THE USE OF MEMBRANE VESICLES IN TRANSPORT STUDIES

Author: **Julia E. Lever**
The Salk Institute
La Jolla, California

Referee: H. R. Kaback
Laboratory of Membrane Biochemistry
Roche Institute of Molecular Biology
Nutley, New Jersey

INTRODUCTION*

Studies using isolated membrane vesicles have dramatically advanced our understanding of energetic and chemical interconversions associated with transport of molecules across cell membranes. The concept of investigating in a cell-free system a vectorial membrane function such as transport, by definition a reflection of membrane integrity, offers considerable additional information not obtainable from studies using intact cells. Membrane carriers can be selectively assayed in a functional state dissociated from complications arising from unknown internal compartmentalization and intracellular metabolism. Furthermore, known magnitudes and polarities of chemical or electrochemical driving forces can be imposed or dissipated across the membrane, allowing a quantitative estimation of their contribution to a membrane transport process. Thus, by dissecting a system and then reconstituting its function in a variety of artificially manipulated experimental situations encompassing a range wider than that encountered in the physiological milieu, a clearer picture of the function of a particular transport system in the living cell can be constructed. The demonstration by Kaback[1] that isolated bacterial membranes could form resealed vesicles which retained active transport functions and subsequent extensive characterization of these systems by Kaback and co-workers[1-6] provided an impetus for the development of similar systems for eukaryotic membranes. This approach has been propagated in many laboratories, yielding detailed information concerning mechanisms for entry of sugars, ions, amino acids, and nucleosides into a variety of bacterial and animal cells.

This review will focus on several ion and nutrient transport systems studied in vesicles comprised of the surface membranes of animal cells. Transport studies using bacterial membrane vesicles have received extensive and authoritative review;[2,5-9] therefore, discussion here of these systems will be limited to selected aspects which illustrate theoretical or empirical concepts and experimental designs relevant to an understanding of transport studies using surface membranes from nucleated animal cells. Recent reviews have evaluated the preparation and use of animal cell plasma membrane vesicles to study nucleoside transport mechanisms[10,11] and intestinal[12] and renal[13,14] transport systems. Within the framework of this survey of current knowledge of nutrient transport mechanisms across animal cell plasma membranes, potentially fruitful approaches to study mechanisms of biological regulation of transport systems will be discussed.

The nature of the membrane preparations and the experimental approaches to investigate transport activity available to the investigator determine the scope and limita-

* The abbreviations used in this paper are *CCCP*, carbonyl cyanide-*m*-chlorophenylhydrazone; *FCCP*, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; iso-Abu, 2-aminoisobutyric acid; TPMP,+ triphenylmethylphosphonium ion; MEF, mouse embryo fibroblasts; Me_2SO, dimethylsulfoxide; MES, 2-*N*-morpholino ethane sulfonic acid; SV40, simian virus 40.

tions of these studies. Therefore, a brief summary and evaluation of these procedures will precede discussion of studies of individual transport systems.

ISOLATION OF TRANSPORT-COMPETENT VESICLES FROM ANIMAL CELLS

Summary — Preparation of plasma membrane vesicles for transport studies requires large initial cell batches (10^8 to 10^9 cells), high membrane yield, and production of sealed, osmotically sensitive vesicles under conditions which do not produce functional inactivation. The most promising method currently available for preparation from single cells is homogenization by nitrogen pressurization followed by separation of purified mixed vesicles by velocity sedimentation or more extensive purification of plasma membrane vesicles by isopycnic centrifugation.

In order to carry out meaningful transport determinations, membrane vesicle preparations relatively homogeneous with respect to the transport activity under investigation must be obtained. Thus, at the cellular level, membrane heterogeneity arising from multiple cell types constituting a tissue can be avoided by the use of clonal cell populations propagated in cell culture. Within such cell culture systems, nonuniformity of growth state can contribute to membrane heterogeneity. For example, a complex and striking array of changes in activities of membrane transport systems dependent on such parameters as serum, nutrient, or hormonal depletion, stage of the mitotic cycle, and cell density have been described and are reviewed in References 15 and 16. Ideally these parameters should be controlled as closely as possible during cell growth. Finally, subcellular fractionation methods designed to minimize contamination of surface membrane vesicle preparations by intracellular membranes, organelles, and soluble contaminants are complicated by possible heterogeneity of vesicle subpopulations derived from the plasma membrane. Examples of this type of heterogeneity arise in certain epithelia, such as renal and intestinal cell membranes which can be resolved into functionally discrete populations derived from either the basal-lateral or the brush border surface membrane.[14,17-20] Plasma membrane subfractions may arise in other cell types. Fibroblast plasma membranes have been resolved into two major fractions, one enriched in ($Na^+ + K^+$) ATPase activity, the other enriched in 5′-nucleotidase activity.[11, 21-22] Mixtures of right-side-out and inside-out plasma membrane vesicles from pig lymphocytes have been resolved, using lectin affinity chromatography.[23]

Membrane Fractionation Procedures

In general, methods to obtain membrane vesicles for transport studies require rupture of the cytoplasmic membrane, fragmentation into small osmotically intact sacs or vesicles, and their purification in high yield from the remainder of soluble and membrane components. This discussion of strategies devised to achieve membrane fractionation from tissues, mammalian cell cultures, and tumor cell populations will emphasize advantages and disadvantages of those methods commonly used to obtain vesicles for membrane transport studies.

Membrane vesicle preparations from whole tissues have generally employed a step producing cell lysis under hypotonic conditions in the presence of mechanical shear. Such conditions favor lysis of nuclei and other organelles, which would decrease the purity of the final membrane preparations. Membrane preparations from tissues rather than from isolated cells have been used to study transport properties of kidney and intestine. Both types of epithelia exhibit polarity of plasma membrane function such that two functionally, enzymatically, and morphologically distinct plasma membrane entities (the brush border membrane at the apical or luminal side of the cell and the basal-lateral membrane at the plasma side of the cell) maintain a unidirectional solute

flux across the cell.[14,24] Attempts to study these systems using isolated cells, such as in the studies of Kimmich,[25] have not yet succeeded in retaining the functional transport polarity observed in intact tissue. Animal cell intestinal brush border membranes prepared from intestinal scrapings by a modification of Hopfer et al.[26] of a procedure developed by Forstner et al.[27] have been used in transport studies. Murer et al.[17,18] and Douglas et al.[28] have described methods to separate transport-competent basal-lateral from brush border membrane vesicles of intestine. Similarly, a variety of methods for isolation of renal brush border membranes and basal-lateral membranes have been described.[13,14,20]

Methods for isolation of plasma membranes from other tissue sources for transport studies have been described. Illiano and Cuatrecasas[29] used a modification of the method of Rodbell[30] to prepare adipocyte membrane vesicles from rat epididymal fat pads for glucose uptake studies. Carter and Martin,[31] Carter et al.,[32] and Li and Hochstadt[33] have modified the method of McKeel and Jarrett[34] to prepare sealed vesicles used to study transport. Membranes isolated by the method of Neville[35] have been used for transport studies.[36]

These methods listed thus far suffer disadvantages such as low plasma membrane yield and significant contamination by mitochondria and nucleic acid where tested.

It should be noted that several widely used methods for membrane purification from cell cultures or tissues would not be expected to yield vesicles suitable for transport studies. Thus, the method of Warren et al.[37] and the biphasic dextran-polyethylene glycol method of Brunnette and Till[38] both utilize zinc salts to stabilize plasma membranes from fragmentation, introducing the possibility of heavy metal inhibition of membrane transport functions and producing large surface membrane sheets and ghosts rather than resealed vesicles. However, Colombini and Johnstone[39] have successfully employed a method using zinc ion stabilization and a step producing plasma membrane sheets to purify plasma membrane vesicles in high yield from Ehrlich ascites tumor cells. After removal of zinc by chelation, these preparations retain ($Na^+ + K^+$) ATPase activity and Na^+-stimulated 2-aminoisobutyric acid transport activity.[40,41]

Cell disruption by nitrogen cavitation appears to be the preferred method for vesicle preparation from isolated animal cells, either from cell culture or grown in vivo, in transport studies where retention of native membrane transport and regulatory components is imperative. In this method, first characterized by Hunter and Commerford[42] and Wallach and Kamat,[43] cells suspended in buffer made isoosmotic with sucrose are pressurized to 600 to 800 lb/ in.^{2} in a nitrogen atmosphere. When the pressure is released, cells are extruded through a small orifice. This results in cell homogenization by mild liquid or mechanical shear.[44] It is believed that osmotically sensitive, closed vesicles with right-side-out orientation[45,46] develop by pinching-off from the plasma membrane, while the endoplasmic reticulum forms inverted vesicles encompassing a similar size range.[47] The use of isotonic conditions and stabilizing concentrations (0.1 to 1.0 m*M*) of Ca^{++} preserve nuclei, lysozomes, and mitochondria intact. Other advantages of the method are the use of inert atmosphere, prevention of local heating during cell disruption, and high yield of unilaminar[45] surface membrane vesicles, apparently right-side-out by several criteria,[45] such as a similar pattern of membrane protein iodination catalyzed by lactoperoxidase in membrane vesicles as in intact cells under conditions where the reagents did not penetrate through the membrane[46] and retention by lectin affinity chromatography.[48] The small (0.1 to 0.2 μm) diameter[49] favors retention of vesicles on nitrocellulose filters without excessive lysis or leakiness which would obviate assay of transport using larger diameter fragments in which resealing is thermodynamically unfavorable. Also, the size of vesicles produced by this technique favors more rigorous purification from mitochondrial contamination than other methods. Due to high solute concentration ratios which can be achieved by intact respiring

mitochondria, even 10% mitochondrial contamination can seriously obscure transport determinations using plasma membrane vesicles.

Several methods based on nitrogen pressure homogenization have been reported for isolation of plasma membrane vesicles from mouse fibroblasts,[10,11,22,50-54] thymocytes,[55,56] and lymphoid cells.[57] In a 2 to 3 hr procedure, a mixed population of small plasma membrane and endoplasmic reticulum vesicles can easily be separated from nuclear and mitochondrial fractions of a mouse fibroblast cell homogenate[48,50,53,54] by velocity sedimentation and concentrated by ultracentrifugation at $10^7 \times g \cdot min$. Such purified mixed vesicle preparations, isolated using the method of Lever[48,53,54] which employs centrifugation over a 35% sucrose cushion, achieve 50 to 68% yield of plasma membrane material contaminated with 20% of the total endoplasmic reticulum, estimated by marker enzymes for these membranes. The method of Quinlan and Hochstadt,[51] which uses a dextran®-110 cushion, yields vesicles contaminated with 50% of the total endoplasmic reticulum. These types of preparations are useful in cases where lack of interference from endoplasmic reticulum contamination in transport estimations can be established. Further purification steps to separate plasma membrane from endoplasmic reticulum[45,51,54] involve ultracentrifugation in dextran density gradients, a medium of low osmotic activity, after osmotic lysis of vesicles to release trapped cytoplasmic contaminants. Alternately, zonal centrifugation on sucrose gradients has been used for further membrane purification.[21] Whereas the procedure for preparation of purified mixed vesicles can be successfully applied to a variety of cell types with similar results, i.e., normal and transformed mouse fibroblasts,[48,50] thymocytes,[55] and thyroid cells,[56] methods for extensive purification of plasma membrane vesicles must be optimized for each cell type. This is best achieved by comparing separation by isopycnic centrifugation at a series of Mg^{++} and dextran gradient concentrations. This strategy takes advantage of differing osmotic activity and fixed membrane changes as discussed by Wallach.[58] Yields of total purified plasma membrane from mouse fibroblasts by the method of Quinlan and Hochstadt[51] average only 10%.[51,54] Low yield plus the lengthy preparation time are disadvantages of the method. Furthermore, plasma membranes from either mouse fibroblasts[22,57] or BHK cells[21] subjected to isopycnic ultracentrifugation form at least two discrete bands; one enriched in 5′-nucleotidase activity, the other enriched in (Na^+ + K^+) ATPase activity, both used as plasma membrane markers. It is not apparent whether this reflects true membrane functional mosaicism, analagous to the separation of these markers on opposite faces of renal or intestinal cells, or whether it represents an artifact of preparation.

Reports of affinity-chromatographic methods for plasma membrane purification offer a promising alternative to centrifugation methods. Retention of plasma membranes by Sepharose columns conjugated with lectins such as concanavalin A[59,60] or lentil beans[23,48] have been reported; however, elution of bound material by competitive sugar ligands has not been satisfactory, possibly due to nonspecific hydrophobic interactions.[61] These experiments have been cited as evidence compatible with right-side-out vesicle orientation.[59,60] Soderman et al.[62] have reported retention of plasma membrane material by insulin coupled to agarose columns. A further disadvantage of these affinity chromatography methods is the low capacity for membrane retention. A variation of the selective affinity approach, the use of concanavalin A for stabilization of plasma membrane as sheets to facilitate purification, followed by vesiculation induced by removal of most of the conanavalin A by the competitive sugar ligand α-methylmannoside, has been used by Scarborough[63,64] to prepare vesicles from *Neurospora* used in transport studies. A disadvantage of the method is the inability to remove all concanavalin A bound to vesicles, thus introducing possible perturbation of certain transport systems responsive to this ligand.[55]

A novel approach for separation of small transport-competent erythrocyte membrane vesicles from inactive vesicles in a density gradient, based on changes in intravesicular volume and vesicle density resulting from uptake of the transport substrate, was recently described by Lee and Vidaver.[65] Sedimentation of vesicles after initiation of Na^+-gradient-coupled glycine uptake resulted in the enrichment of glycine transport activity with respect to intravesicular space. Furthermore, the same subpopulation was selectively enriched in Na^+-stimulated alanine transport activity. This approach may be useful in other cell systems for separation of vesicles derived from different plasma membrane domains as in renal and intestinal epithelia.

Techniques to Evaluate Membrane Vesicle Purification

Methods to monitor the distribution of intracellular components and plasma membrane material during subcellular fractionation procedures must be selected according to the particular characteristics of the cell type under investigation. Enzymatic, immunological, and chemical approaches have been used.

Table 1 lists several enzymes which have been used as plasma membrane markers for various cell types. Suitable enzyme markers for intracellular membranes and cytosol have been listed in several reviews.[11,44,65-68] It is necessary to estimate total recovery of marker enzyme activity by assay of each fraction in order to demonstrate that a low specific activity in plasma membrane fractions of a marker for intracellular contamination is due to purification rather than inactivation. Assays should ideally be performed under conditions where vesicles are made permeant to substrates (either by hypotonic shock or addition of detergents) in order to avoid crypticity of internal enzyme active sites. Vesicle preparations from mouse fibroblasts have been evaluated for contamination by mitochondrial inner membrane using succinate cytochrome c reductase activity (EC 1.3.99.1).[69] NADH oxidase (NADH; acceptor oxidoreductase; EC 1.6.99.3) has been used as a marker for endoplasmic reticulum and mitochondrial outer membrane.[69,70] β-Galactosidase has been used as a marker for soluble enzyme entrapment within plasma membrane vesicles.[22] Plasma membrane preparations have been chemically characterized for DNA, RNA, lipid composition, fucose, and glycoproteins in order to evaluate purity.[71] Techniques for selective radiochemical labeling of membranes by biosynthetic means using fucose,[71] chemical methods such as iodination by lactoperoxidase,[46,72] or fluorescent labeling using fluorescamine[73] have been reported. Immunological probes to detect and quantitate plasma membrane material have been used, notably the Cr release assay[74] for surface membrane material. These approaches have certain advantages over enzyme activity as markers, since enzymes can become inactivated during vesicle preparation.

EXPERIMENTAL RATIONALE FOR TRANSPORT STUDIES WITH MEMBRANE VESICLES

Assay Method

The measurement of transport activity of membrane vesicles must be carried out by a rapid and sensitive procedure under conditions which minimize efflux of intravesicular solutes and nonspecific binding of solutes to vesicles. Filtration through nitrocellulose membranes is the usual procedure, but limitations of this method for certain applications have led to the introduction of alternate techniques.

