Membrane Potential and Neutral Amino Acid Transport in Plasma Membrane Vesicles from Simian Virus 40 Transformed Mouse Fibroblasts†

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ABSTRACT: Accumulation of several neutral amino acids in plasma membrane vesicles derived from Simian virus 40 transformed mouse fibroblasts could be driven either solely by electrical potential differences imposed across the vesicle membrane in the presence of sodium ion, or by an imposed chemical difference in sodium ion concentration alone. When both chemical and electrical gradients were imposed together across the vesicle membrane, they were utilized additively to drive amino acid accumulation. Alanine and glycine accumulation in vesicles increased as a function of increasing sodium ion gradients ($Na^+_{out} > Na^+_{in}$) imposed artificially across the membrane. Levels of alanine accumulation driven by a standard initial sodium ion gradient were further increased in direct correlation with increasing magnitudes of artificially imposed interior-negative membrane potentials estimated by the distribution by diffusion across the vesicle membrane of the lipophilic cation triphenylmethylphosphonium. When chemical sodium ion gradients were selectively dissipated by monensin without affecting membrane potential, amino acid accumulation was reduced to a level accountable by membrane potential, assuming a stoichiometry of one positive charge per molecule of amino acid transported. When electrical differences across the membrane were selectively dissipated, the monensin-sensitive component of this stimulation persisted. As a corollary, sodium ion stimulated alanine transport caused an apparent depolarization evident as a stimulation of thiocyanate accumulation. Stimulation of alanine transport by an interior-negative membrane potential or permeant anions required the presence of sodium ion but not a sodium ion gradient. An obligatory chemical contribution was specific for sodium ion although other ions could contribute electrically. Implications of these findings for the transport mechanism and for cellular regulation of this process are discussed.

Variations of the alkali ion gradient hypothesis (Riggs et al., 1958; Christensen, 1960, 1970; Crane, 1960, 1977; Kromphardt et al., 1963; Schultz and Curran, 1970) propose a direct role of electrochemical sodium ion gradients in effecting the coupling to metabolism of certain Na^+-dependent active transport systems. An important question generated from these concepts concerns the contribution of membrane potential to the total driving force for solute accumulation (Gibb and Eddy, 1972).

Membrane vesicles which catalyze solute transport dissociated from intracellular metabolism have provided a useful experimental system to test proposed mechanisms of active transport in bacteria (Harold, 1972; Kaback, 1974, 1976; Hirata et al., 1974; Ramos et al., 1976) and in animal cells (Hopfer et al., 1973; Colombini and Johnstone, 1974a,b; Hochstadt et al., 1974; Murer and Hopfer, 1974; Sigrist-Nelson et al., 1975; Murer et al., 1976; Lever, 1976a,b; 1977a,b).

Techniques for measurement of membrane potential have involved impaling whole cells with microelectrodes (Lassen et al., 1971), qualitative estimates by optical methods (reviewed by Waggoner, 1976), and quantitation of the equilibrium distribution of freely permeant ions (Skulachev, 1971; Rottenberg, 1975) such as triphenylmethylphosphonium ion (Schuldiner and Kaback, 1975; Ramos et al., 1976; Bakker et al., 1976), dimethyldibenzylammonium (Hirata et al., 1973; Altendorf et al., 1975; de Cespedes and Christensen, 1974), chloride (Lassen et al., 1971), rubidium ion in the presence of valinomycin (Lombardi et al., 1973), and thiocyanate (Scarborough, 1976).

In the experiments described here using plasma membrane vesicles isolated from Simian virus 40 transformed fibroblasts, differences in sodium ion concentration and membrane potential were selectively imposed or dissipated across the membrane by experimental manipulation. The electromotive force acting between the intravesicular aqueous compartment and the external solution was measured by the response of quasiequilibrium distributions by diffusion of the lipophilic cation triphenylmethylphosphonium ion. The relationship of these changes to neutral amino acid accumulation via the A system (Oxender and Christensen, 1963) provided evidence that a chemical ion gradient and an interior-negative membrane potential can each contribute separately or additively at the level of the plasma membrane to energize active amino acid transport. This methodology for measurement of transmembrane potential in fibroblast plasma membrane vesicles will also be useful for the detection of electrical potential changes triggered by binding of hormones or other molecules to the plasma membrane or generated by electrogenic enzymatic systems within the plasma membrane.

Experimental Section

Methods

Growth of Cells and Purification of Membrane Vesicles. A clonal line of Simian virus 40 transformed Balb/c 3T3 cells obtained from Dr. Y. Ito, this department, was propagated on plastic sheets in 2 L of Dulbecco's modified Eagle's medium supplemented with 10% calf serum and equilibrated with 10%

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CO_2 in air, as described previously (Lever, 1977a). Membrane vesicles were prepared from 2 to 5 × 10^9 cells by a modification (Lever, 1977a) of the procedure of Quinlan and Hochstadt (1976).

