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Neutral Amino Acid Transport in Surface Membrane Vesicles Isolated from Mouse Fibroblasts: Intrinsic and Extrinsic Models of Regulation

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Membrane transport carrier function, its regulation and coupling to metabolism, can be selectively investigated dissociated from metabolism and in the presence of a defined electrochemical ion gradient driving force, using the single internal compartment system provided by vesiculated surface membranes. Vesicles isolated from nontransformed and Simian virus 40-transformed mouse fibroblast cultures catalyzed carrier-mediated transport of several neutral amino acids into an osmotically-sensitive intravesicular space without detectable metabolic conversion of substrate.

When a Na^+ gradient, external Na^+ > internal Na^+, was artifically imposed across vesicle membranes, accumulation of several neutral amino acids achieved apparent intravesicular concentrations 6- to 9-fold above their external concentrations. Na^+-stimulated alanine transport activity accompanied plasma membrane material during subcellular fractionation procedures. Competitive interactions among several neutral amino acids for Na^+-stimulated transport into vesicles and inactivation studies indicated that at least 3 separate transport systems with specificity properties previously defined for neutral amino acid transport in Ehrlich ascites cells were functional in vesicles from mouse fibroblasts: the A system, the L system and a glycine transport system. The pH profiles and apparent K_m values for alanine and 2-aminoisobutyric acid transport into vesicles were those expected of components of the corresponding cellular uptake system.

Several observations indicated that both a Na^+ chemical concentration gradient and an electrical membrane potential contribute to the total driving force for active amino acid transport via the A system and the glycine system. Both the initial rate and quasi-steady-state of accumulation were stimulated as a function of increasing concentrations of Na^+ applied as a gradient (external > internal) across the membrane. This stimulation was independent of endogenous Na^+, K^+-ATPase activity in vesicles and was diminished by monensin or by preincubation of vesicles with Na^+. The apparent K_m for transport of alanine and 2-aminoisobutyric acid was decreased as a function of Na^+ concentration. Similarly, in the presence of a standard initial Na^+ gradient, quasi-steady-state alanine accumulation in vesicles increased as a function of increasing magnitudes of interior-negative membrane potential imposed across the membrane by means of K^+ diffusion potentials (internal > external) in

Abbreviations used: iso-Abu – 2-aminoisobutyric acid; $TPMP^+$ – triphenylmethylphosphonium ion; SV 40 – Simian virus 40; FCCP – carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

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the presence of valinomycin; the magnitude of this electrical component was estimated by the apparent distributions of the freely permeant lipophilic cation triphenylmethylphosphonium ion. Alanine transport stimulation by charge asymmetry required Na^+ and was blocked by the further addition of either nigericin or external K^+. As a corollary, Na^+-stimulated alanine transport was associated with an apparent depolarization, detectable as an increased labeled thiocyanate accumulation. Permeant anions stimulated Na^+-coupled active transport of these amino acids but did not affect Na^+-independent transport. Translocation of K^+, H^+, or anions did not appear to be directly involved in this transport mechanism. These characteristics support an electrogenic mechanism in which amino acid translocation is coupled to an electrochemical Na^+ gradient by formation of a positively charged complex, stoichiometry unspecified, of Na^+, amino acid, and membrane component.

Functional changes expressed in isolated membranes were observed to accompany a change in cellular proliferative state or viral transformation. Vesicles from Simian virus 40-transformed cells exhibited an increased V_{max} of Na^+-stimulated 2-aminoisobutyric acid transport, as well as an increased capacity for steady-state accumulation of amino acids in response to a standard Na^+ gradient, relative to vesicles from nontransformed cells. Density-inhibition of nontransformed cells was associated with a marked decrease in these parameters assayed in vesicles. Several possibilities for regulatory interactions involving gradient-coupled transport systems are discussed.

Key words: transport mechanisms, amino acids, mouse fibroblasts, plasma membrane vesicles, regulation, SV40 transformation

The noncovalent coupling of active transport systems for certain nutrients to metabolic energy stored in the form of electrochemical ion circuits across the membrane has emerged as a useful concept to visualize an integral feature of the functional specialization of a diversity of biological membranes (1–9). Thus the alkali-ion gradient hypothesis that Na^+-dependent active transport systems for certain organic solutes in intestinal and renal brush border membranes, erythrocytes, and tumor cells (1–4, 6) are driven by electrochemical Na^+ gradients generated by active Na^+ extrusion by the Na^+, K^+-ATPase shares this fundamental biological principle with Mitchell's subsequent proposal that certain respiration-coupled active transport systems in bacterial cytoplasmic membranes are driven by electrochemical proton gradients generated across the membrane by active proton extrusion (5, 7, 8).

Although much of the information which led to these concepts was obtained using intact cells, an important step was provided by the use of isolated membrane vesicles as a system for transport studies (10). This approach, extended to isolated membranes from animal cells (11–16), obviates ambiguities due to such factors as unknown compartmentalization of ions and substrates, reliance on inhibitors, analogs and metabolic depletion, interaction of ionophore probes with intracellular membranes, and possible coupled or passive movement of other ions which complicate the interpretation of cellular uptake data. Such cell-free transport studies, one example of which will be discussed here, are precursors to more detailed future descriptions of these processes at the molecular level, which would require the use of purified membrane macromolecules.

When surface membranes of fibroblasts grown in culture are disrupted using a nitrogen cavitation procedure (17), unilaminar sacs are formed of average diameter 0.1–0.2 μm (18) which can be identified by characteristic enzymatic and other markers and separated from fragments of intracellular membranes, organelles, and soluble contaminants by centrifugation methods (15, 17). These vesiculated plasma membranes are

selectively permeable and retain functional transport carriers for ions and nutrients (15, 16, 18, 19), properties which permit their use to investigate membrane transport mechanisms at the level of flux of molecules between 2 bulk aqueous phases.

Outlined here are some characteristics of the coupling between electrochemical Na^+ gradients, membrane potential, and accumulation of neutral amino acids via functional components of cellular uptake systems, isolated in membrane vesicles prepared from the surface membranes of mouse fibroblasts. As well as providing an experimental confirmation of general predictions of the alkali-ion gradient hypothesis, these observations reveal additional details of the translocation process.

At one level, this interaction between carrier, ion, substrate, and charge asymmetry forms an intrinsic, fixed mode of regulation of solute translocation, dynamically mediated by the structure of the transport component itself. Superimposed on this are extrinsic modes of regulation mediated by genetic expression and cellular and external agents which can also influence these membrane functions. It was noted that alterations in cellular uptake of neutral amino acids, either by cell density-inhibition in nontransformed fibroblasts or accompanying transformation by Simian virus 40, were expressed in the transport properties of their isolated membrane vesicles (16, 19). Possible intrinsic and extrinsic controls of Na^+-dependent amino acid transport are discussed. Some of the experiments discussed here have been published previously (16, 19, 20).

MATERIALS AND METHODS

Cell Culture

Swiss and Balb/c 3T3 mouse fibroblasts transformed by Simian virus 40 (SV 40), Balb/c tertiary mouse embryo cells, and Swiss 3T3 fibroblasts were propagated in Dulbecco's modified Eagle's medium with 10% serum as described previously (16). Cell lines were routinely monitored for absence of mycoplasma contamination.

Preparation of Membrane Vesicles

Cells ($1-9 \times 10^9$) were washed twice with phosphate-buffered saline at room temperature, harvested by scraping with a rubber blade, and washed again. The cell pellet was resuspended in 0.25 M sucrose, 0.2 mM $MgCl_2$, 0.01 M Tris-HCl, pH 7.5, centrifuged at 4,000 × g for 20 min and the cell pellet was resuspended in 100 ml of this solution with $CaCl_2$ substituted for $MgCl_2$. Cells were disrupted by nitrogen cavitation, and previously described centrifugation methods were used to isolate either purified mixed vesicles (16, 20) or purified plasma membrane vesicles (20, 21).

Purified mixed vesicles contained 39–68% of the total 5′-nucleotidase activity, 20 ± 2% of the total NADH oxidase activity, 1.5 ± 0.5% of the total succinate-cytochrome c reductase activity, and 11% of the total cellular protein. Purified plasma membranes from SV 3T3 fractionated after dextran-110 discontinuous gradients (20) contained 20% of the total 5′-nucleotidase activity, 2.6% of cellular NADH oxidase activity, 0.10% of total succinate-cytochrome c reductase activity, and 2% of cellular protein.