The most widely used current method involves termination of uptake by dilution of vesicles in high salt medium followed by rapid filtration through nitrocellulose or cellulose acetate filters with 0.2 to 0.45-μm pore size. Colombini and Johnstone[40,41] used glass fiber filters rather than nitrocellulose. This approach, first utilized by Kaback[1,4] for bacterial vesicles, has been modified for mammalian membrane vesicle studies by

TABLE 1

Several Enzyme Activities Localized in Plasma Membranes of Various Cell Types Used in Membrane Vesicle Transport Studies

Subcellular fraction	Cell source	Enzyme
Plasma membrane	Nontransformed and SV 40-transformed mouse fibroblasts, myoblasts, liver, BHK cells, Ehrlich ascites cells, L cells, lymphoid cells[11,21,39,44,45,51,57,66,67,70]	5′-Nucleotidase (Na^+ + K^+)ATPase Adenylate cyclase Guanylate cyclase
Brush border segment of plasma membrane	Kidney proximal tubule and small intestine of several species[13,14,27,28]	Trehalase Maltase γ-Glutamyl transpeptidase Alkaline phosphatase Leucine aminopeptidase Alanine aminopeptidase HCO_3-ATPase
Brush border of plasma membrane	Small intestine of several species[13,18,27,28]	Sucrase Isomaltase Lactase Cellobiase Peptidases Glucoamylase Phlorizin amylase Lipase Chloesterol esterase
Basal-lateral segment of plasma membrane	Kidney and small intestine of several species[13,14,18,20,27,28]	(Na^+ + K^+)ATPase 5′-Nucleotidase Adenylate cyclase Guanylate cyclase (Ca^{++} ATPase)

variation of the nature of the salt and temperature of the dilution medium. This technique is rapid and convenient for multiple determinations but suffers from potential disadvantages such as gradual leakage of accumulated solute from vesicles during the termination steps, possible interference between the salt composing the filtration buffer and the transport process under study, and saturation and clogging of the filter by relatively low amounts of vesicle protein.

Transport studies in which uptake termination is carried out under conditions where intravesicular solute efflux is appreciable must be interpreted with caution. Hochstadt et al.[11] have recommended the use of warm (37°C) filtration buffer to minimize solute leakage, and several laboratories have utilized these conditions for transport determinations.[11,22,49-51] However, when amino acid leakage from vesicles after dilution in filtration buffer was compared as a function of temperatures, it was observed that a 37°C buffer promoted the highest rate of solute leakage. Figure 1 shows the considerable amino acid leakage from vesicles[54] observed at 37°C compared with 21° and 2°. The use of ice-cold (2°) buffers during filtration minimizes efflux of intravesicular

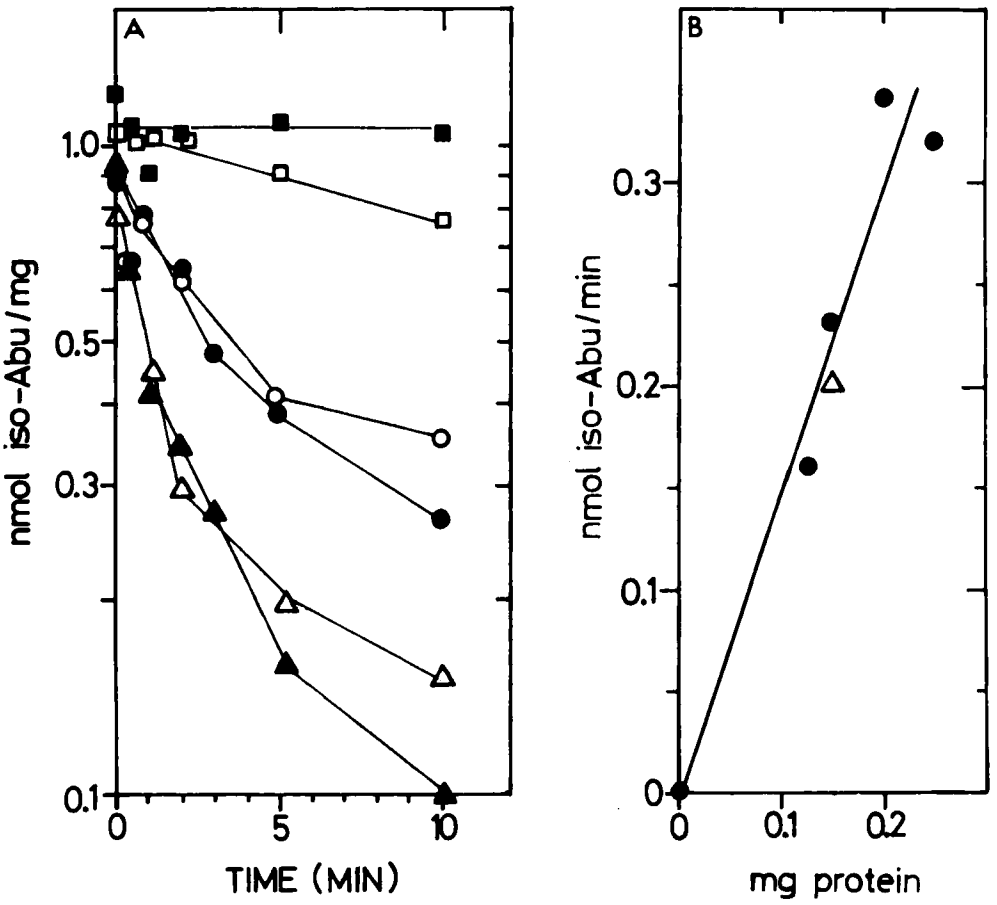

FIGURE 1. Characterization of filtration assay for transport in vesicles from mouse fibroblasts. (A) Efflux of 2-amino-[^{14}C]-isobutyric ([^{14}C]-iso-Abu) acid accumulated in vesicles after 50-fold dilution in 0.8 *M* NaCl, 0.01 *M* Tris, HCl, pH 7.4 (wash buffer) for the indicated time before filtration. Efflux in wash buffer at 21°C (●), 2°C (■) and 37°C (▲) after uptake at 21°C (filled symbols) or after uptake at 37°C (open symbols), (B) linearity of initial rate of [^{14}C] iso-Abu uptake with amount of vesicle protein, using the filtration assay. (△) after addition of 80 μg of bovine serum albumin. (From Lever J. E., *J. Biol. Chem.*, 252, 1990, 1977. With permission.)

contents (Figure 1A). Figure 1B shows the linear relationship between initial rate of amino acid uptake in vesicles and amount of vesicle protein, using the filtration assay.

Since the termination step of the filtration assay is usually carried out using high vacuum within 10 to 20 sec, this places a limit on early time points during kinetic measurements of solute uptake. A further limitation of sampling for kinetic measurements is the small internal volume of vesicles. Internal volumes ranging from 1 to 4 μl/mg membrane protein have been estimated for bacterial[4] and animal cell[51,54] vesicle preparations used in transport. When labeled solute is added to the external solution with zero initial concentration internally, initial rates of uptake must be determined at time points well before equilibration of solutes within the vesicles in order to minimize the rate contribution of the exit process. Equilibration is more rapid the smaller the intravesicular volume. This necessitates measurement of small absolute amounts of solute accumulation which border the limits of sensitivity of the method at amounts of vesicle protein which do not saturate the filter. In order to obviate these problems in measurement of initial rates, an alternate protocol, equilibrium exchange, has been proposed by Stein[75,76] which involves pre-equilibration of vesicles with unlabeled sub-

strate. This approach has been used in studies of sugar transport in vesicles by Hopfer[77] in order to determine kinetic parameters under conditions where the electrochemical Na^+ gradient is maintained constant during tracer flux.

An innovative alternative to the filtration assay has been utilized for bacterial vesicles by Ramos et al.[78] based on a modification of a flow dialysis cell described by Colowick and Womack.[79] Additions are made to membrane vesicles in the assay buffer separated by dialysis membrane from a chamber through which buffer flows at a constant rate before collection in a fraction collector. Changes in diffusible labeled solute concentration due to uptake in vesicles are monitored as a decrease in labeled solute concentration in the fractions collected. This technique permitted detection of changes in intravesicular pH by monitoring uptake of weak acids such as 5,5-dimethyloxazolidine-2,4-dione (DMO).[78,80,81] Excessive leakage of these permeant molecules from vesicles after dilution in wash buffer did not permit their uptake measurement using a filtration assay. The flow dialysis procedure will be a useful alternative to filtration in order to detect equilibrium changes in accumulation above the external concentration of freely permeant molecules under equilibrium conditions, with less perturbation to the system than involved in the filtration assay. However, the appreciable steady-state diffusion time (1.5 min) across the dialysis membrane before changes in the eluate reflect solute concentration changes in the vesicle suspension does not permit rapid kinetic measurements. Furthermore, since the intravesicular volume is a miniscule fraction of the suspension volume even at very high vesicle protein concentrations, this method would not sensitively monitor influx of labeled solute into vesicles in the absence of significant accumulation above the external concentration.

Gel filtration and ion exchange chromatography have been used to monitor transport activity in liposome vesicles. Kasahara and Hinkle[82] used gel filtration of Hg-treated vesicles to measure glucose uptake in reconstituted vesicles which had incorporated band 3 protein of the erythrocyte. Banerjee et al.[83] used ion exchange chromatography to purify and assay the phosphate transporter protein of mitochondrial membrane in reconstituted vesicles. These chromatographic methods share with the filtration assay the risk of leakage of vesicle contents and have the further disadvantage of slow collection time preventing kinetic measurements.

All methods listed thus far to assay transport activity of vesicles rely upon measurement of the translocation step, i.e., movement of labeled solute across the membrane. An alternative approach to rapidly detect carrier function as well as to further probe its molecular aspects is to assay the binding step. Examples of this strategy are the elegant studies in bacterial vesicles of lactose carrier function by monitoring fluorescence changes in a series of dansyl galactosides. These compounds are competitive inhibitors of lactose uptake in *Escherchia coli*, bind the lactose carrier, but are not transported.[6] Several lines of evidence led to the interesting conclusion that binding of the transport substrate was dependent on the energized state of the membrane.[6,84] Rudnick et al.[85,86] used photoreactive azidophenylgalactosides which competitively inhibit *E. coli* lactose transport to provide independent support for the hypothesis that transport substrate binding depends on membrane potential.

Another variation of this general approach is illustrated by studies of binding of ^3H-cytochalasin B, a potent competitive inhibitor of glucose uptake in animal cells,[87,88] which enters the cell and binds to the cytoskeletal system.[89] Observations that high-affinity cytochalasin B binding to membranes was competitively inhibited by sugars transported by the glucose uptake system in erythrocytes and other cells[90] led to the conclusion that a class of high-affinity cytochalasin-binding sites is a sugar-binding protein, possibly identical with the sugar transport protein. This hypothesis formed the rationale for the use of cytochalasin binding or inhibition to monitor sugar transport sites during purification and reconstitution of glucose transport proteins.[82,91,93]

TABLE 2

Evidence for Selective Permeability and Transport Catalytic Activity of Membrane Vesicles

1. Exchange diffusion
2. Temperature dependence of influx and efflux
3. Osmotic sensitivity of accumulated solute
4. Osmotic effects on light scattering, or in larger vesicles, on vesicular volume monitored by light microscopy
5. Substrate saturability of initial rate of uptake, substrate specificity
6. Selective effects of ionophores

Subsequent observations that cytochalasin binding could be dissociated from sugar transport inhibition in certain cases[94,95] questioned this assumption in certain cases. The possibility remains that cytochalasin B influences glucose transport secondary to its binding to a protein distinct from the glucose carrier.

Criteria for Transport Carrier Function

Standard criteria which serve to identify and characterize carrier function in intact cells, such as countertransport stimulation of uptake and efflux, uptake kinetics, competitive interactions among substrates, and pH profiles can be used to demonstrate the corresponding transport carrier activity in vesicles. In addition, osmotic sensitivity of accumulated solute is often used as evidence compatible with enclosure of solute within an intravesicular space rather than binding to fixed sites.

Each of the lines of evidence summarized in Table 2 is not unequivocal, and ideally each new description of a transport system in vesicles should be prefaced by several independent criteria for transport carrier function.

Figure 2 illustrates the experimental demonstration of countertransport stimulation, the strongest evidence for carrier function. This strategy holds either for cells or vesicles, but in intact cells the use of nonmetabolizable substrates is advisable. Vesicles are preloaded with saturating concentrations of unlabeled substrate and then are diluted into suspension medium containing tracer substrate. The experiment may be carried out using alternate combinations. Thus, either labeled substrate or labeled competitive inhibitor of the system may be added to the external solution. In the example shown, alanine was used for preloading and ^{3}H-alanine uptake was measured.[54] In parallel measurements, control vesicles which contain no internal substrate initially were also diluted in the presence of labeled substrate at identical specific activity. These determinations are carried out under conditions where the membrane is de-energized and no active transport occurs which would obscure the countertransport stimulation. Thus, in the case of Na^+-gradient-coupled systems, vesicles must be preincubated with Na^+ in order to dissipate a transmembrane Na^+ gradient which would drive active alanine accumulation. Similarly, in antiport or cotransport systems, gradients of the other, coupled substrate must first be dissipated in order not to obscure exchange diffusion of the test substrate. The apparent stimulation of labeled alanine uptake in the case of vesicles first loaded with unlabeled alanine, compared with controls, can be explained in the following terms, compatible with the existence of a functioning carrier saturable by substrate. In the case of vesicles loaded with unlabeled alanine, the specific activity of internal labeled alanine shortly after initiation of uptake was considerably lower than the specific activity of external labeled alanine. This circumstance effectively minimizes exit of label from the vesicles if exit and entry reaction are mediated by specific carriers saturable by substrate. The result was an apparent enhance-

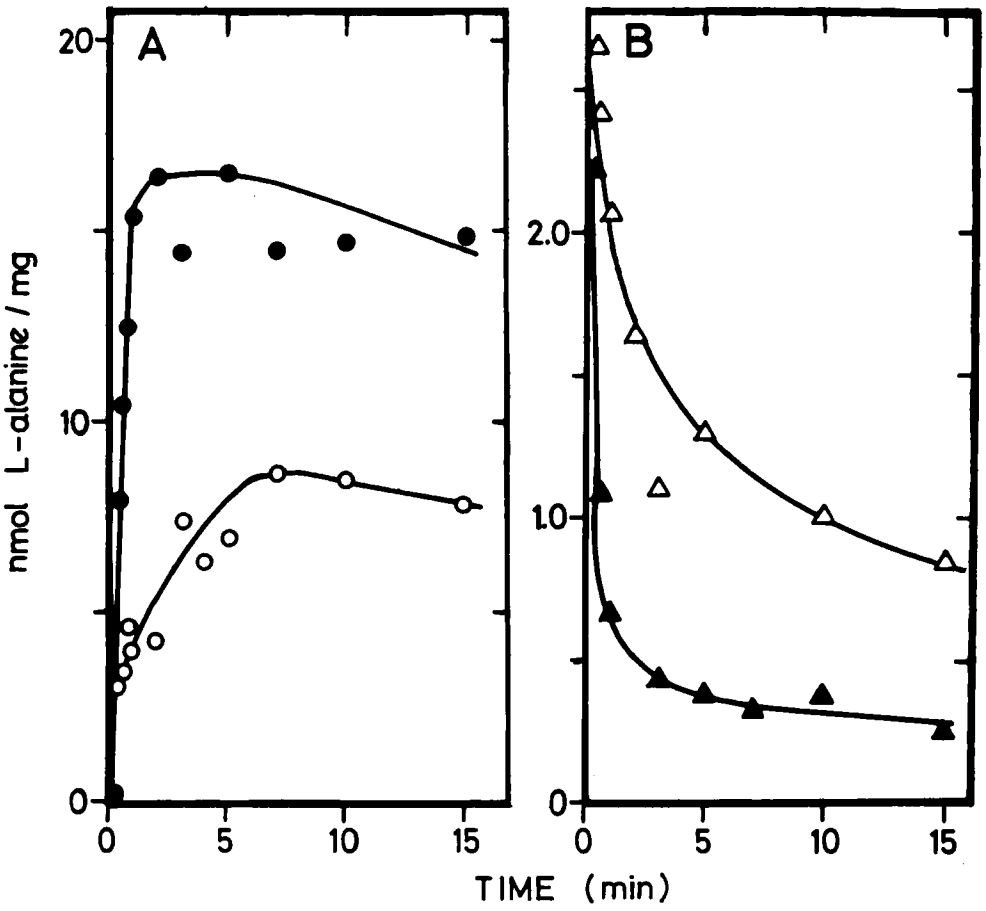

FIGURE 2. Enhancement of Na^+-stimulated L-[^{3}H] alanine uptake (A) and efflux (B) by exchange diffusion in the absence of a Na^+ gradient. (A) Vesicles from Swiss SV3T3 cells were incubated with (●) or without (○) 10 m*M* unlabeled L-alanine in 50 m*M* NaCl for 15 min and then aliquots were diluted to achieve an external suspension medium containing 1.17 m*M* L[^{3}H]-alanine and 50 m*M* NaCl in each case. At the indicated times, vesicles were collected by the filtration assay, (B) vesicles were incubated 15 min with 0.56 m*M* L-[^{3}H]-alanine in 50 m*M* NaCl. Then aliquots were diluted tenfold into 50 m*M* NaCl with (▲) or without (△) 100 m*M* unlabeled L-alanine for the indicated times. (From Lever, J. E., *J. Biol. Chem.*, 252, 1990, 1977. With permission.)

ment of labeled alanine accumulation. Under these conditions, early time points of accumulation reflect mainly the entry process, rather than the equilibrium between exit and entry of label. Stimulation of uptake in preloaded vesicles was evident as a prolonged region of linear uptake before the plateau region was reached, and a transient enhancement of the extent of accumulation of label. Stein[75] has discussed several possible carrier models and their consequences for countertransport stimulation. Countertransport stimulation is specific for pairs of molecules which interact with the same carrier. Thus, in the case of alanine accumulation, countertransport stimulation of uptake was observed if vesicles were preloaded with other amino acids which competitively interact with this transport[52] system. Observation of countertransport stimulation serves as a demonstration that the intra- and extravesicular compartments are not miscible with each other except via the mediation of a membrane component which exhibits a saturable interaction with the substrate.

