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Light-Induced Glutamate Transport in *Halobacterium halobium* Envelope Vesicles. I. Kinetics of the Light-Dependent and the Sodium-Gradient-Dependent Uptake†

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ABSTRACT: During illumination *Halobacterium halobium* cell envelope vesicles accumulate [^{3}H]glutamate by an apparently unidirectional transport system. The driving force for the active transport originates from the light-dependent translocation of protons by bacteriorhodopsin and is due to a transmembrane electrical potential rather than a pH difference. Transport of glutamate against high concentration gradients is also achieved in the dark, with high outside/inside Na^+ gradients. Transport in both cases proceeds with similar kinetics and shows a requirement for Na^+ on the outside and for K^+ on the inside of the vesicles. The unidirectional nature of glutamate transport seems to be due to the low permeability of the membranes to the anionic glutamate, and to the differential cation requirement of the carrier on the two sides of the membrane for substrate translocation. Thus, glutamate gradients can be collapsed in the dark either by lowering the intravesicle pH (with nigericin, or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone plus valinomycin), or by reversing the cation balance across the membranes, i.e., providing NaCl on the inside and KCl on the outside of the vesicles. In contrast to the case of light-dependent glutamate transport, the initial rates of Na^+-gradient-dependent transport are not depressed when an opposing diffusion potential is introduced by adding the membrane-permeant cation, triphenylmethylphosphonium bromide. Therefore, it appears that, although the electrical potential must be the primary source of energy for the light-dependent transport, the translocation step itself is electrically neutral.

The transport of metabolites across cell membranes, against large concentration gradients, can be energized in many species of bacteria by respiration (for recent reviews, see Boos (1974) and Simoni and Postma (1975)). Alternately, in envelope vesicles from photosynthetic bacteria (Hellingwerf et al., 1975) or from extremely halophilic bacteria (MacDonald and Lanyi, 1975), the energy can be supplied by illumination. In many of these cases it has been convincingly argued that the driving force for transport arises from the extrusion of protons from the cells or vesicles, which causes both a pH difference and an electrical potential across the membranes. These gradients, together, create the protonmotive force, postulated by Mitchell (1969) and correspond to what has been called the "energy-rich" state of the membrane. The protonmotive force can originate either directly from electron transport or from the hydrolysis of ATP (Simoni and Postma, 1975). It has been suggested that the transport of metabolites can be coupled to these energy-yielding processes through co-transport or *symport* of the substrates with H^+ (West, 1970; West and Mitchell, 1972, 1973) or with the other cations (for example, Crane, 1965; Schultz and Curran, 1970).

Cell envelope vesicles, prepared from *Halobacterium halobium,* present a very useful system for investigating the energization of amino acid transport (MacDonald and Lanyi, 1975) because a large protonmotive force (Kanner and Racker, 1975; Renthal and Lanyi, 1976) can be achieved by illumination, in the absence of respiratory activity. This effect is due to the action of light on bacteriorhodopsin (Oesterhelt and Stoeckenius, 1971, 1973), found in crystalline arrays (Blaurock and Stoeckenius, 1971; Blaurock, 1975; Henderson, 1975) in regions continuous with the vesicle membrane. The photochemical events which result in the translocation of protons are now understood to involve the cyclic protonation and deprotonation of the chromophore-protein complex (Stoeckenius and Lozier, 1974; Bogomolni and Stoeckenius, 1974). *H. halobium* envelope vesicles containing bacteriorhodopsin can be prepared with unambiguously uniform right-side-out membrane orientation (MacDonald and Lanyi, 1975), although not necessarily under all conditions (Kanner and Racker, 1975). The above properties, and the fact that the composition of the interior contents of the vesicles can be easily

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changed by a loading procedure based on osmotic shock (MacDonald and Lanyi, 1975), has proved a critical advantage in the studies reported in this and the following paper (Lanyi et al., 1976).

Berger and Heppel (1974) have suggested that various amino acids are transported in *Escherichia coli* by one of two types of mechanisms: (1) a respiration- or ATP-hydrolysis-dependent process, sensitive to uncouplers, and therefore probably driven directly by the protonmotive force generated; and (2) an obligately ATP-requiring process which appears to involve another, unknown mode of energy coupling. Light-induced leucine transport in *H. halobium* envelope vesicles belongs in the first of these categories since it is energized by membrane potential, without the necessary involvement of ATP hydrolysis (MacDonald and Lanyi, 1975). This finding was confirmed in whole cells of *H. halobium* as well (Hubbard et al., 1976). We expected that a good representative of a different type of transport would be that of glutamate because, unlike leucine, this amino acid is acidic, and we thought that its transport might belong in the second category because the accumulation of glutamate in *Streptococcus faecalis* was shown to be energized only by ATP hydrolysis (Harold and Spitz, 1975). Since Na^+ stimulation of glutamate transport has been described in *E. coli* (Frank and Hopkins, 1969; Halpern et al., 1973; Miner and Frank, 1974), we were interested also in the cation requirements for the substrate translocation step. In this communication and the following one (Lanyi et al., 1976), we report our findings on the kinetics and the energetics of glutamate transport in *H. halobium* envelope vesicles.

