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Light-Activated Amino Acid Transport Systems in *Halobacterium halobium* Envelope Vesicles: Role of Chemical and Electrical Gradients†

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ABSTRACT: The accumulation of 20 commonly occurring L-amino acids by cell envelope vesicles of *Halobacterium halobium,* in response to light-induced membrane potential and an artificially created sodium gradient, has been studied. Nineteen of these amino acids are actively accumulated under either or both of these conditions. Glutamate is unique in that its uptake is driven only by a chemical gradient for sodium. Amino acid concentrations at half-maximal uptake rates (K_m) and maximal transport rates (V_{max}) have been determined for the uptake of all 19 amino acids. The transport systems have been partially characterized with respect to groups of amino acids transported by common carriers, cation effects, and relative response to the electrical and chemical components of the sodium gradient, the driving forces for uptake. The data presented clearly show that the carrier systems, which are responsible for uptake of individual amino acids, are as variable in their properties as those found in other organisms; i.e., some are highly specific for individual amino acids, some transport several amino acids competitively, some are activated by a chemical gradient of sodium only, and some function also in the complete absence of such a gradient. For all amino acids, Na^+ and K^+ are both required for maximal rate of uptake. The carriers for L-leucine and L-histidine are symmetrical in that these amino acids are transported in both directions across the vesicle membrane. It is suggested that coupling of substrate transport to metabolic energy via transient ionic gradients may be a general phenomenon in procaryotes.

Vesicles prepared from cell envelope membranes of *Halobacterium halobium* by sonication possess features characteristic of the membranes of whole cells; i.e., they have the same orientation as whole cell membranes, they contain purple membrane patches which function in light to pump protons across the membrane, they couple light energy to the formation of ion and electrical gradients, and they accumulate amino acids against large concentration gradients (MacDonald and Lanyi, 1975; Lanyi et al., 1976a,b; Kanner and Racker, 1975). In contrast to whole cells, light-induced ATP formation cannot be demonstrated in these vesicles using the most sensitive techniques available (Lanyi et al., 1976b). A number of workers (Berger and Heppel, 1974; Harold, 1976) have reported that amino acid transport in other bacteria, e.g., *Escherichia coli,* appears to be energized in at least two ways: one in which transport is the result of a protonmotive force or energized membrane state, and the other which directly involves the hydrolysis of ATP. Neither leucine nor glutamate transport in *H. halobium* vesicles is affected by added ATP, or arsenate inside or outside of the vesicles, and it therefore seems unlikely that ATP synthesis (or hydrolysis) is required for transport in vesicles. Whether ATP synthesis (or hydrolysis) is involved in transport in whole cells is not clear.

We have previously reported on the energetics of membrane transport for leucine (MacDonald and Lanyi, 1975) and glutamate (Lanyi et al., 1976a,b) in these vesicles and have shown that the driving force for leucine transport can be separated conceptally into two components, an electrical potential ($\Delta\Psi$)[1] which is generated by the light-activated proton pump (Oesterhelt, 1975), and a chemical sodium gradient (ΔNa^+)[1] which is generated via a Na^+/H^+ antiport system (Lanyi and MacDonald, 1976). Glutamate, in contrast, is transported only in response to a chemical sodium gradient.

The mechanism and structure of bacteriorhodopsin, the proton pump, has been the subject of extensive investigation (Bogomolni and Stoeckenius, 1974; Henderson and Unwin, 1975; Oesterhelt and Stoeckenius, 1973; Racker and Stoeckenius, 1973) but only a limited number of other reports (Kanner and Racker, 1975; Hubbard et al., 1976) have ap-

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[1] Symbols and abbreviations used: $\Delta\Psi$, electrical potential across membrane; ΔpH, ΔNa^+, and ΔK^+, chemical gradients across membrane of H^+ (expressed at pH), Na^+, and K^+, respectively; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; ESR, electron spin resonance.

peared on the transport of other ions and molecules in response to this energy-yielding system.

In this communication we show that 17 of the 18 other amino acids studied are also accumulated against large concentration gradients in vesicles of *H. halobium*. The driving force for this uptake is entirely accounted for by the electrical and/or chemical gradients formed in response to the light-induced proton pump, or to a sodium gradient created artificially in the dark. Several carrier groups are present, which are involved in the transport of two or more amino acids. The carriers are not identical but, with the exception of the carrier for glutamate, share the common feature of responding to either $\Delta\Psi$ or ΔNa^+.

Experimental Procedures

Preparation of Vesicles. Cell membrane vesicles used in this study were prepared from *Halobacterium halobium*, strain R-1. Cultures were grown as previously described (Lanyi et al., 1976a), in 4-L quantities. Cells were harvested by centrifugation, washed once with 4 M NaCl, 0.05 M Tris[1] at pH 6.7, and resuspended in 100 mL of the same solution. Vesicles were produced by sonicating this suspension three times, 3 min each time, with intermittent chilling in an ice bath. Sonication was performed with an MSE 100W ultrasonic disintegrator. Vesicles were purified by differential centrifugation as previously described (MacDonald and Lanyi, 1975; Lanyi et al., 1976a; Kanner and Racker, 1975) and suspended in 4 M NaCl to a final concentration of ca. 20 mg of protein mL^{-1}. Protein was determined by the Lowry method (Lowry et al., 1951) using Sigma crystalline egg white lysozyme as the reference standard.

Before use these vesicles were diluted 100-fold into a 3 M salt solution of the composition desired for the vesicle interior and were reconcentrated to ca. 20 mg of protein mL^{-1} by centrifugation. This abrupt osmotic change permits the vesicles to equilibrate with their environment, apparently by breaking and resealing of the membrane (MacDonald and Lanyi, 1975; Lanyi et al., 1976a). For most experiments, the vesicles in 3 M salt were buffered both inside and out by 1 mM Hepes as previously described (MacDonald and Lanyi, 1975).

