233, 366-380.
Kühn, H., Cook, J. H., & Dreyer, W. J. (1973) *Biochemistry 12,* 2495-2502.
Lardy, H., Reed, P., & Lin, C-H. C. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 1707-1710.
Lowry, O. H., & Passonneau, J. V. (1972) *A Flexible System of Enzymatic Analysis,* p 11, Academic Press, New York, N.Y.
McClennan, D. H. (1970) *J. Biol. Chem. 245,* 4508.
McConnell, D. G., & Scarpelli, D. G. (1963) *Science 139,* 848.
Miki, N., Baraban, J. M., Kierns, J. J., Boyce, J. J., & Bitensky, M. W. (1975) *J. Biol. Chem. 250,* 6320-6327.
Miller, J. A., Paulsen, R., & Bownds, M. D. (1977) *Biochemistry 16,* 2633-2639.
Nace, G. W., Culley, D. D., Emmons, M. B., Gibbs, E. L., Hutchison, V. H., & McKinnell, R. G. (1974) *Amphibians. Guidelines for the Breeding, Care and Management of Laboratory Animals,* National Academy of Sciences, Washington, D.C.
Ostwald, T. J., & Heller, J. (1972) *Biochemistry 11,* 4679-4685.
Randerath, K., & Randerath, E. (1967) *Methods Enzymol. 12A,* 323-350.
Raubach, R. A., Franklin, L. K., & Dratz, E. A. (1974) *Vision Res. 14,* 335-337.
Robinson, W. E., & Hagins, W. A. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1555.
Robinson, W. E., & Hagins, W. A. (1977) *Biophys. J. 17,* 196a.
Robinson, W. E., Yoshikami, S., & Hagins, W. A. (1975) *Biophys. J. 15,* 168a.
Sachs, G., Chang, H. H., Rabon, E., Schackman, R., Lewin, M., & Saccomani, G. (1976) *J. Biol. Chem. 251,* 7690-7698.
Sack, R. E., & Harris, C. M. (1977) *Nature (London) 265,* 465-466.
Schnetkamp, P. P. M., Daemen, F. J. M., & Bonting, S. L. (1977) *Biochim. Biophys. Acta 468,* 259-270.
Sillén, L. G., & Martell, A. G. (1964) *Chem Soc., Spec. Publ. No. 17,* 733.
Smith, H. G., Jr., Stubbs, G. W., & Litman, B. J. (1975) *Exp. Eye Res. 20,* 211-217.
Szuts, E. Z., & Cone, R. A. (1977) *Biochim. Biophys. Acta 468,* 194-208.
Tomita, T. (1970) *Q. Rev. Biophys. 3,* 179-222.
Wheeler, G. L., & Bitensky, M. W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 4238-4242.
Wheeler, G. L., Matuo, Y., & Bitensky, M. W. (1977) *Nature (London) 269,* 822-824.
Woodruff, M. L., Bownds, D., Green, S. H., Morrisey, J. L., & Shedlovsky, A. (1977) *J. Gen. Physiol. 69,* 667-697.
Yau, K.-W., Lamb, R. T. D., & Baylor, D. A. (1977) *Nature (London) 269,* 78.

Coupling of Aspartate and Serine Transport to the Transmembrane Electrochemical Gradient for Sodium Ions in *Halobacterium halobium.* Translocation Stoichiometries and Apparent Cooperativity†

Janos K. Lanyi

ABSTRACT: Cell envelope vesicles, prepared from *Halobacterium halobium,* accumulate aspartate and serine in response to both electrical potential across the membrane, $\Delta\psi$, and chemical potential difference for Na^+, $\Delta\mu_{Na^+} = RT/F \ln (Na_{out}/Na_{in})$. Since $\Delta\mu_{H^+}$ of either positive or negative sign fails to influence the accumulation of amino acids, it was concluded that H^+ is not cotranslocated and thus the transport must be energized solely by cotransport (symport) with Na^+. The electrical ($\Delta\psi$) and the chemical ($\Delta\mu_{Na^+}$) components of the Na^+ gradient could be imposed separately on the vesicles, and the accumulation of aspartate and serine in response to each of these was examined. The ratio of the electrochemical potential differences for the amino acids and for Na^+ in the stationary state gives the coupling ratio, which for efficient coupling corresponds to the translocation stoichiometry. Na^+/aspartate is approximately 2 and Na^+/serine is approximately 1 for transport driven by $\Delta\psi$, but the corresponding values are 4 and 2 for transport driven by $\Delta\mu_{Na^+}$. In the range examined, between 0 and 150 mV, the initial rates of transport are fourth power functions of $\Delta\psi$ for both amino acids tested, but first power (linear) and second power functions of $\Delta\mu_{Na^+}$ for aspartate and serine, respectively. In contrast, the transport rates are hyperbolic functions of the amino acid concentrations, and the K_m values are invariant with $\Delta\psi$ and $\Delta\mu_{Na^+}$. Thus, $\Delta\psi$ and $\Delta\mu_{Na^+}$ seem to have direct regulatory effects on the transport carriers, in addition to their energizing function. The variable stoichiometry and the apparent cooperativity suggest, although do not prove, that the transport carriers for aspartate and serine possess allosteric properties.

Na^+-linked membrane transport systems are widespread among eucaryotic cells (for a recent review, see Crane, 1977), and have been shown to exist also in bacteria (MacDonald & Lanyi, 1975, 1977; Tsuchiya et al., 1977a,b; Tokuda & Kaback, 1977; MacDonald et al., 1977b). It is now generally accepted that these systems accomplish the accumulation of amino acids and sugars by coupling the energetically uphill transmembrane movement of the substrates to the downhill

† From the Extraterrestrial Biology Division, Ames Research Center, National Aeronautics and Space Administration, Moffett Field, California 94035. *Received January 23, 1978.*

movement of Na^+ (*symport,* Mitchell, 1970). The principal difference between mammalian cellular transport and this kind of bacterial transport is in the way an electrochemical gradient of Na^+ across the membranes is achieved: in most mammalian cells through a Na^+/K^+ exchange ATPase (Skou, 1965; Crane, 1977), and in bacteria by a combination of an H^+ pump and H^+/Na^+ *antiport* (Harold & Papineau, 1972; West & Mitchell, 1974; Lanyi et al., 1976a; Lanyi & MacDonald, 1976; Eisenbach et al., 1977; Tokuda & Kaback, 1977; Schuldiner & Fishkes, 1978). In all cases osmotic energy will be conserved in the electrical and the chemical components of the gradient for Na^+ (Mitchell, 1969, 1970). In spite of the elegance of this concept of energy coupling, very little is known about the transport carriers themselves, or of the mechanisms of translocation and coupling.

The exchange of energy between the transported molecules depends on the stoichiometry of the translocation and the efficiency of the coupling. A great deal of effort has been expended on measuring these parameters in various kinds of cells. The stoichiometry, for example, has been determined by comparing the fluxes for Na^+ and substrate (Vidaver, 1964; Curran et al., 1967; Schafer & Jacquez, 1967), and by comparing the magnitudes of stationary state gradients for Na^+ or H^+ and for the transported solutes (Bihler & Crane, 1962; Kashket & Wilson, 1973; Ramos & Kaback, 1977). The ratio of cation to substrate is generally found to be 1 or 2, and losses due to nonspecific leaks are small. Such results form the basis for kinetic models of the transport carriers (Crane, 1977).