Materials and Methods

Halobacterium halobium strain R-1, adapted to the defined medium described below, was grown similarly to the procedure of Danon and Stoeckenius (1974). The growth medium contained per liter: 250 g of NaCl, 20 g of $MgSO_4 \cdot 7H_2O$, 2 g of KCl, 5 g of NH_4Cl, 0.5 g of sodium citrate, 1 ml of glycerol, 15 g of L-malic acid, 10 g of NaOH, 0.1 g of KH_2PO_4, 14 mg of $CaCl_2 \cdot 7H_2O$; the following amino acids, 430 mg of DL-alanine, 400 mg of L-arginine, 50 mg of L-cystine, 1300 mg of L-glutamic acid, 80 mg of glycine, 220 mg of L-isoleucine, 800 mg of L-leucine, 850 mg of L-lysine, 185 mg of L-methionine, 130 mg of L-phenylalanine, 100 mg of L-proline, 305 mg of L-serine, 250 mg of L-threonine, 200 mg of L-tyrosine, 500 mg of L-valine; the following vitamins (sterilized separately), 1 μg of thiamine, 1 μg of folic acid, 1 μg of biotin; and the following trace metals (sterilized separately), 3 mg of $MnSO_4 \cdot H_2O$, 23 mg of $FeCl_2$, 4.4 mg of $ZnSO_4 \cdot 7H_2O$, 0.05 mg of $CuSO_4 \cdot 5H_2O$. The pH was adjusted to 6.8 and the medium was filtered through Whatman No. 1 filter paper before autoclaving. Starter cultures from slants were inoculated into 50 ml of medium in 250-ml flasks and were grown at 37 °C on a rotary shaker, operated at 150 rpm, for 3–4 days, until logarithmic phase of growth. Twenty-milliliter portions were transferred to 500-ml flasks containing 400 ml of medium and incubation was continued as before for 24 h. The flasks were then placed about 1 ft from several 40-W fluorescent fixtures (GE "Cool White") and were incubated further at 37 °C without shaking. In 3–4 days the cultures became deeply purple and were harvested by centrifugation.

The preparation of the cell envelope vesicles, their handling, and the osmotic shock method of loading them with NaCl-KCl solutions (total concentration 3 M) have been described before (MacDonald and Lanyi, 1975). The properties (sidedness, bacteriorhodopsin content, etc.) used to characterize these vesicles were similar to what they were in the earlier publication. When very low or very precisely determined intravesicle NaCl concentration was desired, a different method of osmotic shock was employed. The vesicles, prepared in a small volume of 4 M NaCl, were rapidly injected into a 500-fold excess volume of 3 M KCl, or NaCl-KCl (total concentration 3 M), and the shocked vesicles, recovered by centrifugation, were resuspended in 3 M KCl or NaCl-KCl.

Transport determinations were carried out as previously described (MacDonald and Lanyi, 1975). With the exception of the efflux experiments with pre-loaded vesicles, 3 M KCl at room temperature instead of cold NaCl was used in the present study for stopping uptake and for washing the vesicles. This change was made to better facilitate the complete termination of amino acid uptake since no transport takes place in KCl. Under these conditions, however, the background uptake of amino acids (at zero time or in the presence of inhibitors) was about ten times as high as previously reported (MacDonald and Lanyi, 1975). Reaction mixtures contained 0.025–0.050 mg/ml of vesicle protein (representing a 1:20 to 1:40 dilution of the stock vesicle suspensions) and 1 μCi/ml of [^{3}H]glutamate of 16.3 Ci/mmol specific activity (6×10^{-8} M) or 0.1 μCi/ml of [^{3}H]leucine of 1.0 Ci/mmol specific activity (1×10^{-7} M), unless otherwise stated. When buffers were added, the pH given refers to the pH of the stock buffers, prepared as 1 M solutions, determined with a Corning Triple Purpose electrode.

Possible chemical modification of glutamate during or after uptake was tested as follows. Ten to twenty times the usual amount of vesicles was incubated, with illumination, in 3 M NaCl in the presence of either 0.5 μCi/ml of [^{14}C]glutamate or 20 nmol/ml of unlabeled glutamate. The vesicles were collected either on a Millipore filter disc, similarly to the transport assays, or were separated by centrifugation. The transported substrate was released by incubation with a large volume of distilled water, which fragments the membranes. For autoradiography, authentic L-glutamate was added to the ^{14}C label containing samples and the solution containing the broken vesicles was passed through a small AG2 X8 (Bio-Rad) anion exchange column (Brenner et al., 1965). Essentially all the radioactivity was eluted with 1 N acetic acid and the material was lyophilized. The samples and [^{14}C]glutamate standards were spotted on cellulose TLC plates (Q2 precoated, Quantum Industries) and were run, in separate experiments, with one of the following solvent systems: pyridine–butanol–water (1:1:1); butanol–acetic acid–water (4:1:1); or ethanol–NH_4OH–water (80:4:16). Autoradiographs were made by exposing Kodak x-ray films for 1 week, after which the chromatograms were sprayed with ninhydrin reagent to visualize the standards. Migration of the standards was somewhat retarded in the presence of the vesicle extracts, probably because of the presence of residual salt. In the standards (but not in the samples recovered from the vesicles) a few percent of the radioactivity was found in spots not coincident with the ninhydrin-positive major spot, owing to slow breakdown of glutamate on storage. The samples containing the unlabeled glutamate were centrifuged at 140 000*g* for 3 h to remove vesicle fragments and the supernatant was lyophilized. After dissolution in 0.075 M sodium citrate buffer, pH 3.25, the amino acid composition was determined in a Beckman Model 120C amino acid analyzer, using AA15 resin.

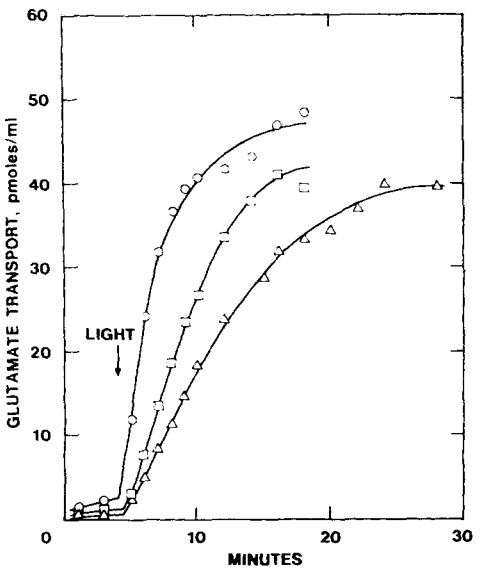

FIGURE 1: Light-induced glutamate transport at different *H. halobium* envelope vesicle concentrations. Vesicles, loaded with 3 M KCl, were suspended in 3 M NaCl containing [^{3}H]glutamate at zero time at the following protein concentrations: (⊙) 0.050 mg/ml; (□) 0.020 mg/ml; (△) 0.010 mg/ml. The maximal level of uptake corresponds to about 75% of the total amount of glutamate added.