A less rigorous criterion for transport versus binding to fixed sites is the demonstration of osmotic sensitivity of accumulated solute. This experiment relies on the as-

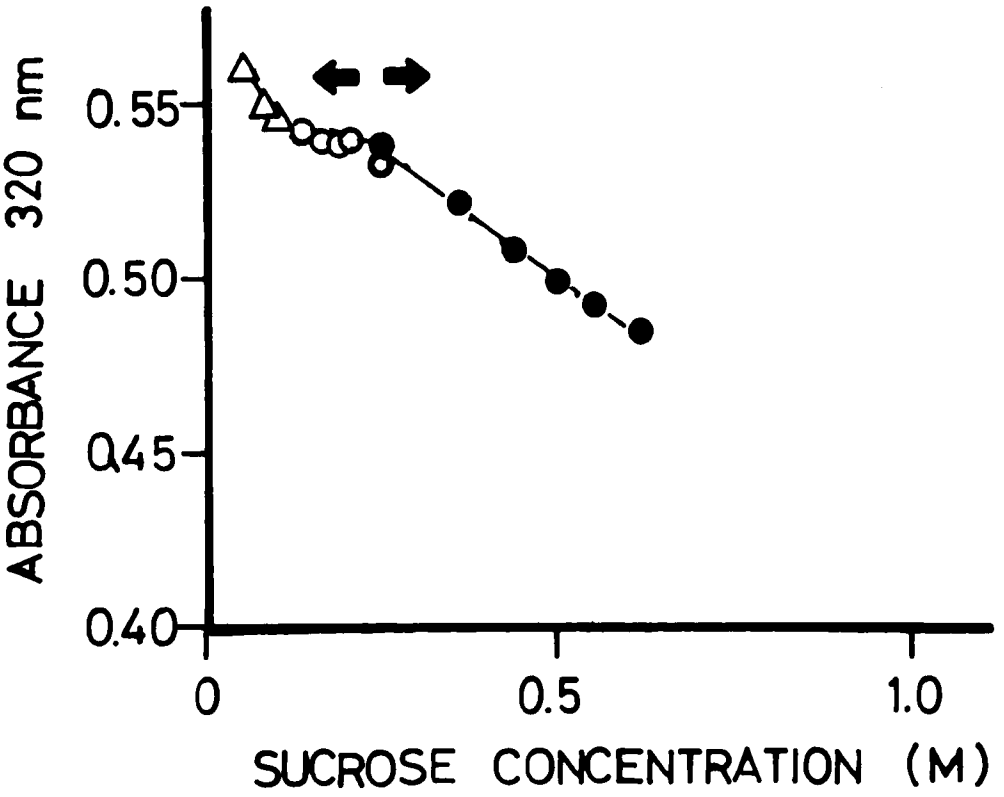

FIGURE 3. Osmotic sensitivity of plasma membrane vesicles from mouse fibroblasts. Vesicles initially contained 0.25 *M* sucrose, 10 m*M* Tris, HCl, pH 7.4 in their interior. The external sucrose concentration, initially 0.25 *M*, was either increased (●) or decreased (○,△) in stepwise increments as indicated. Absorbance at 320 nm was determined. Blank solutions minus vesicles were used as reference at each sucrose concentration. Each symbol refers to a separate aliquot of vesicles.

sumption that vesicles behave as osmometers and shrink and swell in response to changes in the balance between external and internal osmotic activity of a nonpenetrating solute. It should be noted that this approach might not be suitable for certain types of reconstituted phospholipid liposomes which have a structure sufficiently rigid to maintain an intravesicular space apparently insensitive to variation in osmotic pressure.

Several lines of evidence indicate that biological membrane vesicles derived after nitrogen cavitation are osmotically sensitive. Wallach et al.[47] has shown that such membrane preparations undergo changes in buoyant density during centrifugation in media of differing osmotic activity. Furthermore, the average volume of vesicle populations loaded with 0.25 *M* sucrose fluctuates as a function of variation in the external sucrose concentration when volumes were estimated by light scattering measurements (Figure 3). These observations of changes in vesicle volume as a function of osmotic sensitivity of accumulated solute reinforce conclusions based on osmotic sensitivity. In several cases,[22,26,49,53] it has been shown that the amount of solute accumulated at equilibrium in vesicles was a linear function of the osmolarity of the suspension medium, extrapolating to zero accumulation at infinite osmolarity. In such experiments, in addition to independent demonstration of osmotic sensitivity of vesicles volume, it is desirable to provide control experiments demonstrating that the solute used to adjust the osmolarity of the medium does not itself interfere with the transport system or putative binding sites, but is affecting solute accumulation solely by regulation of the

TABLE 3

Some Ionophores and Quasi-Ionophores Used in Transport Studies in Vesicles

Compound	Mol wt	Action[96]
A 21387	523	Ca^{++} ionophore; also mediates flux of other ions such as H^+ or K^+
Sodium monensin	692	Electroneutral Na^+/H^+ exchange primarily
Nigericin	744	Electroneutral K^+/H^+ exchange primarily
CCCP	204.6	Electrogenic H^+ flux; affects electrical membrane potential
FCCP	255	Electrogenic H^+ flux; affects membrane potential
Valinomycin	1111.4	Electrogenic K^+ or Rb^+ flux; affects membrane potential
Gramicidin D	ca. 2000	Quasi-ionophore; forms channels for monovalent cations such as H^+, Na^+, K^+, Rb^+

intravesicular space. Thus, the osmotically active solute should not act as an inhibitor of transport carrier activity, leading to the prediction that initial rates of solute uptake should be relatively insensitive to a similar variation in medium osmolarity.

Ionophores may be used to discriminate between transport and binding. These molecules selectively transport cations across biological and artificial membranes by rapidly forming freely reversible, lipid-soluble ionophore-cation complexes which readily diffuse across lipid barriers.[96] The properties of several ionophores and quasi-ionophores frequently used in transport studies are summarized in Table 3. Ionophores of known cation specificity and transport mode provide a means for insertion of a purified, defined model transport system into a biological membrane in order to test the consequences for solute accumulation via a separate, endogenous biological carrier system. Since ionophores act by facilitating cation permeability and/or electrical potential changes across membrane barriers without otherwise affecting membrane integrity, they would be expected to exert secondary effects on biological transport systems in the same vesicle which are influenced by these electrochemical parameters. Observations of such ionophore effects, described in more detail in subsequent sections, provide an additional line of evidence that retention of the solute in question is due to selective vesicle permeability rather than binding.

Models of Membrane Transport Systems

As a prelude to discussion of results obtained using membrane vesicles, some concepts relevant to an understanding of these experiments are outlined here.

Some terms and definitions

Diffusion

Solutes which exhibit a capacity to randomly penetrate a membrane barrier without forming a complex with a specific membrane component diffuse across the membrane by their intrinsic molecular motion modulated by the thermodynamic constraints of the chemical, electrical, and physical properties of the membrane phase.

Carrier-Mediated Transport

Often referred to as facilitated diffusion, this process refers to movement of solute

across a membrane mediated by the reversible formation of a complex between the solute and a specific membrane component, or carrier, and the thermodynamic constraints of the binding and release of the solute from the carrier. The term carrier used here is not intended to imply that the membrane component rotates or shuttles across the membrane, only that it interacts with the solute. Jardetzky[97] has proposed a visualization of facilitated diffusion in which the carrier is a vectorially oriented multisubunit protein which spans the membrane, enclosing an aqueous channel lined by the solute-binding sites. In this model, the thermadynamic constraints of solute binding and release, polypeptide subunit-subunit interaction, and polypeptide chain conformational change mediate solute transfer across the membrane.

Active Accumulation

This refers to the net carrier-mediated asymmetric distribution of solute across a membrane against its own electrochemical or osmotic gradient.[98] Such a process consumes energy which must be supplied to the system by transduction of energy generated by cellular metabolism. Several mechanisms for energy transduction have been visualized.

Covalent Coupling Hypothesis

In these mechanisms, covalent chemical bond interconversions directly drive active solute accumulation. The transported solute itself may be chemically transformed by a vectorial enzymatic process as it crosses the membrane, as in group translocation. Alternately, the carrier may be reversibly chemically modified to a form with altered affinity for transport solute and/or altered mobility or turnover.

Noncovalent Coupling Hypothesis

In contrast to covalent coupling mechanisms which utilize energy stored in chemical bonds, noncovalent coupling mechanisms utilize energy stored as gradients of thermodynamic functions, usually gradients of electrical potential and/or chemical gradients of other species, which translocate the membrane coupled with the movement of the transport solute under consideration. Such gradients are produced and maintained by a separate active pump system from the transport system in question. Dissipation of the gradient of the coupled species drives active accumulation of the solute in question.

Several possibilities fit this type of mechanism. Active accumulation may be driven by antiport. If entry of one species is coupled to exit of one or more chemical species or electrical charges, then an outwardly directed gradient of one or more chemical species or charge can drive internal accumulation of the coupled solute. Alternately, in coupling by a symport or cotransport mechanism, entry of each species is coupled. Thus an inwardly directed gradient of one chemical or electrical entity energizes internal accumulation of the coupled solute.

The fundamental biological principles represented by such noncovalent coupling mechanisms are a feature of diverse biological membrane-active transport systems. The general concept, first explicitly proposed as the alkali-ion gradient hypothesis in order to explain Na^+-dependent sugar transport in the intestine,[99] was generalized to encompass Na^+-dependent sugar and amino acid transport systems in intestinal and renal brush border membranes and Na^+-dependent amino acid transport systems of Ehrlich cells and erythrocytes.[100-105] It was proposed that these Na^+-dependent organic solute transport systems are driven by electrochemical Na^+ gradients generated by active Na^+ extrusion by the Na^+, K^+ ATPase. Subsequently, Mitchell[106,107] extended the gradient concept to propose that certain respiration-coupled active transport systems in bacterial cytoplasmic membranes are driven by electrochemical proton gradients maintained across the membrane by active proton extrusion.

The essential features of the alkali-ion gradient hypothesis, reviewed recently by Crane,[109] are summarized in Figure 4. Na^+ gradients (external Na^+ > internal Na^+) generated by Na^+, K^+ ATPase activity are coupled via a cotransport mechanism, stoichiometry, and molecular disposition, unspecified, to organic solute accumulation. The magnitude and polarity of organic solute accumulation is dictated by the magnitude and polarity of the electrochemical Na^+ gradient. Anion translocation is not directly coupled to this system; charge compensation, if required, occurs via independent pathways.

Several experimental predictions of this model which can be tested in vesicles are listed in Table 4. Experiments illustrating each of these points will be described in the following sections.

NEUTRAL AMINO ACID TRANSPORT IN MEMBRANE VESICLES

The A and L System Amino Acids

Summary — Active transport of neutral amino acids via the A system occus by means of noncovalent coupling to both the direction and magnitude of a chemical Na^+ gradient and an electrical potential across the membrane. Experimental predictions of this fundamental, unifying concept, formulated as the Na^+ gradient hypothesis, have been verified by studies using membrane vesicles from a variety of cell types. Uptake of L system amino acids in vesicles was much less stimulated by Na^+ and showed less accumulation relative to the external concentration.

Introduction

This was sometime a paradox, but now the time gives it proof.

Hamlet, William Shakespeare

Early studies of amino acid uptake in Ehrlich ascites tumor cell by Riggs et al.[100] perceived the importance of alkali cations in maintaining the capacity of cells to actively accumulate amino acids. They proposed a role for gradients of K^+ and possibly Na^+ in energizing this process. This concept was more explicitly formulated by Crane[99] as the Na^+ gradient hypothesis to explain Na^+-dependent sugar absorption in the intestine. The first clear demonstrations of Na^+-stimulated active amino acid transport were reported for intestinal amino acid transport by Nathans et al.[110] and Csaky,[103] followed by a report by Kromphardt et al.[104] that amino acid transport in Ehrlich ascites cells is controlled by external Na^+. In a classic paper, Oxender and Christensen[111] defined the specificities of two distinct transport systems for neutral amino acid uptake in Ehrlich ascites cells. One, the A system, mediates Na^+-stimulated active accumulation of several neutral amino acids such as alanine, glycine, serine, threonine, and the nonmetabolizable model substrate, 2-aminoisobutyric acid. Another system, the L system, mediates Na^+-independent, nonconcentrative uptake of several branched chain amino acids such as leucine, isoleucine, and valine. The specificities for neutral amino acids of these systems overlap; furthermore, certain amino acids such as methionine are transported equally well by both systems. Whereas the A system produces net concentrative uptake of amino acids, overlapping specificities lead to the consequence that the L system produces net exit of amino acids, driven by the gradients generated by the A system. This energetic interconversion between the A and L systems is a striking feature of neutral amino acid transport.

Additional support for the hypothesis that Na^+ gradients mediate active amino acid accumulation via the A system came from studies by Rosenberg et al.,[112] who demonstrated a relationship between Na^+ and the initial rate and extent of accumulation of

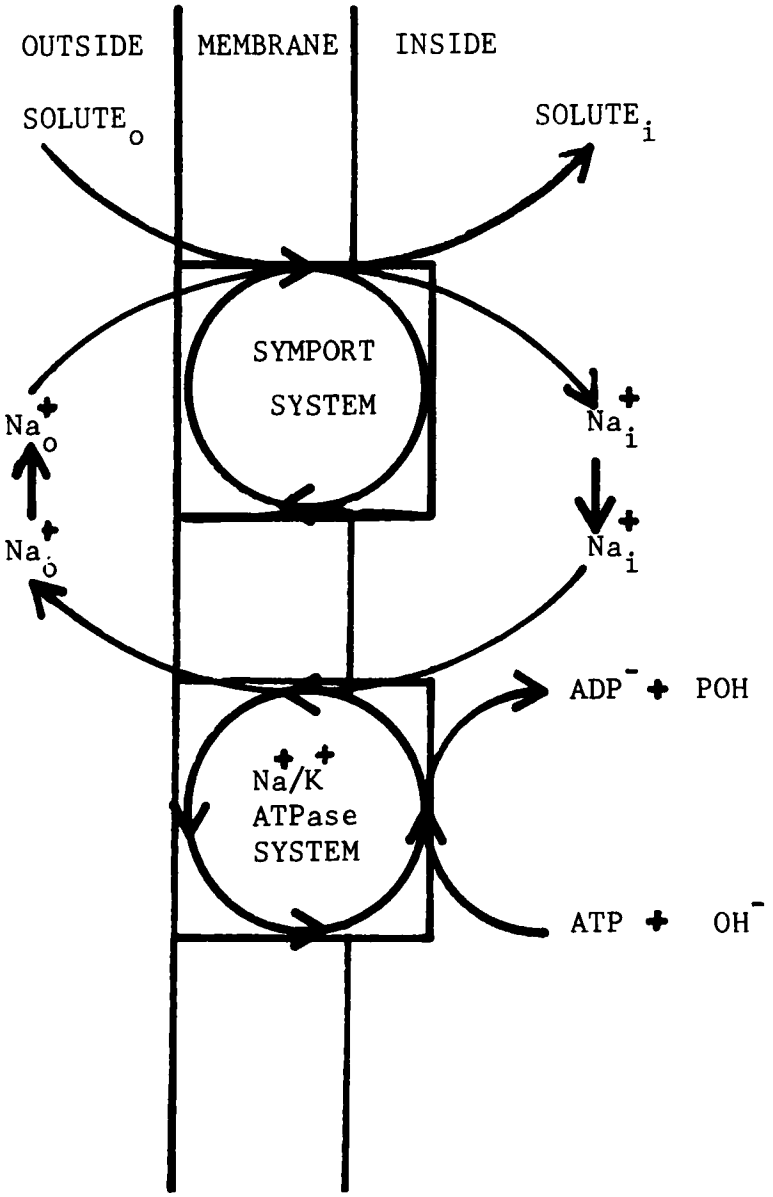

FIGURE 4. The alkali-ion gradient hypothesis.[99] (From Lever, J. E., *J. Cell. Physiol.*, 89, 779, 1976. With permission.)