We have recently developed a system for studying Na^+-linked amino acid transport in *H. halobium* cell envelope vesicles (MacDonald & Lanyi, 1975; Lanyi et al., 1976a,b; MacDonald et al., 1977a). Amino acid transport was shown to be coupled to a Na^+ gradient (MacDonald & Lanyi, 1977), which arises through H^+/Na^+ antiport (Lanyi & MacDonald, 1976; Eisenbach et al., 1977). Since in this system energy can be introduced by illumination (MacDonald & Lanyi, 1975; Renthal & Lanyi, 1976), the size and duration of the gradients can be precisely controlled. In addition, the capacity of K^+ and Na^+ gradients in this system is large and the gradients collapse very slowly (Lanyi & Hilliker, 1976). These advantages make *H. halobium* vesicles particularly appropriate for studying transport energetics. The present report attempts to define the stoichiometry of translocation for Na^+ and aspartate, and for Na^+ and serine, systems which use separate carriers (MacDonald et al., 1977a). The dependence of the transport rates on the magnitude of the Na^+ gradient was also determined. The results are not consistent with a simple model: distinct kinetic differences are observed for transport driven by the electrical and by the chemical components of the Na^+ gradient, and the rate dependencies are nonlinear.

Materials and Methods

Growth of *H. halobium* strain R-1 and preparation of cell envelopes were described earlier (MacDonald & Lanyi, 1975; Lanyi & MacDonald, 1978). The NaCl–KCl concentrations inside the vesicles were adjusted by the osmotic shock method (MacDonald & Lanyi, 1975; Lanyi et al., 1976b), followed by suspension and storage at the NaCl–KCl concentrations desired for the interior. The vesicles remained active at 4 °C for several weeks. The transmembrane electrical potential that could be achieved by illumination increased somewhat with storage of the vesicles.

Transport of amino acids and triphenylmethylphosphonium ion ($TPMP^+$)[1] was determined either by the filter assay described before (MacDonald & Lanyi, 1975; Lanyi et al., 1976b) or by the flow-dialysis technique (Schuldiner et al., 1976). Uptake of acetate or methylamine could be determined only with flow dialysis. Assay mixtures for the filtration method contained 10 mM Mes or Hepes buffer, at pH 5.0 to 8.3, 3 M NaCl or NaCl–KCl mixture, 1 to 10 μCi radioactively labeled substrate, at the specific radioactivities indicated, and 0.05 to 0.2 mg/mL vesicle protein as measured by the Lowry method (Lowry et al., 1951). Transport was assayed at 30 °C, after at least 5 min thermal equilibration of the buffer. Flow dialysis was carried out similarly to the procedure described by Schuldiner et al. (1976), but in a chamber thermostated at 30 °C. Provisions were made to illuminate the chamber with a GE ELH 300-W lamp, through a Corning 3-68 cut-off filter, an Optical Industries, Inc. heat-reflective mirror, and 7.5 cm of water. The upper part of the dialysis chamber was loaded with 0.8 mL of buffer containing 10 mM Mes, pH 5.0 or 6.2, 3 M NaCl, 2–10 μCi radioactive substrate at the specific radioactivities indicated, and 1.5 to 10 mg/mL vesicle protein. The lower part of the chamber was separated by a Spectrapor no. 1 dialysis membrane from the upper part. Sodium chloride solution (3 M), buffered at the same pH, was pumped through the lower part of the chamber, at flow rates of 1 to 4 mL/min, depending on the experiment. Fractions of 2 to 3 mL were collected and 1.00 mL from each fraction was mixed with 8 mL of scintillation fluid containing toluene–Triton X-100 (2:1) and 0.4% 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene (BBOT). The mixtures cleared after 30-min storage at 0 °C and the radioactivities were determined in a Packard Tri-Carb scintillation spectrometer.

Transmembrane electrochemical potential differences for transported substances were calculated from the following formula (Mitchell, 1969):

$$\Delta\tilde{\mu}_i = m\Delta\psi - \Delta\mu_i = m\Delta\psi - \frac{RT}{F}\ln(C^i_{out}/C^i_{in}) \qquad (1)$$

where m is the net charge of the transported species i, $\Delta\psi$ is the electrical potential across the membrane, C^i_{out} and C^i_{in} are the external and internal concentrations of i, and R, T, and F have their usual meaning. The concentration ratios of the accumulated substances were estimated from the radioactive label taken up, the internal volume of the vesicles (3.0 μL/mg protein, MacDonald & Lanyi, 1975; Eisenbach et al., 1977), and the specific radioactivity of the external solution. For acetate and methylamine the formula published by Addanki et al. (1968) was used. $\Delta\psi$ was calculated from $TPMP^+$ uptake (Skulachev, 1971).

Sources of chemicals were as follows. Monensin and the uncoupler 1799 (bis(hexafluoroacetonyl)acetone) were gifts from Dr. E. L. Potter, Eli Lilly and Co. and Dr. B. I. Kanner, respectively. Gramicidin was from Sigma Chemical Co., [^{3}H]-L-serine (2.8 Ci/mmol), [^{3}H]-L-aspartate (16.9 Ci/mmol), [^{3}H]$TPMP^+$ (1.13 Ci/mmol), [^{14}C]acetate (54.7 mCi/mmol), and [^{14}C]methylamine (52.7 mCi/mmol) were from New England Nuclear.

Results

Noninvolvement of Protons in the Translocation of Aspartate and Serine. We have shown earlier (MacDonald et al., 1977a) that Na^+ gradient dependent transport systems (Na^+

[1] Abbreviations and symbols used: $TPMP^+$, triphenylmethylphosphonium ion; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; $\Delta\tilde{\mu}_i$, the electrochemical potential difference for molecular species i across the membrane; $\Delta\mu_i$, the chemical potential difference; $\Delta\psi$, the electrical potential difference, as defined by eq 1; N, the intrinsic translocation stoichiometry; q, the coupling efficiency, as defined by eq 2.

