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Light-Induced Glutamate Transport in *Halobacterium halobium* Envelope Vesicles. II. Evidence That the Driving Force Is a Light-Dependent Sodium Gradient†

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ABSTRACT: Illumination of cell envelope vesicles from *H. halobium* causes the development of protonmotive force and energizes the uphill transport of glutamate. Although the uncoupler, *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone (FCCP), and the membrane-permeant cation, triphenylmethylphosphonium ($TPMP^+$), are inhibitory to the effect of light, the time course and kinetics of the production of the energized state for transport, and its rate of decay after illumination, are inconsistent with the idea that glutamate accumulation is driven directly by the protonmotive force. Similarities between the light-induced transport and the Na^+-gradient-induced transport of glutamate in these vesicles suggest that the energized state for the amino acid uptake in both cases consists of a transmembrane Na^+ gradient ($Na^+_{out}/Na^+_{in} \gg 1$). Rapid efflux of ^{22}Na from the envelope vesicles is induced by illumination. FCCP and $TPMP^+$ inhibit the light-induced efflux of Na^+ but accelerate the post-illumination relaxation of the Na^+ gradient created, suggesting electrogenic antiport of Na^+ with another cation, or electrogenic symport with an anion. The light-induced protonmotive force in the *H. halobium* cell envelope vesicles is thus coupled to Na^+ efflux and thereby indirectly to glutamate uptake as well.

The uphill transport of metabolites into cells and organelles is an energy-requiring process, but the mechanism of coupling energy production to transport is not well understood. According to the chemiosmotic theory of membrane transport (Mitchell, 1970), the energy for transport may be derived in many systems from ionic gradients. In these cases the uphill movement of the substrates being transported is coupled to the relaxation of the ionic gradients in such a way that the energy of the entire system decreases (Mitchell, 1969). It is postulated that this is accomplished by "symport" or "antiport" of the substrates and ions in question; in the former case, both substrate and ion move in the same direction and, in the latter, in the opposite direction across the membrane. The concept of symport and antiport contains the implicit assumption of the obligatory dependence of these movements on one another, and of the existence of specific membrane components which facilitate the translocations. In several microbial systems, symport of amino acids and sugars with H^+ has been demonstrated (Pavlasova and Harold, 1969; West, 1970; Eddy and Novacki, 1971). Since the movements of H^+ across the membrane are originally brought about by the functioning of the respiratory chain (Scholes and Mitchell, 1970; West and Mitchell, 1972; Griniuviene et al., 1974; Lawford and Haddock, 1974), the H^+ gradient thus serves as the means of coupling between the energy-yielding oxidative processes and substrate transport. In other cases substrate movements were found to depend on antiport with phosphate or other anions (Mitchell, 1959; Chappel and Haarhoff, 1967; Meyer and Tager, 1969; LaNoue and Tischler, 1974), the transport being driven by all those processes which give rise to the anion gradients.

Living cells generally contain lower Na^+ concentrations and higher K^+ concentrations than their surroundings and it has been proposed that these gradients are maintained in eucaryotic cells by a Na^+-K^+ exchange ATPase (Skou, 1965; Whittam and Wheeler, 1970), and in procaryotes by preferential Na^+-H^+ antiport (West and Mitchell, 1974). The observation that Na^+ is required for the transport of a number of substrates has given rise to the idea that some substrates may be symported with Na^+, rather than with H^+ (for example, Crane, 1965; Eddy, 1968; Stock and Roseman, 1971; Halpern et al., 1973). If so, the electrochemical potential inherent in the Na^+ gradients could provide the energy required for uphill substrate transport, making such transport energetically coupled to any process which created the Na^+ gradients.

In its simplest concept, this model requires that, whenever the substrate transported is uncharged or carries no net charge, its translocation with Na^+ necessarily results in the transfer of one or more positive charges across the membrane. It should therefore be possible to effect the transport of this class of substrates with either an electrical potential or with a difference in Na^+ concentration on the two sides

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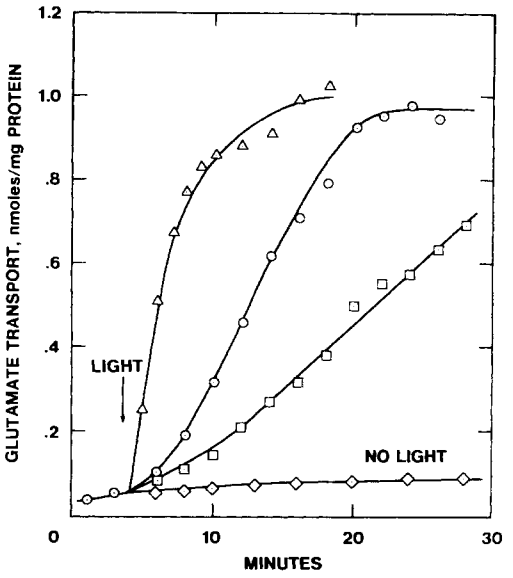

FIGURE 1: Light-intensity dependence of glutamate transport in *H. halobium* envelope vesicles. Vesicles were loaded with 3 M KCl and suspended in 3 M NaCl containing [^{3}H]glutamate at zero times, and the light was turned on at 4 min. Maximal light energy (100%) at the sample chamber was 8 × 10^5 ergs/s; the area illuminated was approximately 1 cm^2; lowered intensities were obtained with neutral density filters. Relative light-intensities: (△) 100%; (⊙) 2.5%; (□) 1%; (◇) 0%.