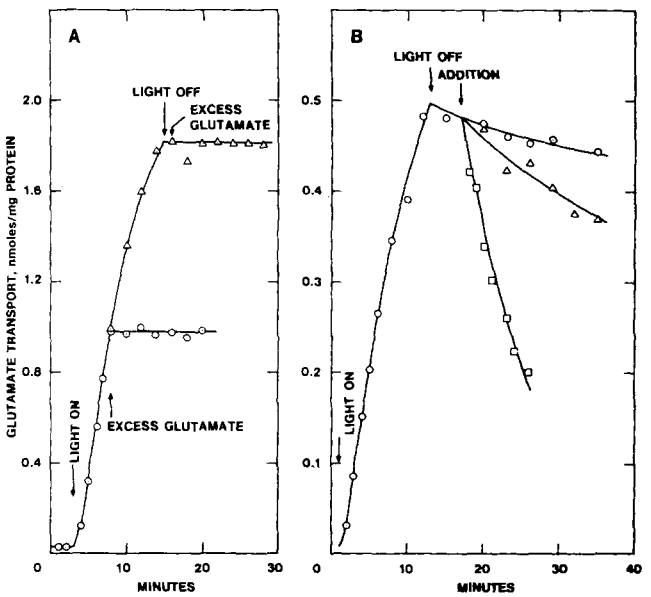

FIGURE 2: Glutamate efflux kinetics during and after illumination. Vesicles were loaded with 3 M KCl and were suspended in 3 M NaCl containing [^{3}H]glutamate at zero time. (A) Addition of nonradioactive glutamate (final concentration 5×10^{-4} M, ca. 10^4-fold excess): at 8 min during illumination (⊙); or at 16 min, after turning off the light (△). (B) Addition of antibiotics after turning off the light: no addition (⊙); valinomycin (final concentration 1×10^{-6} M) and FCCP (final concentration 9×10^{-6} M) added at 17 min (△); nigericin (final concentration 5×10^{-7} M), added at 17 min (□). Valinomycin or FCCP alone had no effect on glutamate efflux. The vesicle suspensions in B were weakly buffered with phosphate (5×10^{-3} M), added to the medium at pH 5.0, because nigericin acts as a K^+/H^+ antiporter only near its pK (Harold, 1970), which appears to be lower than usual at the high salt concentrations used (Lanyi, unpublished observations).

Sources for the chemicals were as follows. Triphenylmethylphosphonium bromide[1] was from K & K Fine Chemicals, gramicidin D, amino acids, vitamins, and malic acid from Calbiochem, and FCCP from Pierce Chemicals. Phenylmercuric acetate and mersalyl were from Sigma. Nigericin was a gift from R. Bogomolni. [^{3}H]Glutamate (L isomer, 24 Ci/mmol) and [^{3}H]leucine (L isomer, 5 Ci/mmol) were from New England Nuclear, and [^{14}C]glutamate (L isomer, 290 mCi/mmol) was from Amersham/Searle.

Results

Influx and Efflux Kinetics for Light-Dependent Glutamate Transport. As in the case of leucine (MacDonald and Lanyi, 1975), illumination of *H. halobium* envelope vesicles in the presence of glutamate, with light of wavelengths above 500 nm, caused the accumulation of the amino acid. The time course of the light-dependent uptake in the presence of varying amounts of vesicles is shown in Figure 1. Glutamate uptake occurs with a short lag which varied between 0.5 and 1.5 min for different vesicle preparations. While the rate of transport increases with the increasing amounts of vesicles, the total amount of glutamate accumulated is nearly constant and represents most of the glutamate added. The final glutamate concentration gradient between the vesicles and the medium thus depends only on the vesicle concentration. Using 3 μl/mg of protein for intravesicle space (MacDonald and Lanyi, 1975), the calculated glutamate gradients are 2×10^4-, 3.3×10^4-, and 5.8×10^4-fold for the three experiments in Figure 1, with 0.05, 0.02, and 0.01 mg/ml of vesicle protein, respectively. Similarly, high gradients (10^4-fold) were observed for respiration-driven glutamate transport in *E. coli* (Frank and Hopkins, 1969).

A possible reason for the unusually high apparent gradients achieved could be chemical modification during or after transport. Autoradiography of thin-layer chromatograms of aqueous extracts from [^{14}C]glutamate-loaded vesicles showed, however, that all the radioactivity recovered from the vesicles after uptake co-migrated with authentic glutamate in three different solvent systems. When similar aqueous extracts were examined on an amino acid analyzer, glutamate was found to be the only ninhydrin-reactive material in amounts greater than trace contamination. It is unlikely, therefore, that glutamate is accumulated in a chemically altered form.

Another possible reason for the high glutamate gradients could be the irreversibility of transport and the low passive permeability of the membranes to glutamate, as suggested by Frank and Hopkins (1969) for *E. coli* and by Harold and Spitz (1975) for *S. faecalis*. Indeed, unlike leucine (MacDonald and Lanyi, 1975), glutamate is retained in the *H. halobium* vesicles after the illumination is terminated. Efflux kinetics are shown in Figure 2. As seen in Figure 2A, adding a large excess of nonradioactive glutamate, either during or after illumination, causes no detectable efflux of the radioactive substrate. Loss of accumulated glutamate can be observed only when the intravesicle pH is lowered and the glutamate carboxyl ion becomes protonated, with loss of its negative charge. This was accomplished by using KCl-loaded vesicles, which were suspended in NaCl, and to which either nigericin or a combination of valinomycin and FCCP was added. In both of these cases the K^+ gradient is expected to be replaced by a H^+ gradient through the efflux of K^+ and the influx of H^+. The latter is indeed evidenced by a dramatic increase in the measured pH outside the vesicles. This technique was used by Harold and Levin (1974)