The purity of each preparation was characterized by marker enyzme analysis (Lever, 1977a). Protein was determined by the method of Lowry et al. (1951). Vesicles were stored at 5 to 9 mg of protein per mL in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.5), in liquid nitrogen.

Transport Assays. Amino acid, 3-*O*-methylglucose, or thiocyanate uptake by membrane vesicles was assayed at 21 °C in 100 μL of 0.125 M sucrose, 10 mM Tris-phosphate (pH 7.5), 5 mM $MgCl_2$ (standard incubation mixture) and other additions as indicated. Transport was terminated by dilution with 5 mL of 0.8 M NaCl–10 mM Tris-HCl (pH 7.5) (wash buffer) at 2 °C and filtration through a 0.45-μm cellulose nitrate filter (Schleicher and Schuell, Selectron) followed by 5 mL of wash buffer, as described previously (Lever, 1976a,b). Radioactivity of dried filters was measured by scintillation counting in toluene–Liquifluor.

Triphenylmethylphosphonium uptake was also determined using these conditions except that 0.5-μm cellulose acetate filters (Millipore, type EH) were used, as discussed by Schuldiner and Kaback (1975).

The internal volume of vesicles was measured by accumulation of 0.5 mM 3-*O*-methyl[^{3}H]glucose using the filtration assay. The measured efflux of 3-*O*-methylglucose in wash buffer indicated less than 20% loss of internal solute during vesicle collection. Measurement of internal volume was proportional to the amount of vesicle protein. The average value of 1 μL/mg was used in calculations of internal solute concentration for purified mixed vesicles.

Materials

[^{3}H]Triphenylmethylphosphonium bromide (114 and 440 Ci/mol) (Schuldiner and Kaback, 1975) and unlabeled triphenylmethylphosphonium bromide were generous gifts from Dr. H. R. Kaback, Roche Institute of Molecular Biology, Nutley, N.J. Potassium [^{14}C]thiocyanate (11.8 fmol/cpm), L-[2,3-^{3}H]alanine (39 fmol/cpm), 2-amino[1-^{14}C]isobutyric acid (91 fmol/cpm), L-[*methyl*-^{3}H]methionine (30 fmol/cpm), L-[4,5-^{3}H]leucine (33 fmol/cpm), [2-^{3}H]glycine (30 fmol/cpm), and L-[G-^{3}H]glutamine (39 fmol/cpm) were purchased from the Radiochemical Centre, Amersham, England.

Monensin and nigericin were generously supplied by Dr. G. L. Smith, Lilly, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was from Dr. P. Heytler, du Pont de Nemours, Wilmington, Del. Valinomycin and ouabain were purchased from Sigma. Other chemicals were purchased as reagent or Ultra Pure grade.

Results

Uptake of Both Triphenylmethylphosphonium Ion and Amino Acids Is Stimulated by Conditions Expected to Generate an Interior-Negative Membrane Potential. Vesicles were loaded with 50 mM potassium chloride and then diluted in the presence of the electrogenic ionophore valinomycin to a 5 mM external potassium ion concentration at a constant 50 mM external NaCl concentration. Time courses of triphenylmethylphosphonium uptake (Figure 1A) indicated that the apparent tenfold potassium ion diffusion potential thus artificially generated (Harold et al., 1974; Pressman, 1976) caused the accumulation of triphenylmethylphosphonium ion to an apparent intravesicular concentration about sixfold higher than its external concentration. The accumulation of alanine (Figure 1B), glycine (Figure 1C), glutamine (Figure 1D), and 2-aminoisobutyric acid (Figure 1E) paralleled and exceeded the stimulation observed for triphenylmethylphosphonium distribution under these conditions. A tenfold potassium ion diffusion potential in the presence of a Na^+ gradient (external > internal) stimulated a transient 7- to 13-fold accumulation of these amino acids. In comparison, a four- to fivefold accumulation of amino acids was stimulated by a sodium chloride diffusion potential in the absence of a potassium ion diffusion potential. Accumulation both of triphenylmethylphosphonium ion and amino acids decayed during incubation, a reflection of the dissipation of the potassium ion diffusion potential.

