Light-Induced Leucine Transport in *Halobacterium halobium* Envelope Vesicles: A Chemiosmotic System†

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ABSTRACT: *Halobacterium halobium* cell envelope vesicles accumulate L-[^{14}C]leucine during illumination, against a large concentration gradient. Leucine uptake requires Na^+ and is optimal in KCl-loaded vesicles resuspended in KCl–NaCl solution (1.5 *M*:1.5 *M*). Half-maximal transport is seen at 1×10^{-6} *M* leucine. In the dark the accumulated leucine is rapidly and exponentially lost from the vesicles. The action spectrum and the light-intensity dependence indicate that the transport is related to the extrusion of protons, mediated by bacteriorhodopsin. Since light gives rise to both a pH gradient and an opposing transmembrane potential (interior negative), it was possible to distinguish which of these components is responsible for providing the energy for leucine transport. The following results were obtained under illumination: (1) membrane-permeant cations and valinomycin or gramicidin greatly inhibited leucine transport without altering the pH gradient; (2) buffering both inside and outside the vesicles eliminated the pH gradient while enhancing leucine transport; (3) dicyclohexylcarbodiimide increased the pH gradient without affecting leucine transport; (4) arsenate did not inhibit leucine uptake. A diffusion potential, established by adding valinomycin to KCl-loaded vesicles, caused leucine influx in the dark. These results suggest that the leucine transport system is not coupled to ATP hydrolysis, and responds to the membrane potential rather than to the pH gradient. The Na^+ dependence of the transport and the observation that a small NaCl pulse causes transient leucine influx in the dark in KCl-loaded vesicles, resuspended in KCl, even in the presence of *p*-trifluoromethoxycarbonylcyanide phenylhydrazone or with buffering, suggest that the translocation of leucine is facilitated by symport with Na^+.

Halobacterium halobium cell envelopes have been shown to include differentiated regions which, after isolation, appear as purple membrane sheets (Stoeckenius and Kunau, 1968; Oesterhelt and Stoeckenius, 1971). These structures contain 25% lipids (Oesterhelt, 1972; Kushwaha et al., 1975) and a single kind of protein, termed bacteriorhodopsin (Oesterhelt and Stoeckenius, 1971), which is arranged in a hexagonal crystalline array in the plane of the membrane (Blaurock and Stoeckenius, 1971). Upon illumination, the chromophore—retinal (Oesterhelt and Stoeckenius, 1971; Kushwaha and Kates, 1973)—undergoes reversible bleaching (Oesterhelt and Stoeckenius, 1973; Oesterhelt and Hess, 1973; Stoeckenius and Lozier, 1974), and the photochemical events have been correlated with the vectorial release and uptake of protons (R. Lozier and W. Stoeckenius, personal communication). Light-dependent translocation of protons has been observed in both intact *H. halobium* cells (Bogomolni and Stoeckenius, 1975) and in artificial, reconstituted systems (Racker and Stoeckenius, 1974; Racker and Hinkle, 1974).

H. halobium cells have been found to utilize light for the synthesis of ATP under anaerobic conditions (Danon and Stoeckenius, 1974). ATP production can also be observed in reconstituted systems on illumination, when purple membrane and mitochondrial ATPase are incorporated into lipid vesicles (Racker and Stoeckenius, 1974). The purple membrane thus appears to be part of a photophosphorylating system far simpler than that of plants or photosynthetic bacteria. Since in *H. halobium* cells the sites for light absorption and for ATP synthesis seem to be in physically separate regions on the cell envelope, Stoeckenius and coworkers have suggested (Oesterhelt and Stoeckenius, 1973; Danon and Stoeckenius, 1974) that this system could be used to test the validity of the chemiosmotic hypothesis of Mitchell (1972). The hypothesis proposes that the respiratory chain generates a pH gradient and/or an electrical potential across the plasma membrane and that this gradient, or protonmotive force, is utilized to synthesize ATP and to drive the transport of various molecules across the membrane, together with or counter to the transport of protons. Thus, it would appear that if in the *H. halobium* membrane the respiratory chain is replaced, all or in part, by a light-activated proton pump, which is capable of activating sites of ATP synthesis solely through chemical or electrical potential, or of transporting substrates against a large concentration gradient, the principle of the chemiosmotic energy coupling will be clearly demonstrated.

An energy-requiring process in bacteria is the translocation of substrates across the plasma membrane against concentration gradients. A number of such "uphill" transport systems have been described in detail and those which are linked to respiratory activity are either driven by ATP hydrolysis (Simoni and Shallenberger, 1972; Berger, 1973), or are directly coupled to electron transport through an "energized state" (Pavlosova and Harold, 1969; Kaback and Barnes, 1971; Klein and Boyer, 1972). Harold and coworkers found that a diffusion potential imposed on K^+-loaded *Escherichia coli* vesicles by a K^+-ionophore resulted in the influx of amino acid and sugars (Hirata et al., 1974), and proposed that this substrate translocation is energized by a potential gradient (interior negative) according to the mechanism postulated by the chemiosmotic theory. Similar results were obtained in *Streptococcus lactis* cells (Kashket

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and Wilson, 1973) and in *Staphylococcus aureus* (Niven et al., 1973). Kaback and coworkers have disputed this suggestion and have given evidence in favor of a direct redox reaction linked model (Kaback and Barnes, 1971; Lombardi et al., 1974). Recently, some of the differences between these views have been reconciled (Kaback, 1974). It was thus of interest to study amino acid transport in *H. halobium* as it seemed likely that the energy required for this process could be supplied by light. The advantages of this system are that a pH gradient and/or a membrane potential can be established and abolished at will, and many of the energy-transducing events which accompany the illumination of bacteriorhodopsin are understood (Stoeckenius and Lozier, 1974). Thus, we have attempted to determine unambiguously whether the amino acid transport across the membranes of this organism is driven by ATP hydrolysis and/or directly by protonmotive force, and also whether the transport requires the simultaneous movement of protons (Pavlosova and Harold, 1969; West, 1970; Eddy and Novacki, 1971) or of other cations.