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

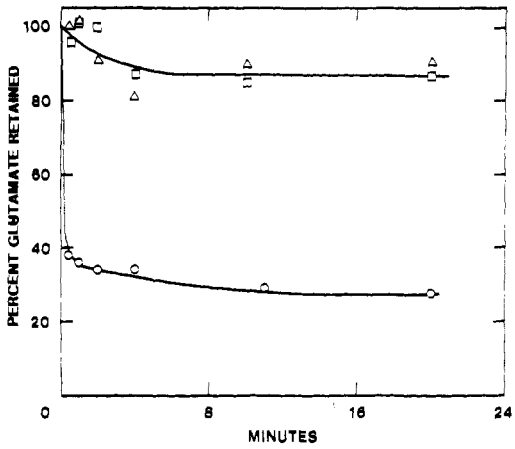

FIGURE 3: Efflux of glutamate from pre-loaded vesicles, in the dark. Vesicles were loaded with 3 M NaCl or KCl and [^{3}H]glutamate (100 μCi/ml) plus carrier glutamate (to a final concentration of 10 mM) and were suspended at 1:1000 dilution in either 3 M NaCl or KCl. Symbols: (⊙) NaCl-loaded vesicles suspended in KCl; (⊡) NaCl-loaded vesicles suspended in NaCl; (▲) KCl-loaded vesicles suspended in KCl.

to study lactate efflux from *S. faecalis* cells. Our results are analogous to theirs: under these conditions glutamate efflux from *H. halobium* envelope vesicles is rapid (Figure 2B). It should be noted that simply lowering the pH in the vesicle suspension and allowing the pH across the membranes to equilibrate should protonate the glutamate carboxyl ion and thus cause efflux as well. Slow glutamate loss is indeed seen at pH 5 (Figure 2B). However, the transport rate and the stability of the vesicles are much impaired below pH 5 and it was not possible to carry out uptake experiments at lower pH values.

We had considered that the unidirectional transport observed may have been due not only to the low permeability of the membranes to the anionic glutamate, but also to the unequal cation distribution across the membranes, as employed in the transport experiments. Efflux of glutamate in the dark was examined therefore, in vesicles pre-loaded with either 3 M NaCl or 3 M KCl, together with radioactive glutamate of very low specific activity (total glutamate concentration 10 mM) in order to minimize binding effects inside the vesicles. The data in Figure 3 show the efflux of the amino acid when the vesicles were suspended at high dilutions to create a large downhill glutamate gradient. Significant efflux in Figure 3 is observed only for NaCl-loaded vesicles suspended in KCl, which corresponds to a cation distribution opposite to that employed in the uptake studies (Figures 1 and 2). Even under these conditions, the efflux of glutamate is only 70–75% complete, probably reflecting the fact that Na^+ gradients decay rapidly in these vesicles (Lanyi et al., 1976). Results similar to those in Figure 3 at 30 °C were obtained at 10 °C as well. When NaCl-loaded vesicles were suspended in 3 M NaCl, very little efflux of glutamate was observed. An equal volume of 3 M KCl was then added, which resulted in the partial loss of glutamate from the vesicles, probably terminated by the equilibration of the cations across the membranes. Repeated additions of KCl caused further limited glutamate efflux. It appears, therefore, that there is a requirement both for Na^+ on the inside of the vesicles and for K^+ at higher concentrations than that of Na^+ on the outside of the vesicles, for glutamate efflux. This conclusion is supported by results from other experiments, in which gramicidin was added to KCl-loaded vesicles suspended in NaCl in the dark after light-induced uptake (not shown). Gramicidin is expected to cause the rapid equilibration of Na^+ and K^+ across the membranes (Harold, 1970), resulting in great dilution of the intravesicle K^+ and thus in having essentially only NaCl on both sides of the membranes. At 1×10^{-7} M gramicidin, which completely abolishes light-dependent transport when added before illumination, a rapid (<1 min) and small (about 20% of uptake) glutamate efflux was observed, with no further change. When the experiment was repeated with KCl-loaded vesicles, suspended in 1.5 M NaCl–1.5 M KCl, the initial efflux of glutamate upon the addition of gramicidin was somewhat more, about 35% of uptake, but again no further efflux was observed.

Effect of Buffering, pH, and NaCl–KCl Balance on Light-Dependent Transport. When *H. halobium* envelope vesicles are illuminated, a pH gradient develops across the membranes (alkaline inside) and an electrical potential (negative inside) is established (Renthal and Lanyi, 1976). Niven and Hamilton (1974) reported that in *Staphylococcus aureus* cells glutamate uptake could be driven by an artificial pH gradient across the cell membrane. Similarly, Harold and Levin (1974) showed that a pH gradient could drive lactate uptake in *S. faecalis*. In order to establish whether or not this mechanism applies to *H. halobium* envelope vesicles, we carried out transport experiments in the presence of phosphate buffer. Buffering at pH 5 and 6 inhibits transport, particularly when the inside of the vesicles is also buffered (not shown). However, this effect is related to pH rather than to buffering itself, since at pH 7 glutamate uptake is not diminished, even at high phosphate concentrations (0.1 M). Since under these conditions a pH difference does not develop across these membranes (Renthal and Lanyi, 1976), glutamate transport in *H. halobium* envelope vesicles cannot be due to pH differences only.