Aliquots of 1 ml of vesicles were stored suspended in 0.25 M sucrose, 0.01 M Tris-HCl, pH 7.5, in liquid nitrogen.

Enzyme Assays

Subcellular fractions of vesicle preparations were assayed as described previously

(20) for the following marker enzymes: 5′ nucleotidase (5′-ribonucleotide phosphohydrolase; EC 3.1.3.5), a marker for plasma membrane (17, 22), NADH oxidase (NADH: oxidoreductase, EC 1.6.99.3), a marker for endoplasmic reticulum and outer mitochondrial membrane (23), and succinate-cytochrome c reductase (EC 1.3.99.1), a marker for the inner mitochondrial membrane (24). Na^+, K^+, Mg^{2+}-dependent ATPase activity (ATP phosphohydrolase, EC 3.6.1.3), a plasma membrane marker (22), was estimated as the increase in initial rate of ATP hydrolysis relative to mixtures lacking KCl, using the method described previously (20). Protein was determined by the method of Lowry et al. (25).

Membrane Vesicle Transport Assays

Incubations were carried out at 21°C in 100 μl volumes containing 40–250 μg membrane vesicle protein, 0.125 M sucrose, 10 mM Tris-phosphate, pH 7.4, 5 mM $MgCl_2$ and other additions as indicated. Uptake was terminated by dilution with 5 ml of 0.8 M NaCl, 0.01 M Tris-HCl, pH 7.5 (wash buffer) at 2°C, immediate filtration through a 0.2 μm or 0.45 μm pore size nitrocellulose filter (Schleicher & Schuell, 2.5 cm diameter), and filtration of an additional 5 ml of wash buffer, as described previously (16, 20). Nonspecific adsorption to vesicles was determined at zero-time by adding labeled substrate after dilution of vesicles. Radioactivity of dried filters was measured by scintillation counting in toluene-Liquifluor (New England Nuclear Corp.). The apparent initial rates of transport were proportional to the amount of vesicle protein up to 250 μg and not affected by addition of nonspecific protein. Minimal leakage of accumulated substrate was observed after 50-fold dilution of vesicles in wash buffer at 2°C, but efflux was appreciable after dilution in wash buffer at 37°C.

For assay of triphenylmethylphosphonium ion ($TPMP^+$) uptake, 2.5 cm diameter, 0.5 μm pore size cellulose acetate filters (Millipore type EH) were used because of high blank values due to binding of this substrate to nitrocellulose.

Binding of $TPMP^+$ to membranes was estimated after osmotic lysis of vesicles as less than 8 pmol per mg. Efflux of $TPMP^+$ during collection of vesicles within 20 s was minimal; t½ of efflux was 2.5 min under these conditions. Accumulated $TPMP^+$ was sensitive to the osmolarity of the suspension and proportional to the amount of vesicle protein.

Analysis of Accumulated Radioactivity

Radioactive amino acids accumulated in vesicles after collection by filtration were eluted with H_2O, concentrated by lyophilization and analyzed by thin-layer chromatography as described previously (20).

Intravesicular Volume

The internal volume of vesicles was measured by accumulation of 0.5 mM 3-0-methyl-^{3}H-glucose using the filtration assay. The measured efflux of 3-0-methylglucose in wash buffer indicated less than 20% loss of internal solute during collection of vesicles. An average value of 1 ± 0.2 μl/mg protein of purified mixed vesicles was used in calculations of internal solute concentrations. Estimated internal volumes were proportional to the amount of vesicle protein.

Materials

^{3}H-$TPMP^+$ bromide*, 114 Ci/mol and 440 Ci/mol (26), and unlabeled $TPMP^+$ bromide were generous gifts from Dr. H. R. Kaback, Roche Institute of Molecular Biology,

Nutley, New Jersey. Potassium ^{14}C-thiocyanate (11.8 fmol/cpm), L-2,3-^{3}H-alanine (39 fmol/cpm), 2-amino-1-^{14}C-isobutyric acid (iso-Abu) (91 fmol/cpm or 61 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 donated by Dr. G. L. Smith, Lilly, U.K. and carbonyl-cyanide p-trifluoromethoxyl phenylhydrazone was from Dr. P. Heytler, du Pont de Nemours, Delaware. Valinomycin, ouabain, and oligomycin were purchased from Sigma.

RESULTS

Criteria for Carrier-Mediated Amino Acid Transport in Vesicles

Several lines of evidence indicated that specific plasma membrane carriers in vesicles mediate amino acid influx and efflux. When Na^+ was preequilibrated across the membrane to abolish active accumulation, countertransport (27), a standard criterion for carrier-mediated exchange diffusion, could be demonstrated both by increased uptake of labeled alanine when vesicles were preloaded with unlabeled alanine (Table I) and by increased efflux of labeled alanine accumulated in vesicles when unlabeled alanine was added to the suspension medium (20). Stimulation was specific for pairs of substrates of this carrier (Table I). Accumulated amino acid was retained in a selectively permeable compartment enclosed by vesicle membranes with negligible binding to membranes, as shown by its sensitivity to variation in the osmotic pressure of the external suspension medium (16). Both influx and efflux were temperature-dependent (20) such that the rate of solute translocation in both directions was increased as the temperature was increased over the range 2–37°C. Transport of alanine, leucine, glutamine, or methionine was not associated with metabolic conversion of substrate detectable by thin-layer chromatography of labeled material extracted after accumulation in vesicles.

TABLE I. Countertransport Stimulation of Amino Acid Transport in Vesicles

Amino acid		Intravesicular labeled amino acid			
Preloaded[2]	External	30 sec	1 min	5 min	15 min
		nmol/mg			
L-alanine, 10 mM	^{3}H-ala, 1.7 mM	9.5	14	15	13.5
–	^{3}H-ala, 1.7 mM	3.1	3.7	6.3	7.1
iso-Abu, 9 mM	^{14}C-iso-Abu, 1.1 mM	2.0	2.8	5.8	6.3
–	^{14}C-iso-Abu, 1.1 mM	2.4	3.1	3.2	4.9
iso-Abu, 9 mM	^{3}H-ala, 0.2 mM, plus iso-Abu, 0.9 mM	0.50	0.51	0.27	0.42
–	^{3}H-ala, 0.2 mM, plus iso-Abu, 0.9 mM	0.08	0.27	0.25	0.59
glycine, 10 mM	^{3}H-ala, 0.2 mM, plus glycine, 1 mM	0.33	0.68	0.81	0.41
–	^{3}H-ala, 0.2 mM, plus glycine, 1 mM	0.26	0.41	0.61	0.50
Leucine, 10 mM	^{3}H-ala, 0.2 mM, plus leu, 1 mM	0.47	0.38	0.62	0.57
–	^{3}H-ala, 0.2 mM, plus leu, 1 mM	0.08	0.45	0.44	0.55
Methionine, 10 mM	^{3}H-ala, 0.2 mM, plus met, 1 mM	0.43	0.45	0.57	0.72
–	^{3}H-ala, 0.2 mM, plus met, 1 mM	0.47	0.51	0.20	0.30

[2]Vesicles were incubated 15 min with or without the indicated unlabeled amino acid and 50 mM NaCl and then aliquots (44–52 μg) were diluted 10-fold to a 100 μl volume containing 50 mM NaCl and the indicated external labeled and unlabeled amino acids.

The Na^+-stimulated transport activity was associated with plasma membrane fragments. Plasma membrane fractions recovered from dextran-110 discontinuous gradients represented 20% of the total plasma membranes, purified 21-fold as estimated by the marker enzyme 5'-nucleotidase, and were associated with 20% of the total Na^+-stimulated iso-Abu transport activity, 9.2-fold increased in specific activity (20). Only 1.4% of the total transport activity was associated with endoplasmic reticulum, 5.5-fold reduced in specific activity with respect to the homogenate and 50-fold reduced with respect to plasma membranes. Other fractions, including nuclear and mitochondrial, showed no detectable Na^+-stimulated transport activity. Similar results were obtained for the subcellular distribution of Na^+-stimulated alanine activity.