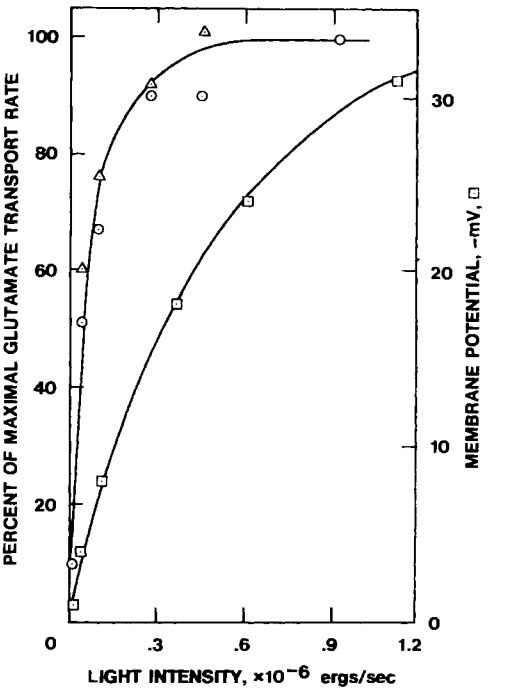

FIGURE 2: Light-intensity dependence of membrane potential, light-induced glutamate transport and post-illumination glutamate transport. Experimental conditions were similar to those in Figure 1. Values for steady-state membrane potentials during illumination were obtained from fluorescence changes in a cyanine dye, as described elsewhere (Renthal and Lanyi, 1976). Light-dependent transport rates were calculated from the linear part of the uptake curves (such as shown in Figure 1), disregarding the initial lag periods at low light intensities. For post-illumination transport, the vesicles were illuminated for 10 min, followed by a 1-min dark period, after which the [^{3}H]glutamate was added. Symbols: (⊙) light-induced glutamate transport; (△) post-illumination glutamate transport; (□) membrane potential.

of the membrane. We have reported evidence consistent with this model for leucine transport in *Halobacterium halobium* cell envelope vesicles (MacDonald and Lanyi, 1975), confirmed recently by Hubbard et al. (1976) in whole cells, in which the accumulation of the substrate appears to be driven by the electrical potential (interior negative) developed by the functioning of bacteriorhodopsin as a light-induced proton pump (Oesterhelt and Stoeckenius, 1973; Stoeckenius and Lozier, 1974; Bogomolni and Stoeckenius, 1974). Alternatively, transport in this system can be driven by an artificial Na^+ gradient. When the substrate is transported as an anion, however, symport with Na^+ could involve a 1:1 stoichiometry of charges and would, thus, result in an electrically neutral translocation. One might expect that in this case the uphill movement of the substrate could be brought about only by a Na^+ gradient across the membrane, and not directly by membrane potential. As described in the preceding paper (Lanyi et al., 1976), the Na^+-gradient-dependent glutamate transport in *H. halobium* envelope vesicles appears to fit this model of electrically neutral translocation, and yet the accumulation of this substrate during illumination depends on the electrical potential (interior negative) developed. The involvement of the electrical potential during the light-induced transport may, of course, be indirect. For example, Kaback and coworkers have recently proposed that in *Escherichia coli* the accessibility of the galactoside transport carrier binding-site toward its substrates is greatly increased in the presence of a transmembrane electrical potential (Reeves et al., 1973; Schuldiner et al., 1975). In *H. halobium*, however, we find that the kinetic parameters measured (K_T and V_{max}) are very similar for light-dependent and for Na^+-gradient-dependent glutamate transport (Lanyi et al., 1976), even though only the former depends on the presence of an electrical potential. On the other hand, glutamate translocation could be coupled to electrical potential if the potential were to cause the formation and maintenance of a Na^+ gradient, which in turn could drive the transport of glutamate. We will examine this second possibility in some detail in this report.

The *H. halobium* envelope vesicles are metabolically inert; hence glutamate transport is initiated entirely by illumination and the subsequent ionic gradients generated. Since the energization by illumination may be terminated abruptly, we are able to temporarily separate the energization and the translocation phases of transport. In discussing the results, we make use of the terms, "energized state", and "driving force". These refer not only to the primary source of energy, i.e., the light-dependent translocation of protons, but also to the mechanism by which this process is coupled to glutamate transport. No a priori assumptions are made about the nature of the energized state or the driving force for transport, except that the latter has a "magnitude", as reflected in varying rates of glutamate transport.

Materials and Methods

Procedures for growing *H. halobium* cells and the preparation and loading of the cell envelope vesicles have been described in previous reports (MacDonald and Lanyi, 1975; Lanyi et al., 1976).

Transport assays with [^{3}H]glutamate were carried out as in the preceding paper (Lanyi et al., 1976). Na^+ fluxes were also determined under the usual transport assay conditions, but in the presence of 10 μCi/ml of ^{22}Na. The amounts of vesicles were about 20 times those used in the glutamate transport assays (1 mg/ml of membrane pro-

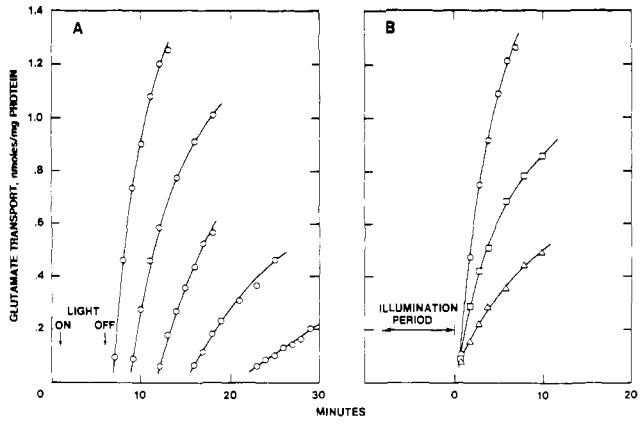

FIGURE 3: Post-illumination transport of glutamate. Vesicles were loaded with 3 M KCl, suspended in 3 M NaCl at zero time. (A) Post-illumination transport and the decay of the driving force in the dark. Illumination was for 5 min, followed by incubation in the dark for 1, 3, 6, 10, and 17 min, and addition of [^{3}H]glutamate, in separate experiments. (B) Dependence of post-illumination transport on the length of prior illumination. Illumination was for (△) 0.5 min; (□) 1 min; and (⊙) 5 min, followed by incubation in the dark for 1 min and addition of [^{3}H]glutamate.