2-aminoisobutyric acid in intestine. The identification in certain cell types[113] of the Na^+, K^+ ATPase, a primary active transport system which maintained Na^+ and K^+ gradients across the plasma membrane at the expense of intracellular ATP, was a major step in describing the linkage of membrane phenomena to intracellular metabolism.

Studies in intact cells of the nature of the energy sources utilized for A system transport led to conflicting interpretations. In a series of elegant studies of glycine uptake in pigeon erythrocytes and their ghosts, Vidaver[114] obtained evidence in support of noncovalent coupling of glycine entry to Na^+ gradients, with a stoichiometry of two Na^+ per glycine molecule transported. In addition, Vidaver[114] provided the first evidence that glycine entry in erythrocytes was further enhanced when an interior-negative

TABLE 4

Experimental Verification of Predictions of Gradient Hypothesis Using Vesicles

1. Facilitated diffusion demonstrated by countertransport when $u_{N_a} +$, the driving force due to the Na^+ gradient is dissipated by Na^+ preincubation.
2. Active accumulation in vesicles is stimulated by imposition of a Na^+ gradient (external > internal).
3. Quasi-steady state levels of accumulation depend on the magnitude of the external Na^+ concentration.
4. Accumulation depends on the polarity of the Na^+ gradient such that substrate accumulates *trans* to the face of the membrane exposed to the higher Na^+ concentration.
5. Affinity/velocity effects of Na^+ on the kinetics of solute transport.
6. Addition of ionophores such as monensin, which are expected to dissipate a Na^+ gradient, cause decreased solute accumulation.
7. Active accumulation of solute and Na^+ stimulation of uptake can be dissociated fom Na^+, K^+, ATPase activity.
8. Coupling of solute translocation to Na^+ gradients occurs at the level of the plasma membrane.
9. Charge compensation, if required, occurs independently. Anion translocation is not directly coupled to solute translocation. If Na^+-solute cotransport results in translocation of positive charge, uptake will be stimulated by an interior-negative membrane potential.

membrane potential was imposed by means of a Cl^- Donnan potential. Subsequently Schafer and Jacquez[115] calculated a stoichiometry of 1:1 for cotransport of Na^+ and 2-aminoisobutyric acid in Ehrlich ascites cells, and Gibb and Eddy[116] provided evidence for a contribution by interior negative membrane potential for this transport system. However, other observations were cited, i.e., hydrolysis of ATP,[117] as evidence that a direct contribution of metabolic energy to the process could not be excluded. For example, in contrast to Eddy's[118] demonstration that metabolically poisoned cells could maintain active neutral amino acid transport in the presence of Na^+ gradients, Schafer and Heinz[119] showed that even when Na^+ gradients were collapsed or inverted, metabolically active cells could still actively transport amino acids. In retrospect, these observations can be rationalized in terms of failure to account for the energetic contribution of an electrical membrane potential. Subsequent careful studies ruled out a direct contribution of ATP to the process.[120] Ultimate experimental proof of the gradient hypothesis requires quantitation of the energy available from ion gradients and electrical potentials across the cell membrane and demonstration of the utilization[121] of these driving forces by the transport system (coupling efficiency). Such quantitative estimations in intact cells were severely hampered by uncertainties as to the compartmentalization of internal ions and substrates[122] and as to the true value of the electrical potential[123] difference across the membrane. Heinz et al.[121] have questioned the validity of the use of Cl^- distributions to measure membrane potential. Additional sources of energization of amino acid have been proposed, including noncovalent coupling to proton gradients,[124] potassium ion gradients,[100] group translocation via γ-glutamyl transferase,[125] and utilization of reducing equivalents generated via an oxidoreduction system in the plasma membrane.[126] The use of membrane vesicle systems was designed to obviate these difficulties in order to provide a direct and definitive test of alternate models of energetics of amino acid transport.

Ehrlich Ascites Cells

Although amino acid transport in vesicles had been reported in studies of proline uptake or binding in renal tubule membranes by Hillman and Rosenberg,[127] and in extensive studies of Na^+-dependent glycine uptake in erythrocyte membrane vesicles,[128] the first characterization of Na^+-dependent amino acid transport in a highly purified plasma membrane vesicle system was provided by Colombini and Johnstone.[40,41] In these studies, 100 to 200 mg of plasma membrane vesicles were purified from approximately 10^{10} Ehrlich ascites cells using a Polytron® homogenization procedure based

on stabilization of plasma membranes with 1 m*M* $ZnCl_2$, followed by removal of Zn by addition of 1 m*M* EDTA.[39] Plasma membranes obtained by this procedure were relatively free from contamination by nuclei and mitochondria and retained 35% recovery of total functional Na^+, K^+-ATPase activity, 20-fold purified over the homogenate.[39] Membranes were stored frozen at −20°C in 15% dimethylsulfoxide. Based on data reported for 3-*O*-methyl glucose accumulation by these preparations, an intravesicular volume of 0.15 μl per mg protein could be calculated, which is somewhat lower than the values of 1 to 3 μl per mg protein reported for nitrogen cavitation vesicles from mouse fibroblasts.[41] Characterization of the purity of these preparations indicated less than 2% contamination with mitochondria as estimated by oxygen uptake, 3% contamination by endoplasmic reticulum as indicated by determination of NADH-cytochrome c reductase activity, a marker for endoplasmic reticulum and mitochondrial outer membrane in certain cells, and 2.5% of total cellular RNA.[39]

In their first report of 2-aminoisobutyric acid uptake in plasma membrane vesicles from Ehrlich ascites cells, Colombini and Johnstone[40] demonstrated that transport of this nonmetabolizable amino acid showed a specific requirement for Na^+. Uptake in vesicles was saturable, with an apparent K_m for 2-aminoisobutyric acid of 3.7 m*M*. They showed that amino acid influx and efflux were time and temperature dependent. Accumulated 2-aminoisobutyric acid was sensitive to the osmotic strength of the suspension medium and capable of exchange diffusion with other amino acids which had been shown to share this uptake system in intact cells. In this first report, transport measurements were made using vesicles which had been incubated with NaCl for 30 min at 37°C before addition of labeled substrate and uptake determination. This manipulation would be expected to dissipate a Na^+ gradient across the membrane and thus, as revealed in subsequent studies in this and other systems, markedly reduce amino acid accumulation. Accordingly, when gramicidin D was added in order to test the effect of dissipation of any cation gradient across the membrane, no inhibitory effect was observed. In a subsequent report,[41] Colombini and Johnstone revised this transport protocol. Vesicles preincubated with one alkali cation were centrifuged and then resuspended in the presence of labeled substrate and a different alkali cation. By this means it was demonstrated that a Na^+ gradient (external > internal) across the membrane stimulates amino acid accumulation in vesicles against a concentration gradient. The nonmetabolizable glucose analogue, 3-*O*-methyl-D-glucose, shown not to accumulate above its external concentration in intact cells, as well as the radioactive tracer cations $^{22}Na^+$ and $^{86}Rb^+$, reached the same apparent steady state accumulation in vesicles relative to their external concentration. By contrast, 2-aminoisobutyric acid accumulation exceeded this level by twofold in the absence of an imposed Na^+ gradient across the membrane. Upon imposition of a Na^+ gradient (external > internal) across the membrane, a marked three- to fourfold stimulation of amino acid uptake was observed which was reduced by inclusion of Na^+ within the vesicles, but not affected by preloading of vesicles with other monovalent cations. Consistent with these findings, it was further observed that Na^+ gradient-stimulated uptake was reduced by gramicidin D. However, this antibiotic acts as a channel for several cations, including Na^+, H^+, K^+, and Li^+. Therefore, the basis for the effects of gramicidin on amino acid transport in this experiment cannot be solely ascribed to dissipation of a Na^+ gradient. The relationship as a function of time between the dissipation of the Na^+ gradient (measured with $^{22}Na^+$) and amino acid accumulation suggested that, assuming a 1:1 stoichiometry, amino acid accumulation in vesicles exceeded the energy provided by the magnitude of the Na^+ gradients. Evidence for electrogenic accumulation of 2-aminoisobutyric acid was obtained in experiments using vesicles loaded with K^+ and then suspended in the presence of external Na^+, creating gradients $K^+_i > K^+_o$ and $Na^+_o > Na^+_i$. In this situation, addition of valinomycin, a K^+ specific ionophore, produced

about a 30% increase in amino acid accumulation compared to samples lacking ionophore; however, control experiments in which the K^+ gradient was dissipated were not provided for comparison. A complex relationship between external pH and time course of amino acid accumulation raised the possibility of a role of pH gradients in contributing to the driving force for transport. Furthermore, the twofold amino acid accumulation observed in the absence of Na^+ gradients, together with observations of a direct inhibitory effect of ouabain on amino acid accumulation in vesicles and the presence of measurable levels of ATP bound to these membrane preparations, did not allow dissociation of Na^+, K^+ ATPase activity from a direct role in the functioning of this system. This important series of papers presenting the key parameters of an in vitro system in vesicles to investigate energy coupling of amino acid transport did not rule out other proposed schemes for the energetics of amino acid transport.

Christensen and co-workers[126] have proposed an additional source of energization of 2-(methylamino)-isobutyric acid, a model substrate for the A system, in Ehrlich ascites cells expressed in their isolated plasma membrane vesicles. Observations that the inhibition of uptake of this amino acid in intact cells treated with metabolic poisons such as dinitrophenol and iodoacetate could be reversed after addition of phenazine methosulfate plus ascorbate, taken together with the demonstration of inhibition by quinacrine, an inhibitor of plasma membrane-associated NADH dehydrogenase activity, were interpreted in favor of a scheme in which NADH shuttles from mitochondria to the plasma membrane, donating reducing equivalents to an unidentified acceptor to provide amino acid transport energization supplemental to that provided from ion gradients. Preliminary studies of 2-(methylamino)-isobutyric acid uptake in membrane vesicles[126] produced results opposite to that predicted from this model. Amino acid uptake was stimulated over 50% by quinacrine and by carbonyl cyanide trifluoromethoxyphenylhydrazone, but inhibited 50% by NADH. The interpretation of these results is complex and must wait further characterization of the system, including the sidedness and purity of the vesicle preparation, inhibitor effects on membrane integrity, flux of other ionic species which could affect membrane potential, and characterization of the unidentified putative electron acceptor of the plasma membrane.

Johnstone and Bardin[129] have provided evidence for reconstitution of A system transport activity from plasma membranes of Ehrlich ascites cells in liposomes formed from soybean phospholipids. A modification of the cholate dialysis procedure of Kagawa and Racker[130] was used. Time and temperature-dependent uptake of 2-aminoisobutyric acid into liposomes was observed. In addition, exchange diffusion of accumulated 2-aminoisobutyric acid with methionine but not leucine was presented as evidence for carrier-mediated transport. However, no evidence for active accumulation in vesicles could be demonstrated, and stimulation of uptake by a Na^+ gradient was variable.

Cecchini et al.[131] have reported a leucine binding activity in isolated Ehrlich ascites cell plasma membranes treated with Triton® X-100. Binding activity was not expressed in the absence of detergent and showed saturability by phenylalanine, another substrate of the L system.

Mouse Fibroblast Cell Cultures

Transport studies using membranes isolated from cells grown in culture provide an additional dimension; a system for investigation of the mechanism of interaction of the transport system with processes which regulate cell proliferation. Mouse 3T3 fibroblast cultures have been used as a model system to investigate regulation of cell proliferation by serum, growth factors, hormones, and viral transformation.[15,16] Fluctuations in nutrient and ion transport as well as in intracellular levels of cyclic nucleotides have been implicated in cellular responses to these agents.[15,16] Uptake of neutral amino

acids via the A system appears to reflect the growth state and hormonal environment of mouse fibroblasts[132] and other cell types, showing decreased activity in quiescent cells. The rate of uptake of amino acids predominantly transported via the L system increased as cells became quiescent.[132]

Mixed vesicles isolated from SV40-transformed 3T3 mouse fibroblasts were shown to catalyze Na^+-stimulated uptake of 2-aminoisobutyric acid,[22,49,53,54] alanine, glycine, glutamine, and methionine[54] (Figure 5) in the absence of metabolism.

Such mixed vesicle preparations contained about 60% of the plasma membrane marker, 5′-nucleotidase, and were contaminated with 4% of total mitochondrial marker, 13% of lysozomes, 24% of endoplasmic reticulum, and 13% of a marker for soluble enzyme.[22,53] Na^+-stimulated 2-aminoisobutyric acid transport activity accompanied plasma membrane material during subcellular fractionation.[22,54]

A central observation of these initial characterization was that the enhancement of amino acid accumulation by Na^+ was greatest when Na^+ was added together with amino acid substrate at the time of initiation of uptake. If vesicles were preincubated with NaCl before addition of labeled amino acid, uptake was significantly decreased compared with vesicles to which NaCl was added at zero time (Figure 5). This finding suggested that amino acid accumulation was stimulated by a Na^+ gradient across the membrane.

Additional experiments established the validity of using membrane vesicles to investigate the corresponding cellular uptake systems. The competitive interactions among neutral amino acids for transport into vesicles[54] resembled those described for intact Ehrlich ascites tumor cells, as shown in Table 5. The specificity profile shown for alanine and 2-aminoisobutyric acid uptake into vesicles shown in Table 5 suggested that these two amino acids share a common carrier for transport into vesicles with the specificity properties described for the A system. The pattern of inhibition of leucine uptake in vesicles was that expected for transport via the L system. Inhibition of labeled methionine uptake by unlabeled amino acids was consistent with previous views that methionine is transported by both the A and the L systems. Studies in intact cells had established that alanine is transported by both the A system and the ASC system.[105] *N*-Methyl-alanine was used as a tool to distinguish between these two systems in vesicles. *N*-methyl amino acids are not substrates of the ASC system. Results in Table 5 indicate the *N*-methylalanine is as effective as alanine in reducing labeled alanine uptake in vesicles, suggesting that alanine uptake in vesicles is not mediated by the ASC system under these conditions. A separate carrier system in addition to the A system, has been shown to mediate glycine uptake in certain cells in a Na^+-dependent mechanism.[105] Glycine showed minimal effectiveness in reducing the uptake of the labeled amino acids tested in Table 5, yet glycine uptake was greatly enhanced by a Na^+ gradient (Figure 5). The relative contribution of Na^+-dependent and Na^+-independent pathways for amino acid uptake in vesicles was estimated by replacing NaCl with choline Cl (Table 5). Approximately 15% of total uptake of alanine and methionine, 20% of the total 2-aminoisobutyric acid uptake, and 50% of leucine uptake occurred by Na^+-independent mechanisms. The lesser stimulation observed for leucine uptake by Na^+ gradients presumably represented that component of its uptake mediated by the A system.