Competitive interactions among neutral amino acids for transport into vesicles, summarized in Table II, resembled those previously described for uptake in Ehrlich

TABLE II. Specificity of Na^+ Gradient-Stimulated Amino Acid Uptake in Vesicles

Unlabeled amino acid 5 mM	Initial rate of uptake of radioactive amino acid[2]			
	^{3}H-ala	^{3}H-met	^{3}H-leu	^{14}C-iso-Abu
	% control[c]			
No addition	100	100	100	100
L-alanine	14	37	90	18
D-alanine	79	75	85	71
L-methionine	15	26	74	27
D-methionine	83	22	28	78
L-leucine	66	42	42	68
D-leucine	83	38	30	86
iso-Abu	31	78	71	31
D-glutamine	87	71	79	82
N-methyl-DL-alanine	21	68	62	20
Glycine	80	90	82	78
L-phenylalanine	87	51	37	70
L-glutamine	27	36	59	38
L-isoleucine	92	48	40	89
L-histidine	58	28	53	48
L-serine	18	32	62	31
L-valine	63	31	38	73
L-proline	29	34	50	26
L-threonine	56	45	47	75
L-tryptophan	69	34	8	66
L-cysteine	22	23	33	25
L-sarcosine	44	36	81	32
L-homoserine	25	28	46	22
1-aminocyclopentane-1-carboxylic acid	20	31	32	30
minus Na^+[b]	15	14	50	20

[a]The initial rate of uptake was measured 30 sec after the addition of 50 mM NaSCN and either 0.2 mM L-[methyl-^{3}H] methionine (30 fmol/cpm), 0.2 mM L-[2,3-^{3}H] alanine (43 fmol/cpm), 0.2 mM L-[4,5-^{3}H] leucine (33 fmol/cpm) or 0.17 mM [1-^{1}C] iso-Abu (91 fmol/cpm).

[b]50 mM KSCN was substituted for 50 mM NaSCN.

[c]100% represented 3 nmol/min·mg for alanine uptake, 1.7 nmol/min·mg for methionine uptake, 1 nmol/min·mg for leucine uptake and 1 nmol/min·mg for iso-Abu uptake. Results were averaged from duplicates with a range of ± 15%.

ascites cells (28, 29) and indicated that the A and L systems and a glycine system were functional in vesicles. N-methyl-DL-alanine was as effective as L-alanine in inhibiting L-^{3}H-alanine transport, which suggests that the ASC system (29) does not contribute appreciably to alanine transport in vesicles at this pH. Furthermore, pH profiles for the initial rate of transport of iso-Abu and alanine into vesicles (20) resembled those of cellular uptake in Ehrlich ascites tumor cells (28), with optimal uptake at pH 7.4. The similarity between K_m values obtained for Na^+-dependent iso-Abu uptake in intact cells (30) and those measured in vesicles further confirmed the identity of the transport carrier activity assayed in vesicles as a component of the corresponding cellular uptake system (Table III). The maximal velocity (V_{max}) values for iso-Abu transport into vesicles, after correction for recovery of membrane protein, resembled V_{max} values reported for iso-Abu uptake in these cell lines (30), an indication that vesicles retain intact a large proportion of functional transport carriers which operate in living cells.

TABLE III. Kinetic Parameters of Sodium Gradient-Stimulated Amino Acid Transport in Vesicles*

Substrate	Na^+ concentration	Amino acid transport	
		K_m	V_{max}
	(mM)	(mM)	(nmol/min per mg)
Balb/c SV3T3 membranes:			
iso-Abu	0	5	2.4
	50	1.33	2.9
Swiss SV3T3 membranes:			
Alanine	0	10 ± 2	20
	1	2.4	12
	10	1.7	15
	50	1.2	15
Balb/c tertiary mouse embryo fibroblasts (subconfluent):			
iso-Abu	50	1.1 ± 0.4	1 ± 0.2

*Vesicles were incubated 30 sec with various concentrations of ^{3}H-alanine or iso-^{14}C-Abu at the indicated Na^+ concentrations. For alanine uptake, SCN^- was used as counterion, at a constant concentration of 50 mM maintained by addition of KSCN. For iso-Abu uptake, Cl^- was the counterion, maintained at 50 mM by the addition of choline Cl. Results were analyzed by inverse-reciprocal plots. The transport activity of vesicles from confluent mouse embryo fibroblasts was too low to permit accurate determination of these parameters.

Concentrative Amino Acid Transport in Vesicles is Coupled to an Electrochemical Na^+ Gradient

The alkali-ion gradient hypothesis, reviewed recently by Crane (9) and summarized in Fig. 1, leads to the expectation that amino acid accumulation is dictated directly as a function of the vectorial sum of the direction and magnitude of the electrochemical Na^+ gradient and membrane potential; in intact cells, this driving force is maintained by the electrogenic Na^+ pump but in vesicles these gradients can be artificially manipulated and tend to dissipate with time.

When a Na^+ gradient (external > internal) was artificially created across the membrane at zero-time by adding Na^+ together with substrate to the external suspension medium, both the initial rate and steady state of alanine and glycine uptake were marked-

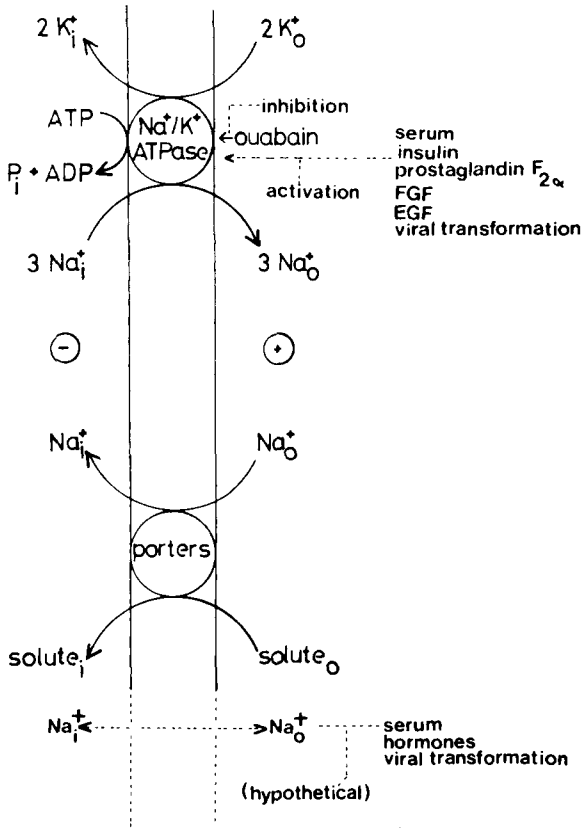

Fig. 1. Characteristics of Na^+-dependent transport into vesicles predicted by the "sodium gradient" hypothesis: some possible targets for regulation.

ly increased, as shown in Fig. 2, A and B. No appreciable stimulation of accumulation was observed after similar imposition of gradients of chloride salts of K^+, choline$^+$, Li^+, Tris$^+$, Mg^{2+}, Rb^+, or Ca^{2+} across the membrane (20); Na^+ gradients similarly enhanced active transport of iso-Abu, glutamine, and methionine (16, 20); maximal accumulation driven by external 50 mM NaCl achieved apparent internal concentrations 6- to 7-fold above external concentrations of these amino acids. By contrast leucine uptake showed much less stimulation by a Na^+ gradient (Fig. 2C). System L may partially mediate Na^+-independent transport of alanine, as recently described for iso-Abu transport (31).

Conditions expected to dissipate the Na^+ gradient, such as the addition of monensin, an ionophore which catalyzes an electroneutral Na^+/H^+ exchange across the membrane (32), or incubation of vesicles with Na^+ for 15 min before adding amino acid (16), decreased both the initial rate and steady state of accumulation of these amino acids, as shown for alanine and glycine in Fig. 2.

The nature of the major counteranion increased the initial rate and maximal extent of Na^+-stimulated amino acid accumulation in the order $NO_3^- > SCN^- > Cl^- > SO_4^{2-}$ (20), which reflects their relative permeability to biological membranes (33). This stimulation by SCN^- is illustrated in Fig. 2.