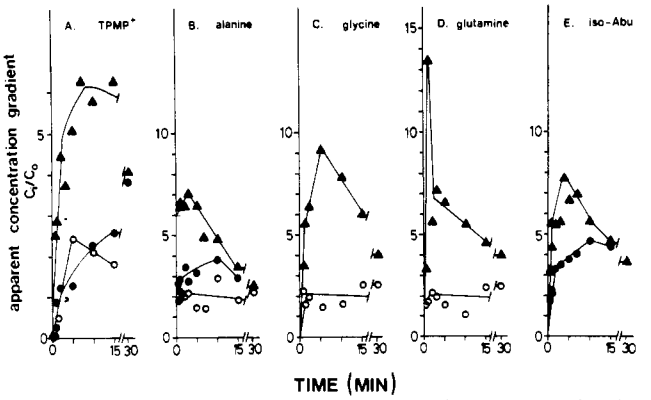

FIGURE 1: Stimulation of triphenylmethylphosphonium ion and amino acid uptake in the presence of a valinomycin-induced potassium ion diffusion potential: (panel A) 0.1 mM [^{3}H]triphenylmethylphosphonium bromide; (panel B) 0.2 mM L-[2,3-^{3}H]alanine; (panel C) 0.2 mM [2-^{3}H]glycine; (panel D) 0.2 mM L-[G-^{3}H]glutamine; (panel E) 0.17 mM 2-amino[1-^{14}C]isobutyric acid. Aliquots of 10 μL of vesicles at 6.3 mg of protein per mL were first incubated for 15 min with: 2% ethanol, 3% dimethyl sulfoxide, and 50 mM KCl (●); 90 μM valinomycin, 2% ethanol, and 50 mM KCl (▲); or 90 μM valinomycin, 2% ethanol, 50 mM KCl, 40 μM nigericin, and 3% dimethyl sulfoxide (○). At time zero, a tenfold potassium ion gradient was generated by adding 10-μL aliquots of vesicles to 90-μL aliquots of the standard incubation mixture containing 50 mM NaCl and the indicated labeled solute; the final external potassium ion concentration was 5 mM. Uptake was measured by filtration at the indicated times.

From the electrochemical equilibrium distribution of a freely permeant lipophilic cation (Skulachev, 1971) such as triphenylmethylphosphonium ion ($TPMP^+$), membrane potential, $\Delta\psi$, can be calculated according to the Nernst equation.

$$\Delta\psi = -2.3(RT/F) \log ([TPMP^+_{in}]/[TPMP^+_{out}])$$

Since lipophilic cations differ in their permeability to various biological membranes (Schuldiner and Kaback, 1975), several criteria must be satisfied as a prerequisite for their use to measure transmembrane potential. Compelling justification for the filtration method employed here for determination of membrane potential by triphenylmethylphosphonium distributions was provided by several observations. Internal triphenylmethylphosphonium concentrations approached the known external concentration under two different conditions expected to specifically collapse membrane potential generated by potassium ion potentials in the presence of valinomycin: the further addition of nigericin (Figure 1A) or the addition of external potassium ion equal to the internal potassium ion concentration (Figure 2A and Table I). Nigericin, which promotes a nonelectrogenic potassium ion/proton exchange (Ashton and Steinrauf, 1970), reduced the apparent internal triphenylmethylphosphonium ion (Figure 1A) and amino acid (Figures 1B–D; Table I) concentration almost to the concentration present externally when added to vesicles with valinomycin at the time of imposition of the potassium ion po-

TABLE I: Electrogenic Amino Acid Translocation: Sodium Dependence.

Additions[a]		Apparent concn gradient,[b] C_i/C_o	
Ionophores	Salts, 50 mM	$TPMP^+$	Alanine
Valinomycin	NaCl	4.7	8.5
Valinomycin	Choline Cl	6.4	2.1
Valinomycin	NaCl + KCl	1.3	4.8
Valinomycin	Choline Cl + KCl	1.4	1.6
Valinomycin + monensin	NaCl	5.9	4.6
Valinomycin + monensin	Choline Cl		2.6
Valinomycin + monensin	NaCl + KCl	1.1	1.8
Valinomycin + nigericin	NaCl		2.6
Valinomycin + nigericin	Choline Cl		1.6

[a] Vesicles at 7.6 mg of protein per mL were first incubated for 15 min with 50 mM KCl and the indicated ionophores in 1% dimethyl sulfoxide; ionophore concentrations were 90 μM valinomycin, 27 μM sodium nigericin, and 14 μM sodium monensin. Then separate aliquots of vesicles were diluted tenfold and incubated either with 20 μM [^{3}H]triphenylmethylphosphonium ion ($TPMP^+$) and 50 mM each of the indicated chloride salts (38 μg of vesicles per aliquot) or with 0.2 mM L-[2,3-^{3}H]alanine and 50 mM of each chloride salt (76 μg of vesicles per aliquot). This manipulation resulted in a tenfold potassium ion diffusion potential (internal > external), with the exception of those incubations indicated where 50 mM KCl was present in the external suspension medium after dilution of vesicles, and a tenfold dilution in ionophore concentration. Maximum accumulation of each radioactive solute was estimated from several time points taken 2 to 5 min after dilution. [b] Ratio of intravesicular solute concentration, estimated by the filtration assay, to its external concentration.