The membranes of extremely halophilic bacteria require high concentrations of salt for retaining integrity (Stoeckenius and Rowen, 1967; Lanyi, 1971, 1972a, 1974, 1976) and envelope vesicles become leaky to large molecules below 2 M NaCl or KCl (Lanyi, 1972a). Accordingly, 3 M salt was employed in all transport experiments. Specific requirements for NaCl or KCl, such as suggested by the efflux experiments (Figure 3), could be tested, however, by altering the [NaCl]/[KCl] ratio. In these experiments the vesicles were illuminated for 5 min before adding [^{3}H]glutamate, in order to eliminate the short lag in uptake, described previously (Figure 1). Initial transport rates, obtained in this manner for KCl-loaded vesicles suspended in various NaCl–KCl solutions, are plotted in Figure 4A. As seen in the graph, transport requires extravesicle NaCl >0.2 M and reaches a maximal rate above 1.4 M. Including NaCl (up to 1 M) in the interior of the vesicles causes long lags (up to 10 min) in uptake during illumination (not shown), which will be discussed in the following paper (Lanyi et al., 1976). Double-reciprocal plots of the initial rates of transport at varying glutamate concentrations are shown in Figure 4B, obtained as in Figure 4A, at 0.8 M NaCl–2.2 M KCl and at 1.4 M NaCl–1.6 M KCl. Transport of glutamate appears to follow Michaelis–Menten type kinetics in both NaCl–KCl solutions. At both Na^+ concentrations (and up to 3 M in other experiments) the same K_T value of $1.2–1.3 \times 10^{-7}$ M glutamate is obtained, indicating that the NaCl dependence of transport shown in Figure 4A is not due to a change in affinity of the carrier for the substrate. This is similar to the case of proline in *Mycoplas-*

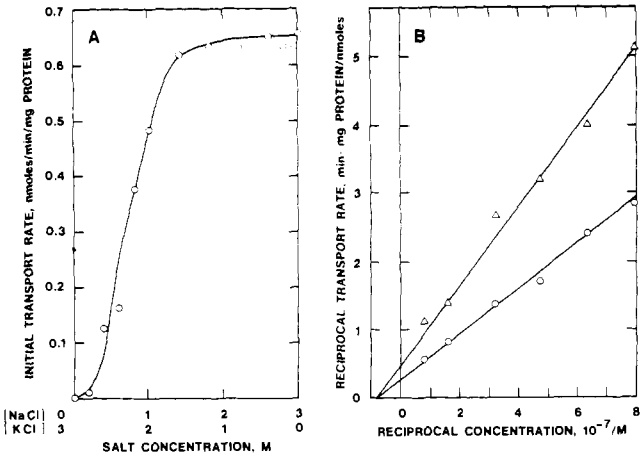

FIGURE 4: Dependence of the light-induced glutamate transport on the external [NaCl]/[KCl] ratio. Vesicles, loaded with 3 M KCl, were suspended in various NaCl–KCl solutions (total salt concentration 3 M) and transport was initiated 5 min after the beginning of illumination, by adding [^{3}H]glutamate. (A) Initial rates of transport vs. [NaCl]/[KCl] concentration. No correction was made for a small amount of KCl introduced with the vesicles into the transport assay mixtures. (B) Double-reciprocal plots of initial transport rates and glutamate concentrations at (⊙) 1.4 M NaCl–1.6 M KCl; and (▲) 0.8 M NaCl–2.2 M KCl.

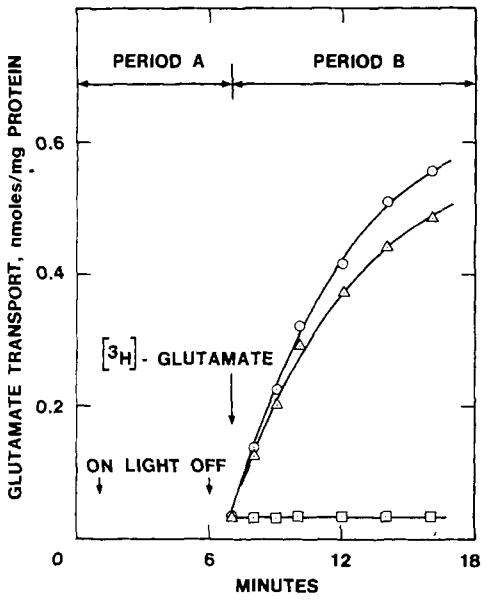

FIGURE 5: Dependence of post-illumination glutamate transport on the presence of NaCl and KCl during and after illumination. Vesicles, loaded with 3 M KCl, were suspended in 0.2 ml of salt solution at zero time and were incubated in the light between 1 and 6 min and in the dark between 6 and 7 min (period A). At 7 min, 0.8 ml of prewarmed salt solution and [^{3}H]glutamate were added and uptake was followed (period B). Symbols: (⊙) period A, 3 M KCl; period B, 2.4 M NaCl–0.6 M KCl; (▲) periods A and B, 2.4 M NaCl–0.6 M KCl; (⊡) periods A and B, 3 M KCl.

ma phlei (Hirata et al., 1974) but unlike many other Na^+-dependent transport systems (for example, Wong et al., 1969; Stock and Roseman, 1971). The V_{max} for glutamate transport in *H. halobium* vesicles is calculated to be 3.7 nmol min^{-1} mg^{-1} of membrane protein from the data in Figure 4B but is somewhat variable in different preparations. This value is near that observed for the light-induced transport of glutamate in whole cells of *H. halobium* (A. Danon, personal communication). For comparison, respiration-driven glutamate transport in *E. coli* vesicles was reported at rates of 4.0 nmol min^{-1} mg^{-1} of protein (Kaback, 1972) and in *Bacillus subtilis* at rates of 38 nmol min^{-1} mg^{-1} of protein (Konings et al., 1972).

In the following paper (Lanyi et al., 1976) we report that illumination of *H. halobium* envelope vesicles produces an "energized state" for glutamate transport, which persists for several minutes after the light is turned off. This observation enabled us to determine whether the Na^+ dependence of the light-induced transport is due to the energizing process or to the translocating process. In these experiments the vesicles were illuminated in salt solutions of different [KCl]/[NaCl] ratio from those in which glutamate transport was tested. Vesicles were loaded with 3 M KCl, suspended in a small volume of 3 M KCl (Figure 5), and were illuminated for 5 min. One minute after the light was turned off, a large volume of 3 M NaCl was added, bringing the total volume to the value usually used in transport experiments, and the final Na^+ concentration to 2.4 M. At the same time [^{3}H]glutamate was added and uptake was followed. Under these conditions glutamate accumulation was somewhat higher than in the control experiment, where 2.4 M NaCl–0.6 M KCl was employed during both illumination and transport periods. As found before (Figure 4A), no transport was seen in the absence of NaCl, when KCl was added after illumination rather than NaCl. It appears from these results that the vesicles can be energized by illumination in either NaCl or KCl solutions. The Na^+ requirement for uptake on the exterior of the vesicles therefore must be due to the translocation step or possibly to the energy coupling process.