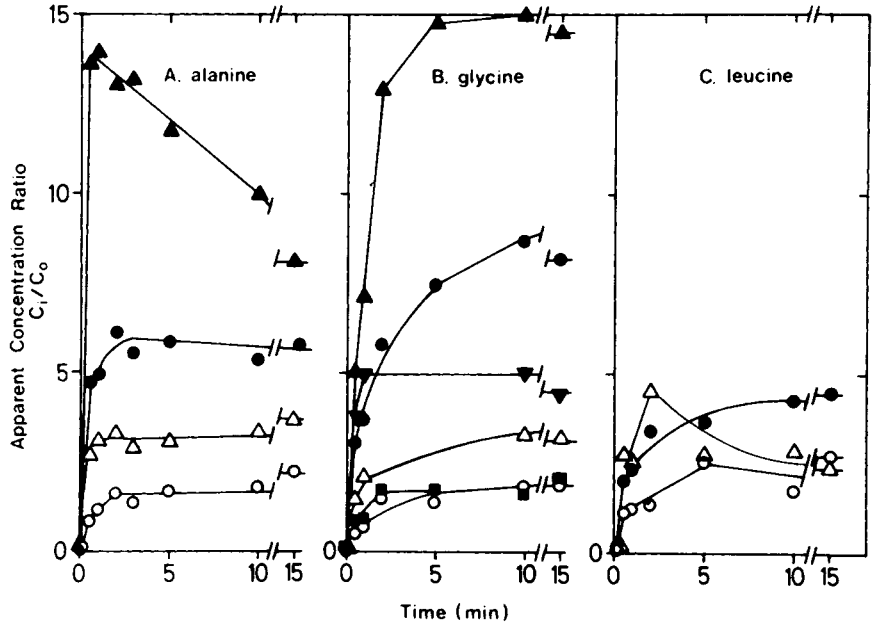

Fig. 2. Na^+ gradient-dependence of A) L-[2,3-^{3}H]alanine, B) [2-^{3}H] glycine, and C) L-[4,5-^{3}H] leucine transport into vesicles. Na^+, choline, or K^+ salts and the indicated 0.2 mM labeled amino acid were added at zero-time: ▲) 50 mM NaSCN; ●) 50 mM NaCl; ○) 50 mM choline chloride; △) 50 mM NaCl plus 14 μM monensin in 1% Me_2SO; ▼) 50 mM NaSCN plus 14 μM monensin in 1% Me_2SO; ■) 50 mM KSCN. Control experiments (not shown) indicated that 1% Me_2SO had no effect on uptake.

Although vesicles retained functional Na^+, K^+-ATPase activity, the Na^+ gradient-stimulated amino acid transport could be dissociated from this activity (20). Addition of ouabain for 20 min to vesicle suspensions completely blocked K^+-dependent ATP hydrolysis in the presence of concentrations of ATP, KCl, $MgCl_2$ and NaCl required for optimal Na^+, K^+-ATPase activity yet did not inhibit Na^+-stimulated alanine transport in similarly treated aliquots of the same vesicle preparation. Addition of 5 mM KCl and 2 mM ATP did not stimulate amino acid transport.

Sensitivity of amino acid transport to variation in external Na^+ was most pronounced at low amino acid concentrations, as shown previously (20). Interpretation of these data by Lineweaver-Burk analyses, summarized in Table III, revealed that substitution of Na^+ for choline$^+$ decreased the apparent K_m for alanine or iso-Abu transport into vesicles with minimal effects on V_{max} when a counteranion was present at a constant concentration. The apparent K_m was decreased as the Na^+ concentration was increased.

At 0.2 mM alanine, both the initial rate and quasi-steady state of alanine transport increased as a function of increasing NaSCN gradients when the SCN^- concentration was maintained constant by the addition of KSCN, as illustrated in Figs. 3 and 4. When the Na^+ gradient was short-circuited by the addition of monensin, quasi-steady-state accumulation was greatly diminished and was rendered independent of variation in Na^+ concentration. By contrast, the increased initial rate of alanine uptake as a function of Na^+, a measure of carrier activity plus driving force, persisted although diminished in the presence of monensin and showed an apparent saturation above 50 mM Na^+. Therefore, in the absence

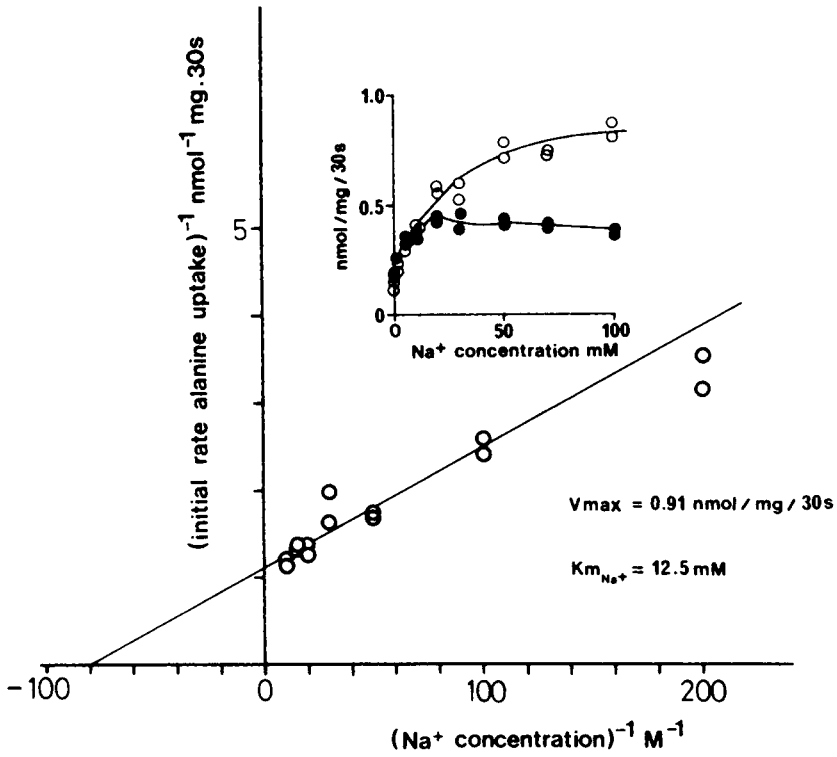

Fig. 3. Stimulation of the initial rate of alanine transport into vesicles as a function of external Na^+ concentration. At zero-time, the indicated concentrations of NaSCN and 0.2 mM L-[2,3-^{3}H]alanine were added to aliquots of 120 μg vesicles (Balb SV3T3) treated with either 1% Me_2SO (○) or 14 μm monensin in 1% Me_2SO (●). The total SCN^- concentration was maintained constant at 100 mM by addition of KSCN so that the Na^+/K^+ ratio was varied. Uptake was determined after 30 sec.

of its chemical concentration difference across the membrane, Na^+ influences the affinity of alanine for the membrane transport component. A predictable uncertainty in the quantitative interpretation of these results is the unmeasured dissipative Na^+ flow resulting from the expected coupled and uncoupled leaks during the course of amino acid uptake; whereas the external Na^+ concentration is constant, the internal concentration, and thus the magnitude of the Na^+ gradient, varies. However, several observations pertinent to this point indicate that the relationship between quasi-steady-state solute accumulation in vesicles and the external Na^+ concentration can roughly approximate a thermodynamic equilibrium between the amino acid accumulation ratio and the driving force.

Dissipation of driving force should allow only transient accumulation, with efflux of accumulated amino acid resembling an overshoot. At true steady state, this would lead to collapse of gradients of Na^+ and amino acid across the membrane. No overshoot of amino acid accumulation was observed within the 15 min time interval shown, either with purified mixed vesicles or purified plasma membrane vesicles, in the presence of a NaCl gradient. When monensin was added to vesicles at 15 min after amino acid transport was initiated to accelerate dissipative Na^+ flow, amino acid efflux was observed (Fig. 5). This indicated that a residual Na^+ gradient persisted at 15 min and that the apparent amino

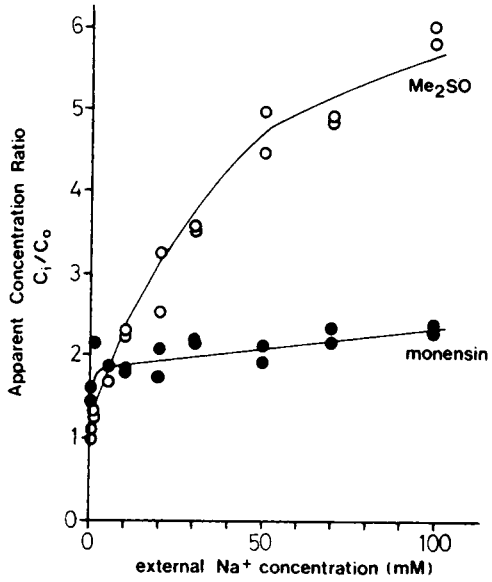

Fig. 4. Dependence of quasi-steady-state levels of alanine accumulation in vesicles on the external Na^+ concentration imposed as a Na^+ gradient. Symbols and conditions as in Fig. 4, but uptake was measured after 10 min.