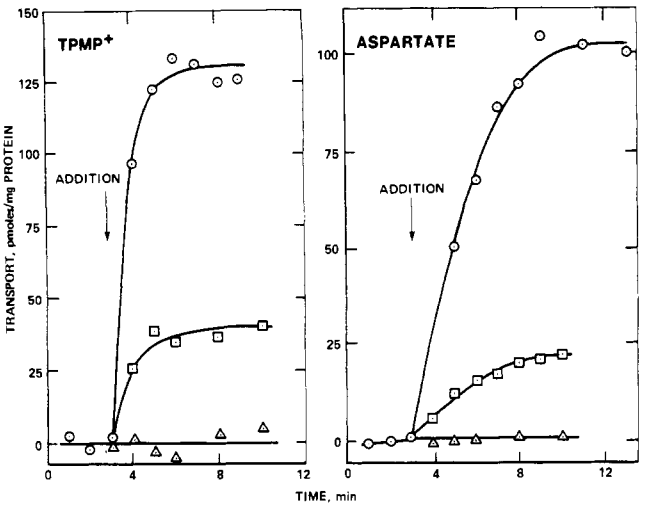

FIGURE 1: Uptake of $TPMP^+$ and aspartate in response to a K^+-diffusion potential. Vesicles, loaded with 2 M KCl–1 M NaCl, were suspended in 3 M NaCl, 10 mM Mes, pH 6.2 (0.1 mg/mL protein). The assay mixtures contained 4.4×10^{-7} M [^{3}H]$TPMP^+$ (0.5 μCi/mL), or 3.0×10^{-8} M [^{3}H]aspartate (0.5 μCi/mL), or 1.8×10^{-7} M serine, not shown (0.5 μCi/mL). At the indicated time, either valinomycin alone (⊙) (5×10^{-6} M), or the uncoupler 1799 (▲) (1×10^{-5} M), or both (⊡) were added. The following potentials were calculated at the steady state: with valinomycin, $\Delta\psi = -113$ mV, $\Delta\tilde{\mu}_{Asp} = 266$ mV, $\Delta\tilde{\mu}_{Ser} = 140$ mV; with valinomycin and 1799, $\Delta\psi = -83$ mV, $\Delta\tilde{\mu}_{Asp} = 200$ mV, $\Delta\tilde{\mu}_{Ser} = 111$ mV.

TABLE I: Independence of Amino Acid Accumulation from the Chemical Potential Difference for Protons.[a]

gradient	magnitude (mV) no addition	magnitude (mV) gramicidin, 1×10^{-6} M
$\Delta\psi$	−99	0
$\Delta\mu_{H^+}$	46	80
$\Delta\tilde{\mu}_{H^+}$	−145	−80
$\Delta\tilde{\mu}_{Asp}$	222	0
$\Delta\tilde{\mu}_{Ser}$	83	0

[a] Gradients calculated from parallel flow-dialysis experiments. Assay mixtures contained 3 M NaCl, 10 mM Mes (pH 5.0), 5 mg/mL vesicle protein, and 1.1×10^{-6} M [^{3}H]$TPMP^+$ (12.5 μCi/mL), or 1.14×10^{-4} M [^{14}C]acetate (6.25 μCi/mL), or 7.4×10^{-7} M [^{3}H]aspartate (12.5 μCi/mL), or 4.5×10^{-6} M [^{3}H]serine (12.5 μCi/mL). Gradients were generated by illumination at approximately 1×10^6 ergs cm^{-2} s^{-1}.

symport) exist for all the transported amino acids in *H. halobium* envelope vesicles. The absolute dependence of transport on the presence of Na^+ suggested that this organism contains no additional H^+ dependent carriers. This possibility was explored more carefully in the present work. Direct demonstration of substrate-dependent ^{22}Na influx and its quantitation (Stock & Roseman, 1971; Tokuda & Kaback, 1977) is not possible in this system, because of the very high concentrations of NaCl necessary for transport (MacDonald & Lanyi, 1975; MacDonald et al., 1977a).

Hamilton and co-workers had devised an indirect test for H^+-dependent transport systems, which consists of establishing a K^+-diffusion potential across *Staphylococcus aureus* cell membranes, and adding an uncoupler (proton conductor). The result is that protons enter the cells, until $\Delta\psi = \Delta\mu_{H^+}$ and thus $\Delta\tilde{\mu}_{H^+} = 0$, giving zero net electrochemical driving force for H^+ (Niven et al., 1973; Niven & Hamilton, 1973, 1974). A transported substrate which depends on electrogenic symport with H^+ should not be accumulated under these conditions, but, since $\Delta\psi \neq 0$, a driving force remains for substrates coupled to other cations. An adaptation of this experiment for *H. halobium* vesicles is shown in Figure 1. When the vesicles are loaded with 2 M KCl–1 M NaCl and are suspended in 3 M NaCl (K^+ in/out = 200, corresponding to a diffusion potential of −133 mV), addition of valinomycin causes the uptake of both $TPMP^+$ and aspartate. At the stationary state, an electrical potential of −113 mV and an aspartate electrochemical potential difference of 266 mV are calculated from the distribution of the radioactive labels (eq 1). After correction for the small chemical gradient of Na^+ (which will be a minor component of the driving force under these conditions), the ratio of aspartate potential to electrical potential is calculated to be 2.05. The corresponding value for serine, from a similar experiment (not shown), is 1.09. Addition of the uncoupler 1799 together with valinomycin is expected to cause H^+ influx, resulting in the acidification of the vesicle interior. Under these conditions the accumulation of $TPMP^+$ gives a somewhat reduced $\Delta\psi$ value of −83 mV. In separate flow-dialysis experiments the electrical potential was observed to decrease, under similar conditions, from −112 mV to −86 mV. Uptake of methylamine is induced by the uncoupler, giving $\Delta\mu_{H^+}$, of −59 mV. The protonmotive force, $\Delta\tilde{\mu}_{H^+}$, is thus reduced in the presence of the uncoupler to about 30% of $\Delta\psi$. However, the electrochemical gradients for aspartate (Figure 1) and serine (not shown) are not correspondingly reduced. The ratios of the electrochemical potentials for the amino acids (corrected for the small $\Delta\mu_{Na^+}$ present) to the electrical potential are unchanged: 2.00 for aspartate and 1.13 for serine. These results indicate that (a) the translocations of the symport cation (Na^+) and the amino acids are coupled to one another in the ratio of 2 for aspartate and 1 for serine under these conditions, and (b) coupling of transport is to $\Delta\psi$, with little or no contribution by $\Delta\mu_{H^+}$.

Another method of testing the involvement of protons in aspartate and serine transport is provided by the observation (Helgerson & Lanyi, manuscript in preparation) that at pH values near 5 and in 3 M NaCl gramicidin abolishes the light-induced electrical potential in *H. halobium* vesicles, while $\Delta\mu_{H^+}$ is raised. Light-induced uptake of $TPMP^+$, acetate, aspartate, and serine into these vesicles was followed in parallel flow-dialysis experiments at pH 5.0, and the magnitudes of the gradients during the stationary state are shown in Table I. Gramicidin is seen to completely abolish $\Delta\psi$, while the total electrochemical gradient, $\Delta\tilde{\mu}_{H^+}$, decreases by only about 45%. In this experiment, the ratio of the potentials for the amino acids to the electrical potential is 2.24 for aspartate and 0.84 for serine, giving coupling ratios of 2 and 1 for these amino acids, as obtained in Figure 1. As indicated in Table I, gramicidin completely abolishes the light-induced accumulation of both amino acids, consistent with the conclusion that the driving force consists entirely of $\Delta\psi$, with no effect of $\Delta\mu_{H^+}$.

Separation of the Electrical and the Chemical Components of the Sodium Gradient across the Membranes. The above results indicate that the major, and probably the sole, cation cotransported with aspartate and serine in *H. halobium* is Na^+. The results further suggest that the translocation of both of these amino acids responds to $\Delta\psi$ and thus involves net charge movement. Conditions were sought where transport could be investigated in response to the electrical potential, $\Delta\psi$, alone and to the sodium chemical potential difference, $\Delta\mu_{Na^+}$, alone.

