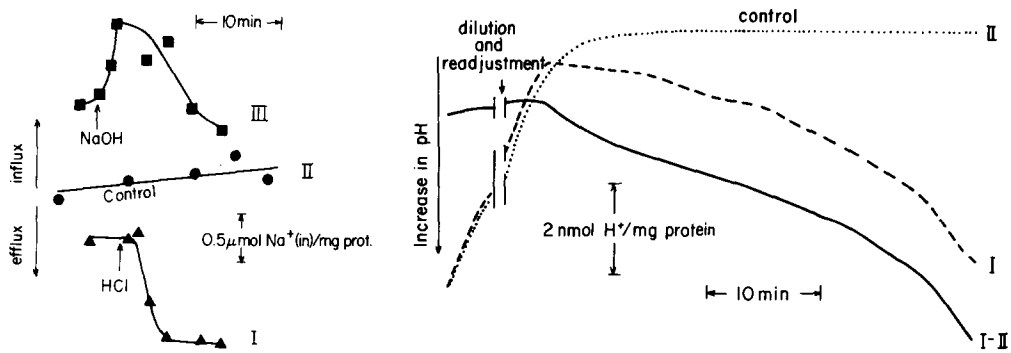


Fig. 8. A pH-driven Na^+ flux in the dark. Sub-bacterial particles, loaded with $^{22}NaCl$, were diluted 4-fold with 4 M NaCl. After 30 min of incubation at room temperature either 1.0 μmol HCl (curve I) or 0.23 μmol NaOH (curve III) were added. The external pH was monitored in parallel with the Na^+ efflux measurement. Specific activity, 3200 cpm/μmol Na^+(in). Curve I (▲), 10.4 μl HCl (0.1 M) were added to 230 μl of a suspension of vesicles (4.8 mg/ml) in 4 M NaCl where indicated in the figure. Curve II (●), no additions. Curve III (■), 2.3 μl NaOH (0.1 M) were added to 230 μl of a suspension of sub-bacterial particles (4.8 mg/ml) in 4 M NaCl where indicated in the figure.

Fig. 9. Proton transport in the dark induced by Na^+ gradient formation. Sub-bacterial particles (4.4 mg/ml) were suspended in a solution of 4 M NaCl, 0.5 mM PIPES, and 0.5 mM MES (pH 6.5) and treated as described in the text. In both curves the dilution itself changed the pH, and the pen of the recorder was shifted back manually to the same point. Temperature, 20°C. Curve I (- - - - -), dilution from 1.8 to 3.3 ml with a solution of 4 M KCl, 0.5 mM PIPES, and 0.5 mM MES (pH 6.5). Curve II (. . . . .), as in curve I but with NaCl replacing KCl.

tively, to an inhibition of the Na^+/H^+ antiport system at low pH as was suggested for *E. coli* [25].

The data presented suggest that the movements of Na^+ and H^+ are reciprocal and that the proton gradient is the driving force for Na^+ flow. If this conclusion is correct, it should be possible to demonstrate that a Na^+ gradient will bring about a flux of protons in absence of illumination. Fig. 9 describes the pH change which accompanies the formation of a Na^+ gradient in the dark. For 21 min the pH of suspension of sub-bacterial particles was followed by a pH meter, and the radioactivity in the vesicles was assayed. After this time, the vesicles were rapidly diluted from 1.8 to 3.3 ml either in buffered 4 M KCl (Fig. 9, curve I) or in buffered 4 M NaCl (curve II). From the difference between these curves we observe that the formation of sodium gradient ($[Na^+]_{in} > [Na^+]_{out}$) caused a net influx of protons, preceded by a Na^+ efflux (not shown in the figure). (The dilution itself affected the pH, but the difference between the curves shows that alkalinization of the external medium is caused only by the sodium gradient.)