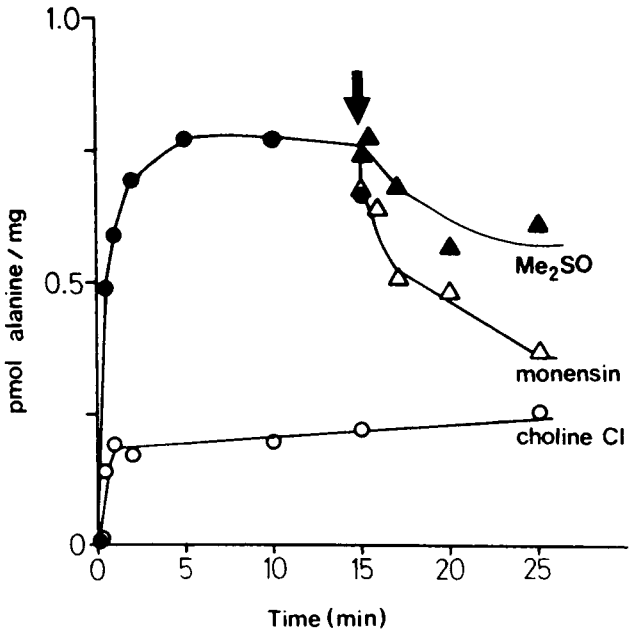

Fig. 5. Efflux of accumulated iso-Abu induced by the addition of monensin. Aliquots of 120 μg vesicles were incubated with 0.2 mM ^{3}H-alanine and either 50 mM NaCl (●) or 50 mM choline Cl (○). Arrow indicates addition at 15 min of either 2% Me_2SO (▲) or 30 μm monensin in 2% Me_2SO (△).

acid accumulation in vesicles was in equilibrium with the Na^+ gradient. By contrast, amino acid accumulation stimulated either by a transient K^+ diffusion potential facilitated by valinomycin or by a gradient of the Na^+ salt of a highly permeant anion such as SCN^- did show a gradual overshoot pattern. Surprisingly, Lineweaver-Burk inverse-reciprocal plots of the initial rates of Na^+-stimulated amino acid uptake in vesicles as a function of amino acid concentration did not show the curvature expected from increased coupled Na^+ influx at increased amino acid concentrations. Furthermore, the logarithm of the observed quasi-steady-state accumulation of alanine was a linear function of the logarithm of the external Na^+ concentration at a constant external amino acid concentration (20). Presumably, Na^+ influx under certain experimental conditions is not fast enough in these membranes to contribute a measurable source in variability, but can be accelerated when charge compensation across the membrane is greatly facilitated by experimental manipulation. Other membrane systems, such as intestinal brush border membranes, which exhibit rapid overshoot of Na^+ stimulated glucose and alanine uptake (12) may contain endogenous systems to facilitate Na^+ influx, such as the proposed Na^+/H^+ antiport system (37).

Membrane Potential and Amino Acid Transport

Amino acid translocation into vesicles was stimulated by an interior-negative membrane potential. This response specifically required the presence of Na^+. Figure 6 shows that when the internal lumen of vesicles was loaded with K^+ in the presence of valinomycin, a K^+-specific ionophore (32), and then vesicles were diluted to achieve a 10-fold lower external K^+ concentration at the time of addition of amino acid and external Na^+, a

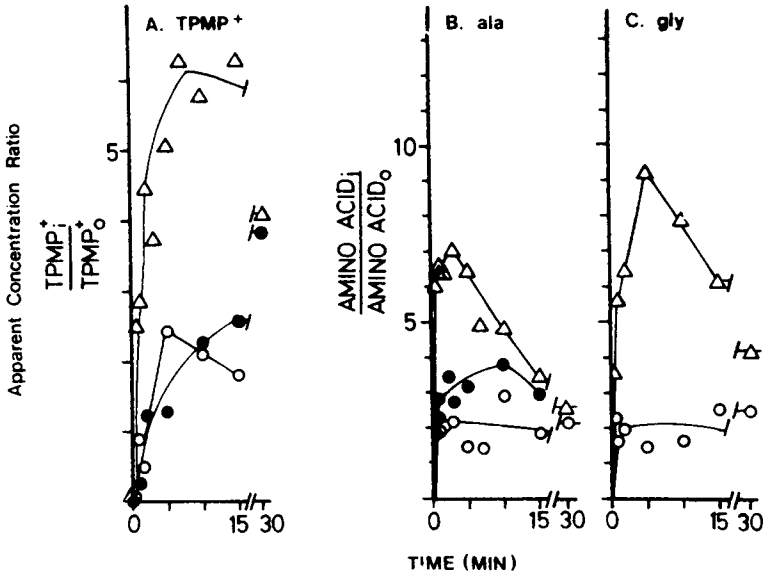

Fig. 6. Stimulation of Na^+-dependent amino acid and lipophilic cation accumulation by imposition of an interior-negative membrane potential. A) 0.1 mM ^{3}H-triphenylmethylphosphonium bromide; B) 0.2 mM ^{3}H-alanine; C) 0.2 mM ^{3}H-glycine. Aliquots of 10 μl of vesicles (6.3 mg per ml) were preincubated 15 min with: •) 2% ethanol and 3% MeSO; △) 90 μM valinomycin, 2% ethanol, 3% Me_2SO, and 50 mM KCl; or ○) 90 μM valinomycin; 40 μm nigericin, 2% ethanol, 50 mM KCl, and 3% Me_2SO. Then, vesicles were diluted 10-fold into 100 μl volumes containing 50 mM NaCl and the indicated labeled substrate, for the times shown.

striking transient enhancement of alanine and glycine accumulation was observed. Such a K^+ diffision potential (internal > external) and the large selective increase in K^+ permeability mediated by translocation of the positively-charged K^+-valinomycin complex (32) would be expected to create a transient internal-negative membrane potential in vesicles which were otherwise relatively impermeable to ions, of a magnitude dictated mainly by the K^+ potential, (34, 35).

This assumption was verified in these preparations using the apparent distributions of the lipophilic cation $TPMP^+$ as shown in Fig. 6A. Theoretically, from the distribution of a cation which freely distributes across the membrane according to electrical effects, membrane potential, $\Delta\psi$, can be calculated according to the Nernst equation:

$$\Delta\psi \frac{-RT}{nF} \ln \frac{TPMP^+_i}{TPMP^+_o} = -58.8 \log \frac{TPMP^+_i}{TPMP^+_o}$$

From the 6-fold apparent accumulation of lipophilic cation, a membrane potential of −45.8 mV could be calculated.

Several observations indicated that this lipophilic cation distributed according to electrical effects rather than binding to the membrane or specific transport (Lever JE, in preparation). The accumulation of lipophilic cation showed a positive correlation with the K^+ dilution factor under these conditions (Lever JE, in preparation). Furthermore, the combination of nigericin and valinomycin has been shown to collapse membrane potential in similar situations (36) and would be expected to abolish $TPMP^+$ accumulation. When nigericin plus valinomycin were added to K^+-loaded vesicles before dilution, $TPMP^+$ did not accumulate beyond an apparent internal concentration equal to its external concentration. Similarly, amino acid accumulation was dissipated. The nonelectrogenic ionophore nigericin added to K^+-loaded vesicles did not induce $TPMP^+$ accumulation above its internal concentration nor did it stimulate amino acid transport. Thus K^+ influences this distribution electrically, by its electrogenic translocation only, but is not chemically coupled either to lipophilic cation or amino acid accumulation. Finally, when K^+-loaded vesicles were diluted with valinomycin in the presence of an external K^+ concentration equal to its internal concentration, no lipophilic cation accumulation was observed. Retention of $TPMP^+$ in membranes due to binding was negligible as estimated after osmotic lysis.

Significantly, the response of amino acid translocation to stimulation by an interior-negative membrane potential specifically required Na^+. Since Na^+ was not required for generation of an interior-negative membrane potential by these manipulations, as shown by the lack of Na^+-dependence of $TPMP^+$ accumulation (Table IV), the Na^+ requirement must be at the level of amino acid translocation.