Transport Assay. The ability of vesicles to incorporate amino acids was determined as before (MacDonald and Lanyi, 1975). A water-jacketed reaction vessel, containing from 0.5 to 1.5 mL of reaction mixture, was kept at a constant temperature (usually 24 °C) with a circulating water bath. Illumination was accomplished when necessary with a 300-W quartzline lamp (General Electric ELH). The light beam was passed through a Corning 1-75 short pass filter and a Corning 3-68 cutoff filter and focused on the reaction vessel. Light intensity was measured in the center of the reaction vessel with a YSI radiometer and was adjusted with an appropriate neutral density filter to 2×10^6 ergs s^{-1} cm^{-2}. The same apparatus was used for experiments in which transport was driven by a sodium gradient, except that the reaction was run in the dark. In this case vesicles loaded with, and suspended in, 3 M KCl were added directly to 3 M NaCl in the reaction vessel to start the reaction. The stock vesicle suspension volume was never more than 1% of the reaction mixture volume, so the external KCl concentration in this system did not exceed 0.03 M.

At appropriate time intervals after initiating the experiment, either by adding the vesicles or turning on the light, 0.1-mL samples were withdrawn with a Carlsberg pipet, quickly injected into 2 mL of 3 M NaCl and collected on a prewetted (with distilled water) nitrocellulose filter (Matheson-Higgins, 24 mm, 0.45-μm porosity). The filter was washed 5× with 1-mL washes of 3 M NaCl and then placed in a scintillation vial and dried. Seven milliliters of scintillation fluid (0.4% 2,5-bis[2-(*tert*-butylbenzoxazolyl)]thiophene in toluene) was added and the radioactivity retained on the filters was determined in a Packard liquid scintillation spectrometer. Counting efficiency was ca. 20% for tritium and 80% for carbon-14. Counting times to give a percent standard deviation of not more than 2.5% were always used.

Determination of Kinetic Constants. The kinetic constants, K_m (substrate concentration giving half-maximal transport rate) and V_{max} (maximal transport rate), for light-energized transport were determined with vesicles containing buffered 1.5 M KCl–1.5 M NaCl and suspended in the same medium. After illumination for 10 min, varying concentrations of the appropriate amino acid were added to the illuminated suspension and the uptake rate was determined by taking samples, at 1-min intervals, for 4 min. Because of a variable amount of background uptake, especially with the less polar amino acids such as phenylalanine and tryptophan, initial rates were difficult to measure and sometimes necessitated repeated determinations. Presoaking the filters in 1 mM solutions of the amino acid reduced this variation but did not eliminate it. Variations were also observed with the lot of filters being used. When possible, rates were determined using all points, but the initial uptake rate decreased rapidly in some cases after the first 2 min and necessitated using only the first two time points. In general five or more substrate concentrations were used for determination of the kinetic constants, and, when possible, they were chosen to fall on both sides of the K_m. The kinetic constants were calculated by least-squares linear regression analysis of the relation of the reciprocal of the substrate concentration to the reciprocal of the rate of uptake. These calculations were always checked graphically and are only reported if both methods of analysis are consistent. Calculated data were considered satisfactory if a correlation coefficient of 0.98 or higher was obtained.

Temperature Effects. A study of the effect of temperature on the entry and exit of amino acids was carried out in buffered 1.5 M KCl–1.5 M NaCl using vesicles with these same internal concentrations of salts. Temperature was monitored in the reaction vessel with a YSI 42SC tele-thermometer. The exit reaction was followed after illuminating a tenfold concentrated vesicle suspension for a time (ca. 15 min) sufficient to accumulate the amino acid to a steady-state concentration at 30 °C and then transferring 0.1 mL of this illuminated suspension to 0.9 mL of an identical salt solution equilibrated to the desired temperature, containing no amino acid and not illuminated. Less than a minute was required for the temperature to reequilibrate after adding the vesicles. Amino acid exit was followed by sampling in the same manner as for the uptake studies.

Nature of the Accumulated Amino Acids. ^{14}C-labeled amino acids accumulated by vesicles in response to illumination were extracted in the following manner. Vesicles were allowed to accumulate the amino acid in the light for 8 min and were then collected on a filter and washed with 3 M NaCl. The filter was transferred to a small beaker and extracted with two, 1-mL volumes of water. These aqueous extracts were evaporated to dryness in vacuo and redissolved in 50 or 100 μL of water. Ten microliters of this mixture was spotted on thin-layer chromatography plates and these plates were developed in the appropriate solvent. Radioautograms were made by exposing the plates to x-ray film for 3 to 5 days. The film was developed and the chromatogram sprayed with a ninhydrin solution to detect the bulk amino acid. The systems used were: cellulose plates

(Quantum Industries, Q2), butanol-acetic acid-water (25:4:10); silica gel plates (Quantum Industries Q5), methyl ethyl ketone-pyridine-water-acetic acid (70:15:15:2); and DEAE-cellulose plates (Baker-flex), methanol-water-pyridine (20:5:1).

Chemicals. ^{3}H- and ^{14}C-labeled amino acids for uptake studies were purchased from New England Nuclear Corp. and were adjusted to the desired specific activities with unlabeled L-amino acids obtained from Sigma Corp. ^{14}C-labeled amino acids used for the chromatographic analysis of the accumulated amino acids were purchased from Amersham/Searle Corp. All other chemicals were standard reagent grade.