Materials and Methods

Halobacterium halobium R-1 strain (made available to us by W. Stoeckenius) was grown in shake cultures according to the method of R. A. Bogomolni and W. Stoeckenius (personal communication). The growth medium contained the following per liter: 250 g of NaCl, 20 g of $MgSO_4 \cdot 7H_2O$, 2 g of KCl, 0.2 g of $CaCl_2$, and 10 g of peptone, adjusted to pH 7. Optimum aeration was achieved when 700-ml quantities of medium were used in 2-l. flasks and the cultures were placed on a rotary shaker operated at 150 rpm. Growth was at 37° for 5 to 8 days, under illumination by two 400-W fluorescent fixtures (GE "Cool White"). The cell envelopes were prepared essentially according to a procedure developed by E. Racker (personal communication). Cells were harvested by centrifugation, washed with a solution of the salts contained in the growth medium (⅓ vol), washed again with ⅓ vol of 4 *M* NaCl–0.05 *M* Tris-HCl (pH 7), and were resuspended in 1/30 vol of the same solution. The cell suspension was sonicated for 4 × 15 sec with a Branson LS-75 Sonifier, DNase was added (ca. 0.2 mg/ml), and the lysate was diluted fivefold with the same solution. Unbroken cells and large particulate debris were removed by centrifugation at 4500*g* for 20 min, and the envelopes at 46,000*g* for 30 min. The reddish supernatant was discarded. The purple-red pellet was washed with an equal volume of 4 *M* NaCl containing no buffer, and was resuspended in 4 *M* NaCl at 1 to 2 mg/ml protein concentration, followed by a final centrifugation at 4500*g* for 20 min to remove any residual whole cells. Various preparations of envelopes contained 2.5 ± 0.4 nmol of bacteriorhodopsin/mg of protein, equal to 6 to 7% of the total envelope protein. From 60 to 80% of the pigment recovered was in the envelope fraction.

Electron micrographs of thin sections and freeze-etch preparations (performed kindly by W. Stoeckenius) showed that the envelope vesicles were single-walled spheres, fairly uniform in size, of about 0.5 μm diameter and that a large majority of the vesicles were right-side out.

The water-permeable space in the envelope vesicles, determined with ^3H_2O and [^{14}C]dextran according to Hunter and Brierley (1969), was approximately 3 μl/mg of protein.

The integrity and "sidedness" of the envelope vesicles were also ascertained by determining DPNH-menadione reductase activity in the presence and absence of Triton X-100. As described earlier for *Halobacterium cutirubrum* (Lanyi, 1972), a related organism, this enzyme is located on the inside surface of the plasma membrane and, in appropriately prepared envelope vesicles and whole cells (Lanyi, 1973), it is inaccessible to the substrate, while mechanical breakage or detergents release all the enzyme activity. By this criterion the *H. halobium* envelope preparations used were more than 85% right-side out (87 ± 7%). In contrast, the slowly sedimenting reddish envelope fragments, not collected, were about 50% right-side out, indicating that these structures were more or less randomized. Different envelope preparations showed several-fold differences in light-induced proton efflux and leucine transport activity (the former increasing considerably with aging), which were apparently not related to any of these measured properties.

The NaCl/KCl ratio inside the envelope vesicles was manipulated by subjecting the preparations to osmotic shock in the desired salt solution. The vesicles in 4 *M* NaCl were centrifuged at 46,000*g* for 30 min, the pellet was suspended in a 3 *M* salt solution, containing NaCl and KCl in the desired ratio (for the interior), and the vesicles were collected again and resuspended in a 3 *M* salt solution of the desired NaCl/KCl ratio (for the exterior). The effectiveness of this method was tested as follows. Vesicles, loaded with KCl as described, were suspended in 3.0 *M* NaCl and centrifuged, and the pellet was resuspended in distilled water. Atomic absorption analysis for K (Lanyi and Silverman, 1972) and the Lowry procedure yielded values for KCl and protein contents, from which an average KCl concentration of 2.9 ± 0.6 mol/l. of intravesicle water was calculated. It appears, therefore, that the vesicles had equilibrated osmotically and most or all the internal NaCl was replaced by KCl.

Protein was determined either by resuspending envelope vesicles in Biuret reagent containing 0.5% Triton X-100 and reading absorbance at 560 nm (Lanyi, 1971) or by the Lowry method (Lowry et al., 1951). In the latter case a white precipitate appeared, caused by the presence of KCl, which was removed by centrifugation. Bacteriorhodopsin was determined spectrophotometrically, after isolating purple membranes from the envelope vesicles by resuspension in distilled water, centrifuging at 46,000*g* for 30 min, and washing the pellet with water, which treatment removed nearly all material but the purple membranes (Stoeckenius and Kunau, 1968). A molar extinction coefficient at 570 nm of 63,000 was used; the very small amount of light scattering of the preparations was ignored.