Amino acid accumulation increased as a function of the magnitude of membrane potential generated under these conditions, estimated by $TPMP^+$ accumulation, as shown in Fig. 7. Since in this experiment, assay of amino acid accumulation and generation of membrane potential were independent of the metabolic functions of the cell, amino acid transport stimulated by an electrochemical Na^+ gradient involves a direct and obligatory coupling of the translocation step to membrane potential at the plasma membrane.

Although this positive correlation demonstrated that the energy from the electrical potential difference across the membrane contributes to the driving force for amino acid transport, amino acid accumulation driven by both a Na^+ gradient and an imposed electrical difference always exceeded the energy provided by the total electrical component alone. Thus, Table IV shows that in the presence of an initial driving force

TABLE IV. Sodium Requirement for Electrogenic Amino Acid Translocation*

Additions		Apparent concentration gradient, C_i/C_o	
		$TPMP^+$	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 + nigericin	NaCl	–	2.6
Valinomycin + nigericin	Choline Cl	–	1.6

*Vesicles (7.6 mg/ml) were preincubated 15 min with 50 mM KCl and the indicated ionophores in 1% Me_2SO: valinomycin, 90 μM; nigericin, 27 μM; and sodium monensin, 14 μM. Then, 38 μg aliquots were diluted 10-fold with respect to KCl and ionophore concentrations and incubated with 20 μM ^{3}H-triphenylmethylphosphonium and 50 mM of the indicated chloride salt; 76 μg aliquots were similarly diluted and incubated with 0.2 mM ^{3}H-alanine and 50 mM chloride salts. Maximum accumulation was estimated at 2–5 min after dilution. C_i/C_o refers to the ratio of the observed intravesicular solute concentration to its external concentration.

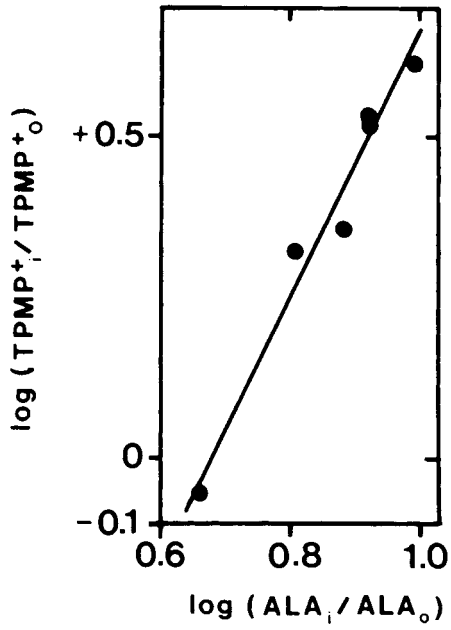

Fig. 7. Correlation of levels of quasi-steady-state alanine accumulation stimulated by a Na^+ gradient with magnitudes of interior-negative membrane potential estimated by ^{3}H-TPMP accumulation in vesicles. Vesicles were incubated with valinomycin and 50 mM KCl as in Fig. 7, and then 10 μl aliquots were diluted to various final volumes in the range 20–200 μl which contained various final external KCl concentrations in the range 50–2.5 mM, 50 mM NaCl, and either 0.1 mM ^{3}H-triphenylmethylphosphonium bromide or 0.2 mM L-^{3}H-alanine. Maximal accumulation was estimated by the filtration assay.

provided by 50 mM external Na^+ plus a 10-fold K^+ dilution potential a maximal apparent alanine accumulation of 8.5-fold was achieved representing −54.6 mV, whereas a membrane potential of −39.5 mV was estimated from the apparent 4.7-fold triphenylmethylphosphonium ion accumulation assuming a coupling ratio of 1.

When monesin was added to short-circuit the Na^+ gradient without affecting membrane potential, alanine accumulation decreased from 8.5-fold to 4.6-fold, a value compatible with the electrical driving force estimated by the apparent 5.9-fold $TPMP^+$ accumulation under these conditions. This clearly demonstrates that amino acid accumulation is driven by the sum of contributions from an electrical potential and a chemical difference in Na^+ concentration across the membrane. These parameters can vary independently but Na^+ is required for the response to charge asymmetry. Under these conditions the electrical potential does not arise from the Na^+ gradient itself; in whole cells this potential may arise from electrogenic gradients of other ions. This would be in contrast with proton-coupled transport systems, in which proton gradients contribute to membrane potential (7, 38).

Inactivation of Carrier Activity

Alanine transport activity stimulated by a Na^+ gradient was specifically inactivated by treatment of vesicles with p-chloromercuribenzenesulfonate, as shown in Fig. 8. The Na^+-independent alanine transport and Na^+-stimulated glycine transport into vesicles were relatively insensitive to this reagent. Both the initial rate and quasi-steady state of

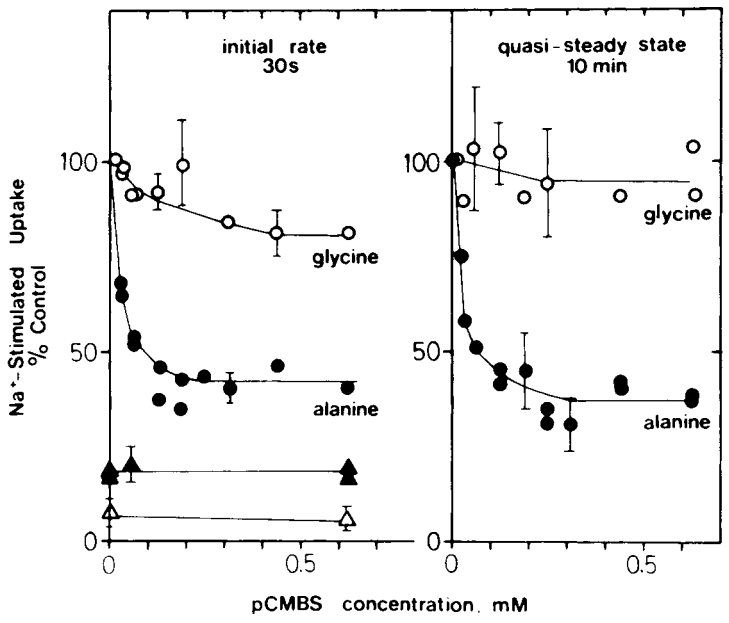

Fig. 8. Inactivation of the alanine carrier by treatment of vesicles with p-chloromercuribenzenesulfonate. Aliquots of 56 μg vesicles were treated 15 min with the indicated concentrations of p-chloromercuribenzenesulfonate, then diluted 2-fold into incubation mixtures containing 0.2 mM ^3H alanine (●, △) or 0.2 mM ^3H-glycine (○, ▲) and either 50 mM NaCl (○, ●) or 50 mM choline chloride (▲, △). Uptake was measured after 30 sec and 10 min. Initial rates of 100% represented 2.1 nmol/mg/30 sec for glycine and 820 pmol/mg/30 sec for alanine. Quasi-steady-state accumulation of 100% represented 2.5 nmol/mg for glycine and 1.2 nmol/mg for alanine.

alanine transport showed parallel inactivation as a function of inhibitor concentration.

The relative insensitivity of quasi-steady-state glycine accumulation driven by a Na^+ gradient to this sulfhydryl reagent indicates that the ability of the membrane to maintain a Na^+ gradient is not affected by this reagent. These results suggest that the presence of Na^+ induces a selective conformational change in the alanine carrier to expose sulfhydryl groups. Inactivation of Na^+-stimulated alanine transport was also observed in the presence of monensin (not shown).

Physiological Regulation of Amino Acid Transport

The activity of certain nutrient transport systems fluctuates in a complex array with changes in cellular physiological state in many instances, such as the stimulation of proliferation of resting nontransformed mouse fibroblasts by serum or mitogenic hormones (39–41), after viral transformation (41, 42) and the triggering of the blast transformation of lymphocytes (43, 44). The V_{max} of iso-Abu transport activity of nontransformed mouse fibroblast cultures diminishes as cultures become quiescent and increases accompanying viral transformation (42).