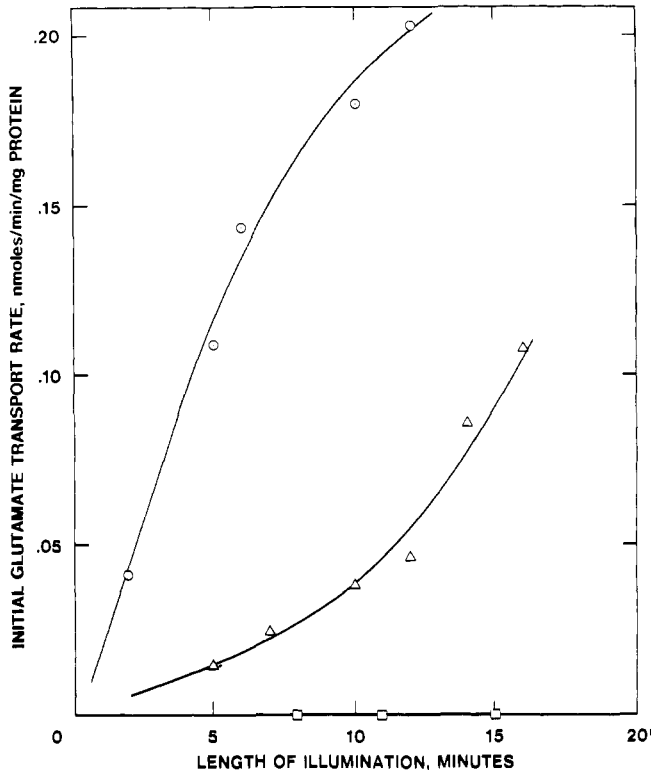

FIGURE 4: Dependence of post-illumination glutamate transport on conditions during illumination. Vesicles were loaded with 3 M KCl, suspended in 3 M NaCl, and were illuminated for the indicated periods of time. The suspensions were incubated in the dark for 1 min, [^{3}H]glutamate was added, and the uptake rates were determined. Symbols: (⊙) light-intensity 8×10^5 ergs/s; (△) 2.6×10^4 ergs/s; (□) 8×10^5 ergs/s, in the presence of 4×10^{-3} M TPMP$^+$.

tein). One-tenth-milliliter aliquots of the reaction mixture were added to 1 ml of 3.5 M NaCl at room temperature on Millipore filter discs pre-soaked in 3.5 M NaCl, and the disks were washed 3 times with 1 ml of 3.5 M NaCl. This procedure represents a compromise between: (a) minimizing the background radioactivity, due either to ^{22}Na tightly bound to the filter discs and the vesicles, or carried over because of insufficient dilution, and (b) maximizing the exchangeable radioactivity retained inside the vesicles, by avoiding excessive washing. Assuming 3 μl/mg of protein for accessible vesicle volume (MacDonald and Lanyi, 1975), ca. 50% of the expected radioactivity at equilibrium is retained.

^{22}NaCl (in H_2O solution, carrier-free) was purchased from New England Nuclear Co.

Results

Light-Intensity Dependence of Glutamate Transport. The accumulation of [^{3}H]glutamate in *H. halobium* envelope vesicles was followed at different light intensities. The time course of uptake, shown in Figure 1, indicates that at decreased light intensities glutamate transport proceeds with increased lags and somewhat decreased rates. Transport is not proportional to the incident light energy: after the initial lag period, the uptake rates are unexpectedly high at low light intensities. These post-lag transport rates are plotted vs. light intensity in Figure 2, together with the membrane potentials (interior negative) developed under these conditions. The latter were estimated from fluorescence changes of the cyanine dye, 3,3-dipentyloxadicarbocyanine (Sims et al., 1974; Renthal and Lanyi, 1976). The light-intensity dependence of the membrane potential is similar to the light dependence of the pH gradients developed (MacDonald and Lanyi, 1975), both effects showing half-saturation near $3-4 \times 10^5$ ergs/s. High rates of glutamate transport were observed at light intensities much below this value (Figure 2), although only after increasing lag periods.

As discussed in the preceding paper (Lanyi et al., 1976), the driving force for light-dependent glutamate transport originates from the electrical potential developed during illumination. Unlike the case of leucine, reported in a previous study (MacDonald and Lanyi, 1975), however, glutamate transport appears not to be a *direct* consequence of the light-induced membrane potential. Rather, transport appears to be energized in this case by a process which is cumulative in time.

Post-Illumination Transport of Glutamate. Since the energization of the envelope vesicles can be accomplished even at very low light intensities, after a lag followed by increasing rates of glutamate accumulation (Figure 1), it follows that partially energized states must persist for measurable lengths of time and are cumulative. We tested, therefore, the possibility that the vesicles retain the driving force for glutamate transport *after* illumination. In these experiments, the vesicles were illuminated for 5 min and [^{3}H]glutamate was added after increasingly long incubation times in the dark. As shown in Figure 3A, the vesicles were able to accumulate glutamate for 5–10 min after the light was turned off. In a similar manner, the kinetics of the establishment of the dark-persistent driving force was determined. Vesicles were illuminated for various lengths of time (Figure 3B) and, after 1 min of incubation in the dark, glutamate transport was initiated. The data in Figure 3B indicate that the magnitude of the driving force for transport increases over several minutes of illumination.