Na^+-Gradient-Dependent Glutamate Transport. The Na^+ requirement on the initial side of glutamate translocation, that is, either on the outside of the vesicles for light-dependent uptake (Figures 4A and 5) or on the inside of the vesicles for efflux in the dark (Figure 3), raises the possibility that glutamate transport in *H. halobium* envelope vesicles involves symport with Na^+, in a manner analogous to leucine transport in the same system (MacDonald and Lanyi, 1975), and to the transport of various substrates in a number of bacterial (Wong et al., 1969; Stock and Roseman, 1971; Halpern et al., 1973; Miner and Frank, 1974) and eucaryotic systems (for example, Wheeler and Christensen, 1967; Eddy et al., 1967; Eddy, 1968; Murer and Hopfer, 1974). Vesicles were loaded, therefore, with KCl–NaCl solutions (total concentration 3 M), using what is described under Methods as the injection method, with amounts of NaCl calculated to give Na^+_{out}/Na^+_{in} ratios of >500, 200, and 40 when the vesicles were suspended in 3 M NaCl. Glutamate uptake was followed in the dark, immediately after adding the vesicles. As shown in Figure 6A, Na^+-gradient-dependent transport was obtained under these conditions, and for the highest Na^+ gradient used, the uptake was comparable in magnitude with the light-dependent transport. On the basis of the curves in Figure 6A, and a slow dark uptake observed in vesicles loaded with KCl by the pelleting and resuspension method (MacDonald and Lanyi, 1975), the NaCl content of the latter vesicles can be estimated at 0.1–0.2 M, which is considerably higher than originally intended.

Similarly to light-dependent transport (Figure 4B), varying of the glutamate concentration under these conditions also produced Michaelis–Menten type kinetics and a K_T value of approximately 1.3×10^{-7} M glutamate (Figure

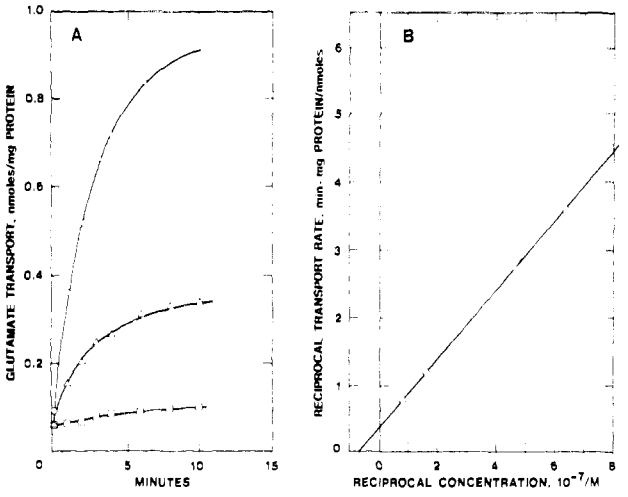

FIGURE 6: Na^+-gradient-induced glutamate transport. Vesicles were loaded by the injection method (see under Methods) with either 3 M KCl or with KCl–NaCl solutions to give the indicated Na^+ gradients at zero times, after suspension in 3 M NaCl containing [^{3}H]glutamate. Glutamate uptake was followed in the dark, immediately after adding the vesicles. (A) Kinetics of transport. Symbols: (⊙) Na^+-gradient > 500×; (▲) Na^+-gradient set at 200×; (⊡) Na^+-gradient set at 40×. (B) Double-reciprocal plots of initial transport rates (Na^+-gradient > 500×) and glutamate concentrations.

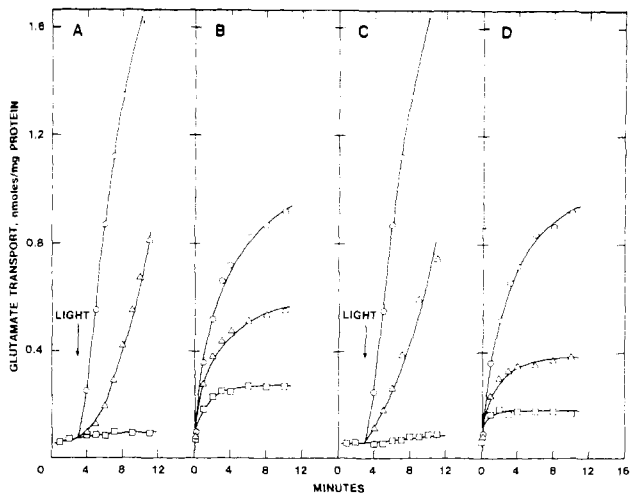

FIGURE 7: Effect of FCCP and $TPMP^+$ on light-dependent and Na^+-gradient dependent transport. Vesicles were loaded with 3 M KCl and were suspended in 3 M NaCl containing [^{3}H]glutamate at zero time. When following light-induced uptake (A and C), the light was turned on at 3 min and the inhibitors were added at this time. When following Na^+-gradient dependent uptake in the dark (B and D), the vesicles were added to the NaCl solution (Na^+_{out}/Na^+_{in} > 500) at the same time as the inhibitors. (A and B) Effect of FCCP. Symbols: (⊙) no addition; (▲) 1×10^{-6} M FCCP; (□) 3×10^{-6} M FCCP. The effect of the ethanol added with the inhibitor (<0.5% v/v) was very small. (C and D) Effect of $TPMP^+$. Symbols: (⊙) no addition; (▲) 3×10^{-3} M $TPMP^+$; (□) 4×10^{-3} M $TPMP^+$

6B). Maximal initial rates (V_{max}) were 2.9 nmol min^{-1} mg^{-1} of protein, about 80% of the light-induced rates (Figure 4B) in the same vesicle preparation. The correspondence between Na^+-gradient dependent and light-dependent transport kinetics thus appears to be reasonably good.