Several possibilities for cellular regulation of neutral amino acid transport are suggested by the demonstrated characteristics of energy coupling of this process. Notably, the activity of the Na^+, K^+-ATPase transport system, which directly utilizes cellular ATP to maintain cellular K^+ and Na^+ gradients and generate an interior-negative membrane potential as demonstrated in certain cell systems (45, 46), appears to be a primary target for regulation as summarized in Fig. 1. The "Na^+ pump" of nontransformed mouse fibroblast cultures is rapidly stimulated by serum (47), physiological concentrations of insulin, and prostaglandin $F_{2\alpha}$ (48) which initiate cell proliferation (49), or by low, mitogenic concentrations of fibroblastic growth factor (FGF) or epidermal growth factor (EGF) (Lever JE, and Jimenez de Asua L, in preparation), by a mechanism which does not require cyclic nucleotide fluctuation or protein synthesis (48). Also, increases in Na^+, K^+-ATPase transport activity accompany viral transformation in certain cases (50). On the basis of this indication that cytoplasmic membrane potential and Na^+ gradients are regulated by certain hormones, possibly by their direct interaction with the membrane, it can be postulated that rapid and transient regulation of those transport systems which are coupled to Na^+ or electrical gradients can be accomplished either by this means or by other unidentified electrogenic membrane systems.

Amino acid transport activity in vesicles can be evaluated in the presence of a standard Na^+ gradient, independently of Na^+, K^+-ATPase activity. This evaluation should permit detection of regulatory changes in the amino acid carrier itself or in other membrane constituents which may affect uptake. Furthermore, the contribution of proteins, lipids or hormones removed during membrane isolation which are necessary to maintain increased carrier activity might be detected by reconstitution to membrane vesicles.

Transport activities of vesicles isolated from nontransformed and SV 40-transformed mouse fibroblasts were compared (16), as shown in Fig. 9 and Tables III and V. Both an increased maximal velocity of Na^+-dependent iso-Abu transport and increased quasi-steady-state accumulation were noted in vesicles from SV 40-transformed cells compared with those from proliferating nontransformed cells (Fig. 9). These parameters were decreased in vesicles from confluent nontransformed cells. The initial rates of adenosine uptake, relatively invariant with growth or transformed state (51), were compared in addition to marker enzymes, to indicate variation in transport-specific activity due to unsealed vesicles or different distributions of transport-competent vesicles during purification from

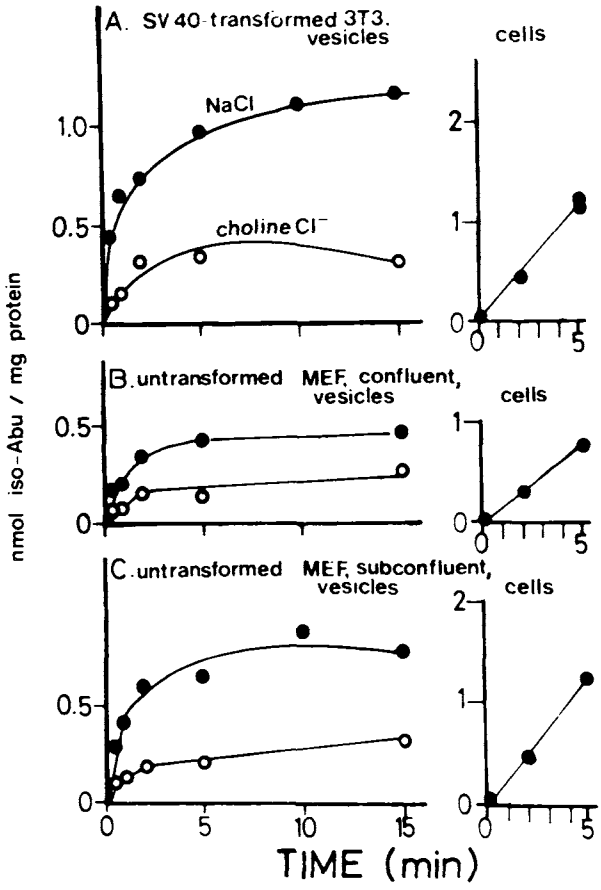

Fig. 9. Changes in the initial rate and quasi-steady state of iso-Abu accumulation in isolated membrane vesicles and uptake by intact cells which accompany cell-density growth inhibition or SV 40 transformation of mouse embryo fibroblasts. Vesicles were incubated for the indicated times with 0.17 mM ^{14}C-iso-Abu and either 50 mM NaCl (•) or 50 mM choline chloride (○). Uptake of 0.06 mM ^{3}H-iso-Abu in Dulbecco's modified Eagle's medium minus amino acids and minus serum by cells attached to 30 mm petri dishes at 37°C was determined according to a procedure described previously (40). Total cellular protein of each dish was measured (25). M.E.F.) Balb/c tertiary mouse embryo fibroblasts.

each cell type. Furthermore, rates of uridine uptake in vesicles, a cell density-dependent transport function (51) expressed in isolated membranes (52), were also compared. These transport specific activities, summarized in Table V, indicate that Na^+-independent iso-Abu transport activities showed much less variation than Na^+-dependent activities.

The changes in iso-Abu transport activity of cell populations expressed in vesicles as alterations in V_{max} could represent either changes in the number or mobility of A system carrier molecules and/or the net driving force acting on the system. Such changes would alter cellular uptake of these amino acids even at high, saturating amino acid concentrations. The changes which also affect quasi-steady-state accumulation should be largely independent of the number of carriers, but represent the net driving force acting on the carriers. Since a standard Na^+ gradient driving force was artificially applied to the membrane, the latter changes probably represent changes in membrane Na^+ permeability.

TABLE V. Specific Activities of Transport Systems in Membrane Vesicles*

Source of membranes	No. of preparations	Transport specific activity[a] iso-Abu Na^+-dependent	iso-Abu Na^+-independent	Adenosine	Uridine
		pmol/min per mg protein[b]			
Balb/c M.E.F.[c]					
(confluent)	5	440	560	1.7	0.10
(subconfluent)	3	1,300	810	2.6	0.20
Balb/c SV3T3					
(confluent)	3	960	870	2.9	0.19
(subconfluent)	2	2,300	930	2.0	0.19
Swiss 3T3 K	2				
(confluent)	2	190	210	1.3	0.04
(subconfluent)	2	440	600	1.7	0.26
Swiss SV3T3					
(confluent)	2	660	530	2.2	0.32
(subconfluent)	2	480	687	1.9	0.13

*Recovery of protein and marker enzymes for mixed plasma membrane preparations from each source was described previously (16, 19).

[a]Incubations were 30 sec and 1 min with 50 mM NaCl and either 1 mM iso-^{14}C-Abu, (0.061 pmol/cmp), 0.5 μM ^{3}H-adenosine (9×10^{-5} pmol/cpm), or 0.2 μM ^{3}H-uridine (3.9×10^{-5} pmol/cpm), using 70–170 μg vesicle protein per 100 μl of incubation mixture. Initial rates of uptake for each substrate were assayed at concentrations below their K_m values to minimize entry by simple diffusion. The Na^+-independent rate of iso-Abu uptake was determined using 50 mM choline chloride substituted for NaCl. This value was subtracted from uptake observed using 50 mM NaCl, to give the Na^+-gradient-dependent uptake.

[b]Results were averaged from duplicate determinations repeated 2 or 3 times on each preparation. Variation was ± 10–25% among duplicates.

[c]M.E.F. – mouse embryo fibroblasts.

Since K_m values for iso-Abu transport, which vary as a function of Na^+ concentration as shown above, were not altered in these preparations, it seems likely that the interaction of Na^+ with the carrier is not altered.

As a preliminary indication of differences in Na^+ permeability, vesicles from these cell types were loaded with iso-Abu in the presence of a standard initial Na^+ gradient, and then efflux was measured after dilution, maintaining a constant external Na^+ concentration, as shown in Fig. 10. The observed efflux would depend on the levels and mobility of iso-Abu carriers as well as the internal iso-Abu and Na^+ concentrations at the time of dilution. Efflux from vesicles from confluent normal cells was faster than from those prepared from subconfluent normal cells and slower than efflux from SV 40-transformed cell membranes. As expected, addition of monensin at the time of dilution increased iso-Abu efflux from all types of preparations. Interestingly, monensin eliminated differences in efflux between confluent and subconfluent nontransformed cells, an indication that their efflux differences were mainly due to a relatively higher membrane Na^+ permeability in confluent cell membranes. The increased efflux maintained in vesicles from SV 40-transformed cells after addition of monensin indicates that iso-Abu carriers are also increased in these cells relative to nontransformed cells.