Results

Effect of Temperature. The rapid loss of L-[^{3}H]leucine from vesicles at 30 °C after termination of illumination or on the addition of unlabeled leucine (MacDonald and Lanyi, 1975) indicates that an exchange of leucine takes place across the vesicle membrane during the normal course of uptake. The exit rate of an amino acid will influence its rate of accumulation, and indeed this must be a major factor in the regulation of the pool size of amino acids in these vesicles. The exit reaction for glutamate, on the other hand, appears to be entirely prevented by the presence of a sodium gradient (Lanyi et al., 1976a,b), and, since the entry of glutamate is dependent on the sodium gradient, the exit rate is very low under normal conditions. Carrier mediated exit of leucine and perhaps all other amino acids also could be inhibited by the sodium gradient which would provide the asymmetry for unidirectional transport. In this case the observed exit rate (greater for the less polar amino acids) would be due to the nonpecific permeability of the vesicle membranes. If this were so, there should be a much greater activation energy (E_a) for entry than for exit (Jain, 1972). This possibility was examined by determining the E_a for entry and exit of leucine and histidine as shown in Table I. The Arrhenius plots show a break around 20 °C for entry of leucine and histidine, whether energized by light or by a sodium gradient, or for exit. The activation energies calculated for entry rates are two to three times higher for temperatures below 20 °C. For the exit rates, the E_a is greater *above* 20 °C than below. The simplest explanation for this observation is that below ca. 20 °C membrane leakiness plays an increasing role in the exit of amino acids because the movement of the amino acid carrier is increasingly restricted. Thus the E_a for exit below 20 °C is low, while the E_a for entry, which represents active transport, is high. Above 20 °C there is a close similarity in the activation energy for both entry and exit of leucine. It is of interest that Andersen (1975) reports a similar temperature effect for the transport of glutamate by membrane vesicles of *Halobacterium salinarium*, energized by respiration. Plachy et al. (1974) reported a change at 23 °C in the ESR spectrum of spin labels incorporated into liposomes formed from *H. cutirubrum* lipids, which they attributed to a change in the orientation of the polar head groups of the phospholipids.

For subsequent studies on the kinetics of uptake of other amino acids, 24 °C was chosen as the reaction temperature because it was higher than the highest "break point" for the data in the Arrhenius plots, but as low as possible to minimize the effect of the exit reaction on the net uptake rate.

Rate Constants for the Transport of 19 Amino Acids. Table II shows the kinetic constants for uptake of 19 commonly occurring L-amino acids, using either a prearranged, or a light-generated sodium gradient, as the driving force. The data are presented as substrate concentration giving half-maximal uptake rate (K_m) and the maximal uptake rate (V_{max}), obtained by plotting the reciprocal of amino acid concentration against the reciprocal of the initial uptake rate.

TABLE I: Effect of Temperature on L-Leucine and L-Histidine Uptake and Exit in Membrane Vesicles of *H. halobium*.[a]

Amino acid	Driving force	Activation energy (E_a) (cal mol^{-1}) 0 to 20 °C	20 to 40 °C
L-Histidine	Light	36 038	11 894
	Na^+	22 034	13 361
	Exit	6 516	21 819
L-Leucine	Light	28 899	16 820
	Na^+	30 505	19 099
	Exit	9 282	19 952

[a] For light-induced uptake, intra- and extra-vesicle concentration was 1.5 M NaCl, 1.5 M KCl, 1 mM Hepes buffer, pH 6.0. For sodium gradient driven uptake, intravesicle salt concentration: 0.01 M NaCl, 2.99 M KCl, 1 mM Hepes, pH 6.0. Initial reaction volume: 0.6 mL containing ca. 100 μg of vesicle protein ml^{-1}. L-[^{3}H]Leucine concentration for uptake studies was 9.1 μM and for exit studies, 11 μM (sp act. 2 Ci mmol^{-1}). L-[^{3}H]Histidine concentration for uptake study was 1 μM (sp act. 0.909 Ci mmol^{-1}) and for exit study was 45 μM (sp act. 0.088 Ci mmol^{-1}). The vesicle suspension was equilibrated at the desired temperature for at least 5 min. After illumination for 5 min, the substrate was added. One-hundred microliter samples were taken 0.5, 1, 2, 3, and 4 min after adding the substrate. Sodium-gradient-driven uptake was started by adding the vesicles to the reaction mixture in the dark and sampling as for the light-driven uptake. Exit rate was measured in the dark, using vesicles which had been loaded with the amino acid by illumination of a 10× concentration of vesicles at 30 °C for at least 15 min. To start the exit experiments, 1 volume of these illuminated vesicles was transferred to 9 volumes of salt solution at the desired temperature in the dark. Sampling times and procedures were the same as in the uptake experiment. The E_a values are obtained from a linear regression analysis of the data plotted according to the Arrhenius equation.

The data show that, for most amino acids, the K_m for uptake is approximately the same whether the uptake is driven by light-induced or a prearranged sodium gradient. The uptake of some amino acids (for example, asparagine, methionine, and phenylalanine) shows higher K_m's when driven by a prearranged sodium gradient than when driven by light, suggesting different carrier mechanisms (see Figure 1). Glutamate differs from this general pattern in that its uptake is energized only by ΔNa^+. Tyrosine is transported by both conditions, but a complete set of kinetic constants was not determined. Cysteine transport was not observed but it may inhibit its own carrier and thus be difficult to detect (see below). Both V_{max} and K_m vary somewhat in repeat determinations. For instance, although kinetic data for leucine were among the easiest to obtain due to its high V_{max}, the standard deviation from six determinations of K_m was ±0.9 μM ($\bar{X}$ = 1.4 μM). Differences in lots of vesicles contributed only slightly to the variation in K_m but to a considerable extent to the variation in V_{max}.

In the case of light-induced arginine and lysine transport, there is some evidence to support the idea that more than one protein or protein state is involved in transport. Here the data analyzed by Lineweaver-Burk plots are best described by two intersecting regression lines. The significance of this observation is not yet clear. Histidine, which shares a common carrier with lysine and arginine (see below), on the other hand, shows normal kinetics for both driving forces. Other data presented below also show that the uptake of histidine differs from that of lysine and arginine and support the idea that

TABLE II: Kinetic Constants for Light-Induced and Sodium-Gradient Induced Uptake of 19 L-Amino Acids by Membrane Vesicles of *H. halobium*.[a]