The kinetics of the generation of driving force in these vesicles, which gives rise to post-illumination transport, was examined further in Figure 4. As in the previous graph, vesicles were illuminated for increasing lengths of time and, after 1 min of incubation in the dark, glutamate transport was followed. The slopes of the transport curves (as in Fig-

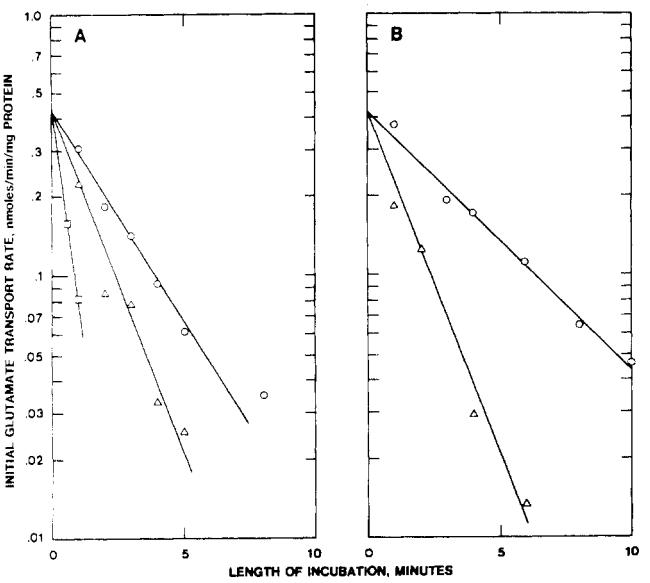

FIGURE 5: Time-dependent decrease of the post-illumination driving force for glutamate transport. Vesicles were loaded with 3 M KCl, suspended in 3 M NaCl, and were illuminated for 10 min. When inhibitors were present, they were added when the light was turned off. Incubation in the dark was continued for up to 10 min. In separate experiments, [^{3}H]glutamate was added at the indicated times for determining the initial transport rates. (A) Decay of the driving force in the presence of FCCP: (⊙) ethanol, 0.5% (v/v), equivalent to that added with FCCP; (△) 3×10^{-6} M FCCP; (□) 9×10^{-6} M FCCP. (B) Decay of the driving force in the presence of TPMP$^+$: (⊙) no addition; (△) 2×10^{-3} M TPMP$^+$.

ure 3B) yielded initial rates, shown plotted vs. the length of illumination in Figure 4. At maximal light intensities, the vesicles can be fully energized for transport by 5–15 min of illumination, depending on the vesicle preparation used. Since we infer the existence of the driving force only through the ability of the vesicles to accumulate glutamate in the dark, it is not clear whether the apparent saturation after this period of illumination is due to saturation of the driving force or to the limited capacity of the glutamate translocation system. At very low light intensities (Figure 4), the capability of the vesicles for post-illumination glutamate transport increases more slowly and shows a lag period, similar to the kinetics of glutamate transport *during* illumination (Figure 1). TPMP$^+$, a membrane-permeant cation[1] (Skulachev, 1971), abolishes the effect of light in causing post-illumination transport (Figure 4), as in the case of light-induced glutamate transport (Lanyi et al., 1976). Post-illumination transport was also tested at various light intensities, after illumination for 10 min. As seen in Figure 2, the light-saturating characteristics of post-illumination transport and of transport during illumination are about the same. Thus, glutamate transport in the dark, dependent on previous illumination, and glutamate transport during illumination show a number of similarities, suggesting that they reflect the properties of a single energy coupling system.

The kinetics of the dark decay of the light-induced driving force was studied in the presence of various inhibitors of transport. Vesicles were illuminated for 5 min, the light was turned off, and the inhibitor was added. The vesicles were then incubated in the dark for up to 10 min, and transport was initiated at various times in separate experiments, by adding [^{3}H]glutamate. The initial rates of the post-illumination transport were determined from the uptake data obtained (similar to those in Figure 3A) and are shown, plotted vs. the length of post-illumination incubation, in Figure 5. The dark decay of glutamate transporting ability appears to be exponential within the accuracy of the method (Figure 5). The half-life for the process is 1.5–3.5 min, depending on the vesicle preparation. When the vesicles are incubated at 0 °C rather than at 30 °C after illumination, post-illumination transport (tested after rewarming) persists for much longer periods of time, with a half-life of over 30 min. The presence of the uncoupler FCCP (Figure 5A) or TPMP$^+$ (Figure 5B), at concentrations similar to those inhibitory to light-dependent glutamate transport (Lanyi et al., 1976), results in steeper decay curves. The curves in Figure 5 extrapolate to the same value at zero time of incubation (the time when the inhibitors were added). Lack of inhibition at zero time may reflect a time dependence for the action of FCCP and TPMP$^+$. These agents do not show a lag for inhibition, however, in accordance with the previous observation that they are inhibitory to transport at very short times after addition (Lanyi et al., 1976). Hence, the kinetic data suggest that the decay of the post-illumination driving force is more rapid, but transport itself is not inhibited, in the presence of FCCP or TPMP$^+$.