Effect of Inhibitors on Light-Induced and Na^+-Gradient-Induced Amino Acid Transport. The extremely halophilic bacteria possess a functional electron transport chain (Lanyi, 1968, 1969; Cheah, 1969, 1970). The possible involvement of endogenous substrate oxidation in transport was tested by examining glutamate uptake in the presence of 2 mM KCN (buffered at pH 7 with 0.01 M phosphate), both under the conditions described in Figure 1 for light-dependent transport and in Figure 6A for Na^+-gradient-dependent transport. No effect on glutamate accumulation was observed with this inhibitor, even though KCN abolishes respiration in these organisms at the concentration used (Lanyi, 1968, 1972b). Mercurials, particularly phenylmercuric acetate, have been found to inhibit respiratory enzymes in extremely halophilic bacteria (Lanyi, 1972a). Incubation of the envelope vesicles with 1×10^{-4} M phenylmercuric acetate or mersalyl for 5 min, however, has no effect on light-induced glutamate uptake, suggesting that exposed sulfhydryl groups are not involved in this transport system.

Gramicidin, an antibiotic which increases the permeability of membranes to Na^+ and K^+ (Harold, 1970), is expected to cause the relaxation of cation gradients. The ability of the vesicles to accumulate glutamate due to a Na^+ gradient was indeed found to decline rapidly in the presence of very low concentrations of gramicidin (1×10^{-8} M).

FCCP acts as an upcoupler of oxidative phosphorylation and can increase the permeability of various membranes to protons (Harold, 1970). Since the primary effect of illumination on bacteriorhodopsin in *H. halobium* is to cause a translocation of protons (Oesterhelt and Stoeckenius, 1973; Bogomolni and Stoeckenius, 1974; Stoeckenius and Lozier, 1974), shown both in whole cells (Bogomolni et al., in preparation) and in envelope vesicles (Kanner and Racker, 1975; Renthal and Lanyi, 1976), FCCP was expected to inhibit the light-induced transport of glutamate. The results in Figure 7A show that FCCP is indeed inhibitory: 1×10^{-6} M FCCP causes reduced transport rate and a lag, which together resemble transport kinetics at very much reduced light intensities (Lanyi et al., 1976), while 3×10^{-6} M FCCP completely abolishes uptake. On the other hand, if the Na^+-gradient-induced transport of glutamate in the dark is dependent directly on Na^+ flux and not on secondary movements of H^+, caused by the possible presence of a Na^+/H^+ antiport system (West and Mitchell, 1974), then such transport should not be inhibited by FCCP. The experiment is complicated by the fact that the downhill influx of Na^+ into these vesicles and therefore the relaxation of Na^+ gradients is much accelerated in the presence of FCCP (Lanyi et al., 1976). Nevertheless, the results, shown in Figure 7B, are highly suggestive, as they indicate that the *initial* rates of the Na^+-gradient-induced transport are either not inhibited or only slightly inhibited at FCCP concentrations at which the initial rates of the light-dependent transport are highly inhibited. A more detailed kinetic analysis of such results, described in the following paper (Lanyi et al., 1976), also suggests that the effect of FCCP on the Na^+-gradient-induced transport consists solely of accelerating the decay of the driving force. The accumulation of glutamate in the vesicles under these conditions thus appears to be due to the chemical-gradient-dependent flux of Na^+ rather than to secondary movements of H^+. Similar conclusions were reached for the Na^+-gradient-induced transport of leucine in this system (MacDonald and Lanyi, 1975).

If glutamate transport in *H. halobium* is by Na^+ symport, as suggested by the results of this study, the stoichiometry of the two species which interact with the transport carrier (and of any other ions involved) determines the net electrical charge associated with the translocation process. Since glutamate in solution is anionic in the pH region in-

vestigated, a 1:1 stoichiometry could give zero net charge transported, while higher Na^+/glutamate ratios would correspond to positive charge translocation. Ideally, this question should be explored by direct measurements of the ion and substrate fluxes (see, for example, Eddy, 1968). In the *H. halobium* system, however, only an indirect approach, using the permeant cation $TPMP^+$, was possible. Lipid bilayers and biological membranes are highly permeable to this type of lipophilic cation, and hence its addition to vesicles can cause the development of a diffusion potential, positive inside (Skulachev, 1971). The magnitude of such membrane potential, which depends on the permeability difference between $TPMP^+$ and other ions, must be considerable in *H. halobium* envelope vesicles, since $TPMP^+$ effectively abolishes light-induced membrane potentials of more than 100 mV, of the opposite sign (Renthal and Lanyi, 1976). As expected, the initial rates of the light-induced glutamate uptake were inhibited by $TPMP^+$ (Figure 7C) in the same manner as by FCCP, consistent with the idea that the effect of both of these agents is a reduction in the magnitude of the driving force, equivalent to lowering light intensities. As with FCCP, the effect of $TPMP^+$ on the Na^+-gradient-induced transport of glutamate is complicated by the fact that $TPMP^+$ also accelerates the decay of the Na^+ gradient. As seen in Figure 7D, however, the *initial* rates of glutamate transport are not greatly affected by $TPMP^+$, at concentrations which are highly inhibitory to light-induced uptake. More detailed analysis of the kinetics of $TPMP^+$ inhibition (Lanyi et al., 1976) confirmed this conclusion. The case of glutamate should be contrasted with that of leucine in these vesicles, which, when transported by Na^+ gradients under similar conditions, is expected to be accompanied by a net positive charge (MacDonald and Lanyi, 1975). The experiment in Figure 7D was repeated with [^{3}H]leucine and the results, given in Figure 8, show that the initial rates of the Na^+-gradient-dependent leucine transport are inhibited by $TPMP^+$, as is light-induced transport of this amino acid (MacDonald and Lanyi, 1975). Thus, it appears that, when glutamate (but not leucine) transport is driven by a Na^+ gradient, a positive interior membrane potential is not inhibitory, hence the net charge translocation is zero. Light-driven glutamate transport, on the other hand, is clearly inhibited by $TPMP^+$ and FCCP and, hence, appears to depend on the membrane potential developed under these conditions.