Na^+ movement in association with Cl^-

Although the data presented are consistent with a Na^+/H^+ antiport, another possibility to consider is a Na^+/Cl^- symport mechanism. (It needs to borne in mind that the only ions present in the incubation medium with vesicles are Na^+, Cl^-, OH^-, and H^+, in addition to the organic complex ions used as buffers). A Na^+/Cl^- symport mechanism does not appear likely a priori, since it would not be expected to depend on $\Delta\tilde{\mu}_{H^+}$. Nevertheless we examined whether the rate and extent of Cl^- uptake was consistent with such a mechanism.

As with Na^+ influx, an influx of Cl^- could be observed only for a short time (later, after the particles became partially loaded with Cl^-, only an efflux could be detected). We followed the influx of Cl^- in the light and in the dark, using the procedure reported in Fig. 4 for Na^+. The kinetics obtained were very similar to those in Fig. 4. There was no light-induced influx of Cl^-.

The initial effluxes of Na^+ and Cl^- obtained in a separate paired experiment were 9.1 and 3.2 $\mu mol \cdot h^{-1} \cdot mg^{-1}$, respectively (no influx or efflux was observed in the dark). The fact that the initial rate of the Na^+ loss is about three times faster than that of the Cl^- does not favour a Na^+/Cl^- symport mechanism. The efflux of Cl^- is probably a passive transport driven by the electrical gradient across the membrane.

Discussion

The data presented in this paper support the idea firstly suggested by Lanyi et al. [10] that in *H. halobium* the mechanism operating to maintain low cellular Na^+ levels is a Na^+/H^+ antiport. The energy source for this process in organisms grown at low O_2 tension appears to be the $\Delta\tilde{\mu}_{H^+}$ established by the operation of the light-dependent proton pump. This conclusion is reached for the following reasons, based largely on experiments with vesicles prepared from purple cells. (1) The rate of Na^+ loss, like the rate of proton influx, is a function of light intensity (Table I). (2) Na^+ efflux in absence of illumination can be induced by acidification of the medium, whereas Na^+ uptake occurs upon its

alkalinization (Fig. 8). Thus the direction of Na^+ transport is dependent on the direction of the pH gradient in absence of illumination. (3) ATP does not appear to be involved in Na^+ efflux, since inhibitors of ATP synthesis do not abolish Na^+ efflux (Fig. 3 and Table I) and loading the vesicles with ADP plus P_i does not stimulate Na^+ efflux. (4) Uncouplers which destroy or decrease the $\Delta\tilde{\mu}_{H^+}$ also reduce Na^+ efflux (Fig. 3 and Table I). (5) Imposition of a Na^+ gradient causes a movement of protons in a direction opposite to the direction of the Na^+ gradient (Fig. 9). (6) Upon illumination of the purple vesicles, a period of rapid acidification of the medium is followed by a slower realkalinization period in Na^+ media only (Fig. 6). This alkalinization and the Na^+ efflux are similarly affected by uncouplers (Fig. 5), ATPase inhibitors (Fig. 7) and variations in light intensity. Such a result is consistent with a Na^+/H^+ exchange process. A simple outline of the mechanism of operation of such a Na^+/H^+ exchange was presented above. Similar models for Na^+ transport have been suggested for *E. coli* [23,25], *Streptococcus faecalis* [12,26,27], and *H. halobium* [10].

The observations that ATPase inhibitors such as arsenate and DCCD either stimulate or are without effect on Na^+ efflux indicate that both the proton leak through the ATPase (or ATP synthesis) and Na^+ influx derive their energy from the proton electrochemical gradient. At a low rate of proton pumping (i.e. low light intensity) the proton leak through the ATPase (or the proton uptake due to phosphorylation) is sufficient to inhibit Na^+ efflux, while at a high rate of pumping there is no observable inhibitory effect.

The dependence of the proton pump on light intensity needs to be considered when examining the effects of ATPase inhibitors and uncouplers on Na^+ efflux and on H^+ influx. For example, with very high light intensities (2000 W/m^2) alkalinization of the medium following acidification is not observed, in contrast to the effect shown in Fig. 5 at 1250 W/m^2. Presumably H^+ pumping is so rapid that it obliterates the slower reaction of Na^+/H^+ exchange.