	Light-induced transport		Sodium-gradient-induced transport		Light-induced transport in an all NaCl system	
L-Amino acid	K_m (μM)	V_{max}[b]	K_m (μM)	V_{max}[b]	K_m (μM)	V_{max}[b]
Arg	1.0[c]	446	12	303	2.7	45
Lys	2.0[c]	592	4.2	268	3.3	59
His	3.9; 6.8	430; 190	5.6	116	8.7	71
Asn	10.3	89	59; 55	64; 70	3.6	19
Gln	13; 32	78; 39	5.5; 11	20; 33	10	25
Aspartate	25	1875	30	1686	2.3	211
Glutamate	0.13	3700	0.13	2900		0
Ala	4.2; 9.6	485; 1007	10; 9.1	301; 163	11	107
Gly	4.5	746	4.6	481	2.6	28
Thr	5.0	288	16; 2.4	397; 93	2.7	35
Ser	2.9; 3.2	1031; 1069	6.8; 10	504; 874	7.6	34
Leu	1.4 ± 0.9[d]	2706	2.8; 1.0	1224	3.7	52
Val	2.4	892	2.4	829	2.0	15
Ile	1.1	930	2.7	814	2.2	19
Met	3.7; 3.7	1208; 1240	10; 9.0	767; 1120	1.4	30
Phe	6.4; 8.3	37; 46	39; 29	90; 67	3.8	23
Tyr	33	8.3				
Trp	2.3	71	3.3; 5.2	96; 61	4.1	6.2
Pro	0.8; 0.7	63; 27	0.8; 1.4	28; 74	1.2	9.2
Cys		<1.0				

[a] In the light-induced system, vesicles were loaded with 1.5 M NaCl, 1.5 M KCl, 1 mM Hepes buffer, pH 6.0, and suspended in 0.6 mL of the same solution. They were equilibrated for several min at 24 °C and then illuminated for 10 min before adding the substrate. Samples were taken at 0.5, 1, 2, 3, and 4 min after adding the substrate. In the sodium-gradient-driven system, vesicles loaded with 3 M KCl were added to 0.6 mL of 3 M NaCl containing the substrate. Sampling was as for the light-induced study. Vesicle protein concentration was 125 μg mL^{-1} for all experiments except those with tryptophan and phenylalanine (320 μg mL^{-1}), tyrosine (1300 μg mL^{-1}), and cysteine (725 μg mL^{-1}). In the all NaCl system, vesicles were loaded with, and suspended in, 3 M NaCl. They were not preilluminated before addition of the amino acid. [b] V_{max} in units of pmol min^{-1} (mg of protein)$^{-1}$. [c] See Discussion. [d] SD of six determinations.

multiple carriers are present for lysine and arginine. This does not necessarily mean that histidine is transported on only one carrier, and considerably more work needs to be done to clarify this point.

Illuminated vesicles develop two driving forces which could permit transport, a large electrical potential ($\Delta\Psi$), which is produced by the light-driven proton pump, and a chemical gradient for sodium (ΔNa^+), generated by Na^+/H^+ antiport (Lanyi and MacDonald, 1976). Since vesicles in the experiments reported in Table II were preilluminated for 10 min before adding the amino acid, the uptake rates used for the calculation of K_m reflect both of these driving forces.

The alternative of using vesicles which were not preilluminated was rejected because lags were observed in transport, which were greater for some amino acids than for others, and the resulting variability in the initial uptake rates made the results obtained under these experimental conditions difficult to interpret. The possible error inherent in this choice is partly countered by carrying out the kinetic analysis in an all-sodium system (see below); we assume that only $\Delta\Psi$ drives the transport. However, for leucine, the K_m for light-driven transport in nonpreilluminated vesicles is 2.4 μM, in preilluminated vesicles 1.4 μM, and for sodium-gradient driven transport it is 1.9 μM. The closeness of values is consistent with the idea that at least one carrier is common for these three conditions. It does not eliminate the possibility of separate additional carriers for each mechanism or separate carriers with similar K_m's.

The data in Table II show that the K_m's for uptake driven by a sodium gradient are not greatly different from the K_m's for light-induced uptake. This prompted an examination of the light-driven reaction in an all-sodium system (Table II, columns 5 and 6). Since H^+ and Na^+ are the only cations present and the concentration of Na^+ inside is equal to the concentration outside of the vesicles, transport must be energized only by the electrical component of any sodium gradient established by the light-induced proton pump. The data on transport kinetics under these conditions agree quite well with those shown in column 1 and 2, except that the V_{max} is considerably lower in every case. The data do not completely rule out the involvement of K^+ in the uptake system since trace K^+ is always present in 3 M NaCl. However, the amount is too small to permit the efflux of Na^+ needed to establish a sodium concentration difference of sufficient magnitude in the time period observed to account for the rate of uptake. Glutamate, which has been shown by other means to be transported only in response to a chemical sodium gradient, is not accumulated at all in the all-sodium system.

The data in Table II support the idea that there is both a chemical and an electrical component in the driving force for amino acid transport, but the magnitude of the light-driven uptake rate cannot be clearly accounted for by combining the sodium-gradient driven uptake rate (Table II, column 2) with the uptake rate due to $\Delta\Psi$ (column 3). The difficulty in obtaining initial uptake rates (i.e., 0 to 10 s) with the method used may account for some of the discrepancy, but the concentration of K^+ in the reaction mixture is likely to be more important. This is born out by the data presented in Figures 1A and 1B which show the effect of Na^+:K^+ ratios on the V_{max} and K_m for glycine and methionine. In this experiment, the internal ion