TABLE II: Effect of Ion Gradients on the Accumulation of Triphenylmethylphosphonium Ion and Alanine.[a]

	Apparent concn ratio, C_i/C_o			
	$TPMP^+$		Alanine	
Addition	5 min	10 min	5 min	10 min
Choline Cl	4.7 ± 0.4	3.6 ± 0.15	1.4 ± 0.02	1.8 ± 0.06
NaCl	3.1 ± 0.06	2.8 ± 0.25	4.2 ± 0.09	4.3 ± 0.17
NaSCN	3.1 ± 0.32	3.1 ± 0.3	7.6 ± 0.02	7.1 ± 0.08
KSCN	3.2 ± 0.2	2.8 ± 0	1.9 ± 0.11	1.9 ± 0.11
$NaNO_3$	3.1 ± 0.15	3.4 ± 0.15	7.6 ± 0.14	7.1 ± 0.19
Na_2SO_4	2.52 ± 0.03	1.9 ± 0.08	3.2 ± 0.08	3.3 ± 0.04
KCl	3.4 ± 0.2	2.5 ± 0.3	1.3 ± 0.18	2.0 ± 0.02

[a] Accumulation of 20 μM [^{3}H]triphenylmethylphosphonium or 0.2 mM L-[2,3-^{3}H]alanine was estimated at 5 and 10 min after twofold dilution of 170-μg aliquots of vesicles into the standard incubation mixture containing labeled substrate plus the indicated 50 mM salt; Na_2SO_4 concentration was 25 mM. No ionophores were added.

TABLE III: Effect of Ionophores, Inhibitors, and Uncouplers on Triphenylmethylphosphonium Ion Accumulation Generated by an Anion Gradient.

Addition[a]	$TPMP^+$ apparent concn ratio, C_i/C_o, 5 min
1% dimethyl sulfoxide, 10 mM KCl minus NaCl	3.19 ± 0.14
27 μM nigericin, 10 mM KCl minus NaCl	2.87 ± 0.05
50 mM NaCl	2.43 ± 0.01
1 mM ouabain,[b] 50 mM NaCl	2.3 ± 0.05
2 mM 2,4-dinitrophenol, 50 mM NaCl	2.5 ± 0.1
20 mM sodium arsenate, 50 mM NaCl	2.3 ± 0.18
2 mM KCN, 50 mM NaCl	2.43 ± 0.36
1.25 mM pCMBS,[b,c] 50 mM NaCl	1.23 ± 0.06

[a] Aliquots of vesicles were diluted twofold into the standard incubation mixture containing the indicated additions plus 20 μM [^{3}H]triphenylmethylphosphonium bromide ($TPMP^+$). The transient anion gradient generated under these conditions caused accumulation of $TPMP^+$. The apparent ratio of the intravesicular $TPMP^+$ concentration to its external concentration was estimated by filtration after 5 min. Results obtained using duplicate samples are shown as the average ± range. Similar values were obtained in several determinations using independent vesicle preparations. [b] Vesicles were pretreated 15 min with inhibitor. [c] pCMBS, *p*-chloromercuribenzenesulfonate.

tential. The combination of these two ionophores results in the collapse of membrane potential under analogous conditions (Kessler et al., 1976) presumably by short circuiting the potassium ion gradient and would be expected to dissipate triphenylmethylphosphonium accumulation. Accumulation of triphenylmethylphosphonium was not observed when nonelectrogenic potassium ion efflux was induced by nigericin alone in potassium ion loaded vesicles (not shown). This result indicated that triphenylmethylphosphonium distributed according to electrical forces and was not directly influenced by potassium ion gradients. Furthermore, the permeant anion [^{14}C]thiocyanate did not accumulate in vesicles during valinomycin-induced potassium ion efflux (not shown). Uptake of triphenylmethylphosphonium ion into fibroblast plasma membrane vesicles could also be driven by permeant anion diffusion potentials and did not require the presence of ionophores or lipophilic anions (Table II). The accumulation of this lipophilic cation was not appreciably influenced by the chemical component of sodium ion gradients (Table I), variation in external pH in the range pH 5–9, omission of phosphate (not shown), or the addition of ionophores such as monensin (Table I) or nigericin (Table III) which catalyze electroneutral cation exchange (Pressman, 1976). Additional evidence that triphenylmethylphosphonium ion freely distributes across the membrane by diffusion was provided by observations that *p*-chloromercuriphenylsulfonate does not inhibit permeation of this lipophilic cation into vesicles although this reagent blocked its accumulation above the external concentration. Also lipophilic cation accumulation driven by anion diffusion potentials was insensitive to ouabain and several metabolic and oxidative inhibitors (Table III). Retention of triphenylmethylphosphonium in membranes due to binding was estimated as less than 3 pmol/mg at 20 μM external triphenylmethylphosphonium, i.e. <15% of the internal volume. Internal triphenylmethylphosphonium was sensitive to changes in the osmolarity of the external suspension medium and was proportional to the amount of vesicle protein. Efflux of accumulated triphenylmethylphosphonium during collection of vesicles by dilution in wash buffer at 2 °C was minimal; $t_{1/2}$ of efflux under these conditions was 2.5 min.

The lack of stimulation of amino acid influx by potassium ion efflux in the presence of nigericin (Lever, 1977a) provided evidence that potassium ion is not directly coupled to amino acid translocation by an antiport mechanism; thus, it may be concluded that the electrical potential but not the chemical potential of potassium ion gradients can drive active neutral amino acid transport.