The first of these conditions can be achieved by illumination of the vesicles in 3 M NaCl. In such an experiment a sustained constant protonmotive force develops (Renthal & Lanyi, 1976), but the exit of Na^+ from the vesicles cannot be exten-

TABLE II: Coupling Ratios for Aspartate and Serine.[a]

		light-driven transport		$\Delta\mu_{Na^+}$-driven transport[b]	
pH	$-\Delta\psi$ (mV)	$\Delta\tilde{\mu}_{Asp}/-\Delta\psi$	$\Delta\tilde{\mu}_{Ser}/-\Delta\psi$	$-\Delta\mu_{Asp}/\Delta\mu_{Na^+}$	$-\Delta\mu_{Ser}/\Delta\mu_{Na^+}$
5.50	128	2.20	0.96	1.63	1.51
6.20	136	2.21	0.90	1.75	1.57
6.90	137	2.12	0.86	1.50	1.45
7.80	123	2.24	0.93	1.28	1.41
8.30	117	2.28	0.92	1.35	1.40

[a] From filter-assays, as described in Figure 2A for $\Delta\psi$-driven transport, and in Figure 2B for $\Delta\mu_{Na^+}$-driven transport. $\Delta\psi$ calculated from [^{3}H]TPMP$^+$ accumulation. Buffering was with 10 mM Mes between pH 5.5 and 6.2, and with 10 mM Hepes between pH 6.9 and 8.3. [b] Vesicles contained 2.96 M KCl and 38 mM NaCl and were suspended in 3 M NaCl in the dark (Na^+ out/in = 79, corresponding to $\Delta\mu_{Na^+}$ = 110 mV).

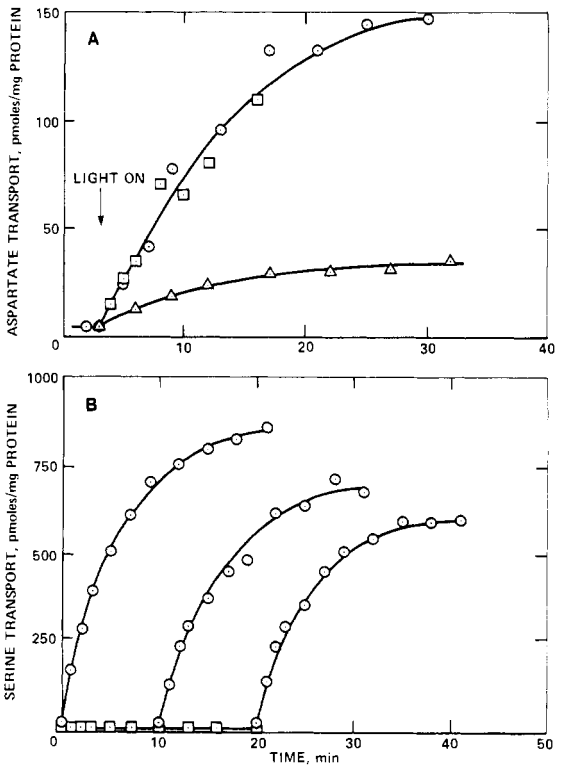

FIGURE 2: Separation of the electrical and the chemical potential differences for Na^+ as driving forces for transport. (A) Electrical potential alone. Vesicles, loaded with 3 M NaCl, were suspended in 3 M NaCl, 10 mM Mes, pH 6.2 (0.1 mg/mL protein), with [^{3}H]aspartate or [^{3}H]serine (not shown) at concentrations as in Figure 1. Light intensity was approximately 1×10^6 ergs cm^{-2} s^{-1}. Symbols: (⊙) no inhibitor; (⊡) monensin, 1×10^{-6} M; (△) 1799, 1×10^{-5} M. (B) Chemical potential of Na^+ alone. Vesicles, loaded with 2.99 M KCl and 11.5 mM NaCl, were suspended in 3 M NaCl (Na^+ out/in = 260, corresponding to $\Delta\mu_{Na^+}$ = 140 mV), containing 10 mM Mes (pH 6.2) and [^{3}H]serine as in Figure 1 (0.0625 mg/mL protein). Symbols: (⊙) no inhibitor; (⊡) monensin, 1×10^{-6} M, present in an experiment initiated at $t = 0$. For transport initiated at $t = 0$ (coincident with the suspension of the vesicles), at $t = 10$ min, and at $t = 20$ min, maximal $\Delta\mu_{Ser}$ values of −189 mV, −182 mV, and −177 mV were calculated, respectively.

sive, because of lack of a counterion (Lanyi & MacDonald, 1976; MacDonald & Lanyi, 1977). Figure 2A shows aspartate transport induced by illumination in 3 M NaCl at pH 6.2. The system reaches "static head" (Essig & Caplan, 1968) in about 25 min for aspartate and 15 min for serine, where the amino acid gradient is just balanced by the electrochemical gradient of Na^+ ($\Delta\tilde{\mu}_{Na^+}$). Transport is inhibited by the uncoupler 1799, as well as by other uncouplers, but appears to be unaffected by monensin. Since the latter agent facilitates the electroneutral exchange of Na^+ and H^+, it is expected to reduce any $\Delta\mu_{Na^+}$ present to the magnitude of $\Delta\mu_{H^+}$, which is very small at pH 6.2 (Helgerson & Lanyi, manuscript in preparation). Similarly, little or no inhibition of serine transport by monensin is observed under these conditions (not shown). The results indicate that in these experiments $\Delta\psi$ is the principal driving force for the transport of aspartate and serine, and $\Delta\mu_{Na^+}$ contributes little or nothing to $\Delta\tilde{\mu}_{Na^+}$ under these conditions.

A chemical potential difference for Na^+ is obtained when vesicles loaded with 3 M KCl are suspended in 3 M NaCl. Amino acid transport proceeds in the dark under such conditions, with no other source of energy (Lanyi et al., 1976b; MacDonald et al., 1977a). Figure 2B shows serine transport in response to $\Delta\mu_{Na^+}$ of 140 mV, calculated from the Na^+ concentrations inside and outside the vesicles (eq 1). After 15 min $\Delta\mu_{Ser}$ is seen to reach a limiting value of −189 mV. When transport is initiated 10 or 20 min after suspension of the vesicles, the stationary state value of $\Delta\mu_{Ser}$ is somewhat reduced, but the change is small enough to warrant the conclusion that $\Delta\mu_{Na^+}$ is essentially constant during the first 15–20 min of transport. The accumulation of serine under these conditions is completely inhibited by monensin (Figure 2B). Since no detectable amount of $TPMP^+$ uptake takes place in response to the gradients of Na^+ and K^+ in such experiments (this work, results not shown; and Helgerson & Lanyi, manuscript in preparation), an electrical potential of significant magnitude (>10 mV, estimated) does not develop. In this experiment, therefore, the sole driving force for transport is $\Delta\mu_{Na^+}$.