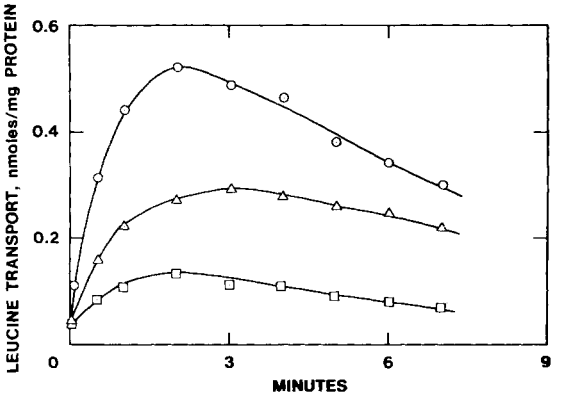

FIGURE 8: Effect of $TPMP^+$ on the Na^+-gradient dependent leucine transport. Conditions as under Figure 8D, but in the presence of [^{3}H]leucine. Symbols: (⊙) no addition; (▲) 1×10^{-3} M $TPMP^+$; (⊡) 2×10^{-3} M $TPMP^+$.

Discussion

The results in this study indicate that, as leucine (MacDonald and Lanyi, 1975), glutamate is accumulated during the illumination of *H. halobium* envelope vesicles. The source of energy for glutamate transport appears to be the electrical potential developed across the membranes (Renthal and Lanyi, 1976), due to the light-dependent photochemical events in bacteriorhodopsin and the consequent translocation of protons (Oesterhelt and Stoeckenius, 1973; Bogomolni and Stoeckenius, 1974; Stoeckenius and Lozier, 1974). Thus, even though Niven and Hamilton (1974) obtained glutamate transport in *S. aureus* cells solely by pH gradients, buffering of the vesicles both inside and outside at pH 7 during illumination did not diminish glutamate transport in our studies. On the other hand, addition of FCCP and $TPMP^+$, which can discharge electrical potential (Harold, 1970: Skulachev, 1971), were highly inhibitory (Figures 7A and 7C). Our results can, therefore, be interpreted on the basis of the chemiosmotic theory (Mitchell, 1970; Harold, 1972) in which the protonmotive force generated is the source of energy for transport (and for other energy-requiring processes). According to our scheme, the substrate translocation takes place through a carrier remote from the site of the proton ejection, i.e., the bacteriorhodopsin-containing purple patches on the membrane, and energy coupling is effected through the electrical component of the protonmotive force.

Glutamate transport in *H. halobium* is unidirectional, as in the *E. coli* (Frank and Hopkins, 1969) and *S. faecalis* (Harold and Spitz, 1975) systems, but unlike in the case with *B. subtilis* (Konings et al., 1972), and thus extremely high glutamate gradients are achieved. The evidence can be rationalized on the basis of a model of different ionic requirements for the transport carrier on the two sides of the membrane. Thus, both downhill glutamate efflux from the vesicles and light-dependent uphill transport into the vesicles require high concentrations of Na^+ (and low concentrations of K^+) on the side of the membrane where the translocation is initiated and in both cases high concentrations of K^+ (and low concentrations of Na^+) are required on the other side. Na^+ requirement or stimulation has been observed for respiration-driven glutamate transport in *Halobacterium salinarium* (Stevenson, 1966) and in *E. coli* (Frank and Hopkins, 1969; Halpern et al., 1973; Miner and Frank, 1974), although not in *M. phlei* (Prasad et al., 1975), and for other substrates in various bacterial systems (Wong et al., 1969; Stock and Roseman, 1971). In *H. halobium*, at least, such Na^+ requirement is in accord with the observation that Na^+ gradients can drive transport in the dark at nearly full rates, through what appears to be direct coupling with the Na^+ flux. The Na^+ requirement on the side of the membrane where the translocation is initiated may thus be due entirely to symport of glutamate with this cation. Such symport should be driven either by a Na^+ gradient (as in Figure 6A), or by a glutamate gradient in the absence of a Na^+ gradient. The latter was not observed (Figure 3), however, and therefore the requirement for K^+ on the far side of the membrane must be for reasons other than to maintain osmotic pressure. Indeed, specific intracellular K^+ requirement or K^+ stimulation for substrate uptake has been observed for glutamate in *E. coli* (Halpern et al., 1973) and for other amino acids in a marine pseudomonad (Thompson and MacLeod, 1971, 1974) and in Erlich ascites tumor cells (Eddy et al., 1967; Eddy and Hogg, 1969). In all of these cases it was suggested that the binding

of K^+ on the opposite side of the membrane accelerates the cycling of the carrier. Our results in *H. halobium* are consistent with this idea.

The stoichiometry of the glutamate–Na^+–carrier complex, in our scheme for the transport system, determines the net charge translocated per glutamate molecule. Lack of inhibition of the initial rates of the Na^+-gradient-dependent glutamate transport by $TPMP^+$ (Figure 7D) suggests that the translocation is electrically neutral and thus depends only on the chemical gradient of Na^+. On the other hand, $TPMP^+$ effectively inhibits the light-dependent transport of this amino acid (Figure 7C). This, inhibition by FCCP (Figure 7A), and lack of inhibition by buffering suggest that the light-dependent process must be energized by electrical potential. If charge translocation is not involved in the glutamate translocation step, the mode of coupling of the transport with the light-induced membrane potential is not clear. Three possible explanations can be advanced to resolve this problem: (1) the light-dependent and the Na^+-gradient-dependent transport proceed through different carriers, energized by different means. In this case the similarity of kinetic parameters (Figures 4B and 6B) is merely fortuitous. (2) The two kinds of transport utilize a common glutamate carrier, but it is energized differently in the presence of a Na^+ gradient or an electrical potential. (3) The two kinds of transport have, in fact, the same mechanism and the role of light (and thus of the electrical potential) is to create and maintain a Na^+ gradient. In the following paper (Lanyi et al., 1976) we present evidence in favor of the third model.

Acknowledgments

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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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