Light-dependent glutamate transport is affected by the pH, although not by buffering per se (Lanyi et al., 1976), the latter result suggesting that light-dependent pH gradients across the membrane are not obligatory for transport. Post-illumination glutamate transport was examined in vesicles buffered inside and outside with phosphate or arsenate at various pH values. Table I compares the rates of light-dependent glutamate transport, the rates of the formation of post-illumination driving force (obtained from the initial slopes of curves plotting post-illumination transport rate vs. length of illumination, such as Figure 4), and the half-lives for the decay of the post-illumination driving force (obtained from data similar to those in Figure 5). Lowering the pH in either buffer system (inside and outside the vesicles) decreases both the rate of glutamate accumulation and the rate of the development of the post-illumination driving force and accelerates the decay of the energized state. Near pH 7, however, little effect of the buffer is observed, even at high buffering capacities (0.1 M phosphate), indicating that the post-illumination driving force does not require the presence of pH differences across the membranes. No specific effect of phosphate vs. arsenate is evident in Table I.

It is very unlikely that post-illumination glutamate transport is due to the persistence of any ATP synthesized in the vesicles during illumination. Attempts to detect ATP in these vesicles, before and during illumination, were unsuccessful even though the detection limit for ATP was less than 10 pmol/mg of protein, which is far below the amount of glutamate transported (unpublished experiments).

Kinetics of Light-Dependent pH Gradients and Electrical Potentials in Envelope Vesicles. The time course of the development of the light-induced energized state for transport and its persistence in the dark after illumination (Figure 3) raises the question of the kinetics of the development and relaxation of the protonmotive force itself, under these conditions. Techniques for measuring the chemical (pH difference) and electrical (membrane potential) components of the light-induced protomotive force in these vesicles have been developed (Renthal and Lanyi, 1976): the former can

[1] Abbreviations used: TPMP$^+$, triphenylmethylphosphonium cation; FCCP, *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone.

Table I: Effect of Buffering[a] and pH on the Energization of Glutamate Transport.

Buffer	Concn (M)	pH	Rate of Transport (%)	Rate of Formation[b] (%)	Half-Life[c] (min)
None		6.5–7.0	100	100	2.4
Phosphate	0.01	5.0	24		0.8
		5.5	53	25	1.8
		6.0	71	53	5.5
		7.0	100	100	5.6
	0.1	7.0	99	94	4.6
Arsenate	0.01	5.0	33		
		6.0	72	53	2.0
		7.0	83	98	5.4

[a]Buffer present both inside and outside the vesicles. [b]Rate of formation of post-illumination driving force (%). [c]Half-life of post-illumination driving force (min).

be measured in the external medium with a fast pH electrode, the latter by monitoring fluorescence changes of a cyanine dye (Sims et al., 1974). The fluorescence changes were calibrated with K^+ diffusion potentials of known magnitude, established by the addition of valinomycin to vesicle suspensions (Renthal and Lanyi, 1976). Fluorescence and pH traces, obtained for unbuffered and buffered (0.01 M phosphate, pH 7) vesicles, containing 3 M KCl and suspended in 3 M NaCl, are shown in Figure 6. The traces indicate that, while buffering prevents the development of a pH difference across the membrane during illumination, it increases rather than decreases the electrical potential attained. The development of both pH change and membrane potential is rapid after the light is turned on (half-rise times in Figure 6 are 20 and 1–2 s, respectively), and the relaxation of these gradients after the light is turned off is similarly fast. The kinetics of the changes in pH and electrical gradients are, therefore, not consistent with either the development or the decay of the energized state for glutamate transport, both of which exhibit half-rise times of several minutes (Figures 3–5). This result also suggests that it is very unlikely that the existence of protonmotive force constitutes, in itself, the energized state for glutamate transport in the envelope vesicles.

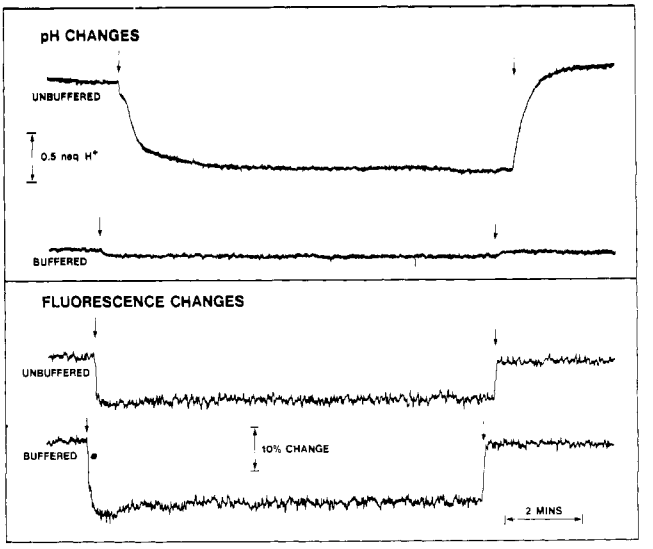

FIGURE 6: Traces of pH changes and the fluorescence of a membrane-potential indicating dye during illumination of *H. halobium* envelope vesicles. The membrane potential was followed by measuring the fluorescence changes of a cyanine dye (Sims et al., 1974). The procedure for pH and fluorescence determinations has been described elsewhere (Renthal and Lanyi, 1976). Vesicles were loaded with 3 M KCl, with or without 1×10^{-2} M phosphate, pH 7.0, and were suspended in 3 M NaCl, with or without buffer. Arrows indicate when the light was turned on and off. The small apparent decrease in pH with buffered vesicles was also observed for buffered NaCl without vesicles; hence it is ascribed to an electrode light response.