If active neutral amino acid transport stimulated by a sodium ion gradient involves a direct, obligatory coupling of the translocation step to membrane potential, then amino acid

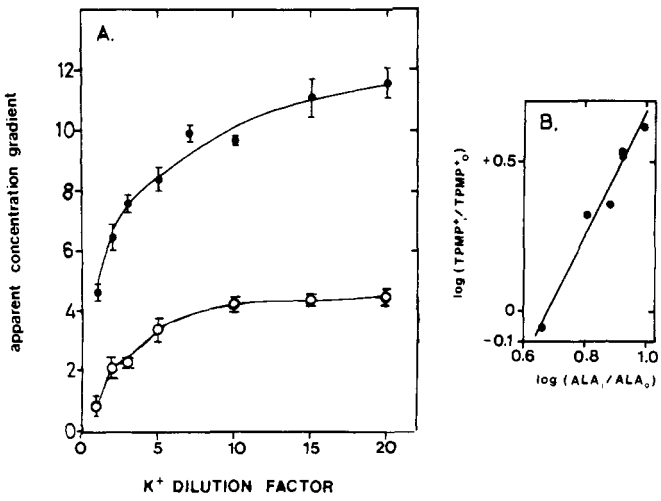

FIGURE 2: (A) Accumulation of triphenylmethylphosphonium ion and L-alanine as a function of potassium ion dilution factor in the presence of valinomycin. Vesicles (7.5 mg of protein/mL) were first incubated for 15 min in 90 μM valinomycin, 2% ethanol, and 50 mM KCl. Then potassium ion gradients of various magnitudes were generated by adding 10-μL aliquots of vesicles to volumes from 10 to 190 μL of the standard incubation mixture containing 50 mM NaCl and either 0.1 mM [^{3}H]triphenylmethylphosphonium bromide (○) or 0.2 mM L-[2,3-^{3}H]alanine (●). Uptake of each labeled solute was measured after 5 min. Accumulation observed when internal and external potassium ion concentrations were made equal by twofold dilution of vesicles (preincubated as above) into incubation mixtures containing 50 mM KCl in addition to 50 mM NaCl and labeled solute is represented by a dilution factor of 1.0. (B) Correlation of the logarithm of the apparent concentration ratio of triphenylmethylphosphonium accumulation with the logarithm of the apparent concentration ratio of L-alanine accumulation. Data were obtained from the experiment shown in Figure 2A.

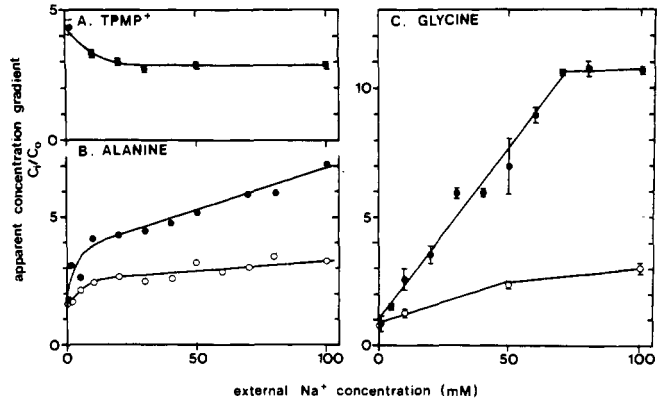

FIGURE 3: Ion gradient stimulated accumulation of triphenylmethylphosphonium ion and amino acids: effect of external sodium ion concentration: (panel A) 20 μM [^{3}H]triphenylmethylphosphonium bromide; (panel B) 0.2 mM L-[2,3-^{3}H]alanine; (panel C) 0.2 mM [2-^{3}H]glycine. Aliquots of 170 μg of vesicles containing either 1% dimethyl sulfoxide (●) or 14 μM sodium monensin in 1% dimethyl sulfoxide (○) were diluted twofold into aliquots of 100 μL of the standard incubation mixture containing 0 to 100 mM NaCl, 0 to 100 mM choline chloride to maintain a constant 100 mM chloride ion concentration, and the indicated labeled solute. By this means, various initial magnitudes of sodium ion gradients and a constant initial chloride ion gradient were artificially generated. Apparent accumulation of labeled solute was estimated by filtration at 5 min after dilution.

accumulation should increase as a function of membrane potential estimated by triphenylmethylphosphonium distribution. Furthermore, this accumulation should be dissociable from the metabolic activity of the cell and independent of the means by which membrane potential is generated.

Data presented in Figure 2 provide evidence that both triphenylmethylphosphonium and alanine distribute across the membrane as a function of an interior-negative membrane potential. When vesicles loaded with 50 mM potassium chloride were diluted in the presence of valinomycin to achieve a range of external potassium ion concentrations from 2.5 to 50 mM at a constant external sodium ion concentration of 50 mM, the apparent intravesicular accumulation of triphenylmethylphosphonium and alanine (Figure 2A) increased as a function of the potassium ion dilution factor. In this protocol, sodium, chloride, and potassium ion gradients dissipate with time according to their respective permeabilities and are not measured. Goldman's constant field equation (Goldman, 1943; Hodgkin, 1958) predicts that this experimental manipulation of transmembrane sodium and potassium ion gradients together with the selective large increase in potassium ion permeability mediated by valinomycin should produce an interior-negative potential of a magnitude mainly determined by the potassium ion gradient.