These results indicate that conditions can be devised where a sustained constant driving force, consisting of either $\Delta\psi$ alone or $\Delta\mu_{Na^+}$ alone, can be imposed on the vesicles.

Translocation Stoichiometries for Aspartate and Serine. The ratio of the magnitudes of electrochemical potential differences for the amino acids and for Na^+ gives numerically the extent of coupling between the two gradients. Essig & Caplan (1968) derived a relationship, which for aspartate and serine transport in *H. halobium* takes the following form

$$\frac{\Delta\tilde{\mu}_{\text{amino acid}}}{\Delta\tilde{\mu}_{Na^+}} = \frac{m\Delta\psi - \Delta\mu_{\text{amino acid}}}{\Delta\psi - \Delta\mu_{Na^+}} = -qN \qquad (2)$$

where q is the efficiency of coupling ($0 \leq q \leq 1$); N is the intrinsic translocation stoichiometry, Na^+/amino acid, generated by the particular mechanism of the coupling process, and is probably an integer. Unless all the coupling coefficients for the transport are known (Essig & Caplan, 1968), q cannot be separated from the stoichiometry. The assumption is generally made that when the coupling ratio is an integer, e.g., 1 or 2, q equals one.

The coupling ratio, qN, has been determined for aspartate and serine transport at different external pH, in response to

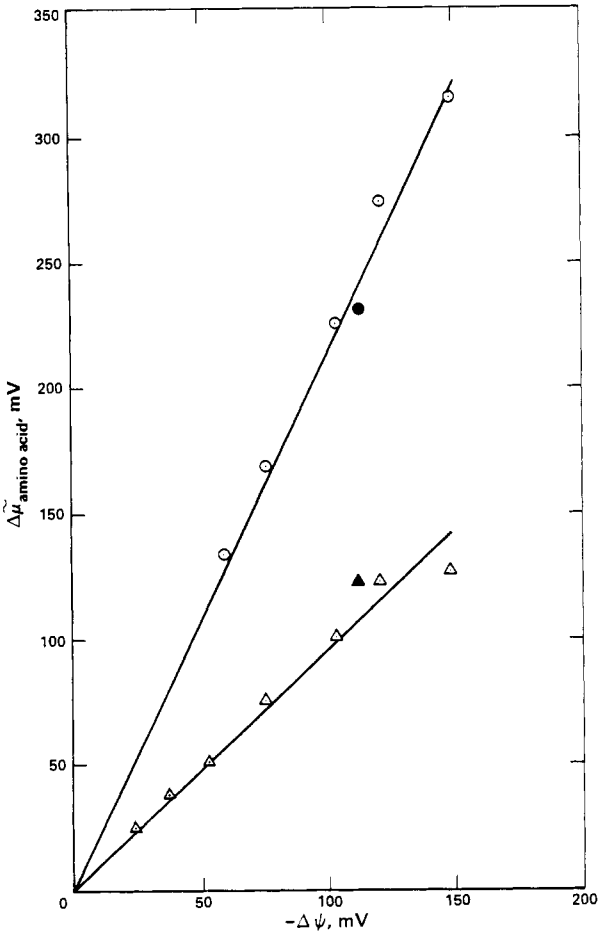

FIGURE 3: Amino acid accumulation in response to electrical potential. Procedure as described under Figure 2A. Different light intensities were achieved with neutral density filters (A = 0.7-2.0). $\Delta\psi$ determined from [^{3}H]TPMP$^+$ uptake in parallel experiments. Symbols: (⊙) aspartate uptake; (▲) serine uptake; (● and ▲) aspartate and serine, respectively, driven by K$^+$-diffusion potential (from Figure 1). All the points but the lowest three on the serine curve were obtained from filter assays. The latter three points were calculated from flow-dialysis experiments. Conditions were as described under Materials and Methods; assay mixtures were similar to those in Table I for flow dialysis.

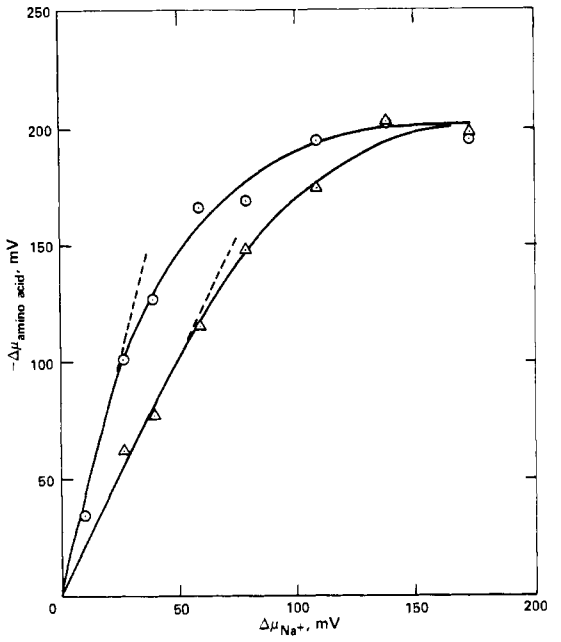

FIGURE 4: Amino acid accumulation in response to chemical potential difference for Na$^+$. Vesicles, loaded with different KCl-NaCl mixtures (total concentration 3 M), were suspended in 3 M NaCl, 10 mM Mes (pH 6.2), as described under Figure 2B. The $\Delta\mu_{Na^+}$ values were calculated from the Na$^+$ concentration ratios at the time of the suspension. Symbols: (⊙) aspartate uptake; (▲) serine uptake. Interrupted lines are tangents, drawn to fit the initial segments of the curves, with slopes of 4 for aspartate and 2 for serine.

$\Delta\psi$ alone and to $\Delta\mu_{Na^+}$ alone. As shown in Table II, unlike in *E. coli* (Ramos & Kaback, 1977), the coupling ratios in *H. halobium* are pH independent for $\Delta\psi$-driven transport. The approximate values of 2 for aspartate and 1 for serine confirm the results obtained under different conditions (Figure 1 and Table I). When the driving force imposed on the vesicles is $\Delta\mu_{Na^+}$, the coupling ratios are not integers, and a mild decrease with increasing pH is observed (Table II). The pH-dependent decrease of the coupling ratio probably reflects an overestimation of $\Delta\mu_{Na^+}$ at higher pH, because of the somewhat more rapid collapse of the Na$^+$-gradient under these conditions (Lanyi & Hilliker, 1976).

The data in Table II were obtained at fixed potential differences for Na$^+$. The magnitudes of electrical and chemical potentials for Na$^+$ could be manipulated, the former by varying the light intensity in experiments similar to that in Figure 2A, and the latter by using vesicles loaded with different KCl-NaCl mixtures in experiments similar to that in Figure 2B.

Aspartate and serine accumulation at various $\Delta\psi$ is shown in Figure 3. A constant relationship between $\Delta\tilde{\mu}_{amino\ acid}$ and $\Delta\psi$ is obtained up to $\Delta\psi = -150$ mV. The slopes of the lines are 2.14 for aspartate and 0.96 for serine, suggesting that under these conditions (a) the coupling efficiency, q, is constant and probably equals 1, and (b) the translocation stoichiometry N (Na$^+$/amino acid) is 2 for aspartate and 1 for serine. The amino acid gradients achieved in response to a K$^+$-diffusion potential (Figure 1) are consistent with the rest of the data in Figure 3, where $\Delta\psi$ is generated by proton movement.