Leucine uptake was determined in a magnetically stirred chamber (Titrator assembly, TTA31, Radiometer), thermostated at 30°, which contained a pH electrode (Corning Triple Purpose Combination). Changes in pH were recorded with a Radiometer Model TTT2 pH meter connected to a 10-in. recorder. The chamber was illuminated with a GE EJL Quartzline lamp, using a 10-cm pathlength water-filled heat filter, a 700-nm short-pass filter (Optics Technology), and a Corning 3-68 cut-off filter. For the action spectrum a series of interference filters (Baird-Atomic, bandwidth ca. 10-nm) were used. When the chamber contained 1 ml of liquid, the cross section illuminated was approximately 1 cm^2; under these conditions the light energy at the chamber was 8 × 10^5 ergs/sec (determined with a Kettering Model 68 radiant energy meter). Typically, the reaction mixture contained vesicles equivalent to 0.2–0.4 mg/ml of protein and 0.5 μCi of [^{14}C]leucine. The experiments were started by either adding the leucine or opening

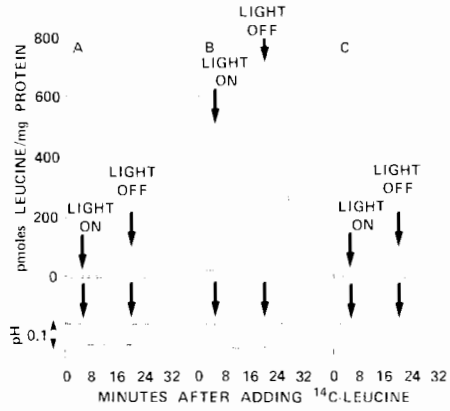

FIGURE 1: Leucine uptake and pH changes during illumination of *H. halobium* envelope vesicles. The vesicles were suspended in 1×10^{-3} *M* Hepes (pH 6.1) plus 3.0 *M* salt to give 0.22 mg/ml of protein: (A) KCl-loaded vesicles suspended in KCl; (B) KCl-loaded vesicles suspended in 2.0 *M* NaCl–1.0 *M* KCl; and (C) NaCl-loaded vesicles suspended in NaCl.

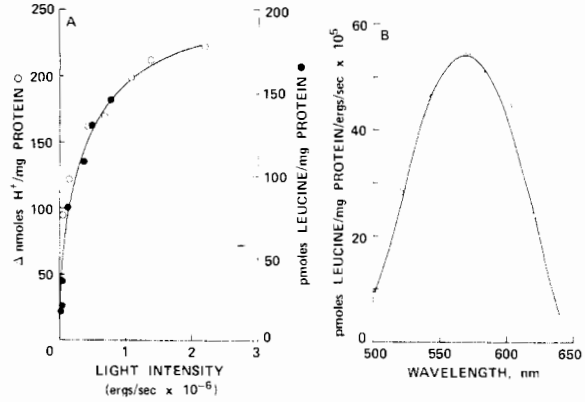

FIGURE 2: (A) Light-intensity dependence of leucine transport and H^+ extrusion. KCl-loaded vesicles were suspended in 2.5 *M* KCl–0.5 *M* NaCl; (○) change in external $[H^+]$ per milligram of membrane protein; (●) [^{14}C]leucine taken up per milligram of membrane protein. (B) Action spectrum for leucine uptake; conditions as in A. Monochromatic light was achieved by using interference filters; light intensities were 0.4–1.25 $\times 10^5$ ergs/sec.

a shutter in the light beam. If buffered, the suspension contained 1×10^{-3} *M* Hepes,[1] adjusted to give pH 6.0 in water at the same dilution. In 3 *M* KCl solutions the actual pH readings were 0.4 to 0.5 higher and rather variable. During the experiments 100-μl aliquots were quickly delivered into a Millipore filter apparatus containing 1 ml of 10 m*M* leucine solution in 3 *M* NaCl (chilled) above a prewetted 24-mm Millipore filter disk, pore size 0.45 μm. Suction was turned on and the filter disk was washed with 5×1 ml portions of cold 3.0 *M* NaCl. The disks were dried for 30 min at 70° in 20-ml polyethylene vials, and 7 ml of toluene containing 0.4% 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene was added. Radioactivity retained on the filters was determined in a Packard liquid scintillation spectrometer. Counting efficiency was 80% for ^{14}C and 25% for ^{3}H.

Sources for the chemicals were as follows. *N,N'*-Dicyclohexylcarbodiimide was from Schwarz/Mann, Ph_3MePBr and Bu_4NBr[1] were from K&K Fine Chemicals, valinomycin and gramicidin D from Calbiochem, DNase and 2-heptyl-4-hydroxyquinoline *N*-oxide from Sigma, and FCCP[1] from Pierce Chemicals. Bacteriological peptone was from Wilson Diagnostics, Inc., [^{14}C]leucine (L isomer, 240 mCi/mmol) was from International Chemical and Nuclear Corp., [^{3}H]leucine (L isomer, 42.5 Ci/mmol), [*carboxyl*-^{14}C]dextran (4.73 mCi/g), and ^3H_2O were from New England Nuclear. D-Leucine was a gift from Glen Pollock.

Results

When *H. halobium* envelope vesicles were illuminated with light at wavelengths above 500 nm, H^+ efflux and leucine uptake were observed. These effects were investigated as functions of the NaCl/KCl ratio. In Figure 1, pH changes and [^{14}C]leucine uptake on illumination are shown for KCl-loaded vesicles resuspended in KCl, KCl-loaded vesicles resuspended in NaCl–KCl (2:1), and for NaCl-loaded vesicles resuspended in NaCl. Because of the well-known salt dependence of halophilic enzymes (Larsen, 1967; Kushner, 1968; Lanyi, 1974), the total salt concentration was kept at 3.0 *M*. It is seen in Figure 1 that H^+ efflux proceeds in the presence of either NaCl or KCl, although KCl has a stimulatory effect. Leucine uptake is influenced much more dramatically by changing the cation. Thus, for leucine uptake there appears to be an absolute requirement for Na^+, even though NaCl alone gives poor incorporation. Optimal transport is observed at an intermediate NaCl/KCl ratio. As seen in Figure 1, the time course of the pH changes at the beginning and the end of the illumination period was much faster than the uptake and loss of leucine. A membrane potential, measured in this system (Renthal and Lanyi, manuscript in preparation) with the aid of a fluorescent cyanine dye (Sims et al., 1974), followed the kinetics of the pH changes.