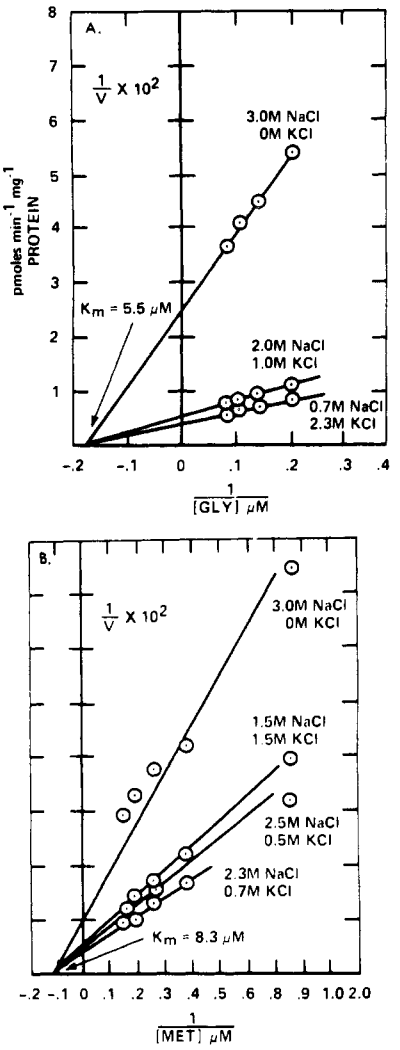

FIGURE 1: Effect of increasing [Na^+] on the K_m and V_{max} for L-methionine and glycine in membrane vesicles of *H. halobium*. Vesicles containing 3 M NaCl were added to 0.8 mL of illuminated salt mixtures. Samples were taken at 1-min intervals for 4 min, and the uptake rate was calculated from the linear portion of the curve, as for data in Table II. Vesicle concentration was 240 μg of protein mL^{-1}. (A) Lineweaver-Burk double-reciprocal plot of the uptake of glycine by 3 M NaCl loaded vesicles at three different external concentrations of NaCl and KCl. (B) Lineweaver-Burk double-reciprocal plot of the uptake of methionine by 3 M NaCl loaded vesicles at four different concentrations of NaCl and KCl.

concentration was 3 M NaCl and only the external ion ratio was varied. There is only a slight effect of ion ratio on the K_m for transport of the two substrates, but a large effect of external [Na^+] on the V_{max}. The maximal values are obtained for a Na^+:K^+ ratio close to 1.

A more extensive investigation of the effects of Na^+:K^+ ratios both inside and outside of the vesicles (data not shown) indicate that at low external [Na^+] there is no uptake of any amino acid regardless of the internal ion concentration, but at 0.1 M NaCl many amino acids show an appreciable (but not maximal) rate of uptake. Apart from demonstrating that sodium is required, the data further indicate that K^+:Na^+ ratio affects the uptake of some amino acids more than others, and that this effect is dependent on the ion ratio inside of the vesicles. With K^+-loaded vesicles, the uptake rate increases with the external [Na^+] presumably because the large initial sodium gradient is the driving force. In vesicles containing Na^+, at equal or greater concentration than K^+, the uptake rate depends only on external [Na^+] for some amino acids (e.g., arginine, lysine, asparagine, proline, tryptophan), but for others (e.g., methionine, valine, isoleucine, serine, threonine, and aspartic acid) K^+ also appears to be stimulatory and a Na^+:K^+ ratio near one is optimal.

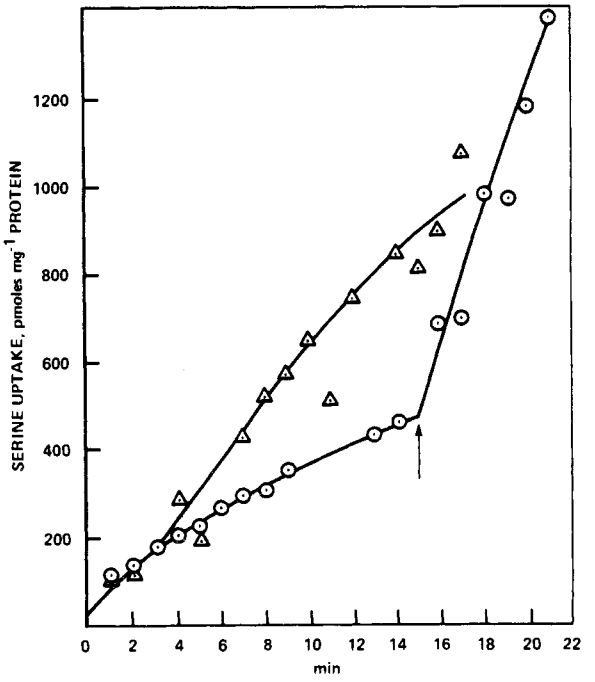

FIGURE 2: L-Serine uptake at low potassium concentrations. Effect of adding K^+. Vesicles loaded with NaCl were suspended in 3 M NaCl. Serine was added to a final concentration of 7 μM and the light was turned on to start the experiment. At 15 min, 12 μL of 2.9 M KCl was added, arrow (final concentration of KCl, 0.08 M). Vesicle concentration 380 μg of protein mL^{-1}. Symbols: (⊙) ultrapure NaCl (Ventron Corp); (▲) reagent grade NaCl.

The stimulatory role of K^+ on the vesicle exterior is shown by its effect on the uptake of serine in Figure 2. When ordinary reagent grade NaCl is used, a slow but increasing rate of uptake occurs after an initial 4- to 5-min lag. When ultrapure (10 μM K^+ in 3 M NaCl) NaCl (Ventron Corp.) is used, little or no secondary increase of uptake rate is obtained. This could be explained if the rate of accumulation of serine is limited by K^+ which functions as a counterion. If a small amount of KCl (0.08 M final concentration, see arrow, Figure 2) is added to vesicles which have accumulated serine in the light in this all-sodium system, there is a rapid increment in the accumulation of serine. This supports the idea that K^+ is required externally to permit the efflux of Na^+ and permit the establishment of ΔNa^+.

When the K^+:Na^+ ratio inside of the vesicles is varied but the outside ratio is kept constant and equimolar, i.e., varying both the steepness and direction of the initial chemical gradient for sodium (or potassium), four patterns are observed as shown in Figure 3. For amino acids like serine (Figure 3A), vesicles loaded with K^+ show rapid uptake which levels off after about 10 min. The high uptake rate is attributable to transport in response to both the preformed Na^+ gradient and to the $\Delta\Psi$ generated by the light-induced proton pump. For vesicles containing a Na^+:K^+ ratio internally and externally equal (1.5 M NaCl-1.5 M KCl), a biphasic curve is obtained. This demonstrates the operation of two driving forces for the uptake of serine, $\Delta\Psi$ which produces the first rise, and ΔNa^+ which produces the second after several minutes. For vesicles containing only sodium chloride, the initial rise parallels that for