Based on the partial inhibition of Na^+ efflux by $TPMP^+$, Lanyi et al. [10] suggested that the Na^+/H^+ exchange mechanism is electrogenic. $TPMP^+$ is a permeable cation [28] and in its presence $\Delta\psi$ decreases [22]. In their experiment Lanyi et al. [10] used a high concentration of $TPMP^+$ (10 mM) which, according to our observations, might alter the membrane properties besides abolishing the membrane potential. Furthermore, Renthal and Lanyi [22] themselves claimed that 0.7 mM $TPMP^+$ is enough to abolish the membrane potential. On repeating this experiment with 10 mM $TPMP^+$ we found that the $TPMP^+$ decreased the $^{22}Na^+$ counts by half even in the dark before the illumination, indicating the presence of a leaky membrane. This effect was not observed when we used a concentration of 1 mM $TPMP^+$. With this concentration of $TPMP^+$ we again observed an inhibition of Na^+ efflux, but this inhibition occurred only after 10 min of efflux. Before this no inhibition by $TPMP^+$ was observed. Thus we feel that the results with $TPMP^+$ are equivocal. Whether removal of a potential difference eventually diminishes Na^+ transport, or whether $TPMP^+$ has some other deleterious effect, remains to be established.

Although subject to severe uncertainties an attempt can be made to estimate the maximum and minimum rates of H^+ influx (and hence Na^+ efflux) by the antiport system. From the upper curve in Fig. 5, the initial rate of acidification

is 8 μmol $H^+ \cdot h^{-1} \cdot (mg\ protein)^{-1}$. Since this represents the net value, which includes also the back flow of protons of 4 μmol $H^+ \cdot h^{-1} \cdot (mg\ protein)^{-1}$ (taken from the initial rate of alkalinization in Fig. 5) the overall upper limit of proton influx is 12 μmol $H^+ \cdot h^{-1} \cdot (mg\ protein)^{-1}$. Considering there is probably some component leak, this must represent an upper limit. The lower range of H^+/Na^+ exchange can be calculated from the rate of alkalinization upon removal of illumination; a value of 5 μmol $H^+ \cdot h^{-1} \cdot (mg\ protein)^{-1}$ is obtained. This value represents a lower limit since the gradient will decrease when illumination ceases. Thus despite uncertainties we can place Na^+/H^+ exchange between 12 and 5 $\mu mol \cdot h^{-1} \cdot (mg\ protein)^{-1}$ from computation of the H^+ influx. In comparison with values for H^+ influx, estimates of the rate of Na^+ loss have yielded an average value of 7 $\mu mol \cdot h^{-1} \cdot (mg\ protein)^{-1}$. These data taken together suggest that the stoichiometry of the exchange process may be 1 : 1. However, in view of the uncertainties involved in the computation of the H^+ influx, it would be premature to accept this value with certainty. A higher stoichiometric ratio (as suggested by Lanyi et al. [10]) is not excluded.

Other types of cation transport systems have been reported in various bacteria. The Na^+/Na^+ self-exchange mechanism reported by Harold and coworkers [29] for *S. faecalis* cannot account for the present observations nor is it likely to result in a net loss of Na^+. The Na^+/K^+ exchange mechanism described by Schultz and Solomon [30] in *E. coli* is also eliminated because Na^+ loss in our preparation occurs in the absence of K^+. Nor do our data support an ATP driven K^+/Na^+ exchange mechanism as was suggested for *S. faecalis* [31,32] since neither ATP nor K^+ appear to be required for net Na^+ efflux. The possibility of a neutral NaCl efflux mechanism is also unlikely because Cl^- loss is too slow to accompany Na^+ loss and such a mechanism would not be expected to be dependent on the $\Delta\tilde{\mu}_{H^+}$.

In conclusion, the observations reported in this communication provide the basis for a suggested exchange mechanism whereby *H. halobium* maintains its low cellular Na^+ in face of its milieu of 4 M Na^+, and are entirely in line with the chemiosmotic hypothesis [33].

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