Different results are obtained when the driving force for transport is a chemical potential difference for Na$^+$. As shown in Figure 4, the relationship between $-\Delta\mu_{amino\ acid}$ (which is equal to $\Delta\tilde{\mu}_{amino\ acid}$ under these conditions) and $\Delta\mu_{Na^+}$ is not linear between 0 and 170 mV, but deviates downward from the expected line, indicating decreased efficiency, q, at high $\Delta\mu_{Na^+}$. Furthermore, the initial, linear segments of the curves exhibit slopes of approximately 4 for aspartate and 2 for serine. The increased accumulation of the amino acids in these experiments, relative to those where the driving force is $\Delta\psi$ (Figure 3), would be trivial if the Na$^+$ gradient caused the development of $\Delta\psi$ through electrogenic H$^+$/Na$^+$ exchange (Lanyi & MacDonald, 1976). However, as mentioned above, no evidence for such electrical potential is measurable with TPMP$^+$ uptake under these conditions. The results suggest that when transport occurs in response to $\Delta\mu_{Na^+}$ (a) the coupling efficiency, q, decreases with the increasing gradient and (b) the translocation stoichiometry, N, determined at small $\Delta\mu_{Na^+}$, where the efficiency is highest, is changed from 2 to 4 for aspartate, and from 1 to 2 for serine.

Dependence of Amino Acid Transport Rate on the Electrochemical Potential Difference for Na$^+$. The magnitude of amino acid fluxes should be directly proportional to an expression containing a linear combination of the terms $\Delta\tilde{\mu}_{Na^+}$ and $\Delta\tilde{\mu}_{amino\ acid}$ (Essig & Caplan, 1968). Rottenberg (1976) derived a relationship which predicts that, under certain kinetic conditions (initial $\Delta\tilde{\mu}_{amino\ acid}$ near zero and amino acid concentration near the K_m), the rate is expected to be proportional to $\Delta\tilde{\mu}_{Na^+}$ alone. The expected linear relationship of transport

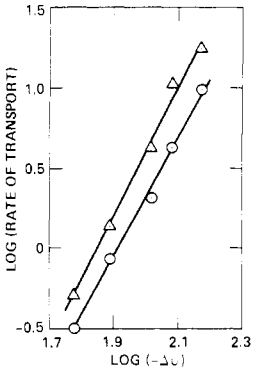

FIGURE 5: Dependence of the log of initial transport rate on the log of electrical potential. Procedure as described under Figure 2A. The vesicles were incubated with the amino acids for 8 min in the dark for passive equilibration, energized transport was then initiated by illumination. Rates were calculated from the first 4 min of transport, from points taken at 1-min intervals, and given in pmol min^{-1} (mg of protein)$^{-1}$. Symbols: (⊙) aspartate uptake; (▲) serine uptake. The slopes of the log–log plots, which correspond to the exponents of the $\Delta\psi$ dependence, are: 3.70 for aspartate and 4.01 for serine.

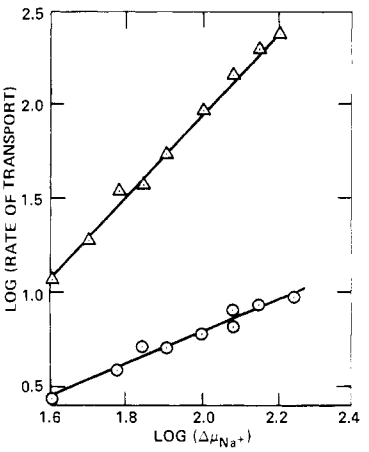

FIGURE 6: Dependence of the log of initial transport rate on the log of chemical potential difference for Na^+. Procedure as described under Figure 2B. Initial rates determined as in Figure 5. Symbols: (⊙) aspartate; (▲) serine. The slopes of the log–log plots, which correspond to the exponents of the $\Delta\mu_{Na^+}$ dependence, are: 0.86 for aspartate; and 2.18 for serine.

TABLE III: Kinetic Constants (K_m) for Aspartate and Serine Transport[a] at Varying Electrical and Chemical Gradients for Na^+.

driving force	magnitude (mV)	K_m for Asp (M)	K_m for Ser (M)
$\Delta\psi$	−104	4.8×10^{-7}	
	−121	4.1×10^{-7}	5.9×10^{-6}
	−149	3.8×10^{-7}	6.4×10^{-6}
$\Delta\mu_{Na^+}$	70	3.0×10^{-6}	6.2×10^{-6}
	120	3.2×10^{-6}	7.0×10^{-6}
	160	3.0×10^{-6}	4.0×10^{-6}

[a] The K_m values determined from experiments similar to those described in Figures 2A, 2B, 3, and 4, but carried out at different amino acid concentrations. Rates and intercepts in double-reciprocal plots obtained according to the least-squares method.

rate to $\Delta\tilde{\mu}_{Na^+}$ was tested under a variety of conditions, including those described by Rottenberg (1976). In all cases $\Delta\tilde{\mu}_{amino\ acid}$ represented a negligibly small contribution to the driving force for transport.

Initial uptake rates for aspartate and serine were estimated from the first few minutes of transport, where the rates were linear with time. The electrical potentials were determined from the steady-state distribution of $TPMP^+$. Results with cyanine dyes had shown earlier that these steady-state values of $\Delta\psi$ are established with a $t_{1/2}$ = 5–10 s from the beginning of the illumination (Renthal & Lanyi, 1976; Lanyi & MacDonald, 1976). Figure 5 shows data obtained for $\Delta\psi$-driven transport under conditions where $\Delta\mu_{Na^+} = 0$ (as in Figure 2A). Unexpectedly, the rates are nonlinear with $\Delta\psi$, but when the logarithms of the rates are plotted against the logarithms of $\Delta\psi$ (Figure 5), the curves become linear with slopes of approximately 4, indicating that the dependencies can be described as power functions, with exponents of 4 for both aspartate and serine. The experiments in Figure 5 were carried out at amino acid concentrations $^1/_{10}$th of those yielding half-maximal transport. The fourth-power dependence of the rates on $\Delta\psi$ is not appreciably changed over a wide range of amino acid concentrations, up to several times the K_m values (not shown). More detailed kinetic studies at different $\Delta\psi$ values yielded the result that initial transport rates are hyperbolic with amino acid concentration, as described before (Lanyi et al., 1976b; MacDonald et al., 1977a), and that the K_m values for the amino acids remain essentially unchanged with varying $\Delta\psi$ (Table III). This finding is demanded by self-consistency for the data and indicates that the causes of the nonlinearity are not fortuitous kinetic effects.