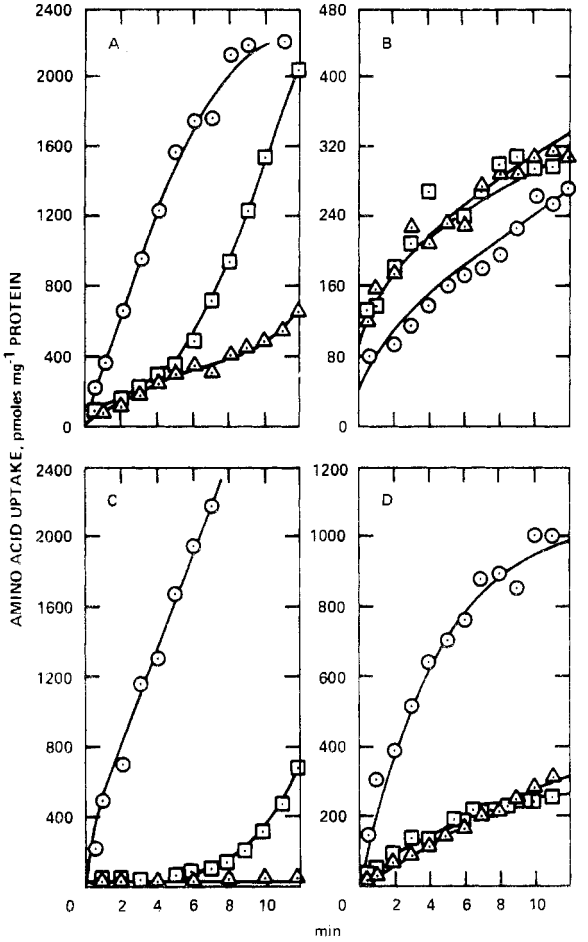

FIGURE 3: Effect of intravesicle NaCl:KCl ratios on the light-driven uptake of L-serine, L-glutamine, L-aspartate, and L-glutamate in membrane vesicles of *H. halobium*. Vesicles loaded with 3 M NaCl, 1.5 M NaCl-1.5 M KCl, or 3 M KCl were added to illuminated cuvettes containing 0.8 mL of 1.5 M NaCl-1.5 M KCl, buffered with 1 mM Hepes, pH 6.0. Initial amino acid concentrations were: (A) L-[^{3}H]serine, 9 μM, sp act. 0.15 Ci mmol^{-1}; (B) L-[^{3}H]glutamine, 5 μM, 0.235 Ci mmol^{-1}; (C) L-[^{3}H]glutamate, 9 μM, 0.045 Ci mmol^{-1}; (D) L-[^{3}H]aspartate, 13 μM, 0.476 Ci mmol^{-1}. The vesicle concentration was ca. 375 μg of protein mL^{-1}. Symbols: (▲) intravesicle content 3 M NaCl; (⊡) intravesicle content 1.5 M NaCl, 1.5 M KCl; (⊙) intravesicle content 3 M KCl. The uptake data for L-serine are reduced by one-third for convenience of plotting

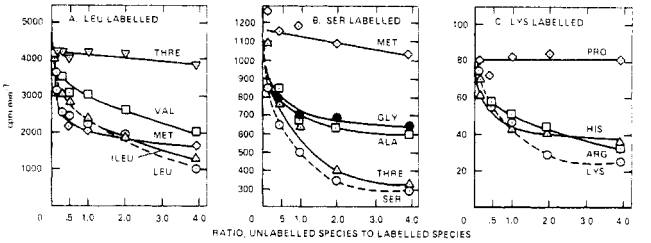

FIGURE 4: Inhibition of the light-induced uptake of L-[^{3}H]lysine, L-[^{3}H]serine, and L-[^{3}H]leucine by several other amino acids. The rate of uptake of the labeled amino acids is plotted against the ratio of the unlabeled to labeled amino acid. In each case, the labeled amino acid is present at the same concentration throughout the experiment. The mixture 1.5 M NaCl-1.5 M KCl buffered with 1 mM Hepes was present inside and outside of the vesicles. Experiments were started by adding labeled amino acid and inhibitor amino acid to vesicles which had been preilluminated for 5 min. (A) L-[^{3}H]Leucine, 5 μM, sp act. 2 Ci mmol^{-1}, vesicle concentration 150 μg of protein mL^{-1}; (B) L-[^{3}H]serine, 5 μM, sp act. 0.178 Ci mmol^{-1}, vesicle concentration 187 μg of protein mL^{-1}; (C) L-[^{3}H]-lysine, 5 μM, sp act. 0.212 Ci mmol^{-1}, vesicle concentration 367 μg of protein mL^{-1}. Dotted lines are for inhibition of uptake rate of amino acid containing a radioisotope by the same amino acid lacking the radioisotope.

the 1.5 M NaCl-1.5 KCl vesicles but the second rise occurs considerably later, reflecting the longer time required for generation of a ΔNa^+. The transport system for glutamine, on the other hand, must use $\Delta\Psi$ as a major component of its driving force. Thus ΔNa^+, which is present initially in KCl-loaded vesicles, or is generated on prolonged illumination, does not contribute appreciably to the rate of uptake and therefore the uptake rates at all three external/internal ion ratios are nearly equal (Figure 3B).

Figure 3C shows the response of glutamate transport to these three internal/external ion ratios. This transport system has been discussed in detail elsewhere (Lanyi et al., 1976a,b) but is presented here for comparative purposes. It differs from the others in that uptake in response to $\Delta\Psi$ alone is entirely lacking. Figure 3D shows the response of the uptake system for aspartate under similar conditions. It is similar to the uptake system for serine in response to $\Delta\Psi$, i.e., when vesicles are loaded with 3 M NaCl. When loaded with 1.5 M NaCl and 1.5 M KCl, on the other hand, there is only a very small secondary response to the generation of a chemical gradient for sodium.