Another important criterion for the use of membrane vesicles as a system to study transport, in addition to the standard lines of evidence (Figure 2, Table 2) proving the existence of specific carrier systems, is to demonstrate recovery of total functional transport sites after membrane isolation. Obviously, if the major pathway of transport studied in vesicles is only a minor pathway operating in the intact cell, extrapolation of results obtained in vesicles to describe cellular transport mechanisms is risky. A

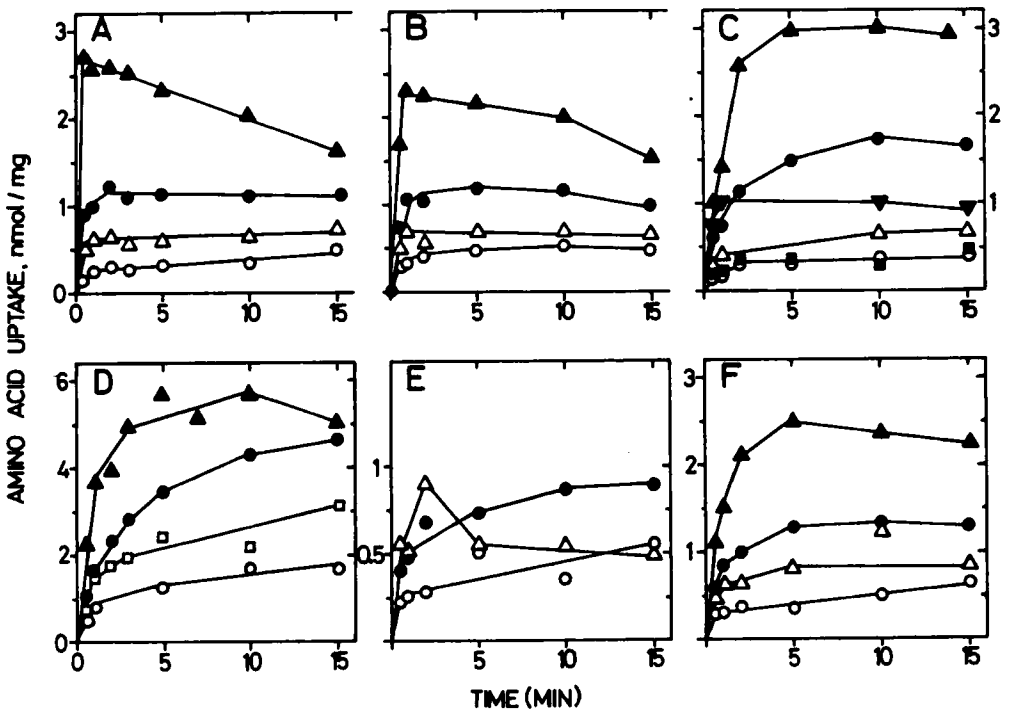

FIGURE 5. Stimulation of neutral amino acid uptake by a Na^+ gradient and inhibition by monensin. (A) ^{3}H-alanine, 0.2 m*M*, (B) ^{3}H-methionine, 0.2 m*M*, (C) ^{3}H-glycine, 0.2 m*M*, (D) ^{14}C-2-aminoisobutyric acid, 0.17 m*M*, (E) ^{3}H-leucine, 0.2 m*M*, (F) ^{3}H-glutamine, 0.2 m*M*. Substrate and Na^+, K^+, or choline salts were added at zero time. ▲, 50 m*M* NaSCN; ●, 50 m*M* NaCl; O, 50 m*M* choline chloride substituted for Na^+, △, 1 μg of monensin in 1% Me_2SO and 50 m*M* NaCl; □ vesicles were incubated with Na^+ 15 min before uptake was measured at 50 m*M* external NaCl; ▼, 50 m*M* NaSCN plus 1 mg of monensin in 1% Me_2SO; ■, 50 m*M* KSCN. 1% Me_2SO had no effect on uptake. (From Lever, J. E., *J. Biol. Chem.*, 252, 1990, 1977. With permission.)

maximal velocity (V_{max}) of 2.1 nmol mg^{-1} min^{-1} was observed for Na^+-stimulated transport of 2-aminoisobutyric acid into membrane vesicles from SV40-transformed mouse fibroblasts.[48] After suitable correction for recovery of plasma membrane material relative to total cellular protein, this value compares favorably with rates which have been reported for transport of this nonmetabolizable analogue by intact SV40-transformed 3T3 mouse fibroblasts.[133] This indicates that close to 100% of the 2-aminoisobutyric acid transport sites are recovered functionally intact in isolated membrane vesicles.

Contribution of an Electrochemical Na^+ Gradient

Several studies were directed towards investigation of the nature of the contribution of Na^+ to the amino acid transport process. In these experiments, Na^+ gradients of various initial magnitudes (external > internal) were set up across the membrane at zero time by adding Na^+ together with substrate to the external suspension medium of vesicles. The following lines of evidence indicate that a difference in Na^+ concentration across the membrane serves as a primary, but not only, driving force energizing amino acid accumulation in vesicles:[54]

1. A NaCl gradient imposed across the membrane increased both the initial rate and quasi-steady state of accumulation of several amino acid substrates of the A

TABLE 5

Specificity of Na^+ Gradient-Stimulated Amino Acid Uptake in Vesicles from SV40-Transformed Mouse Fibroblasts

Unlabeled Amino Acid (5 m*M*)	Initial rate of uptake of radioactive amino acid[a] (% control)			
	^{3}H-alanine (0.2 m*M*)	^{3}H-methionine (0.2 m*M*)	^{3}H-leucine (0.2 m*M*)	^{14}C-2-amino-isobutyrate (0.17 m*M*)
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
2-aminoisobutyric acid	31	78	71	31
D-glutamine	87	71	79	82
2-dansyl-alanine	85	—	79	94
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] Uptake was measured 30 sec after the addition of labeled and unlabeled substrates and 50 m*M* NaSCN to vesicles.

[b] KCN was substituted for NaSCN.

From Lever, J. E., *J. Biol. Chem.*, 252, 1990, 1977. With permission.

system in vesicles (Figure 5). Based on estimates of 0.8 to 1.1 μl/mg protein, maximal accumulation of these amino acids in vesicles reached six- to sevenfold above their external concentration.[54] By contrast, a choline Cl gradient of the same initial magnitude stimulated only a one- to twofold accumulation of these amino acids. Accumulation in vesicles maintained a relatively constant plateau within the 5 to 10 min interval after initiation of uptake. As expected, leucine uptake showed much less stimulation by a Na^+ gradient (Figure 5E), since it is predominantly transported by the Na^+-independent L system.[111]

2. Stimulation of amino acid accumulation by ion gradients was specific for Na^+ salts when tested at a concentration of 50 m*M*. Other monovalent cations tested at a 50 m*M* initial gradient — K^+, Rb^+, choline, and Tris chloride salts — were not stimulatory.[22,49,54] A slight (50%) stimulation of accumulation was observed after substitution of LiCl gradients for NaCl gradients.[54]
3. Stimulation of amino acid accumulation increased as a function of external Na^+ concentration, tested at a constant thiocyanate concentration maintained by the

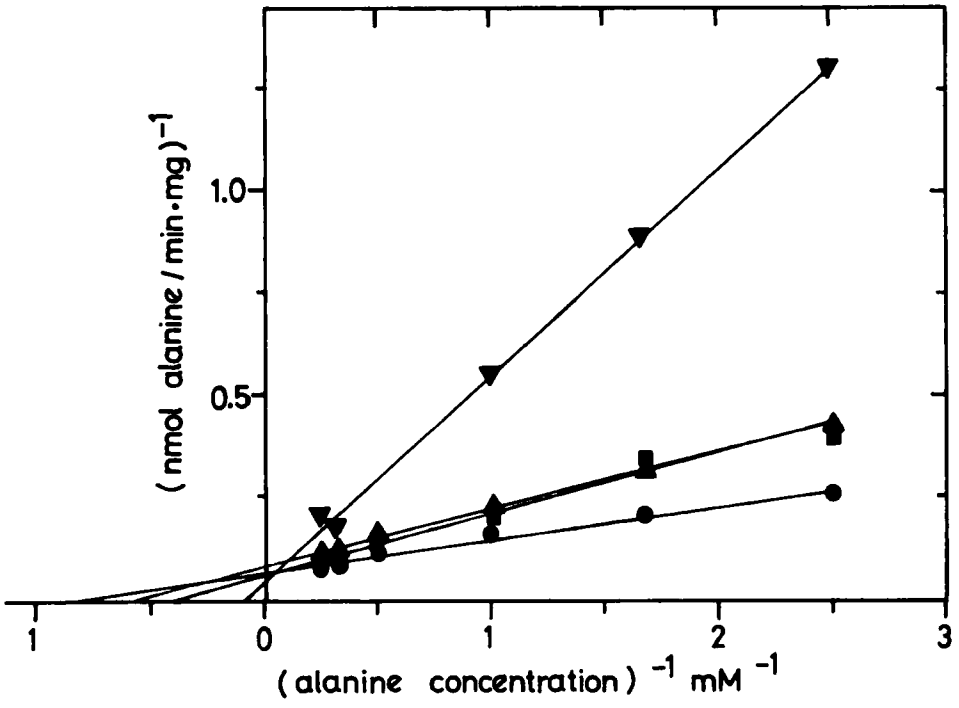

FIGURE 6. Effect of a Na^+ gradient on alanine uptake as a function of alanine concentration. Uptake was determined 30 sec after addition of labeled alanine (0.4 to 4.0 m*M*) with the indicated salts. ○, 50 m*M* NaSCN; ▽, 50 m*M* KSCN; ▲, 10 m*M* NaSCN plus 40 m*M* KSCN; ■, 1 m*M* NaSCN plus 50 m*M* KSCN. Results are the sum of Na^+-dependent and -independent rates. (From Lever, J. E , *J. Biol. Chem.*, 252, 1990, 1977. With permission.)

addition of KSCN.[54] Na^+ effects on the initial rate of uptake as a function of amino acid concentration were expressed mainly as effects on the apparent K_m for amino acid uptake in vesicles. Figure 6 shows that an apparent K_m of 10 m*M* was observed when KSCN replaced NaSCN. When the external Na^+ concentration was increased in the range of 1 to 50 m*M*, the apparent K_m for alanine accumulation decreased from 2.4 m*M* to 1.2 m*M*. Effects of Na^+ on maximal velocity of alanine uptake were less pronounced. Similar effects of Na^+ on K_m for transport into vesicles were observed for 2-aminoisobutyric acid[22,52] and glutamine uptake in this system.[54]

Both initial rate and quasi-steady state accumulation of alanine and glycine in vesicles increased as a function of the external Na^+ concentration. Figure 7 shows that when external Na^+ concentration was varied in the range 0 to 100 m*M* at a constant external thiocyanate concentration of 100 m*M* maintained by the addition of potassium thiocyanate, quasi-steady state alanine accumulation increased from one- to sixfold, relative to the external alanine concentration assuming an internal volume of 1 μl/mg protein. Similar results were observed when chloride was used as counterion.[134]

When the logarithm of the apparent alanine accumulation after subtraction of Na^+-independent uptake at quasi-steady state in vesicles was plotted vs. the logarithm of the external Na^+ concentration, a straight line with a slope of 0.7 was obtained (Figure 7, inset).

4. Addition of monensin,[96] an ionophore which has been shown to catalyze an electroneutral exchange of Na^+ for H^+, and thus should dissipate a Na^+ gradient

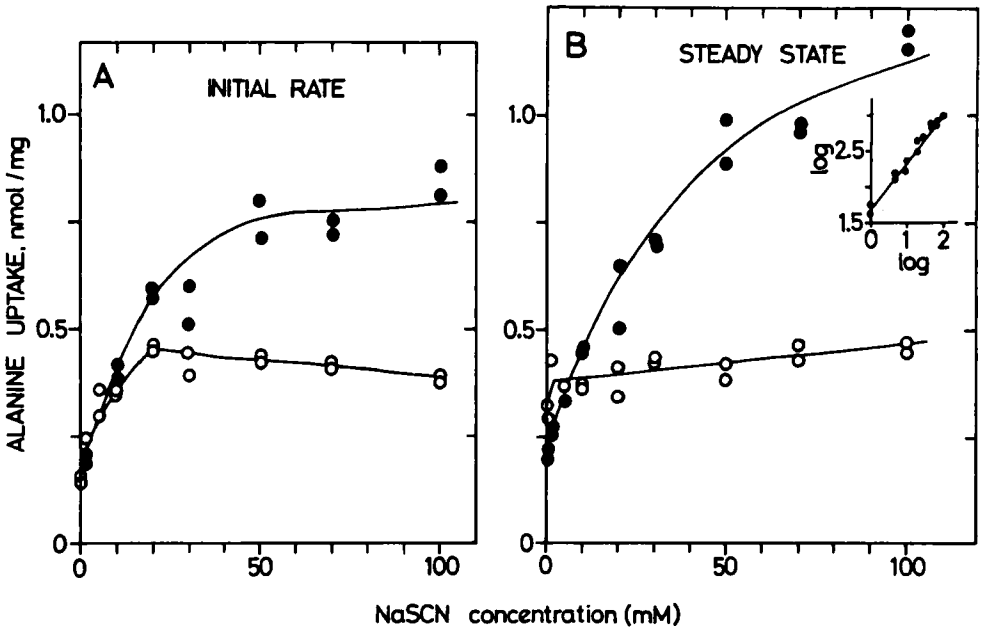

FIGURE 7. Effect of a Na^+ gradient on (A) initial rate of L-alanine uptake and (B) steady state accumulation of alanine, as a function of external Na^+ concentration. Uptake was measured 30 sec (A) and 10 min (B) after addition of labeled alanine and thiocyanate salts. Total SCN^- concentration was maintained at 100 m*M* by addition of KSCN, and the Na^+/K^+ ratio was varied. ●, 1% Me_2SO; ○, 1 μg of monensin in 1% Me_2SO. Inset, logarithm of Na^+-dependent alanine uptake (with Na^+ independent values subtracted) at 10 min (pmol/mg) vs. the logarithm of the external Na^+ concentration (m*M*). (From Lever, J. E., *J. Biol. Chem.*, 252, 1990, 1977. With permission.).

across the membrane, decreased the stimulation of amino acid accumulation by an imposed Na^+ gradient.[54,134] Monensin did not abolish the stimulation of the initial rate of alanine uptake (Figure 7), but the decreased accumulation persisting in the presence of monensin reached an apparent plateau at Na^+ concentrations above 50 m*M* with half maximal stimulation observed at 10 m*M* NaSCN.

By contrast, monensin collapsed the stimulation of quasi-steady state alanine accumulation by Na^+ gradients at all Na^+ concentrations tested (Figure 7). The effects of monensin on the time course of accumulation of several amino acid substrates of the A system are illustrated in Figure 5.

5. Endogenous Na^+, K^+, ATPase activity of the plasma membrane could be dissociated from Na^+-gradient-stimulated amino acid transport activity of vesicles.[54] Addition of ouabain, a cardiac glycoside which specifically inhibits the Na^+,K^+, ATPase transport system,[135] did not affect Na^+-stimulated alanine transport activity in vesicles when assayed either in the presence or absence of concentrations of ATP, KCl, $MgCl_2$, and NaCl required for optimal expression of ATPase activity. Control experiments using the same vesicle preparation demonstrated that this concentration of ouabain was effective in completely inhibiting the endogenous K^+-dependent ATP hydrolysis, the enzymatic manifestation of the Na^+, K^+, ATPase transport system. Furthermore, addition to vesicles of substrates of the Na^+, K^+, ATPase system such as KCl and ATP did not stimulate Na^+-coupled alanine transport activity in vesicles.[54]

Similarly, Na^+-stimulated alanine transport activity could be dissociated from mitochondrial functions. Several uncouplers and respiratory inhibitors — oligomycin, 2,4-

dinitrophenol, antimycin A, and carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone — had minimal or no effect when tested for inhibition of alanine transport in vesicles.[54]

Several aspects of this experimental approach using artificially constructed Na^+ gradients in vesicles should be noted. Although the external Na^+ concentration is constant, the intravesicular Na^+ concentration gradually increases during the course of amino acid uptake due to leakage of Na^+ across the membrane by pathways both coupled and uncoupled to amino acid flux. In these protocols, the intravesicular Na^+ concentration is not measured and thus the magnitude of the Na^+ gradient across the membrane at quasi-steady state is not known. Since the Na^+ gradient driving force is gradually dissipated during the course of amino acid accumulation, it would be expected that only transient amino acid accumulation would be observed in vesicles. At true steady state, ions and amino acids would be uniformly distributed across the membrane. However, several observations indicate that the relationship between quasi-steady state amino acid accumulation and the known external Na^+ concentration may be utilized to estimate the thermodynamic equilibrium between the amino acid accumulation ratio and the driving force derived from an electrochemical Na^+ gradient. Amino acid accumulated in vesicles maintains a relatively constant plateau value during the 5 to 15 min interval after initiation of uptake.[53,54] No overshoot of accumulation was observed in this interval, in contrast to reports demonstrating rapid loss of 2-aminoisobutyric acid accumulated in vesicles after 2 min.[22,49] However, addition of monensin during this interval produced efflux of amino acid accumulated in vesicles.[52] Thus, a residual Na^+ gradient persists during the plateau interval used to measure quasi-steady state accumulation and the apparent maximal amino acid accumulation in vesicles was in equilibrium with the Na^+ gradient. In the inverse reciprocal plots shown in Figure 6, curvature expected from increased coupled Na^+ influx at increased amino acid concentrations is not evident. Presumably, Na^+ influx does not create a measurable source of variability under the experimental conditions used here. It seems likely that the 37°C temperatures used in experiments where overshoot phenomena were pronounced[22,49] facilitate Na^+ influx and amino acid leakage from vesicles,[54] producing a more transient amino acid accumulation.