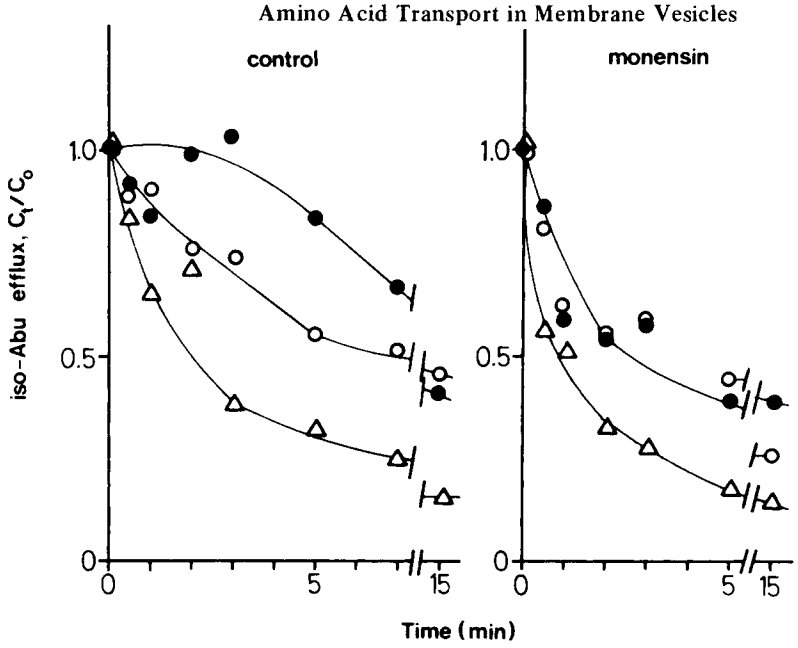

Fig. 10. Efflux of iso-Abu accumulated in vesicles from nontransformed and SV 40-transformed mouse fibroblasts. Vesicles from subconfluent (•) or confluent (○) cultures of nontransformed mouse embryo fibroblasts or SV 40-transformed cultures (△) were incubated 15 min with 50 mM NaCl and 0.17 mM ^{14}C-iso-Abu. Then efflux was measured after addition of A) 2% Me_2SO or B) 30 μM monensin in 2% Me_2SO and 10-fold dilution with respect to ionophore and labeled substrate. C_t/C_o represents the ratio of solute at the indicated times relative to that observed at zero-time. 100% was 1.2 nmol/mg (•), 0.42 nmol/mg (○), and 1.2 nmol/mg (△).

DISCUSSION

The properties of these neutral amino acid transport systems of isolated membrane vesicles from mouse fibroblast cell lines provide experimental confirmation of some of the general predictions derived from the alkali-ion gradient hypothesis for active transport of several organic solutes in animal cells as formulated by Christensen (1, 2), Crane (3, 9), Heinz (4) and others. It could be clearly demonstrated that the accumulation of several neutral amino acids across the plasma membrane is unequivocally coupled to the direction and magnitude of a driving force specifically and directly provided by a Na^+ gradient imposed across the membrane. The nature of this noncovalent energy coupling dictated that amino acids translocate by movement of a positively-charged complex and accumulate electrogenically *trans* to the face of the membrane exposed to the higher Na^+ concentration. Thus, it was further established that the ability to utilize electrical potential differences across the menbrane as an additional driving force for transport of these amino acids required Na^+ and is an integral feature of this mechanism. The molecular basis of the coupling process appeared to be an increased affinity of the carrier for the amino acid substrate directly induced by interaction of Na^+ with the membrane, concurrent with the formation of a positively charged translocating complex.

The experimental support on which these conclusions are based depends upon: 1) the ability to rapidly collect vesicles under conditions where efflux of intravesicular

solutes and nonspecific binding are minimal, 2) the demonstration that transport carrier activity in membrane vesicles fulfils standard criteria which serve to identify specific transport systems in the cell, such as countertransport stimulation of uptake and efflux, saturation kinetics of rates of uptake, competitive interactions, and pH profiles, 3) the ability of vesicles to maintain a maximal accumulation of an amino acid above its external concentration which reflects the quasi-steady-state response of the carrier to the magnitude and direction of a specific ion-gradient driving force, this accumulation representing a specific response since a variety of experimental conditions designed to collapse the driving force also reduced the apparent internal amino acid concentration to its known external concentration, and 4) interpretation of the effects of ionophore probes based on their known actions characterized in artificial and biological membranes (32).

In addition to the relationship between electrical currents, gradients of several ions, and amino acid transport in vesicles, studies were directed to several additional questions concerning the translocation mechanism beyond the general predictions of the alkali-ion gradient hypothesis. Do other ions interact with the carrier to directly influence this mechanism? How is the utilization of driving force for transport apportioned between electrical and chemical components? Are these components utilized by separate or interdependent processes? Does the uncomplexed carrier bear a net charge? Does Na^+ induce a conformational change in the carrier?

Although a chemical role for other physiological ions has been proposed (2, 53), Na^+ was the only ion which could be demonstrated to make a chemical contribution to the driving force for neutral amino acid transport in vesicles. The Na^+ did not make an appreciable electrical contribution under these conditions, and in fact caused a small depolarization. The K^+ could only contribute electrically to the driving force. Efflux of internal K^+ by endogenous pathways or catalyzed by the nonelectrogenic ionophore nigericin did not affect amino acid uptake but electrogenic efflux of K^+ mediated by valinomycin markedly enhanced influx and accumulation of amino acids by specific generation of a measurable interior-negative membrane potential as confirmed by suitable control experiments. Also, external K^+ did not stimulate amino acid transport in the absence or presence of Na^+. This observation can be rationalized because cellular chemical K^+ gradients directionally oppose Na^+ gradients and would tend to diminish the driving force from Na^+ gradients if K^+ interacted directly with the carrier. Proton gradients could not be demonstrated to be obligatorily coupled to this process as shown by the lack of inhibition by proton conductors such as nigericin, 2,4-dinitrophenol, and FCCP and the relative insensitivity of quasi-steady-state neutral amino acid accumulation to external pH. The initial rate of Na^+-dependent uptake showed a distinctive pH profile with maximal uptake at external pH 7.4 but Na^+-independent uptake was relatively pH-independent in this range (20). Since this is the same pH as the buffer enclosed within the vesicles, no pH gradient would be expected when maximal uptake was observed. Predictably, H^+ gradients could contribute electrically to the transport process and also make a secondary, nonobligatory chemical contribution as identified by the pH profile of Na^+-dependent uptake in vesicles.

An important observation was that stimulation of amino acid uptake by interior-negative membrane potential, and conversely, dissipation of membrane potential by amino acid transport, required the presence of Na^+. The Na^+-dependent utilization of energy derived from charge asymmetry did not require a gradient of Na^+ although energy from a Na^+ gradient could also be utilized in an additive manner with that from the electrical contribution. This intimate connection between Na^+ and the response of the carrier to

membrane potential implies that each contributes energetically through a common mechanism: Na^+ is required to convert translocation to an electrogenic process represented by movement of a positively-charged "ternary complex" of Na^+, substrate, and membrane component, stoichiometry and molecular disposition unspecified. From this observation that amino acid translocation is nonelectrogenic in the absence of Na^+, plus previous evidence (53) that the zwitterion seems to be the predominant translocated species, it may be deduced indirectly that the unbound carrier is uncharged at this pH. Since the preferred orientation of an electroneutral carrier would not be influenced by membrane potential, this possible contribution to the total driving force is eliminated. From the fact that the loaded carrier responds to membrane potential in the presence of Na^+, it may be concluded that binding and release of substrate on either side of the membrane are not rate-limiting, as discussed for several theoretical possibilities (54). Probably, the translocation step is rate-limiting, and its mobility increased by Na^+, as predicted from effects of Na^+ on K_m, according to a cotransport kinetic model (55).

Many important details of this mechanism remain to be established, notably the stoichiometry and disposition of interactions between ion, substrate, charge, and membrane component. Studies of the regulation of this transport system indicate that direct hormonal regulation of the active Na^+ pump and also changes in membrane Na^+ permeability may be primary targets to rapidly modulate influx of amino acids, in addition to possible changes in levels of carrier molecules and alterations in membrane fluidity. Since the system responds directly to electrical differences, other electrogenic processes, membrane depolarization, or electrical coupling between different cells could also make important contributions. The relatively small observed fluctuations in Na^+-dependent amino acid transport in cell populations could merely represent an indirect monitor of the primary interactions of certain hormones with the cell membrane. It remains to be established whether this transport regulation is obligatory, permissive, or dissociable from the regulation of cell proliferation.

ACKNOWLEDGMENTS

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