The dependences of leucine uptake and H^+ efflux on light intensity are shown in Figure 2A where maximal amounts of leucine transported and maximal changes in the external H^+ concentration are plotted against light energy. The data indicate that the two processes saturate at similar light intensities, although the amount of leucine uptake is approximately three orders of magnitude lower than the H^+ extruded (a value variable with different vesicle preparations). Leucine uptake was examined at different wavelengths at light intensities near the linear part of the intensity dependence curve in Figure 2A. The resulting action spectrum, shown in Figure 2B, is very similar to the absorption spectrum of bacteriorhodopsin (Oesterhelt and Stoeckenius, 1971), and is consistent with the idea that the light-dependent leucine uptake is mediated by this pigment.

As shown in Figure 1, the leucine taken up during illumination is rapidly lost in the dark. The efflux kinetics for leucine were further investigated, as shown in Figure 3, after (a) turning off the light, (b) adding a large excess of unlabeled L-leucine during illumination, and (c) adding the membrane-permeant cation Ph_3MePBr^+ (Grinius et al., 1970), which can abolish a potential across the membrane, during illumination. The results indicate that in the latter case the efflux rate approaches the influx rate. Both influx and efflux were exponential within experimental error. In the dark or in the presence of unlabeled L-leucine the efflux rates were somewhat slower. It is evident from Figure 3, nevertheless, that even in these cases the observed transport properties of the system are dominated by the rapid efflux of the accumulated amino acid, which must give rise to large fluxes even during no net uptake in the light. Unlabeled D-leucine, added as in Figure 3B, caused no efflux of

[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Ph_3MePBr, triphenylmethylphosphonium bromide; Bu_4NBr, tetrabutylammonium bromide; FCCP, *p*-trifluoromethoxycarbonylcyanide phenylhydrazone.

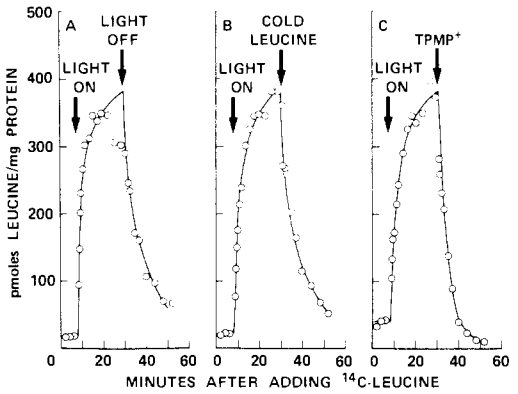

FIGURE 3: Efflux of leucine under various conditions. KCl-loaded vesicles were suspended in 2.5 *M* KCl-0.5 *M* NaCl: (A) efflux in the dark, after illumination; (B) efflux during illumination after adding a 100-fold excess of unlabeled leucine; and (C) efflux during illumination after adding Ph_3MePBr, to a final concentration of 2.5×10^{-3} *M*.

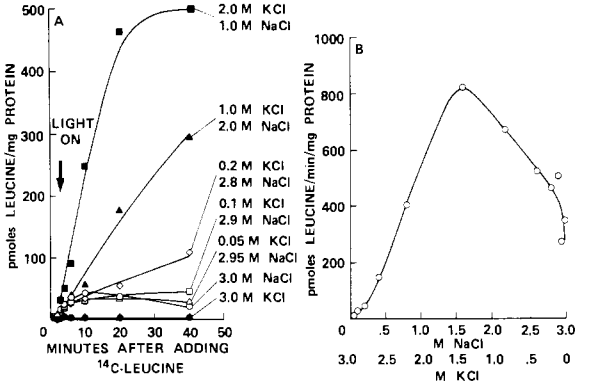

FIGURE 4: NaCl and KCl dependence of leucine transport. (A) Kinetics of leucine uptake for vesicles prepared in and resuspended in the following salt solutions: (⊙) 3.0 *M* NaCl; (△) 2.95 *M* NaCl-0.05 *M* KCl; (⊡) 2.9 *M* NaCl-0.1 *M* KCl; (◊) 2.8 *M* NaCl-0.2 *M* KCl; (▲) 2.0 *M* NaCl-1.0 *M* KCl; (■) 1.0 *M* NaCl-2.0 *M* KCl; (●) 3.0 *M* KCl. (B) Initial rates of leucine uptake for KCl-loaded vesicles suspended in NaCl-KCl solutions at the indicated concentrations.

the radioactive label, indicating that the transport system is stereospecific.

The rapid efflux of leucine gave rise to some doubts about the integrity of the cell envelopes. It was found, however, that when the vesicles were chilled to 0° subsequent to illumination the efflux rate in the dark was greatly diminished.

The rate of transport was examined at several substrate concentrations, using [^{3}H]leucine of higher specific radioactivity than available with ^{14}C. Half-maximal transport was obtained at $0.8–1.2 \times 10^{-6}$ *M* leucine. The high rate of leucine uptake and the limitations of the mechanics of the sampling procedure precluded very precise determinations of initial rates. Thus, detailed examination of transport kinetics was not feasible.

The radioactive label in illuminated vesicles was not precipitated in cold 5% trichloroacetic acid, and over 90% of the label recovered from aqueous extracts of the vesicles cochromatographed with authentic leucine on Gelman ITLC-SG sheets with chloroform–1-butanol–methanol–5 *N* aqueous NH_3 (70:30:17:5). Thus, it is unlikely that leucine is accumulated in a chemically altered form.