Contribution of Interior-Negative Membrane Potential

Na^+-stimulated amino acid accumulation in vesicles was further enhanced when an interior-negative membrane potential was independently imposed by artificial means across the vesicle membrane.[53,54,134] This response to electrical membrane potential specifically required the presence of Na^+.[134] By means of selective imposition and dissipation of electrical and Na^+-gradient driving forces, it was further established that amino acid accumulation could be driven either solely by electrical potential differences imposed in the presence of Na^+ but in the absence of a Na^+ gradient, or by an imposed chemical difference in Na^+ concentration across the membrane in the absence of electrical differences.[134] When both chemical and electrical gradients were imposed simultaneously across the membrane, they could be utilized additively for amino acid accumulation.[134]

An electrical potential difference across the membrane may be generated by various means. Biological systems in most instances generate electrical asymmetry across the membrane by means of vectorial enzymatic systems in the membrane which utilize metabolic energy with an end result that a charged species is pumped across the membrane. Examples of such systems are the Na^+, K^+, ATPase of animal cell plasma membranes[113,116] and the proton pumps of mitochondrial and bacterial membranes.[7,106-108] In vesicles, generation of potentials by endogenous enzymatic pumps can be bypassed and electrical potentials of various magnitudes and polarities can be generated by ar-

tificial manipulation. Vidaver and co-workers[114,136] have produced Donnan potentials in erythrocyte ghosts by replacing Cl^- with 2,4-toluenedisulfonate or glutamate. In plasma membrane vesicles from mouse fibroblasts, interior-negative membrane potentials were generated by two alternate methods. The artificial generation of a permeant anion diffusion potential (external > internal) by addition of anion and relatively impermeant cation to the vesicle suspension medium generates an interior-negative membrane potential without requirement for addition of ionophores.[134] Alternately, transient interior-negative membrane potentials are generated by means of a potassium ion diffusion potential (internal > external) in the presence of valinomycin.[134] Thus, vesicles loaded with K^+ are diluted in the presence of valinomycin to achieve a decreased external K^+ concentration. The cyclic peptide antibiotic valinomycin, a K^+-specific ionophore, causes a large selective increase in membrane permeability to K^+ by forming a positively charged K^+-valinomycin complex which diffuses across the membrane.[96] The magnitude of the electrical potential thus generated is largely dictated by the magnitude of the imposed K^+ gradient since ionophore-facilitated K^+ permeability exceeds the permeability of the other ions present in the solution. The magnitude of electrical potentials generated by these means may be estimated using the distribution by diffusion across the vesicle membrane of the lipophilic cation ^{3}H-triphenylmethylphosphonium ion ($TPMP^+$).[134,137,138] The Nernst equation describes the relationship between membrane potential, $\Delta\psi$, and the apparent accumulation of lipophilic cation in vesicles:

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

Other techniques for estimating membrane potential are qualitative estimates using fluorescence changes[139] or quantitation of the distribution of freely permeant ions such as dimethyldibenzylammonium ion,[140] chloride,[123] $^{86}Rb^+$ in the presence of valinomycin,[141] or labeled thiocyanate.[142]

Various biological membranes differ in their permeability to lipophilic ions. The validity of estimates of membrane potential derived from lipophilic cation distributions rests on the assumption that they freely diffuse across the membrane into a single compartment in response to electrical asymmetry without interaction with specific membrane components. Thus, an appreciable contribution of carrier-mediated equilibration, active transport, or binding to fixed charges would contribute sources of error to estimates of membrane potential. These difficulties may apply to several probes which have been used to estimate membrane potential. Chloride distributions may be partially mediated by specific transport systems in animal cells.[121,143] $^{86}Rb^+$ distributions are complicated by the requirement for addition of valinomycin and specific active Rb^+ transport via the Na^+, K^+, ATPase. Dimethyldibenzylammonium distributions are markedly enhanced by addition of tetraphenylboron, which possibly serves as a carrier or counterion, shedding doubt on estimates using this probe.[144] Compelling justification for the use of $TPMP^+$ distributions to measure the magnitude of membrane potential has been presented for bacterial[138] and mouse fibroblast[134] membrane vesicles. Furthermore, this indicator does not require the addition of ionophores or lipophilic anions, is insensitive to variation in pH from 5 to 9 and does not appear to interact with carriers in the membrane.[134,138] Binding to fibroblast membranes was negligible. Chilling of vesicles to 2°C during dilution in wash buffer and collection by filtration prevented appreciable loss of accumulated $TPMP^+$. The $t_{1/2}$ of efflux was 2.5 min under these conditions.[134]

Results in Figure 8 indicate that Na^+-stimulated amino acid uptake is markedly stimulated by conditions expected to generate an interior-negative membrane potential. In this experiment, vesicles were first loaded with 50 m*M* KCl in the presence of valinomycin. Then, vesicles were diluted to achieve external salt concentrations of 5 m*M* KCl

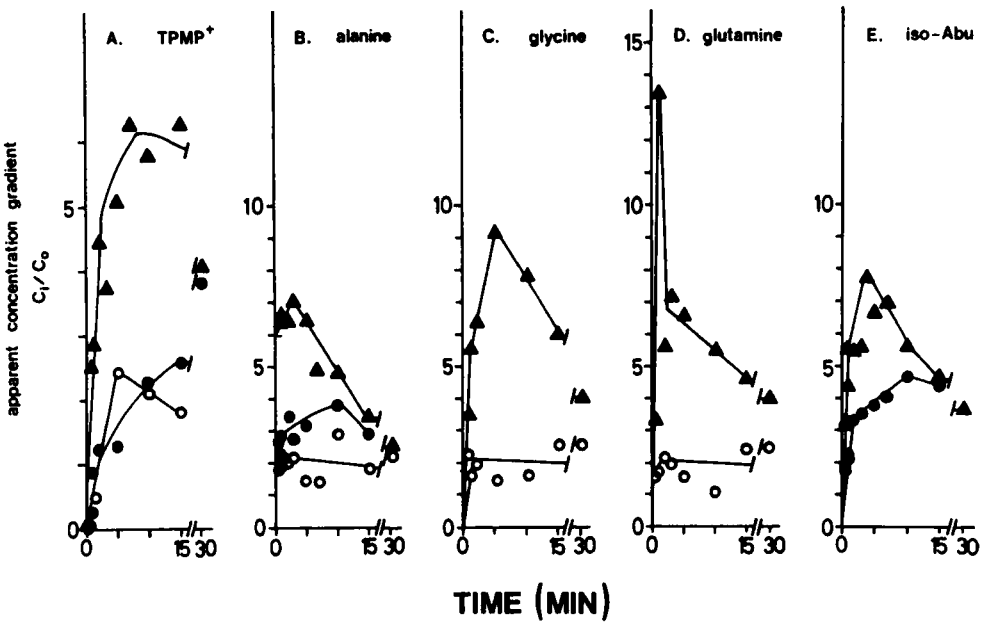

FIGURE 8. Stimulation of triphenylmethylphosphonium ion and amino acid uptake in the presence of a valinomycin-induced potassium ion diffusion potential. (A) 0.1 m*M* [^{3}H] triphenylmethylphosphonium bromide, (B) 0.2 m*M* L-[2,3-^{3}H] alanine, (C) 0.2 m*M* [2-^{3}H] glycine, (D) 0.2 m*M* L-[G-^{3}H] glutamine, (E) 0.17 m*M* 2-amino [1-^{14}C] isobutyric acid. Vesicles were incubated 15 min with 2% ethanol, 3% dimethylsulfoxide, and 50 m*M* KCl (●); 90 μ*M* valinomycin, 2% ethanol, and 50 m*M* KCl (▲); or 90 μ*M* valinomycin, 2% ethanol, 50 m*M* KCl, 40 μ*M* nigericin, and 3% dimethylsulfoxide (○). At zero time a tenfold potassium ion gradient was generated by dilution of vesicles into mixtures containing 50 m*M* NaCl and the indicated labeled solute; the final external potassium ion concentration was 5 m*M*. Uptake was measured by filtration at the indicated times. (Reprinted with permission from Lever, J. E., *Biochemistry*, 16, 4328, 1977. Copyright by the American Chemical Society.)

and 50 m*M* NaCl at the time of addition of external labeled solute. The tenfold K^+ gradient produced by this manipulation, together with the large selective increase in K^+ permeability with respect to that of the other ions, is expected to produce an interior-negative potential of a magnitude dictated by the magnitude of the K^+ gradient. Time courses of $TPMP^+$ accumulation in vesicles, shown in Figure 8A indicated that lipophilic cation reached an apparent intravesicular concentration about sixfold higher than its external concentration. Alanine, glycine, glutamine, and 2-aminoisobutyric acid (Figure 8, panels B to E, respectively) accumulation under these conditions paralleled and exceeded the accumulation of $TPMP^+$. Whereas a NaCl gradient (external > internal) alone stimulated a four- to fivefold accumulation of these amino acids, a valinomycin-induced K^+ diffusion potential superimposed on a NaCl gradient stimulated a transient seven- to thirteenfold accumulation of these amino acids.[134]

Convincing evidence that $TPMP^+$ accumulated in response to an interior-negative membrane potential was obtained using two different methods expected to specifically collapse membrane potentials generated in the presence of valinomycin. The further addition of nigericin, which promotes an electroneutral K^+/H^+ exchange,[146] with valinomycin to vesicles at the time of imposition of the K^+ gradient, would be expected to collapse membrane potential, presumably by short-circuiting the K^+ gradient.[147] This treatment reduced the internal $TPMP^+$ concentration to a value approaching its known external concentration. Furthermore, dilution of vesicles with valinomycin, such that internal and external K^+ concentrations were made equal, also prevented $TPMP^+$ accumulation above its external concentration.[134]

A chemical K^+ gradient per se did not affect either $TPMP^+$ or amino acid accumulation[134] either in the presence or absence of nigercin, an ionophore which would be expected to facilitate K^+ efflux from vesicles but not generate a membrane potential.[146,147]

When tested at a constant tenfold initial K^+ gradient, the effects of valinomycin on Na^+-stimulated amino acid transport varied markedly with valinomycin concentration, reaching a maximal stimulation at a concentration above which no stimulation was observed.[54]

At the optimal valinomycin concentration, the magnitude of $TPMP^+$ accumulation increased as a function of increasing magnitudes of K^+ gradients imposed across the membrane,[134] as predicted if the interior-negative membrane potential is mainly determined by the K^+ gradient. This provides a means of artificially generating a series of magnitudes of interior-negative membrane potentials in order to test effects on amino acid accumulation. It was observed that Na^+-stimulated alanine accumulation also increased as a function of the magnitudes of this series of K^+ gradients in the presence of valinomycin, exceeding $TPMP^+$ accumulation in each case.[134] The logarithm of the apparent accumulation ratio of $TPMP^+$ in vesicles was a linear function of the logarithm of the accumulation of alanine in vesicles. The slope of this correlation indicated that two molecules of alanine accumulated per molecule of $TPMP^+$[134] when tested in the presence of a Na^+ gradient. These data indicate that the electrical potential difference across the membrane contributes to the driving force for amino acid transport. This transduction of energy occurs as a direct consequence of an obligatory coupling of the Na^+-stimulated amino acid translocation step to membrane potential.

Substitution of gradients of NaSCN or $NaNO_3$ for those of NaCl produced a remarkable stimulation of alanine or 2-aminoisobutyric acid accumulation. This stimulation by anion was not observed in the absence of Na^+. Presumaby, amino acid transport activity is enhanced in the presence of SCN^- or NO^-_3 because these anions are more freely permeant to biological membranes than Cl^- and thus are better able to facilitate charge compensation during amino acid translocation. Less permeant anions such as $SO^=_4$ caused inhibition of amino acid transport.

The artificial imposition of a permeant anion gradient across the membrane provides an alternate method to generate an interior-negative membrane potential without addition of ionophores. This was verified by measurements of $TPMP^+$ accumulation.[134] For example, addition of external 50 m*M* Cl^- generated a potential of −39.5 mV as estimated from TPMP accumulation. Membrane potential generated from an anion diffusion potential was less sensitive to the nature of the cation; substitution of Na^+ or K^+ for choline caused a small depolarization, presumably due to inward leakage of cation.

As a corollary, uptake of ^{14}C-thiocyanate in vesicles was increased in the presence of alanine and Na^+.[134] This finding is consistent with the view that alanine transport stimulated by Na^+ causes a transient depolarization by translocation of a positive charge inward.

Na^+ was required for the response of amino acid translocation to an interior-negative membrane potential generated either by anion gradients or valinomycin-induced K^+ diffusion potentials. Table 6 shows that although an interior-negative membrane potential, reflected by TPMP accumulation, was generated in the absence of Na^+, it could not be utilized to stimulate amino acid accumulation.

A chemical difference in Na^+ concentration across the membrane could be utilized to drive active amino acid accumulation in vesicles in the absence of electrical effects. Membrane potential could selectively be dissipated when internal and external K^+ concentrations were made equal in the presence of valinomycin and a Na^+ gradient (Table 6). This could be confirmed by the absence of $TPMP^+$ accumulation above its external

TABLE 6

Electrogenic Amino Acid Translocation: Sodium Dependence

Additions[a]		Apparent conc. gradient[b] (C_i/C_o)	
Ionophores	Salts (50 m*M*)	$TPMP^+$	Alanine
Valinomycin	NaCl	4.7	8.5
	Choline Cl	6.4	2.1
	NaCl + KCl	1.3	4.8
	Choline Cl + KCl	1.4	1.6
Valinomycin + monensin	NaCl	5.9	4.6
	Choline Cl	—	2.6
	NaCl + KCl	1.1	1.8
Valinomycin + nigericin	NaCl	—	2.6
	Choline Cl	—	1.6

[a] Vesicles from SV3T3 were first incubated for 15 min with 50 m*M* KCl and the indicated ionophores in 1% dimethylsulfoxide. Then separate aliquots of vesicles were diluted tenfold and incubated either with 20 μM ^{3}H-triphenylmethylphosphonium ion ($TPMP^+$) and 50 m*M* each of the indicated chloride salts or with 0.2 m*M* L-[2,3-^{3}H] alanine and 50 m*M* of each chloride salt. This manipulation resulted in a tenfold potassium ion diffusion potential (internal > external), with the exception of those incubations indicated where 50 m*M* 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.

concentration. Under these conditions, a four- to fivefold accumulation of alanine was stimulated in the presence of a 50 m*M* NaCl gradient (Table 6). This component of alanine accumulation due to a chemical Na^+ gradient was dissipated after the further addition of monensin (Table 6). Such a demonstration of a chemical contribution of Na^+ confirms predictions based on the specific Na^+-amino acid cotransport model outlined in Figure 4.

Monensin does not affect electrical membrane potential under these conditions as shown by its lack of effect on $TPMP^+$ accumulation in vesicles; therefore, its effects on amino acid transport in the presence of an interior-negative membrane potential (Figure 5 and 7, Table 6) must reflect primarily the dissipation of the chemical Na^+ gradient. The residual amino acid accumulation observed in the presence of monensin reflects the contribution from electrical differences across the membrane.

It can therefore be concluded that the total driving force available for concentrative A system amino acid uptake, μ_{Na^+} can utilize additively or separately an electrical potential difference, $\Delta\psi$, and a chemical difference in Na^+ concentration, such that

$$\mu_{Na^+} = \Delta\psi - 2.3\,(RT/F)\log([Na_o^+]/[Na_i^+])$$

The polarity of the response of Na^+-dependent amino acid transport is formally equivalent to a transport cycle coupled to either movement of positive charge inward or to movement of negative charge outward. The former possibility can be visualized

at the formation of a positively charged complex of Na^+, amino acid and carrier during translocation. In this model, since transport is insensitive to membrane potential in the absence of Na^+ and the amino acid substrate is predominantly translocated as the zwitterion,[124] one would predict that the carrier molecule itself is uncharged. The second model predicts that the free carrier molecule is negatively charged and the translocating complex is uncharged. The preferred orientation of the negatively charged carrier molecule would respond to membrane potential and contribute to the net driving force for transport. Additional evidence will be required to unequivocally decide between these models.