The logarithm of the apparent concentration gradient of alanine at quasi-steady state was a linear function of the logarithm of the apparent concentration gradient of triphenylmethylphosphonium ion with a slope of 2, as shown in Figure 2B. This positive correlation indicates that the energy from the electrical potential difference across the membrane contributes to the driving force for amino acid transport.

The artificial creation of a permeant anion diffusion gradient (external > internal) across the membrane provides an alternate method to generate an interior-negative membrane potential in vesicles as shown by triphenylmethylphosphonium accumulation in the experiments described in Figure 3A and Table II. Chloride translocates across the plasma membrane of the Ehrlich ascites cell mainly by diffusion, although a minor mediated pathway for chloride movement was also demonstrated (Levinson and Villereal, 1976). The quasiequilibrium distributions of triphenylmethylphosphonium ion shown in Table II and Figure 3A indicate that when a chloride gradient was created by dilution of vesicles into the standard suspension medium plus 50 mM choline chloride, an apparent interior-negative membrane potential of about −39.5 mV was generated. Substitution of sodium or potassium ion for choline caused a comparable, small depolarization which presumably resulted from inward leakage of cation. Substitution of gradients of the relatively impermeant anion sulfate for chloride in the presence of sodium ion caused marked depolarization and inhibition of alanine accumulation. A similar magnitude of apparent interior-negative membrane potential induced by anion potentials was also observed with highly purified plasma membrane vesicles which contained less than 2% contamination with endoplasmic reticulum and less than 0.2% mitochondrial contamination. Interestingly, proton conductors do not affect triphenylmethylphosphonium accumulation (Table III). This behavior may indicate that membrane permeability to anions can permit nonelectrogenic proton movement in this system.

Stimulation of Amino Acid Transport by an Interior-Negative Membrane Potential Requires Sodium Ion. Enhancement of amino acid accumulation by a valinomycin-induced potassium ion diffusion potential was insignificant when choline was substituted for sodium ion, as shown in Table I. Since an interior-negative membrane potential, estimated by triphenylmethylphosphonium ion accumulation, was generated under these conditions, sodium ion was not required for triphenylmethylphosphonium ion uptake, as similarly observed by Schuldiner and Kaback (1975).

Similar conclusions could be drawn from the amino acid accumulation driven by interior-negative membrane potentials generated by permeant anion diffusion potentials, as shown

in Table II. Substitution of imposed gradients of the anions thiocyanate or nitrate for those of chloride remarkably stimulated alanine accumulation in the presence of sodium ion gradients (Table II) as shown previously (Lever, 1976, 1977a), presumably because these anions are more permeant to biological membranes in comparison with chloride (Mitchell and Moyle, 1967) and facilitate charge compensation during electrogenic amino acid translocation. No stimulation of amino acid accumulation by electrical gradients generated by permeant anion diffusion potentials was observed when potassium ion was substituted for sodium ion.

These results indicate that sodium ion is required for the response of amino acid translocation to an electrical potential difference across the membrane.

A Chemical Sodium Ion Gradient Can Drive Amino Acid Accumulation in the Presence or Absence of an Independently Imposed Electrical Gradient. An essential feature of the alkali ion gradient hypothesis, reviewed by Crane (1977), requires that solute accumulation is dictated directly by the direction and magnitude of the electrochemical sodium ion gradient; in certain intact cells this gradient is maintained by the vectorial activity of the electrogenic ($Na^+ + K^+$)ATPase (Glynn and Karlish, 1975; Post and Jolly, 1957) located in the plasma membrane. In vesicles both a sodium ion gradient and/or an electrical gradient can be generated independently by experimental manipulation and these gradients dissipate with time.

A major prediction of the alkali ion gradient hypothesis applied to interpret neutral amino acid transport is the positive dependence of steady-state levels of alanine and glycine accumulation in vesicles on the external sodium ion concentration at a constant initial chloride gradient (external > internal), shown in Figure 3.

Monensin, an ionophore which performs an electroneutral sodium ion/proton exchange (Pressman, 1976), was used to selectively short circuit the sodium ion gradient in the presence of an interior-negative membrane potential generated either by a chloride diffusion potential (Figure 3) or by a potassium ion diffusion potential in the presence of valinomycin (Table I). Monensin does not affect electrical membrane potential under these conditions as shown by its lack of effect on triphenylmethylphosphonium accumulation driven by a potassium ion diffusion potential regardless of the presence or absence of sodium ion (Table I). Results in Figure 3 and Table I indicate that monensin decreased the level of alanine accumulation in the presence of an interior-negative membrane potential. Furthermore, amino acid accumulation showed greatly decreased variation as a function of sodium ion concentration. Since the dissipation of alanine accumulation by monensin was achieved without a change in membrane potential, the dissipation of the monensin-sensitive component of alanine accumulation must reflect primarily the collapse of the chemical sodium ion gradient. This also suggests that the chemical difference in sodium ion concentration across the membrane can be utilized to energize active amino acid transport independently of electrical effects.