As indicated in Figure 1, leucine uptake is greatly influenced by the NaCl/KCl ratio in the solution. Two series of experiments were carried out in order to explore this effect. In the first, the salt concentrations were set to be equal inside and outside the vesicles. Leucine uptake kinetics under these conditions are shown in Figure 4A, giving data for 3 *M* NaCl, for increasing KCl/decreasing NaCl (total concentration 3 *M*), and for 3 *M* KCl. Again, in the presence of KCl alone no leucine uptake was seen and NaCl alone gave poor uptake. Optimal leucine transport appears to require both KCl and NaCl. Moreover, the data show that a preexisting salt gradient is not necessary for the transport process. Since the extreme halophiles contain large concentrations of intracellular K^+ (Christian and Waltho, 1962; Lanyi and Silverman, 1972), while the growth medium contains mostly Na^+ (Larsen, 1962), it was natural to expect that the requirement for KCl might be for the inside and the requirement for NaCl might be for the outside. In a second series of experiments KCl-loaded vesicles were used and the exterior NaCl/KCl ratio was varied. The results are shown in Figure 4B as initial rates of leucine uptake vs. NaCl/KCl concentration. Rapid leucine uptake is indeed observed with KCl-loaded vesicles and optimal uptake was reached at a 1:1 NaCl/KCl ratio. There is

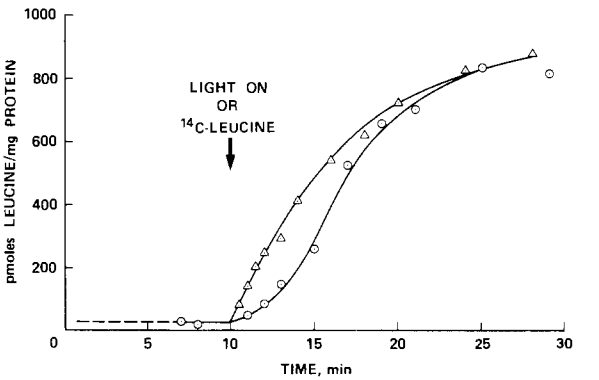

FIGURE 5: Leucine transport by NaCl-loaded envelope vesicles suspended in 2.0 *M* NaCl-1.0 *M* KCl: (⊙) [^{14}C]leucine added at 5 min, illumination began at 10 min; (△) illumination began at 0 min, [^{14}C]leucine added at 10 min.

considerable inhibition of leucine uptake above this optimal salt ratio, indicating that while NaCl is required on the exterior, NaCl alone is not sufficient. Nevertheless, it is clear that extensive leucine transport occurs at high internal K^+ concentration and high external Na^+ concentration. The maximal initial rate of transport in Figure 4B is 0.8 nmol of leucine/min per mg of membrane protein. This value is comparable with those found in *E. coli* vesicles, ca. 1 nmol/min per mg (Kaback, 1972) or in *S. aureus* vesicles, ca. 3 nmol/min per mg (Short et al., 1972) for respiration-linked leucine uptake. The maximal leucine concentration gradient established during illumination was estimated, using 3 μl/mg of protein for water-permeable space, to be approximately 200-fold.

If high concentrations of K^+ were required inside the envelope vesicles, as suggested by the results in Figure 4, it might be expected that NaCl-loaded vesicles would not show rapid leucine uptake. Such vesicles, suspended in 2.5 *M* KCl-0.5 *M* NaCl, in fact incorporated leucine during illumination, but, as shown in Figure 5, exhibited a pronounced lag in uptake. Preincubation of the vesicles in the dark did not shorten this lag, but as Figure 5 shows, preillumination of the vesicles resulted in immediate leucine uptake. A possible explanation of these results might be that K^+ is accumulated in the vesicles during illumination. Attempts to influence the lag in leucine uptake by adding valinomycin or gramicidin, which would facilitate the move-

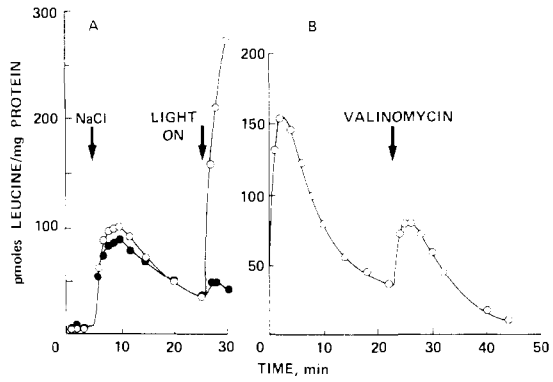

FIGURE 6: Leucine transport by cell envelope vesicles in the dark. (A) Effect of NaCl or KCl addition: (○) KCl-loaded vesicles were suspended in 3.0 *M* KCl, and 0.2 vol of 3.0 *M* NaCl was added; (●) same experiment as before, repeated in the presence of 6×10^{-6} *M* FCCP. After 26 min both preparations were illuminated. (B) Effect of valinomycin; KCl-loaded vesicles were suspended in 2.0 *M* NaCl–1.0 *M* KCl at 0 min. Valinomycin was added at the given time, to 2×10^{-6} *M*.

ment of K^+ across the membrane, were unsuccessful because these agents inhibit the uptake of leucine (as discussed below).