Kinetics of Na^+-Gradient-Dependent Glutamate Transport. The transport of glutamate observed in the dark after a period illumination (Figure 3) resembles the Na^+-gradient-dependent transport of this amino acid, which will take place in the dark (Lanyi et al., 1976). In the latter case the vesicles must contain KCl with little or no NaCl, in order to obtain high outside/inside Na^+ gradients on suspension in NaCl solutions. Incubation of these vesicles in the dark, before addition of [^{3}H]glutamate, should result in the decay of the Na^+ gradient and a corresponding lowering of the transport rates. Vesicles were, therefore, suspended at high Na^+ gradients ($Na^+_{out}/Na^+_{in} > 500$) and were incubated in the dark for various lengths of time before initiating the transport assays. The time course of glutamate accumulation under these conditions is shown in Figure 7A, where it can indeed be seen that the glutamate-transporting ability of the vesicles declines considerably within 10 min of incubation. The initial rates of transport, obtained from the curves in Figure 7A, are plotted vs. the length of incubation before adding [^{3}H]glutamate in Figure 7B. The transport rates decline exponentially, with a half-life of 1.6 min. The vesicle preparation used in these experiments was tested also for post-illumination transport of glutamate (as in Figure 5) and the half-life of the post-illumination energized state is similar, 1.7 min. Further similarities between the decay of the Na^+-gradient-dependent transporting ability and the decay of post-illumination transporting ability of the vesicles are found in the effect of FCCP and $TPMP^+$ on these processes. As seen in Figure 7B, both of these agents accelerate the rate of decay for the Na^+-gradient-dependent transport, as they do for post-illumination transport (Figure 5), but appear not to inhibit glutamate transport itself. Thus, the driving force for glutamate transport decays at the same rate, whether derived from a Na^+ gradient or from previous illumination and this rate is increased in both cases in the presence of FCCP or $TPMP^+$.

The post-illumination driving force for the transport of glutamate decayed very rapidly (<1 min) in the presence of 1×10^{-8} M gramicidin (not shown), similar to the Na^+-gradient-induced transport (Lanyi et al., 1976).

Light-Induced Na^+ Fluxes in H. halobium Envelope Vesicles. The similarity of glutamate transport, energized by illumination, to that caused by artificially provided Na^+ gradients suggested that the source of energy for transport

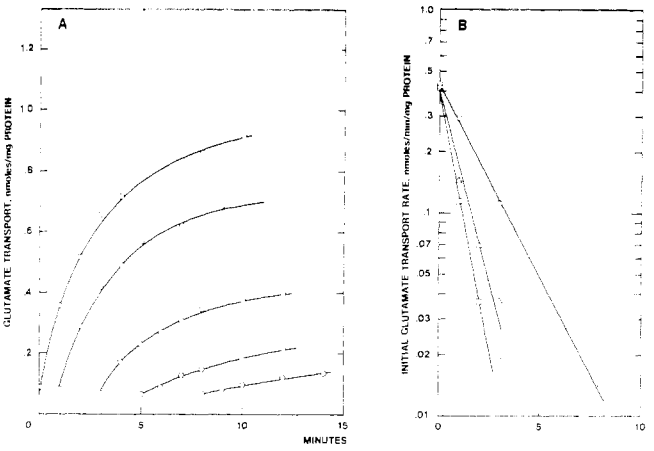

FIGURE 7: Time-dependent decrease of the Na^+-gradient-dependent driving force for glutamate transport. (A) Time course of the decay of the driving force during incubation in the dark. Vesicles were loaded with 3 M KCl by the injection method, described in the preceding paper (Lanyi et al., 1976), and were suspended at zero time in 3 M NaCl ($Na^+_{out}/Na^+_{in} > 500$). In separate experiments [^{3}H]glutamate was added at 0, 1, 3, 5, and 8 min of incubation and the time course of glutamate uptake was followed. (B) Decay of the driving force in the presence of FCCP or $TPMP^+$. Experimental conditions as in A, but in the presence of inhibitors, added at zero time. Symbols: (⊙) no addition; (△) 2×10^{-3} M $TPMP^+$; (□) 1×10^{-6} M FCCP. The latter inhibitor was added to the vesicles in ethanol, giving a final ethanol concentration of <0.2% (v/v), which by itself had a negligible effect.

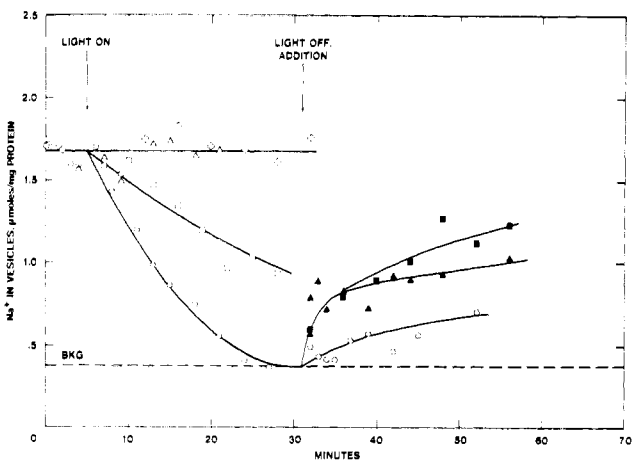

FIGURE 8: Light-induced Na^+ fluxes in *H. halobium* envelope vesicles. Vesicles were loaded with 1 M NaCl–2 M KCl, containing 10 μCi/ml of ^{22}NaCl, and were suspended in 1 M NaCl–2 M KCl at the same specific radioactivity. The transport assay conditions are described under Methods. The background level of radioactivity indicated (BKG) was obtained with the complete assay mixture, but in the presence of 1×10^{-6} M gramicidin, which allowed the removal of all exchangeable ^{22}Na during the NaCl wash of the vesicles. Binding of ^{22}Na to the Millipore filter discs alone accounted for about half the observed background radioactivity. Symbols: (⊙) no inhibitor added; (△) 3×10^{-5} M FCCP added to the vesicles at zero time; (□) 1×10^{-2} M $TPMP^+$ added at zero time; (◇) no inhibitor added, no illumination; (▲), 3×10^{-5} M FCCP added at 31 min, at the end of the illumination; (■) 1×10^{-2} M $TPMP^+$ added at 31 min, at the end of the illumination. The high concentrations of inhibitors used were necessary because these assays were carried out at much higher vesicle protein concentrations than in the glutamate transport experiments.