In order to further explore this possibility, additional evidence that a sodium ion gradient by itself can drive amino acid accumulation was obtained. When vesicles were diluted in the presence of a sodium ion gradient and valinomycin so that the external potassium ion concentration was equal to the internal concentration (Figure 2A; Table I), triphenylmethylphosphonium ion distributed equally across the membrane, in accordance with the zero membrane potential predicted for this condition. In a similar experiment shown in Figure 2A and Table I, membranes were pretreated so that potassium ion was equally distributed across the membrane in the presence of valinomycin at the time of imposition of a sodium ion gradient (external > internal) and initiation of alanine uptake. This method allowed the selective determination of the chemical contribution of a sodium ion gradient to the driving force for alanine transport in the absence of a contribution from an electrical membrane potential. A four- to fivefold accumulation of alanine was stimulated by a 50 mM (external > internal) sodium ion gradient when electrical forces were selectively discharged by these conditions. This component of alanine accumulation observed in the absence of electrical differences was dissipated after the addition of monensin. Thus, it may be concluded that a chemical sodium ion gradient itself can solely drive amino acid accumulation in the absence of an electrical gradient.

An Electrochemical Sodium Ion Gradient and an Interior-Negative Membrane Potential Contribute Additively to the Driving Force for Amino Acid Accumulation. Experiments shown in Figure 2 and Table I indicate that when a sodium ion gradient and an electrical gradient together are imposed across the membrane, both forces can be utilized additively to drive amino acid accumulation. Thus, alanine accumulation in the presence of a sodium ion gradient and an interior-negative membrane potential exceeded the driving force which could be calculated from electrical potential differences alone. As shown in Table I, after imposition of a tenfold (internal > external) potassium ion gradient, a membrane potential of −39.5 mV was estimated using the Nernst equation from the apparent 4.7-fold triphenylmethylphosphonium accumulation, whereas the 8.5-fold alanine accumulation observed under these conditions would require a driving force of −54.6 mV. The further addition of monensin, which does not affect membrane potential, decreased alanine accumulation to the level predictable from the electrical gradient alone (Table I).

Results in Figure 2A show that the additive contribution of a constant initial sodium ion gradient to the driving force for alanine accumulation extends over a range of magnitudes of electrical gradients generated by valinomycin-induced potassium ion gradients. Alanine accumulation stimulated by a sodium ion gradient increased from about 4.5-fold in the absence of an electrical gradient to nearly 12-fold when a 20-fold potassium ion potential generated a 4.5-fold triphenylmethylphosphonium ion accumulation.

Similarly, the residual threefold alanine and glycine accumulation (shown in Figure 3) which persists after the chemical sodium ion gradient contribution was selectively dissipated by monensin is consistent with an electrical driving force estimated from a threefold triphenylmethylphosphonium ion accumulation. In this experiment, the interior-negative membrane potential was generated by a constant chloride diffusion potential. For this reason, triphenylmethylphosphonium ion accumulation does not vary appreciably from 0 to 100 mM external sodium ion concentration under the conditions used in Figure 3, although a slight depolarization was induced by the presence of sodium ion. Thus, due to its apparent low permeability, sodium ion does not seem to generate electrical gradients but causes depolarizing currents; resting potentials in this case are mainly due to the constant chloride gradient. That artificial sodium ion gradients contribute mainly chemically to drive amino acid accumulation is further supported by the marked increase of the monensin-sensitive component of alanine and glycine accumulation as a function of external sodium ion concentration, compared with the greatly decreased dependence of the monensin-insensitive, electrical component of the driving force for amino acid accumulation (Figure 3).

Amino Acid Transport in the Presence of Sodium Ion Affects Membrane Potential. During the course of L-alanine accumulation in vesicles in the presence of a sodium ion gradient, an increased capacity for accumulation of [^{14}C]thiocyanate in vesicles was observed, as shown in Figure 4. No stimulation of [^{14}C]thiocyanate accumulation by L-alanine was observed when choline chloride was substituted for sodium chloride. The distribution of the permeant anion thiocyanate is used here as a measure of depolarization of membrane potentials. Thiocyanate distributions under conditions where it is not accumulated in vesicles are complicated by its significant binding to membranes, estimated as at least 54 pmol/mg at 0.17 mM thiocyanate, which represents 32% of the intravesicular volume. As a corollary, triphenylmethylphosphonium ion accumulation was decreased by L-alanine or glycine uptake in the presence of a sodium ion gradient (not shown).

These results suggest that transport of alanine stimulated by sodium ion causes a transient depolarization by translocation of a positively charged complex.