Illumination of the cell envelope vesicles produces a pH gradient across the membranes and an electrical potential. We therefore examined the effects on leucine uptake of various agents which preferentially influence one or the other of these. The results are given in Table I, as initial rates of leucine accumulation and net amounts of H^+ extruded. The agents used fall into several categories. The cationic membrane-permeable substances include Bu_4NBr and Ph_3MePBr, which are cations of strong bases (Grinius et al., 1970), and valinomycin and gramicidin, which facilitate the diffusion of K^+ and of Na^+ or K^+, respectively, across the membranes. When a transmembrane potential (interior negative) is created these substances are expected to travel across the membranes and abolish the potential. As seen in Table I, the permeant cations and the ionophores greatly inhibit leucine uptake without affecting the pH gradient. Uncouplers such as FCCP, which can carry protons across membranes and which are very effective inhibitors of oxidative phosphorylation, are expected to abolish both electrical potential and pH gradient. Both leucine uptake and pH gradient were eliminated by FCCP (Table I). Neither *N,N'*-dicyclohexylcarbodiimide (Harold, 1972; Danon and Stoeckenius, 1974) nor arsenate (Klein and Boyer, 1972), which have been used as ATPase inhibitors, was found to diminish leucine incorporation even at concentrations higher than that which totally inhibited ATP synthesis in *H. halobium* cells (Danon and Stoeckenius, 1974). Dicyclohexylcarbodiimide, however, was consistently found to increase several-fold the light-induced pH gradient, particularly at pH >6. Table I shows the *N,N'*-dicyclohexylcarbodiimide enhanced net H^+ extrusion similar to that observed in other systems, where it has been suggested that it decreases proton diffusion across membranes (Altendorf et al., 1974). Unlike *N,N'*-dicyclohexylcarbodiimide, arsenate between 10^{-3} and 2×10^{-2} *M* abolishes the pH gradient, since it is a strong buffer at pH 6. The results in Table I indicate, however, that arsenate substantially enhances leucine uptake. The increased rate of transport is probably due to the lack of alkalinization inside the vesicles which may be inhibitory. Thus, in the experiments described above, leucine transport is seen to be completely dissociated from the

Table I: Effect of Various Agents on Leucine Transport and Proton Extrusion during Illumination.[a]

Addition	Concn (*M*)	Leu Uptake (pmol/min per mg of Protein)	ΔH^+ (nmol/mg of Protein)
None		138	16.5
Bu_4NBr	8×10^{-3}	<2	16.2
Ph_3MePBr	7×10^{-4}	44	17.1
	2×10^{-3}	1	16.8
Valinomycin	10^{-6}	95	17.6
Gramicidin D	10^{-8}	14	18.0
	10^{-7}	<1	21.0
FCCP	6×10^{-6}	<1	5.0
	6×10^{-5}		0.8
Dicyclohexylcarbodiimide	4×10^{-5}	136	45.4
Arsenate[b]	10^{-3}	160	4.0[c]
	2×10^{-2}	280	<0.2[c]

[a] KCl-loaded vesicles, suspended in 2.5 *M* KCl–0.5 *M* NaCl, partially buffered with 1×10^{-3} *M* Hepes. [b] Arsenate present both inside and outside the vesicles. [c] Apparent decrease, due to buffering by arsenate.

pH gradient created on illumination. Rather, leucine uptake appears to be dependent on the electrical potential across the membranes. KCN at 10^{-2} *M* and 2-heptyl-4-hydroxyquinoline *N*-oxide at 5×10^{-6} *M*, which are respiratory inhibitors in extreme halophiles at these concentrations (Lanyi, 1968, 1969, 1972), had no effect on either leucine uptake or pH gradient.

The results in Table I show that gramicidin is a much more effective ionophore in *H. halobium* membranes than valinomycin, and shows inhibition of leucine transport at approximately three orders of magnitude lower concentration. Valinomycin is a mobile carrier of K^+, while gramicidin is thought to form channels in the membranes (Hladky and Haydon, 1970). Studies in this laboratory on the dynamics of chain motions in the lipids of extreme halophiles (Plachy et al., 1974) suggest that the extensive chain branching present should hinder the diffusion of small molecules across these membranes. The differences found between the action of valinomycin and gramicidin are consistent with this expectation.

The above results suggest that under appropriate conditions leucine uptake might be induced in the dark. If leucine transport involves Na^+ or K^+ symport the addition of NaCl or KCl to vesicles should cause a small, transient influx of leucine, facilitated by the diffusion potential-limited movement of the cation. Such experiments were carried out, as shown in Figure 6A, by adding NaCl to KCl-loaded vesicles suspended in KCl. In this manner a large downhill gradient of Na^+ toward the interior of the vesicles was created, while the K^+ gradient in the opposite direction was very small (1:0.83). Under these conditions transient uptake of leucine was observed, at an initial rate of approximately 50 pmol/min per mg of protein, which is a substantial fraction of the rate of uptake during illumination at this NaCl/KCl ratio (Figures 4B and 6A). In a similar experiment, adding KCl to NaCl-loaded vesicles, suspended in NaCl, caused no movement of leucine. It is significant that the Na^+ pulse induced leucine influx is not diminished by the uncoupler FCCP at a concentration which abolishes light-dependent leucine uptake, implying that the Na^+ pulse induced leucine flux does not involve H^+ movements. Since the light-dependent H^+ gradient was not entirely eliminated at this

FCCP concentration (6×10^{-6} *M*), however (Table I), the Na^+ pulse experiment was carried out also at higher concentrations, and approximately 50% inhibition of leucine uptake was observed at 6×10^{-5} *M* FCCP. This inhibition is probably not related to the H^+ carrier role of this uncoupler, since the Na^+ pulse dependent leucine uptake was not affected by 2×10^{-2} *M* arsenate (present both inside and outside the vesicles) even though any pH gradient would be very much reduced under these conditions (Table I). Adding HCl to envelope vesicles, equilibrated with 2.5 *M* KCl–0.5 *M* NaCl (inside and outside), producing a pH change from 7.0 to 5.6, resulted in no movement of leucine, in contrast with *Staphylococcus aureus* cells where such H^+ pulse caused isoleucine uptake (Niven and Hamilton, 1974). In the Na^+ pulse experiments, therefore, leucine transport appears to be dependent on the chemical gradient of Na^+ rather than on the electrochemical potential of H^+.