in the former case might also be a Na^+ gradient, created during illumination. Hence, the movements of Na^+ across the envelope vesicle membranes during and after illumination were examined. In these experiments, more NaCl was used than in those designed for the study of glutamate transport, in order to better follow the time course of the Na^+ fluxes. Vesicles were loaded with 1 M NaCl–2 M KCl, containing ^{22}Na, and were suspended in 1 M NaCl–2 M KCl, containing ^{22}Na at the same specific radioactivity. As expected, Na^+ was in equilibrium across the membranes, and the vesicles could be incubated in the dark indefinitely, without any measurable net flux of Na^+. As shown in Figure 8, however, illumination for 20–25 min causes the progressive loss of exchangeable Na^+ from the vesicles. The accuracy of the method does not allow an estimate of the final Na^+_{out}/Na^+_{in} ratio attained, but its value is undoubtedly high since the vesicle Na^+ concentration appears to decrease asymptotically toward the background value. After the light is turned off, the Na^+ gradient relaxes partially, reaching a Na^+_{out}/Na^+_{in} ratio of 4–5 within 15–20 min. Large changes in the Na^+ gradient, such as required for the appearance and disappearance of glutamate transport (Lanyi et al., 1976), are restricted to the last 4–5 min of illumination in Figure 8, and to the first few minutes after the light is turned off. The correspondence between the Na^+ gradients and glutamate transport holds since, when large amounts of NaCl are included in the vesicles, such as in the experiment in Figure 8, glutamate accumulation proceeds only after a long lag (Lanyi et al., 1976), while the post-illumination energized state for transport decays over several minutes of incubation (Figures 3 and 5). When the envelope vesicles were loaded with KCl only, as for the light-induced glutamate transport experiments, which results in low internal Na^+ concentration (ca. 0.1 M), the light-induced efflux of Na^+ occurs at about the same rate as in Figure 8. Because of their lower Na^+ content, however, the vesicles are depleted of ^{22}Na much more rapidly, within 2–3 min (not shown).

In the presence of FCCP or $TPMP^+$, the light-induced efflux of Na^+ is inhibited and the post-illumination influx of Na^+ is accelerated (Figure 8). These results suggest that transmembrane electrical potentials are involved in the movements of Na^+. The data in Figure 8 are best explained by the electrogenic translocation of Na^+ involving either the movement of another cation in the *opposite* direction to the movement of Na^+ (antiport), or the movement of an anion in the *same* direction (symport), with a stoichiometry greater than 1. Efflux of Na^+ from the vesicles could then be driven by illumination and the consequent development of the electrical component of the protonmotive force (interior negative). Na^+ influx by the reversal of this mechanism, after illumination, would be limited by the negative interior potential developed due to this translocation. Either variation of this model accounts for the results in Figure 8 since, in the presence of FCCP or $TPMP^+$, both the light-induced potential and the Na^+-flux-induced potential after illumination are expected to be abolished, thereby resulting in inhibition of Na^+ efflux during illumination and the enhancement of Na^+ influx after the illumination. These results are analogous to those obtained for the production of the energized state for glutamate transport, which was inhibited by FCCP and by $TPMP^+$, and whose decay was accelerated by these agents.

Discussion

Active glutamate transport in *H. halobium* cell envelope vesicles can be induced, with similar kinetics, by two conditions: (1) a Na^+ gradient, artificially provided, at very high Na^+_{out}/Na^+_{in} ratios, or (2) illumination (Lanyi et al.,

1976). In the latter case, the energized state for transport is acquired over several minutes of exposure to light and appears to be due to a process cumulative in time (Figures 3 and 4) unrelated to the kinetics of the establishment of the protonmotive force under these conditions (Figure 6). Glutamate transport proceeds only when the interior of the vesicles contain KCl and the exterior NaCl; increasing amounts of NaCl inside the vesicles lead to increasing lag in light-induced uptake (Lanyi et al., 1976). The ability of the vesicles to accumulate glutamate, i.e., the "energized state", declines exponentially over several minutes of incubation in the dark, both for Na^+-gradient-induced transport and for light-induced transport and, thus, appears to decay by a much slower process than the relaxation of the pH and the electrical gradients after illumination (Figure 6). The decay of the energized state, derived from either illumination or a Na^+ gradient, is accelerated in the presence of FCCP or $TPMP^+$ (Figures 5 and 7), at concentrations which are inhibitory to light-induced transport. Neither of these agents appears to inhibit the translocation of glutamate per se.

These results, together with the observed movement of Na^+ (Figure 8), strongly suggest that the light-dependent energized state for glutamate transport in *H. halobium* envelope vesicles consists of a Na^+ gradient, established during illumination by Na^+ efflux, and decaying after illumination through Na^+ influx. The existence of this light-dependent Na^+-extrusion mechanism is remarkable since, under these conditions, Na^+ movements must take place against both the chemical and electrical potential of this cation. The effects of FCCP and $TPMP^+$ on the movements of Na^+ (Figure 8) are in accord with their effects on the production and decay of the energized state of the vesicles for glutamate transport (Lanyi et al., 1976; Figure 5). The results are best explained by postulating the electrogenic antiport of Na^+ with another cation, or the electrogenic symport of Na^+ with an anion, such as Cl^- or OH^-. With the present information it is impossible to distinguish these two possibilities, although we are inclined to favor the former.