Initial transport rates for aspartate and serine were determined also at different $\Delta\mu_{Na^+}$ values, with $\Delta\psi = 0$, in experiments similar to that described in Figure 2B. The results show that for aspartate the uptake rate is linearly dependent on $\Delta\mu_{Na^+}$, but for serine the dependency is nonlinear. A log–log plot (Figure 6) yields exponents of 0.86 for the aspartate rate dependence and 2.18 for serine. The K_m values for the amino acids were determined at different $\Delta\mu_{Na^+}$ and found to be essentially constant (Table III). Interestingly, the K_m for serine is the same when transport is driven by $\Delta\psi$ and by $\Delta\mu_{Na^+}$, but the K_m for aspartate is increased about tenfold for $\Delta\mu_{Na^+}$-driven transport.

Discussion

The hypothesis of a simple, reversible Na^+/amino acid transport carrier generates some predictions, which were tested for aspartate and serine transport in *H. halobium*. These are: (a) the transmembrane electrochemical potential differences at steady state for Na^+ and for the amino acids have a characteristic relationship to one another, determined by the translocation stoichiometry; (b) the electrical and the chemical terms of the potential difference for Na^+ are equivalent in driving the transport of the amino acids; and (c) when the amino acid gradient is small and the Na^+ gradient is large, the transport rates for the amino acids are linearly dependent on the electrochemical potential difference for Na^+.

The results indicate that at least two of these predictions do not apply to aspartate and serine transport in *H. halobium*. The coupling ratio of sodium to amino acid, calculated from the ratio of electrochemical potential differences in the stationary state (eq 2), doubles for both amino acids when the driving force is supplied as $\Delta\mu_{Na^+}$ rather than as $\Delta\psi$. This variation of the coupling ratios cannot be explained as yet, but may reflect regulatory functions for $\Delta\psi$ and $\Delta\mu_{Na^+}$, in addition to their energizing function. Regulation of the substrate binding site by $\Delta\psi$ and $\Delta\mu_{Na^+}$ in the *Salmonella typhimurium* thiomethyl galactoside carrier has been described by Tokuda & Kaback (1978).

TABLE IV: Kinetic Parameters for Aspartate and Serine Transport.[a]

	$\Delta\psi$-driven transport		$\Delta\mu_{Na^+}$-driven transport	
amino acid	coupling ratio (translocation stoichiometry)	coefficient of cooperativity	coupling ratio (translocation stoichiometry)	coefficient of cooperativity
Asp	2.14	3.70	4.0	0.86
Ser	0.96	4.01	2.0	2.18

[a] Coupling ratios are from Figures 3 and 4 and are defined by eq 2; coefficients of cooperativity (apparent) are from Figures 5 and 6 and are the exponents of the rate vs. potential plots.

The initial transport rate is a linear function of $\Delta\mu_{Na^+}$ for aspartate, second power function for serine, and fourth power function of $\Delta\psi$ for both amino acids tested. Nonlinear kinetics for single substrate site enzyme reactions can be observed under specific conditions, where the system is shifted from one alternate pathway to another at changing substrate concentrations (Ferdinand, 1966). Since apparent affinity constants (K_m) for the amino acids are not influenced by the size of $\Delta\psi$ and $\Delta\mu_{Na^+}$, this kind of explanation for the nonlinearities observed is unlikely. An alternate possibility is that the results reflect cooperative interaction among multiple transport sites, which themselves respond linearly to the driving force.

The calculated coupling ratios (translocation stoichiometries) for Na^+ and amino acids, and the coefficients of (apparent) cooperativities are listed in Table IV for $\Delta\psi$-driven transport and for $\Delta\mu_{Na^+}$-driven transport. The pattern of these kinetic parameters is distinctive: the values assumed are restricted to 1, 2, and 4, and there seems to be an inverse relationship between the stoichiometries and the coefficients of cooperativity. It is premature to speculate on the molecular details of the coupled amino acid and Na^+ translocation process which would generate such a pattern. On the other hand, it is apparent that there is an analogy between these results and those which had given rise to the concept of allosteric regulation in enzymes (Monod et al., 1965). Carrying this analogy to its logical conclusion predicts that the transport carriers investigated are multisite (and possibly multisubunit) structures, in which both variable homotropic interaction (resulting in cooperativity) and variable heterotropic interaction (resulting in the coupling of the translocations of Na^+ and the amino acids) are possible. However, the validity of such an allosteric model for the transport carriers is difficult to decide on the basis of kinetic evidence alone. It is interesting to note that in the thiomethyl galactoside transport system of *S. typhimurium* inward Na^+ movement (Na^+/sugar = 1) is induced not only by the natural substrate, but also by the nontransportable substrate, nitrophenylgalactoside (Tokuda & Kaback, personal communication). The translocations of the substrate and Na^+, normally linked, are thus separable from one another.

Variations in transport stoichiometries have been observed in other systems. Ramos & Kaback (1977) reported that the H^+/substrate translocation ratio is changed from 1 to 2 when the external pH is raised from 5.5 to 7.5. Schuldiner & Fishkes (1978) find that H^+/Na^+ antiport in *E. coli* is electroneutral ($H^+/Na^+ = 1$) at pH 6.6 but electrogenic ($H^+/Na^+ > 1$) at pH 7.5. Although these results could be interpreted in a simpler way, in terms of titration of an acidic site on the carriers (Rottenberg, 1976), the possibility of variation in transport stoichiometry seems to be well established. Another report (Collins et al., 1976) demonstrates the isolation of two mutant strains of *E. coli*, where the transport stoichiometry of H^+/alanine (and of other members of the transport group) is changed from 1 to 2 and 4. These observations, together with those reported here, indicate that the kinetics of coupled transport processes are more complex than previously suspected.

Acknowledgments

The author is grateful to Drs. E. L. Potter and B. I. Kanner for gifts of monensin and the uncoupler 1799, and to Dr. H. R. Kaback for a gift of [^{3}H]TPMP$^+$ which was used in a preliminary stage of this work. Thanks are also due to Drs. L. I. Hochstein, H. R. Kaback, R. E. MacDonald, E. Padan, and S. Schuldiner for valuable discussions, and to Drs. H. R. Kaback and S. Schuldiner for making their manuscripts available before publication. Expert technical assistance by V. Yearwood-Drayton is also acknowledged.

References

Addanki, S., Cahill, F. D., & Sotos, J. F. (1968) *J. Biol. Chem. 243,* 2337.

Bihler, I., & Crane, R. K. (1962) *Biochim. Biophys. Acta 59,* 78.

Collins, S. H., Jarvis, A. W., Lindsay, R. J., & Hamilton, W. A. (1976) *J. Bacteriol. 126,* 1232.

Crane, R. K. (1977) *Rev. Physiol. Biochem. Pharmacol. 78,* 99.

Curran, P. F., Schultz, S. G., Chez, R. A., & Fuisz, R. E. (1967) *J. Gen. Physiol. 50,* 1261.

Eisenbach, M., Sprung, S., Garty, H., Johnstone, R., Rottenberg, H., & Caplan, S. R. (1977) *Biochim. Biophys. Acta 465,* 599.

Essig, A., & Caplan, S. R. (1968) *Biophys. J. 8,* 1434.

Ferdinand, W. (1966) *Biochem. J. 98,* 278.

Harold, F. M., & Papineau, D. (1972) *J. Membr. Biol. 8,* 45.