If leucine uptake during illumination responds to the membrane potential, uptake should occur also in the dark when an appropriate diffusion potential is developed across the membranes. For this purpose KCl-loaded vesicles were resuspended in NaCl–KCl (2:1) (Figure 6B) and leucine uptake was followed immediately. The large transient influx of leucine observed is probably similar in origin to that seen after adding a pulse of NaCl (Figure 6A), although here a large K^+ gradient (downhill toward the exterior) is also created and thus, provided that the permeability of K^+ in these membranes is greater intrinsically than that of Cl^-, a membrane potential (interior negative) could assist the leucine influx. This point cannot be settled since the permeabilities of various ions in *H. halobium* membranes are not yet known. On adding valinomycin, an ionophore with a well-described specificity for K^+, however, the permeability of K^+ alone is increased in the influx of leucine in this case (Figure 6B) is clearly due to the diffusion potential. Similar conditions have been reported to cause amino acid uptake in other systems as well (Asghar et al., 1973; Hirata et al., 1974; Niven et al., 1973; Kashket and Wilson, 1973).

Discussion

Upon illumination, *H. halobium* cell envelope vesicles are able, when NaCl and KCl are present in appropriate concentrations on the inside and the outside, to transport leucine against a considerable concentration gradient. In this system, unlike in others (Lombardi et al., 1974), the energy required for the translocation of leucine is clearly provided by the movement of H^+ across the membrane, as a result of the light-induced cycling of bacteriorhodopsin between its protonated and unprotonated forms (Stoeckenius and Lozier, 1974). The protonmotive force generated consists of two components: a chemical gradient of H^+ and a membrane potential (interior negative). When no phenomena other than proton translocation take place the relative contributions of these two components can be calculated according to Mitchell (1969). Movement of other ions across the membrane complicates the situation. The magnitude of the proton gradient and the membrane potential in this case can be calculated only if the membrane permeabilities of all the ions are known. In general, the movement of other ions diminishes the potential term while permitting the proton gradient to increase (Gromet-Elhanan and Leiser, 1973), and the back-diffusion of H^+ diminishes both potential and proton gradient. Buffering the pH outside and inside the vesicles eliminates the proton gradient without significantly decreasing the electrical potential. Since these two components of the protonmotive force are thus separable, we attempted to find out which is primarily responsible for energizing leucine transport.

The evidence obtained consists of the following: (1) valinomycin in the presence of K^+, gramicidin, or membrane-permeable cations which decrease the membrane potential (Grinius et al., 1970) inhibits leucine transport without affecting the pH gradient; (2) the presence of a nonpermeant buffer, arsenate, inside and outside the vesicles greatly diminishes the pH gradient but does not inhibit leucine uptake; and (3) the addition of valinomycin to KCl-loaded vesicles, suspended in NaCl, which is expected to give rise to a diffusion potential (interior negative), causes influx of leucine in the dark, as it does in membrane vesicles of other bacteria (Hirata et al., 1974). From these results we have drawn the conclusion that, during illumination, leucine is transported by a transmembrane potential, with little or no assistance from the proton gradient formed.

The ATPase inhibitors dicyclohexylcarbodiimide (Harold, 1972) which acts in *H. halobium* (Danon and Stoeckenius, 1974) and arsenate (Klein and Boyer, 1972) do not affect leucine uptake in this system; thus, the transport of the amino acid appears to be coupled *directly* to the light-induced "energization" of the membranes, which (for the purposes of leucine transport) is identified as the development of a membrane potential.

Since in the pH region investigated (6.5 ± 0.5) leucine has no net charge, a plausible hypothesis, which provides a mechanism for the transport of leucine, might be co-transport or *symport* with a cation. This hypothesis presumes a carrier with appropriate binding sites for leucine and a cation and interaction between the binding sites. The specificity of the transport for L-leucine argues in favor of the existence of a carrier. Proton symport has been suggested for sugar transport in *E. coli* (Pavlosova and Harold, 1969); West, 1970) and amino acid transport in yeast (Eddy and Novacki, 1971), while Na^+ symport is found in many eucaryotic systems (Vidaver, 1964; Eddy, 1968; Thomas et al., 1971).

Proton symport of leucine would be influenced by the proton gradient and since this was not found in the *H. halobium* system one might conclude that proton movement is not involved. However, the amount of leucine transported during illumination is only a negligible fraction of the protons ejected (Figure 2A) and the possibility is not quite ruled out that a very small fraction of the back-diffusion of protons is responsible for facilitating leucine influx. Two lines of evidence argue against this mechanism, however. The first is that leucine uptake is absolutely dependent on Na^+ (Figures 1 and 4), even in the presence of adequate amounts of KCl for providing the high salt concentrations generally required by halophilic enzymes (Larsen, 1967; Kushner, 1968; Lanyi, 1974). Even though this Na^+ dependence may conceivably reflect a cation-specific structural stabilization of the leucine carrier system or of other membrane components, such high selectivities are usually not observed for halophilic proteins, where the salt dependency is due to electrostatic screening of charges and/or hydrophobic stabilization (Lanyi, 1974). Rather, it is more probable that the Na^+ dependence of leucine transport is, in fact, due to Na^+ symport with an as yet unknown stoichiometry. The second line of evidence in favor of Na^+ symport originates from the results obtained with a NaCl pulse, where leucine influx was observed in the dark (Figure 6A). In this experiment the addition of NaCl to KCl-loaded en-

velope vesicles, resuspended in KCl, creates a very small downhill K^+ gradient toward the outside and a large Na^+ gradient toward the inside. The transient uptake of leucine observed is probably caused by a limited influx of Na^+, down its concentration gradient, terminated by a diffusion potential (interior positive). Proton symport, if present in this system, would depend on a pH gradient created by Na^+/H^+ antiport, such as found in *E. coli* (West and Mitchell, 1974). However, the presence of FCCP or buffering, both of which would abolish a pH gradient, do not diminish leucine uptake. This lends strong support to the idea that Na^+ is the only symporter ion in the leucine carrier system. On the other hand, these experiments alone do not completely rule out the possibility of proton symport since we cannot unambiguously prove that proton movement has not taken place.