Transport Carrier Groups. A large body of literature (cf. Christensen, 1975) supports the idea that a number of individual amino acids share common carriers in cells and vesicles. In addition, in some cases several carriers exist for single amino acids (Wood, 1975). On the assumption that substrates sharing the same carrier ought to competitively inhibit transport, the uptake of each amino acid was examined in the presence of the other amino acids at 100× concentration, added either singly or in groups. These data are presented in Table III and were obtained using a prearranged sodium gradient since it is simpler than the light-driven system. A similar study of light-induced transport was also made with results not distinguishably different from those presented in Table III as far as carrier grouping is concerned.

The data for competitive inhibition of transport presented in Table III show that there are several groups of carriers: asparagine-glutamine; arginine-lysine-histidine; alanine-glycine-serine-threonine; valine-leucine-isoleucine-methionine; and possibly phenylalanine-tryptophan-tyrosine. This latter group is questionable because the extent of inhibition is much less than expected. It is likely in this case, therefore, that this group shares a common carrier but each amino acid has one or more specific carriers as well. Cysteine strongly inhibits the uptake of a number of amino acids, particularly lysine and histidine, but also the alanine-glycine-serine-threonine and the valine-leucine-isoleucine-methionine groups and proline. The inhibition shows noncompetitive kinetics. Cystine has no effect. The mechanism of inhibition is unclear; -SH reactive inhibitors such as mersalyl or phenylmercuric acetate, shown by Lanyi (1972) to be effective inhibitors of respiratory enzymes in other halobacteria under the same ionic conditions, did not affect the uptake of histidine, leucine, or glutamate even at 10 mM concentration.

Aspartate and glutamate appear to be transported independently of each other and the mechanism of transport of the two amino acids seems to differ (Figure 3).

Christensen (1975) has pointed out the inherent error in the assumption that inhibition of the uptake of one amino acid by another indicates that they share a single common carrier. The data in Table III, thus, do not indicate whether more than one carrier is present in the above groups. These groups were therefore examined in more detail and the results of the competition experiments are shown in Figure 4. The analysis is not

TABLE III: Inhibition of Amino Acid Transport in Membrane Vesicles in *H. halobium*.[a]

Inhibitors	^{14}C or ^{3}H amino acid transported																		
	Arg	Lys	His	Gln	Asn	Asp	Glu	Thr	Ala	Ser	Gly	Leu	Ile	Val	Met	Trp	Phe	Pro
Arg	>90	92	69															
Lys	97	>90	49			0	0	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	13	0	0	15
His	95	90	>90															
Gln	39	+18[c]	17	>90	90	0	0	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	0	0	34	0
Asn				89	>90													
Asp	44	+42[c]	29			>90	+10[c]											
Glu	28	+62[c]	3			+9[c]	>90											
Cys	32	92	89			0	30	85	73	70	84	62	69	56	84	0	0	75
Thr	40	0	12			0	10	>90	80	95	97	8	7	6	38	11	17	0
Ala								90	>90	89	92	39	25	30				
Ser	35	+11[c]	28			0	20	90	75	>90	93	8	0	0	13		0	
Gly								36	74	84	>90	8	13	18				
Leu	5	25	23			+4[c]	30	70	67	77	82	>90	97	97	98	11	0	0
Ile												99	>90	97	98			
Val												97	91	>90	88			
Met	28	0	27									87	80	74	>90	0	0	
Trp	10	25	5			+9[c]	0	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	13	>90	67	0
Phe																50	>90	
Tyr																50	67	
Pro	29	+18[c]	26			+9[c]	0					0[b]	0[b]	0[b]	0[b]	0	0	>90

[a] In this series of experiments, vesicles containing 3 M KCl were added to 3 M NaCl solution containing the labeled amino acid and the inhibitor amino acid at 100 μM concentration. The concentration of the labeled test amino acid was 1 μM. When groups of amino acids were tested together as inhibitor (}), the final concentration of each amino acid was 100 μM. *Iso*-inhibition, i.e., arginine vs. arginine, is given as >90% but was not actually determined as the same information is available from the kinetic constants of Table I. Data are recorded as percent inhibition. Vesicle protein concentration was 125 μg mL^{-1}. [b] Data from light-induced uptake. [c] Percent *stimulation* of uptake rate.

complete, as all other combinations should also be examined, but it suggests that there is more than one carrier in the alanine-glycine-serine-threonine and the valine-leucine-isoleucine-methionine groups. Although from the inhibition data in Table III it seems likely that there is also more than one carrier for the arginine-lysine-histidine group, this is not clear from Figure 4. In each case, a noninhibitory amino acid is included for comparison. In each case, the amino acid sharing a common carrier should depress the uptake of the labeled amino acid in a manner closely similar to the way the unlabeled amino acid depresses the uptake of its labeled isotope (shown as a dotted line). In all cases shown, the K_m's are close (Table II), and unlikely to affect the data. Thus, deviation from the expected pattern must indicate either uptake by alternate carriers or the differential contribution of other factors, such as membrane leakiness.

Nature of the Accumulated Amino Acids. ^{14}C-labeled amino acids accumulated by vesicles in response to illumination were extracted and analyzed as described in Experimental Procedures. At least 90% of the added label was recovered from the vesicles in all cases. For many of the extracted amino acids, migration is altered relative to the standards, and the spots are badly streaked. A similar effect was observed previously with glutamate (Lanyi et al., 1976a) and is presumably due to the NaCl present in the extract. Controls run in the presence of NaCl show similar alterations in migration patterns. The use of DEAE plates reduces the degree of streaking for all but the basic amino acids, but the resolving power of this system is poor (R_f's 60 to 80 for most amino acids). However, comparison of migration patterns under several conditions permits the conclusion with reasonable certainty (identical R_f's in at least two systems) that asparagine, glutamine, aspartate, histidine, isoleucine, valine, methionine, alanine, glycine, threonine, serine, tyrosine, and tryptophan are accumulated in an unaltered form. In all cases, though, the streaking of the extracted amino acid (and the control containing added salt) is sufficient to obscure a small amount (up to 25%) of any altered form which has a close but lower R_f. Because of the extent to which arginine and lysine streaked, no conclusions can be drawn regarding their accumulated state. Proline and phenylalanine give R_f's which are close but not identical with the standards; glutamate and leucine have been reported elsewhere (MacDonald and Lanyi, 1975; Lanyi et al., 1976a) to be accumulated unchanged.