Contribution of pH

Proton gradients make a secondary, nonobligatory chemical contribution to Na^+-dependent amino acid transport as identified by the pH dependence of Na^+ stimulation. pH profiles observed for rates of Na^+-stimulated 2-aminoisobutyric acid or alanine transport into vesicles[54] resembled those previously described for these systems in intact cells (Figure 9).[111] Transport of these amino acids in the absence of Na^+ did not vary appreciably with pH. Proton gradients are not obligatorily coupled to Na^+-stimulated amino acid transport, as shown by lack of inhibition by proton conductors such as nigericin, 2,4-dinitrophenol and FCCP. Since the external pH optimum for Na^+-stimulated amino acid transport in vesicles is the same pH as the buffer enclosed within the vesicles, no pH gradient exists across the membrane under conditions of maximal uptake.

Regulation of Amino Acid Transport

The cellular proliferative state or transformation by Simian virus 40 (SV 40) of mouse fibroblast cultures had a profound effect on A system transport activity in their isolated membrane vesicles. These changes mirror changes in the activity of this system in intact cells. The V_{max} of 2-aminoisobutyric acid transport activity of nontransformed mouse fibroblasts diminishes as cultures reach confluence, and increases in this parameter accompany viral transformation.[133] It remains to be established whether the ability of this transport system to monitor the physiological state of the cell can be dissociated from the regulation of cell proliferation.

Table 7 summarizes transport specific activities observed in membrane vesicles from nontransformed and SV40-transformed cells. Both initial rate and quasi-steady state of Na^+-stimulated amino acid accumulation in vesicles were increased accompanying SV40 transformation (Figure 10). In vesicles from nontransformed cells, both these parameters were decreased as cells approached confluence.[52,53] The initial rates of adenosine transport, which are relatively invariant in these cells, were also measured as an internal control in addition to measurement of marker enzyme specific activity to estimate variation due to unsealed vesicles or altered distributions of plasma membrane vesicles in each preparation. The altered rates of Na^+-stimulated amino acid transport activity in vesicles were expressed as changes in V_{max}. Na^+-independent transport activity showed much less variation. Similar observations of changes in membrane vesicle transport activities accompanying growth regulation were made by Quinlan and Hochstadt[50] for uridine transport in mouse fibroblasts, as also illustrated in internal controls shown in Table 7.

Table 8 lists several possible mechanisms for cellular regulation of Na^+-gradient-coupled transport systems. The Na^+, K^+, ATPase transport system is rapidly activated by serum[148] or physiological concentrations of insulin and prostaglandin $F_{2\alpha}$[149] without requirement for protein synthesis. These observations imply that cytoplasmic membrane potential and Na^+ gradients, which are maintained by the Na^+ pump, are directly

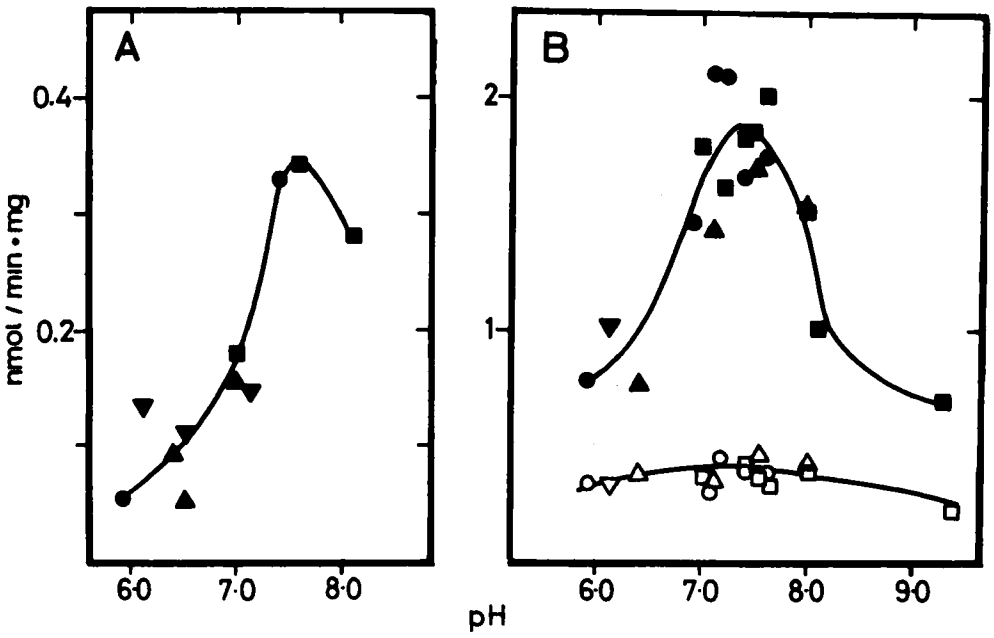

FIGURE 9. Effect of pH on the initial rate of Na^+ gradient-stimulated (A) 2-aminoisobutyric acid and (B) alanine uptake in membrane vesicles. (A) 50 m*M* buffer, 0.17 m*M* 1-[^{14}C]-2-aminoisobutyric acid and 50 m*M* NaCl were added at zero time, (B) 60 m*M* buffer, 0.17 m*M* L-[2,3^{3}H]-alanine and 50 m*M* NaSCN were added at zero time. The Na^+-independent rate, determined using 50 m*M* choline chloride in (A) or 50 m*M* KSCN in (B), shown as open symbols, has been subtracted. (●), Tris-phosphate; (■), Tris-HCl; (▲), potassium phosphate; (▼), 2-[bis(2-hydroxyethyl) amino] ethanesulfonic acid. (From Lever, J. E., *J. Biol. Chem.*, 252, 1990, 1977. With permission.)

regulated by certain hormones, providing a means for rapid and transient regulation of transport systems coupled to Na^+ or electrical gradients.

In addition to regulation of the generation of these gradients, the dissipation of Na^+ and electrical gradients, possibly by an independent pathway, could represent another point of cellular regulation. Since quasi-steady state amino acid accumulation in vesicles should be largely independent of the number of carriers, reflecting instead the net driving force acting on the carriers, changes which affect this parameter in vesicles probably reflect changes in membrane permeability to Na^+. One may draw this conclusion since a standard initial Na^+-gradient driving force is applied to the vesicle assay system. Furthermore, since the apparent K_m for amino acid transport in vesicles, which varies as a function of Na^+ concentration (Figure 6) did not show changes which could be correlated with growth regulation, the interaction of Na^+ with the carrier is probably not altered in these preparations. Comparative effects of monensin on amino acid efflux from vesicles from these cell types reinforced the conclusion that membranes from quiescent nontransformed cells had a higher Na^+ permeability which could account for their amino acid transport differences.[52] Part of the increased amino acid transport activity (V_{max}) in vesicles from SV40-transformed cells could be due to changes in the number or mobility of carriers, since this increased activity persisted when assayed in the presence of monensin.[52]

Possible Role of Protein Kinase in Mediating Permeability Changes in Mouse Fibroblasts

Multiple polypeptides in the plasma membrane of various cell types are substrates for enzymatic phosphorylation by endogenous protein kinases,[150] but the significance

TABLE 7

Specific Activities of Solute Uptake in Isolated Membranes[a]

Source	No. of preparations	Uptake activities[b,c] (pmol/min/mg protein)			
		Na^+-independent iso-Abu	Na^+-gradient-dependent iso-Abu	Adenosine	Uridine
Balb/c tertiary mouse embryo fibroblasts[d]					
Confluent	5	560	440	1.7	0.10
Subconfluent	3	810	1300	2.6	0.20
Balb/c SV3T3					
Confluent	3	870	960	2.9	0.19
Subconfluent	2	930	2300	2.0	0.19
Swiss 3T3K					
Confluent	2	210	190	1.3	0.04
Subconfluent	2	600	440	1.7	0.26
Swiss SV3T3					
Confluent	2	530	660	2.2	0.32
Subconfluent	2	687	480	1.9	0.13

[a] Recovery of protein and marker enzymes for mixed plasma membrane preparations from each source was described previously.[48,53]

[b] Incubations were 30 sec and 1 min with 50 m*M* NaCl and either 1 m*M* ^{14}C-2-aminoisobutyric acid (iso-Abu), 0.5 μM ^{3}H-adenosine or 0.2 μM ^{3}H-uridine. Initial rates of uptake for each solute were assayed at concentrations below their K_m value to minimize entry by simple diffusion. The Na^+-independent rate of amino acid uptake was determined using 50 m*M* choline chloride substituted for NaCl. This value was subtracted from uptake observed using 50 m*M* NaCl, to give the Na^+-gradient-dependent uptake.

[c] Results were averaged from duplicate determinations repeated two or three times on each preparation. Variation was ± 10 to 25% among duplicates.

[d] MEF — mouse embryo fibroblasts.

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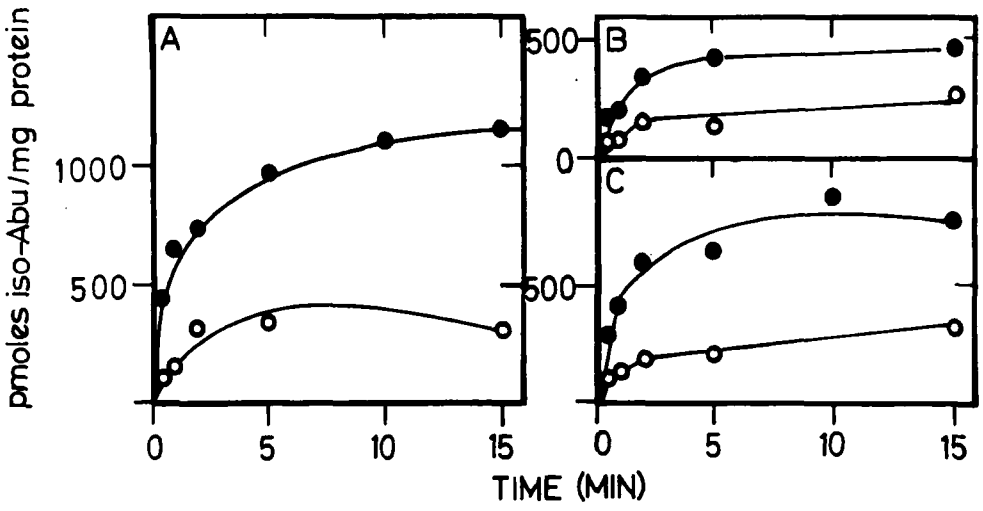

FIGURE 10. Uptake of 2-aminoisobutyric acid (iso-Abu) into membrane vesicles from Balb/c 3T3 cells transformed by SV40 (A), confluent (B), and subconfluent (C) Balb/c tertiary mouse embryo cells as a function of time. 0.17 m*M* ^{14}C-2-aminosobutyric acid and 50 m*M* NaCl (●) or 50 m*M* choline Cl (o) were added at zero time. (From Lever, J. E., *Proc. Natl. Acad. Sci. U.S.A.*, 73, 2614, 1976. With permission.)

of membrane phosphorylation to regulation of membrane function and to growth control is open to conjecture.

Compelling correlative evidence for a role of phosphorylation of specific membrane proteins in regulating membrane permeability has been suggested for the regulation of insulin-stimulated glucose transport in fat cells,[151] the stimulation of Ca^{++} transport by epinephrine in cardiac sarcoplasmic reticulum,[152] rapid triggering of electrical potential changes in postsynaptic neurons by neurotransmitters,[153] the stimulation of Na^+ permeability in erythrocytes by isoproterenol[154] and in toad bladder by aldosterone,[155] and in mediating a decrease in Ca^{++} permeability in rat brain synaptosomes.[156] In mammary epithelial explants,[157] insulin plus hydrocortisone stimulated the phosphorylation of several plasma membrane proteins by both cyclic AMP-dependent and cyclic AMP-independent endogenous protein kinases. Protein kinase activities have been localized in plasma membrane fractions of a variety of cells as well as in soluble, cytoplasmic fractions.[150,158]

Addition of ATP to the external medium of mouse fibroblast cultures resulted in phosphorylation of surface membrane proteins by an endogenous protein kinase activity[159] and triggered complex permeability changes.[160] The physiological significance of these observations is unknown.

As a further step in investigating the functional significance of membrane protein phosphorylation in fibroblast cultures, a cell-free system was developed. Plasma membrane vesicles isolated from either nontransformed or SV40-transformed mouse fibroblasts contained an endogenous protein kinase activity which catalyzed autophosphorylation of membrane proteins in the presence of ATP.[161] When ATP was added to these membrane vesicle preparations, a striking inhibition of transport catalytic activity for amino acids, phosphate ion, and uridine was produced.[161]

Figure 11 shows that previous incubation of vesicles from SV40-transformed fibroblasts with 5 m*M* ATP and 5 m*M* $MgCl_2$ for 20 min abolished their capacity for Na^+-stimulated alanine transport activity. Similar results were obtained using vesicles from nontransformed cells. This effect was not observed when ATP and Mg^{++} were added to vesicles at the time of initiation of the transport assay. The inhibitory effect of ATP and Mg^{++} increased as a function of time of preincubation with vesicles, with maximal

TABLE 8

Possibilities for Cellular Regulation of Gradient-Coupled Systems

1. Regulation of the activity of the Na^+, K^+, ATPase
2. Membrane permeability to Na^+
3. Membrane potential
4. Number of carriers
5. Mobility
6. Modification of activity of carriers
7. Charge dissipation

effect at 20 min. Inhibition of Na^+-stimulated Pi transport and uridine uptake in vesicles were also observed (Table 9).[161]

Although vesicles incubated 20 min with deoxy ATP or ADP also showed alanine transport inhibition, no inhibitory effects were observed after treatment with several other nucleosides or nucleotides (Table 10). Furthermore, the α-β or β-γ-methylene ATP analogues were ineffective (Table 10). Mg^{++} was required for ATP inhibition of transport activity. ATP effects were not due to chelation since pretreatment of vesicles with 5 m*M* EDTA and 5 m*M* $MgCl_2$ produced no transport inhibition (Table 10). Inhibitory activity increased as a function of ATP concentration and showed a pH profile distinct from the pH-dependence of Na^+-stimulated alanine transport in vesicles,[54] as shown in Figure 12. Control experiments indicated that ATP treatment did not induce nonspecific membrane leakiness to other diffusible small molecules.[161] Taken together, these observations suggest a working hypothesis that a Mg^{++}- requiring specific enzymatic ATP hydrolysis acts at the level of the plasma membrane to convert certain membrane transport systems to an inactive form.

When γ-^{32}P-ATP was added to vesicles under the same conditions which produced transport inhibition, label was transferred to multiple plasma membrane proteins, resolvable by polyacrylamide gel electrophoresis. The stability of the introduced phosphoryl residues were those expected of the phosphoserine or phosphothreonine products of protein kinase activity.[161] A protein kinase activity accompanied plasma membranes during purification steps.[161] Membrane-associated protein kinase activity catalyzed phosphorylation both of plasma membrane proteins and of exogenously added phosphate acceptor proteins such as histone. A slight stimulation of membrane phosphorylation by cyclic AMP at pH 6.5 but not at pH 7.5 was noted.[161]

Additional evidence directly correlating membrane phosphorylation with transport changes will be required in order to firmly establish a cause-effect relationship between these phenomena. Furthermore, functional changes created and detected in vesicles must be compared with corresponding changes in membrane permeability and membrane phosphorylation in living cells in order to establish their physiological significance.