Kashket, E. R., & Wilson, T. H. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70,* 2866.

Lanyi, J. K., & Hilliker, K. (1976) *Biochim. Biophys. Acta 448,* 181.

Lanyi, J. K., & MacDonald, R. E. (1976) *Biochemistry 15,* 4608.

Lanyi, J. K., & MacDonald, R. E. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36,* 1824.

Lanyi, J. K., & MacDonald, R. E. (1978) *Methods Enzymol.* (Fleischer, S., & Packer, L., Eds.) Academic Press, New York, N.Y. (in press).

Lanyi, J. K., Renthal, R., & MacDonald, R. E. (1976a) *Biochemistry 15,* 1603.

Lanyi, J. K., Yearwood-Drayton, V., & MacDonald, R. E. (1976b) *Biochemistry 15,* 1595.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193,* 265.

MacDonald, R. E., & Lanyi, J. K. (1975) *Biochemistry 14,*

2882.
MacDonald, R. E., & Lanyi, J. K. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36,* 1828.
MacDonald, R. E., Lanyi, J. K., & Greene, R. V. (1977b) *Proc. Natl. Acad. Sci. U.S.A. 74,* 3167.
MacDonald, R. E., Greene, R. V., & Lanyi, J. K. (1977a) *Biochemistry 16,* 3227.
Mitchell, P. (1969) in *Theoretical and Experimental Biophysics,* Vol. 2, (Cole, A., Ed.), p 160, Marcel Dekker, N.Y.
Mitchell, P. (1970) *Symp. Soc. Gen. Microbiol. 20,* 121.
Monod, J., Wyman, J., & Changeux, J.-P. (1965) *J. Mol. Biol. 12,* 88.
Niven, D. F., & Hamilton, W. A. (1973) *FEBS Lett. 37,* 244.
Niven, D. F., & Hamilton, W. A. (1974) *Eur. J. Biochem. 44,* 517.
Niven, D. F., Jeacocke, R. E., & Hamilton, W. A. (1973) *FEBS Lett. 29,* 248.
Ramos, S., & Kaback, H. R. (1977) *Biochemistry 16,* 4271.
Renthal, R., & Lanyi, J. K. (1976) *Biochemistry 15,* 2136.
Rottenberg, H. (1976) *FEBS Lett. 66,* 159.
Schafer, J. A., & Jacquez, J. A. (1967) *Biochim. Biophys. Acta 135,* 1081.
Schuldiner, S., & Fishkes, H. (1978) *Biochemistry 17,* 706.
Schuldiner, S., Weil, R., & Kaback, H. R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 109.
Skou, J. C. (1965) *Physiol. Rev. 45,* 596.
Skulachev, V. P. (1971) *Curr. Top. Bioenerg. 4,* 127.
Stock, J., & Roseman, S. (1971) *Biochem. Biophys. Res. Commun. 44,* 132.
Tokuda, H., & Kaback, H. R. (1977) *Biochemistry 16,* 2130.
Tokuda, H., & Kaback, H. R. (1978) *Biochemistry 17,* 698.
Tsuchiya, T., Hasad, S. M., & Raven, J. (1977a) *J. Bacteriol. 131,* 848.
Tsuchiya, T., Raven, J., & Wilson, T. H. (1977b) *Biochem. Biophys. Res. Commun. 76,* 26.
Vidaver, G. A. (1964) *Biochemistry 3,* 803.
West, I. C., & Mitchell, P. (1974) *Biochem. J. 144,* 87.

N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, a New Inhibitor of the Mitochondrial F_1-ATPase†

Richard Pougeois, Michel Satre, and Pierre V. Vignais*,‡

ABSTRACT: The ATPase activity of F_1-ATPase and AS-submitochondrial particles is progressively and irreversibly inhibited by *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), a specific carboxyl reagent. Reaction of 1 mol of EEDQ per active site of F_1-ATPase results in a total inactivation of the enzyme. Inactivation of soluble and membrane-bound ATPase by EEDQ is pH and temperature-dependent. pH titration shows that half-maximal inactivation is obtained at pH 7.3–7.5, suggesting that the EEDQ-reactive carboxyl group has an unusually high p*K* value. EEDQ inactivation is markedly increased at temperatures above 10 °C. Protection against EEDQ inactivation of F_1-ATPase is afforded by nucleophiles including mercaptoethanol, dithiothreitol, hydroxylamine, and hydrazine. Protection requires that these compounds are added either prior to or together with the enzyme. When added after EEDQ, the nucleophiles stop the development of the inactivation, without regeneration of the activity. Mg^{2+}, Mn^{2+}, and to a lesser extent Ca^{2+} exhibit a protective effect against EEDQ inactivation. ATP affords slight protection; ADP and AMP are without effect. Partial inhibitions caused by EEDQ and dicyclohexylcarbodiimide are additive. EEDQ does not modify the increase in fluorescence due to the formation of the aurovertin–ATPase complex, but it prevents the quenching effect of ATP on the fluorescent intensity of the aurovertin–ATPase complex. On the other hand, it has no effect on the enhancement of fluorescence of the aurovertin–ATPase complex caused by addition of ADP.

N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)[1], (Figure 1), a highly specific reagent for the activation and modification of carboxyl groups, was introduced by Belleau et al. (1968; also Belleau & Malek, 1968) and characterized initially as a potent depressant of the central nervous system. It has since been used successfully to probe carboxyl groups in serine hydrolases by Belleau et al. (1969) and in the ATPase of the erythrocyte membrane and found to behave in this respect like carbodiimide (Godin & Schrier, 1970). More recently the inhibitory effect of EEDQ on NADH oxidation and membrane-bound ATPase in submitochondrial particles has been briefly reported by Beechey & Cattell (1972). However, these authors did not pursue their investigation as they suspected that EEDQ lacked the required potency and specificity. In this paper, we describe the detailed characteristics of inactivation of isolated F_1-ATPase from beef heart mitochondria by EEDQ. We show that EEDQ acts at, or close to, the same site as dicyclohexylcarbodiimide on F_1-ATPase. It is in fact known that dicyclohexylcarbodiimide binds to two

† From Laboratoire de Biochimie, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires, 38041 Grenoble, and Laboratoire de Biochimie Faculté de Médecine, 38700 Grenoble, France. *Received February 8, 1978.* This investigation was supported by research grants from the Centre National de la Recherche Scientifique (ERA No. 36), the Fondation pour la Recherche Médicale, and the Délégation Générale à la Recherche Scientifique et Technique.

‡ Address correspondence to this author at: Laboratoire de Biochimie, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires, 38041 Grenoble, France.

[1] Abbreviations used: EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; Nbf-Cl, 4-chloro-7-nitrobenzofurazan; Mops, morpholinopropanesulfonic acid buffer; A particles, submitochondrial particles prepared by sonication of beef heart mitochondria in an ammonia solution at pH 9.2; AS particles, submitochondrial particles prepared by treatment of A particles with Sephadex G-50; AS particles are devoided of the natural ATPase inhibitor; F_1-ATPase, mitochondrial ATPase.

0006-2960/78/0417-3018$01.00/0