Our model for the events arising from the illumination of *H. halobium* envelope vesicles is thus summarized as follows. Bacteriorhodopsin, located in the purple membrane patches on the cell envelope (Blaurock and Stoeckenius, 1971), acts as a light-dependent electrogenic H^+ pump (Renthal and Lanyi, 1976), translocating protons from the inside to the outside of the vesicles. If Na^+ is antiported with H^+ ($H^+/Na^+ > 1$), the Na^+ efflux is driven by the protonmotive force generated, and the protons flow in a circular fashion, those returned into the vesicles being re-ejected through the action of light on bacteriorhodopsin. At the same time, K^+ accumulates in the vesicles (Kanner and Racker, 1975; Danon, personal communication), as a result of the electrical potential present (interior negative). As the vesicles are depleted of Na^+, the resulting Na^+ gradient brings about the inward movement of glutamate, through symport of Na^+ with the amino acid (Lanyi et al., 1976). On the other hand, if Na^+ is antiported with K^+ ($K^+/Na^+ > 1$), then Na^+ efflux is driven by electric potential, and the return of H^+ into the vesicles is driven directly by the protonmotive force generated by light. The existence of an electrogenic H^+/Na^+ antiport mechanism is somewhat more attractive since it would explain the rise in extracellular pH, observed under some conditions upon the illumination of intact *H. halobium* cells (Bogomolni and Stoeckenius, 1974; Oesterhelt, 1975). The net effect in either case is the replacement of interior Na^+ with K^+, as in other microbial systems, which depend on respiration or ATP hydrolysis (Schulz and Solomon, 1961; Slayman and Slayman, 1968; Harold et al., 1970). After the light is turned off, the ion movements described above are reversed, but the relaxation of the Na^+ gradient is slow, because Na^+ influx leads to a limiting membrane potential (interior negative), which must be dissipated by the movements of other ions.

Since the intracellular Na^+ concentration in extremely halophilic bacteria, determined by conventional methods, is above 1 M, between $\frac{1}{3}$ and $\frac{1}{4}$ of the extracellular concentration of Na^+ (Christian and Waltho, 1962; Ginzburg et al., 1970; Lanyi and Silverman, 1972), the physiological relevance of a Na^+-gradient-dependent mechanism for amino acid transport in whole cells of *H. halobium* may be questioned. Preliminary results indicate, however, that light-dependent rapid efflux of ^{22}Na, and its subsequent influx in the dark, occurs also in intact *H. halobium* cells. In nonenergized cells (i.e., washed cell suspensions) Na^+ gradients may relax quickly, relative to the time required for the sodium assays; thus, conventional measurements of the intracellular Na^+ concentration are only a reflection of this relaxed state. Moreover, a number of studies have shown that the protein synthesizing apparatus of extremely halophilic bacteria is stable only in low Na^+–high K^+ environments (Bayley and Kushner, 1964; Visentin et al., 1972). The anomaly of this observation is explained if the normal Na^+ concentration in the cells is low.

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Production of High Levels of Phosphorylated F_1 Histone by Zinc Chloride†

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ABSTRACT: Methods have been sought to perturb the level of phosphohistones. $ZnCl_2$ (10 mM) exhibits histone phosphate phosphatase in vivo in HTC cells and leads to hyperphysiological levels of F_1 phosphohistone. Treatment of tissue culture cells with this concentration of $ZnCl_2$ leads to a reduction in medium pH to 6.4. Control experiments have indicated that HTC cells grow efficiently at this pH and that the reduction of pH does not produce the hyperphosphorylated state per se. The optimum conditions for the $ZnCl_2$ effect are described. That the effect of $ZnCl_2$ on the heterogeneity of F_1 histone is due to an effect on phosphorylation was demonstrated by the observation that the entire effect is abolished by treatment with alkaline phosphatase. The site of phosphorylation is in the carboxy-terminal end of the F_1 molecule. The inhibitory effect of $ZnCl_2$ on F_3 phosphorylation in metaphase cells is also described.

Both the rate of F_1 phosphorylation and the amount of the phosphorylated forms of the F_1 histone are much higher in dividing cells than in nondividing cells (Balhorn et al., 1971, 1972a–c). There is a rapid phosphorylation of newly synthesized F_1 histone in S phase coupled with a lower rate of phosphorylation of older F_1 histone (Tanphaichitr et al., 1974; Jackson et al., 1976). Yet another burst of F_1 phosphorylation occurs in metaphase cells occurring to some degree at different sites (Balhorn et al., 1975). The F_3 is also phosphorylated in metaphase cells (Balhorn et al., 1975). Three major proposals have been considered for the function of histone phosphorylation in cell division. These are histone deposition (Tanphaichitr et al., 1974; Dixon et al., 1973), extension of the chromosome prior to replication (Adler et al., 1971, 1972; Stevely and Stocken, 1966, 1968), and condensation of the chromosome in mitosis (Bradbury et al., 1973, 1974; Gurley et al., 1974; Lake, 1973a,b). Recent results (Tanphaichitr et al., 1974; Jackson et al., 1976) provide some support for the first proposal.

On the other hand, a considerable amount of circumstantial evidence has indicated that the processes of chromosome condensation and decondensation are coincidental with the attainment of metaphase F_1 phosphorylation and its subsequent dephosphorylation (Bradbury et al., 1973,

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