The role of K^+ in enhancing leucine transport is uncertain. Although it might be thought that when K^+ is the only cation present inside the vesicles, it provides a Na^+ gradient which favors Na^+ and therefore leucine influx, we found that K^+ is also stimulatory when ion gradients are initially absent (Figure 4A). A study of the effect of internal K^+ concentration is hindered by the fact that NaCl-loaded vesicles show leucine uptake during illumination after a lag period which may be due to light-induced K^+ uptake (Figure 5).

As shown in Table I, dicyclohexylcarbodiimide greatly increases the net proton efflux in the presence of K^+, with no effect on leucine uptake. Although dicyclohexylcarbodiimide has been used in the past as an ATPase inhibitor (Harold, 1972), it is known to restore various energy-dependent processes in vesicles of an ATPase-less mutant of *E. coli* (Bragg and Hou, 1973), and this effect was recently shown to be due to a decrease in the permeability of the membranes to protons (Altendorf et al., 1974). Our observations on the effect of dicyclohexylcarbodiimide on *H. halobium* envelope vesicles are consistent with a decrease in the back-diffusion of H^+. In contrast with the case of *E. coli*, however, where dicyclohexylcarbodiimide also increased membrane potential, as evidenced by the increased influx of proline and of cationic permeant substances (Altendorf et al., 1974), our results suggest that the membrane potential is not increased by dicyclohexylcarbodiimide, perhaps because the back-diffusion of protons is replaced by the movement of other ions, such as K^+.

Acknowledgments

The expert technical assistance of Vernon Yearwood-Drayton is acknowledged. The authors are grateful to Walther Stoeckenius, Peter C. Hinkle, and Roberto Bogomolni for valuable discussions and to Melvin P. Silverman for performing the atomic absorption measurements.

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Kinetic Evidence for the Obligatory Formation of a 30S Initiation Complex in Polyphenylalanine Synthesis Initiated with *N*-Acetylphenylalanyl-tRNA†

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ABSTRACT: The problem of whether the initiation of bacterial protein synthesis involves the obligatory formation of a 30S initiation complex intermediate was examined in a model system with *N*-acetylphenylalanyl-tRNA as initiator tRNA and poly(uridylic acid) as mRNA. The time courses of the formation of the 30S and 70S initiation complex with *Escherichia coli* ribosomes were measured simultaneously by stopping the reaction with dextran sulfate and differentiating the *N*-acetylphenylalanyl-tRNA bound to 30S ribosomal subunits from that bound to 70S ribosomes with RNase I, which hydrolyzes *N*-acetylphenylalanyl-tRNA bound to 30S subunits but not that bound to 70S ribosomes. A maximum in the 30S complex concentration was observed within the first 10–15 sec of the reaction, whereas 70S complex formed more slowly with a slight initial time lag. When an analog computer was programmed with rate constants determined separately for the formation of the 30S initiation complex and for the formation of the 70S complex from preformed 30S complex, kinetic curves very similar to the empirical curves were obtained for the entire time course of the reaction. The results show clearly that formation of the 70S complex obeys the kinetic laws for consecutive reactions, and the 30S complex is, therefore, an obligatory intermediate in the initiation of polyphenylalanine synthesis in the model system.

It is generally accepted that the initiation of bacterial protein synthesis occurs via the formation of a 30S initiation complex composed of the 30S ribosomal subunit, mRNA, and the initiator tRNA (Haselkorn and Rothman-Denes, 1973; Lucas-Lenard and Lipmann, 1971). The best evidence to date for the operation of this pathway is the work of Guthrie and Nomura (1968), which consists of the observation that 70S ribosomes of *Escherichia coli* equilibrate their 50S subunits with free 50S subunits in the course of binding *N*-formylmethionyl-tRNA (fMet-tRNA) but not in binding Val-tRNA. Their experiment, however, is open to an alternative interpretation (Klem and Nakamoto, 1968); moreover, according to studies carried out subsequently by Subramanian and Davis (1971), who took measures to avoid a potential artifact arising from hydrostatic pressure in the technique of sucrose density gradient centrifugation (Infante and Baierlein, 1971), mRNA-free 70S ribosomes readily exchange their subunits with free subunits, independently of protein synthesis.

We have, therefore, investigated the problem of whether there is an obligatory formation of a 30S initiation complex in the initiation of protein synthesis by using a model system with *N*-acetylphenylalanyl-tRNA (AcPhe-tRNA) as the initiator tRNA. This system has been shown to be similar in many essential respects to the natural system, even in its requirement for the three known initiation factors (Bernal et al., 1974a,b; Blumberg et al., 1974; Economou and Nakamoto, 1967; Lucas-Lenard and Lipmann, 1967). In the present study, we examined the problem by making kinetic measurements of the formation of 30S initiation complex simultaneously with that of 70S initiation complex, taking advantage of the observation of Pestka (1968) that RNase I hydrolyzes aminoacyl-tRNA bound to the 30S ribosomal subunit, but not that bound to the 70S ribosome. A

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