Discussion

The data presented show that the mechanism for uptake and concentration of amino acids by vesicles of *Halobacterium halobium* is dependent on the operation of variables: temperature, Na^+:K^+ ratio both inside and outside of the vesicle, the membrane electrical potential, the amino acid tested, and the internal and external concentration of the amino acids. There is an absolute requirement for Na^+. Potassium stimulates the uptake of most (but not all) amino acids when present on either side of the membrane, possibly because it acts as a counterion permitting the overall nonelectrogenic movement of sodium. Its variable effect, however, suggests that a more complicated explanation may be necessary. The well known role of K^+ in the (Na^+-K^+)ATPase. (Jørgensen, 1975) suggests the need for careful analysis of this point even though a Na^+-K^+ pump of this nature has not previously been observed in any procaryote (Epstein, 1975).

Although the evidence presented strongly supports the idea that a Na^+/amino acid symport mechanism is present for transport of all amino acids, the existence of additional transport systems involving H^+ symport (Harold, 1976; Hamilton, 1975) is not ruled out. If so, however, such transport systems must also require Na^+ because in no case was transport observed in 3 M KCl alone.

The presence of a Na^+-linked uptake mechanism for 19 amino acids is a surprising finding considering the infrequency that sodium gradients have been shown to have a detectable effect on amino acid uptake in other bacterial systems (Thompson and MacLeod, 1973; Hamilton, 1975; Henderson, 1971). Thompson and MacLeod attempted to rule out the involvement of Na^+ or K^+ gradients in the accumulation of α-aminoisobutyric acid by a marine pseudomonad which has an obligate requirement for Na^+ for growth and for accumulation of some amino acids. It is not clear from their data whether the uptake of α-aminoisobutyric acid is effected by ΔNa^+ since, although they did not observe a difference in the internal and external concentration of sodium, such gradients may relax very rapidly (Stock and Roseman, 1971) and might escape detection. Obligate Na^+ requirement for the uptake of glutamate by *Escherichia coli* vesicles (Miner and Frank, 1974) and proline by *Mycobacterium phlei* vesicles (Prasad et al., 1975) has also been reported, but little evidence is given that would indicate a sodium-driven mechanism. On the other hand, an adequate explanation for the stimulatory effect of Na^+ has not been put forth and the possibility that this represents a sodium-driven mechanism is attractive.

In eucaryotic cells there is abundant evidence that amino acid and sugar transport involves Na^+ gradients (Heinz, 1972) and the mechanism for generating these gradients has been extensively studied (Murer et al., 1976). Epstein (1975) points out that, in most of the eucaryotic membrane transport systems, the contribution of the sodium gradient has been underestimated due to a failure to recognize the size of the total electrochemical gradient. If the data presented here can be generalized along these lines, it is not unlikely that sodium-gradient-coupled uptake mechanisms for amino acids and perhaps other substrate classes will be found if a careful search for them is made in other procaryotes.

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Subunit Structure of the Orotate Phosphoribosyltransferase–Orotidylate Decarboxylase Complex from Human Erythrocytes†

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ABSTRACT: A complex of orotate phosphoribosyltransferase and orotidylate decarboxylase has been shown to exist in three molecular weight forms (Brown, G. K., Fox, R. M., and O'Sullivan, W. J. (1975), *J. Biol. Chem. 250,* 7352). The smallest of these, of molecular weight 62 000, was subjected to further study. On the basis of the inactivation of the enzyme activities, carried out in the presence of low concentrations of guanidine hydrochloride, and of changes in molecular weight of preparations during aging, it was inferred that the enzyme complex contained more than one type of subunit. This was confirmed by chromatography on Sephadex G-75 after preincubation in guanidine hydrochloride or with guanidine hydrochloride in the elution buffer. It was concluded that the enzyme complex consisted of two types of subunits, two decarboxylase units of molecular weight approximately 20 000 and two further subunits of approximately 13 000. The subunits could be separated and reassociated with partial recovery of both activities. A 40 000 molecular weight form had full decarboxylase activity but no phosphoribosyltransferase activity. Restoration of the 62 000 molecular weight form resulted in restoration of both enzymatic activities. An intermediate species of molecular weight 50 000 representing a combination of the decarboxylase dimer with one of the 13 000 subunits was also demonstrated. This form required the presence of dithiothreitol in order to manifest phosphoribosyltransferase activity. A model of the system has been proposed that accounts for both the different molecular weight forms and also for the deficiency of both activities in the rare inborn error of metabolism, hereditary orotic aciduria.

Hereditary orotic aciduria is a rare inborn error of pyrimidine metabolism in man. It is usually characterized by marked deficiency of two sequential enzymes of the de novo pyrimidine biosynthetic pathway, orotate phosphoribosyltransferase (EC 2.4.2.10) (reaction 1) and orotidylate decarboxylase (EC 4.1.1.23) (reaction 2) (Smith et al., 1972).

$$\text{orotate} + \text{PRibPP}^{\text{l}} \xrightarrow{Mg^{2+}} \text{OMP} + \text{PP} \qquad (1)$$

$$\text{OMP} \rightarrow \text{UMP} + CO_2 \qquad (2)$$

Two variant forms of the disease have also been described, one lacking only orotidylate decarboxylase (Fox et al., 1969), the other having a selective deficiency of orotate phosphoribosyltransferase (Worthy et al., 1974). The two enzymes appear to behave in a coordinated fashion in all mammalian tissues in which they have been studied. Copurification of the enzymes has been observed by a number of investigators, subject to the limitations imposed by the greater instability of the phosphoribosyltransferase (Kasbekar et al., 1964; Appel, 1968; Brown et al., 1975; Grobner and Kelley, 1975). Also, the ratio of the two activities was found to be constant over a wide range in fresh preparations of human hemolysate (Fox et al., 1971). This coordinate behavior of the two enzymes led to the suggestion that the genes coding for them could be linked and that hereditary orotic aciduria could arise from a defect in a regulator gene or that the two enzyme activities might reside

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