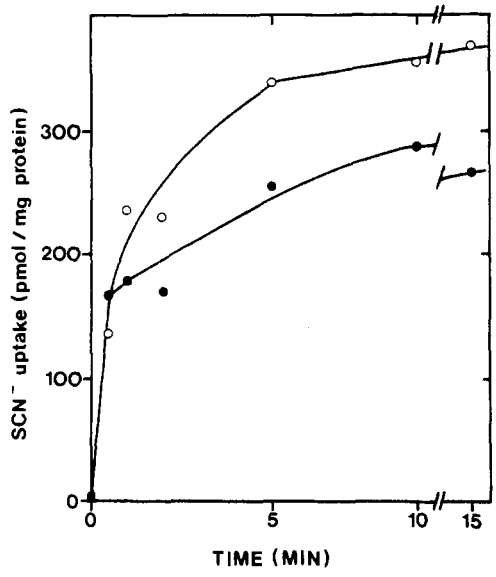

FIGURE 4: Stimulation of labeled thiocyanate ion uptake by L-alanine transport driven by a sodium chloride gradient. Aliquots of 130 μg of vesicles were diluted twofold into the standard incubation mixture containing 0.17 mM potassium [^{14}C]thiocyanate and 50 mM NaCl in the presence (○) or absence (●) of 10 mM unlabeled L-alanine. Thiocyanate uptake was determined by filtration at the indicated times after dilution.

Discussion

The presence in membrane vesicles of functional carriers for neutral amino acids with the transport properties of corresponding cellular uptake processes (Lever, 1976a,b; 1977a) provides a means of testing hypotheses relating carrier-mediated equilibration of amino acids across the plasma membrane to electrochemical ion gradients directly imposed or dissipated by experimental manipulation. These experiments extend previous experimental confirmation (Lever, 1976a,b; 1977a,b) of general predictions of the alkali ion gradient hypothesis, applied to the A system for neutral amino acid transport (Oxender and Christensen, 1963), to show unequivocally that both an electrical potential difference and a chemical difference in sodium ion concentration across the membrane can additively or separately contribute to the net driving force available for concentrative amino acid uptake, $\bar{\mu}_{Na^+}$, where:

$$\bar{\mu}_{Na^+} = \Delta\psi - 2.3(RT/F) \log ([Na^+_o]/[Na^+_i])$$

and $\Delta\psi$ represents the electrical membrane potential.

The polarity of the response of amino acid translocation to membrane potential in the presence of sodium ion leads to two alternate interpretations concerning the mechanism of the transport cycle. If translocation of the amino acid–carrier complex is stimulated by an interior-negative membrane potential in the presence of sodium ion because it is positively charged under these conditions, then the insensitivity of amino acid accumulation to membrane potential in the absence of sodium ion together with evidence (Christensen and Handlogten, 1975) that the zwitterionic form of the neutral amino acid predominantly translocates across the membrane lead to the tentative conclusion that free membrane carrier molecules for neutral amino acids are uncharged. Alternately, Rottenberg (1976) has proposed a model for proton–substrate cotransport which in the case of neutral, zwitterionic substrates would involve a negatively charged uncomplexed carrier site. In this model, the translocating complex of ion, substrate, and carrier site is always uncharged and outward movement of the negatively charged carrier site yielded by dissociation of ion and substrate internally would be facilitated by an interior-negative membrane potential, leading to transport stimulation. The preferred orientation of an uncomplexed negatively charged carrier site would be affected by membrane potential and contribute to the net driving force for transport. Although the lack of observed inhibition of amino acid influx by an interior-negative membrane potential in the absence of sodium ion can be interpreted in favor of the model based on a neutral uncomplexed carrier site, additional evidence will be required to unequivocally establish this point. The lack of stimulation of amino acid transport by an interior-negative membrane potential in the absence of sodium ion rules out the possibility that free carrier sites are positively charged. Since amino acid translocation in the presence of sodium ion responds to electrical differences across the membrane, this implies that the translocation step, rather than binding or release of ion and substrate, is rate limiting for transport, since charge effects would be cancelled in the latter case. Thus, changes in membrane composition which alter mobility of intrinsic proteins should affect the rate of amino acid transport via this system.

A further implication of these findings is that even under conditions where the electrogenic sodium ion pump is inoperative, active neutral amino acid transport could be energized by other, unidentified electrogenic systems in the plasma membrane or by electrical gradients generated by ion pump activities of intracellular organelles (Lehninger et al., 1967). Although amino acid uptake can occur without net accumulation in the absence of driving force, i.e., by facilitated diffusion (Lever, 1977a), it would appear that several points of cellular control operate on this process independently of modification of the carrier itself or its levels: the rate of generation of cellular sodium ion and electrical gradients by means of direct hormonal (Lever et al., 1976) and metabolic (Racker, 1976) regulation of the ($Na^+ + K^+$)ATPase, and the rate of dissipation of these gradients (Lever, 1976a,b; 1977b).

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