Intestinal Epithelial Membranes

A striking feature of the functional organization of the enterocyte, or intestinal epithelial cell, is the polarization of the cell surface into functionally discrete, noninterchangeable and nonintermixing separate plasma membrane domains. The intact enterocyte of the small intestine is positioned such that part of its surface membrane, known as the brush border or microvillus membrane, is facing the lumen of the intestine, whereas at the opposite pole of the cell, the basal or basal-lateral plasma membrane faces blood and lymph vessels (serosal compartment). It has been determined that hydrolytic enzymes such as disaccharidases, alkaline phosphatases, and leucyl naphthylamide hydrolase are confined to the brush border surface, while the (Na^+ + K^+)-dependent ATPase, adenylate cyclase, and 5′-nucleotidase are restricted to the serosal

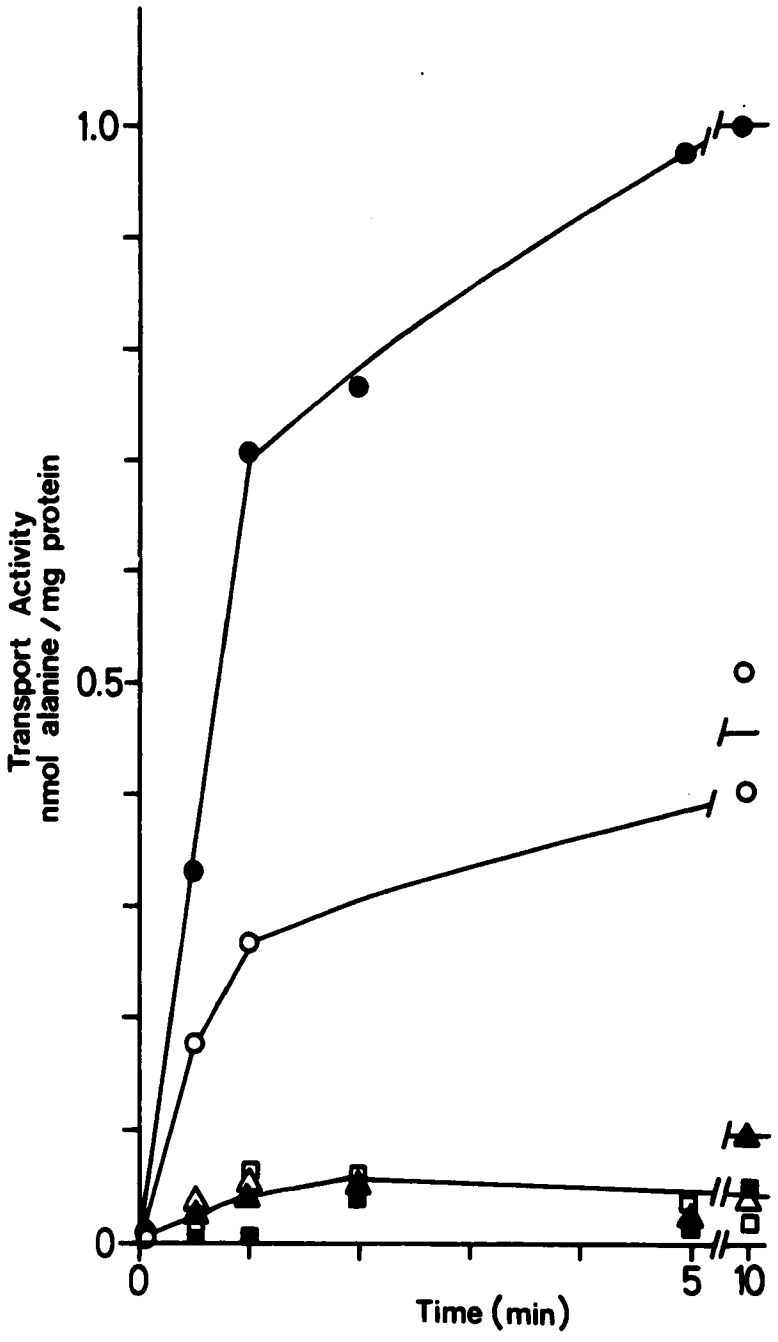

FIGURE 11. Inhibition of alanine transport activity of membrane vesicles by ATP. Vesicles from SV3T3 were incubated 30 min at 37° with (▲, △, ■, □) or without (●, ○) 5 mM ATP and 5 m*M* $MgCl_2$ plus (■, □) or minus (▲, △, ●, ○) 1m *M* cyclic AMP. Then vesicles were diluted five fold into assay mixtures containing 0.2 m*M* ^{3}H-alanine and either 50 m*M* NaCl (filled symbols) or 50 m*M* choline chloride (open symbols) and assayed for alanine transport activity as described previously.[54] Control samples, not pretreated with ATP and Mg^{++}, to which 1 m*M* ATP and 1 m*M* $MgCl_2$ were added at zero time during assay of alanine transport, showed no inhibition of alanine transport activity compared with untreated controls. (From Lever, J. E., *Biochem. Biophys. Res. Commun.*, 79, 1051, 1977. With permission.)

TABLE 9

Inhibition of Several Transport Activities Observed After ATP Treatment

Transport substrate	Relative transport activity after ATP treatment (% control)
Alanine	6
Uridine	< 3
Phosphate	13
TPMP$^+$	103

Note: Vesicles from SV3T3 were treated 30 min with 5 m*M* ATP and 5 m*M* $MgCl_2$ at 37°C. Transport activities for each solute were assayed. Alanine transport activity was determined using a 50 m*M* NaCl gradient and 10 min incubation with 0.2 m*M* ^{3}H-alanine. Uridine transport was measured 15 min after addition of 0.2 μM ^{3}H-uridine. Phosphate ion transport was determined at 10 min after addition of 0.5 m*M* K^+ $^{32}P_i$ in the presence of a 50 m*M* NaCl gradient (external > internal). Uptake of 20 μM ^{3}H-TPMP$^+$ was determined at 15 min after addition in the presence of a 50 m*M* NaCl gradient. Results are average of duplicates expressed as % of transport activity relative to control membranes incubated in the absence of ATP. 100% represents 1 nmol alanine per milligram; 0.17 pmol uridine per milligram; 3.8 nmol phosphate per milligram; 46.3 pmol TPMP$^+$ per milligram.

From Lever, J. E., *Biochem. Biophys. Res. Commun.*, 79, 1051, 1977. With permission.

TABLE 10

Specificity of Alanine Transport Inhibition by ATP

Addition (5 m*M*)	Alanine transport activity (pmol mg^{-1})	
	30 sec	10 min
None	560	840
$MgCl_2$ alone	506	1008
ATP	130	170
DeoxyATP	150	200
ADP	200	300
Adenosine	490	810
GTP	350	710
GMP	380	780
Dibutyryl-2′,3′-cyclic AMP	420	800
α,β-Methylene ATP	486	869
β-γ-Methylene ATP	502	1027
ATP + EDTA	400	790

Note: Vesicles from SV3T3 were incubated 30 min at 37°C with 5 m*M* of the indicated addition plus 5 m*M* $MgCl_2$, then diluted fivefold and assayed for alanine transport activity. From Lever, J. E., *Biochem. Biophys. Res. Commun.*, 79, 1051, 1977. With permission.

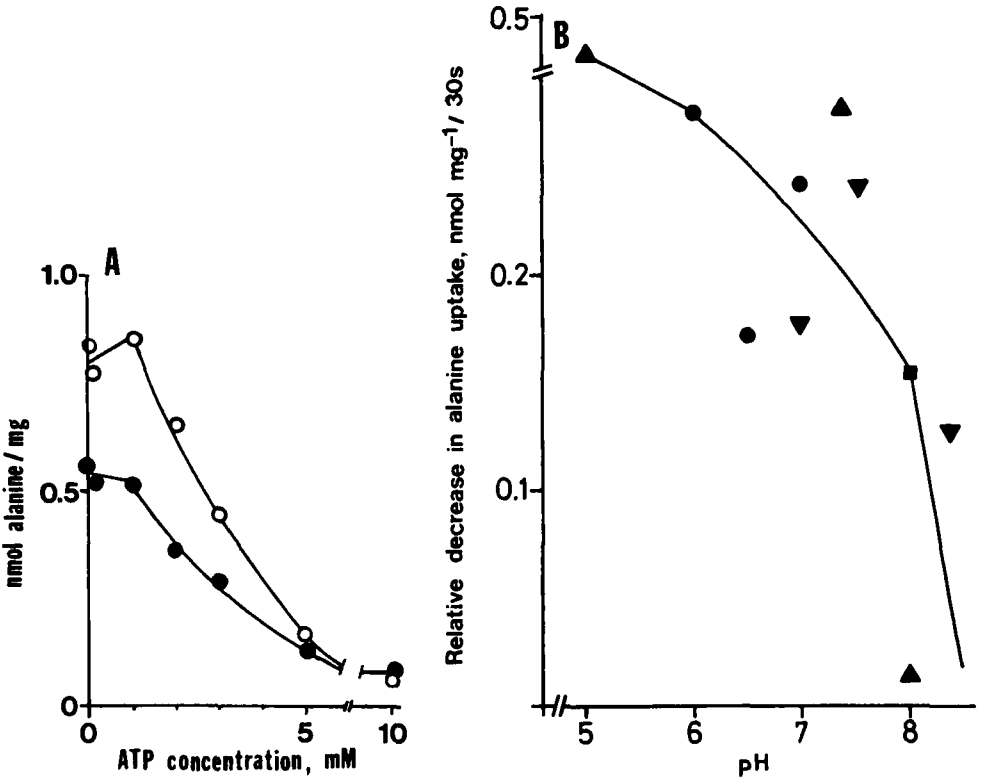

FIGURE 12. (A) Alanine transport inhibition as a function of ATP concentration. Vesicles were treated for 30 min with the indicated concentration of ATP and $MgCl_2$ before dilution and assay of alanine transport at 30 sec (O) and 10 min (o); (B) pH-dependence of alanine transport inhibition by ATP. Vesicles from SV3T3 were incubated 30 min at 37° with 5 m*M* ATP and 5 m*M* $MgCl_2$ in 25 m*M* buffer at the indicated pH. Transport activity observed in incubations at each pH in the absence of ATP and Mg^{++} were subtracted. Alanine transport was assayed[54] 30 sec after dilution of vesicles, using 50 m*M* NaCl, 0.2 m*M* ^{3}H-alanine and 20 m*M* Tris-phosphate, pH 7.5 (▲), Tris-phosphate; (●) 2-[N-morpholino] ethane sulfonic acid; (■), Tris-HCl; (▼), *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid. (From Lever, J. E., *Biochem. Biophys. Res. Commun.*, 79, 1051, 1977. With permission.)

face of the membrane (Table 1).[13,14,24] An important contribution was provided by techniques for the separation of vesiculated enterocyte plasma membranes into two relatively purified populations: one enriched for markers of the brush border surface, the other enriched for markers of the basal surface.[27,28] Subsequent analysis of the transport properties of vesicles of each type of membrane permitted the selective characterization of the transport systems of each face of the cell without interference from the other. Thus, it has been established that brush border membrane vesicles contain Na^+ gradient-coupled systems for amino acid,[162] glucose,[17,26,163] and phosphate[164] transport, as well as a Na^+/H^+ antiport[165] system and a possible Cl^-/H^+ symport system.[166] By contrast, basal lateral vesicles, which contain the bulk of cellular ($Na^+ + K^+$) ATPase activity, are devoid of appreciable Na^+-nonelectrolyte cotransport systems but contain facilitated diffusion systems for amino acids and glucose.[167]

Active alanine transport was first demonstrated in vesicles from the brush border of intestine.[162] Standard criteria such as osmotic sensitivity and countertransport proved that alanine was transported into an intravesicular space. When a Na^+ gradient was imposed across the membrane, a transient overshoot of alanine accumulation in vesicles was observed, with maximal accumulation at less than 30 sec after initiation of uptake. Within 3 min, accumulation decayed to a value one half to one fourth the maximal level, maintaining a relatively constant steady state. Preincubation with Na^+

abolished the overshoot. Presumably the overshoot reflected the response to dissipation of the Na^+ gradient during incubation. No overshoot of uptake was observed for transport of the D-isomer in the presence of a Na^+ gradient. Although intravesicular volume was not measured, these observations suggested transient active accumulation occurred during the overshoot. Stimulation was specific for Na^+. Na^+-stimulated alanine uptake was further stimulated when the permeant anion thiocyanate was substituted for Cl^- as the major counterion, suggesting that amino acid transport was electrogenic, stimulated when charge compensation was facilitated. This concept of charge translocation coupled to amino acid movement was extended by testing the effects of two alternate means for generation of an interior-negative membrane potential: a pH gradient ($H^+_{in} > H^+_{out}$) in the presence of the proton ionophore FCCP, or a K^+ gradient ($K^+_{in} > K^+_{out}$) in the presence of valinomycin. These conditions of electrical asymmetry produced a further transient enhancement of Na^+-stimulated alanine uptake in vesicles. The effects of Na^+ on the kinetics of alanine transport were not evaluated.

By contrast, alanine transport in intestinal basal-lateral membranes was much less stimulated by a Na^+ gradient, showing properties expected of facilitated diffusion.[167]

Possibly the Na^+/H^+ antiport system described for renal and intestinal brush borders[165] facilitates Na^+ gradient dissipation in the experiments where overshoot of alanine accumulation in these membranes was observed. Furthermore, it was demonstrated that addition of Na^+ salts to brush border vesicles resulted in rapid acidification of the medium followed by proton uptake due to the activity of the Na^+/H^+ exchange system and anion transport systems.[165,166] In retrospect, the Na^+/H^+ antiport system would complicate the interpretation of the above experiments in which the effects of a Na^+ gradient or effects of a pH gradient across the membrane in the presence of FCCP were tested,[162] due to coupled effects between H^+ gradients and Na^+ gradients. The Na^+/H^+ exchange system may drive active proton excretion in the small intestine and renal proximal tubule. Preliminary evidence[166] indicated that Cl^- transport across the brush border membrane may be mediated either by an electroneutral chloride/cation exchange or by Na^+/H^+ exchange in combination with Cl^-/H^+ symport. The measured rate of anion uptake in these membranes showed good agreement with the efficiency of these anions in facilitating Na^+-dependent nonelectrolyte cotransport.[166]

Renal Proximal Tubule Plasma Membrane Vesicles

Transport properties and functional specialization of kidney proximal tubule cells show certain remarkable similarities to those of small intestine. Polarization of the plasma membrane into two distinct components — the apical brush border and the peritubular basal-lateral membrane — is also a feature of renal epithelia.[13,14] This plasma membrane asymmetry is reflected in morphological, enzymatic (also see Table 1), and transport[168] differences.[13,14] Whereas several of these properties of kidney and intestinal brush borders are distinctive features unique to these tissues, most general characteristics of basal-lateral membranes resemble those of plasma membranes of other cell types. Exceptions are the A-type transport systems for neutral amino acids, which are Na^+-dependent in most tissues but Na^+-independent in basal-lateral membranes. This specialization permits net amino acids movement across the epithelial cell rather than net amino acid accumulation within the cell.

Transient active accumulation of phenylalanine,[169] L-alanine,[170] L-proline,[171,172] and glycine[172] was characterized in renal brush border membrane vesicles. Several lines of evidence similar to those listed in Table 4 demonstrated carrier-mediated electrogenic amino acid transport[169-171] driven by a Na^+ gradient across the membrane.[169-172] Increasing the Na^+ gradient lowered the K_m[170,171] for amino acid entry with minimal effect on V_{max}. Competitive interactions among neutral amino acids for transport into vesicles[170,172] were of special interest due to their relevance to genetic disorders of renal and intestinal amino acid transport in man: cystinuria, iminoglycinuria, Hartnup dis-

ease, and Fanconi syndrome.[173] Selective competition among individual amino acids was enhanced in the presence of a Na^+ gradient. This was similarly observed for glucose inhibition of amino acid uptake. These findings suggest that sugars and amino acids are coupled electrically. As a consequence, a major source of competitive interaction among these substrates is due to their dissipation of the Na^+ gradient and membrane potential during uptake.

Proline transport in renal basal-lateral membranes was relatively insensitive to Na^+.[168] This finding supports previous views that proline is actively transported across the renal proximal tubule by active Na^+-dependent uptake at the luminal (brush border) membrane face and a Na^+-independent downhill efflux at the contraluminal basal-lateral membrane.

These observations of active amino acid accumulation in renal and intestinal brush border membrane vesicles, dictated by a Na^+ gradient across the membrane, in the absence of their metabolic conversion, together with lack of impairment of amino acid transport in an individual with γ-glutamyl transferase deficiency,[174] tend to disprove the γ-glutamyl cycle hypothesis[125] to explain active amino acid transport. However, definitive proof in favor or against the hypothesis as an alternate or minor pathway of amino acid accumulation remains to be obtained.

PHOSPHATE ION TRANSPORT IN MEMBRANE VESICLES

Summary — Phosphate anion translocation across plasma membrane of fibroblast cultures, renal and intestinal brush border is coupled to Na^+ gradients across the membrane. These mechanisms are distinct from those utilized for P_i transport in erythrocyte and mitochondrial membranes.

Introduction

Regulation of intracellular levels of phosphate ion is an important factor in regulation of rates of glycolysis,[175] oxidative phosphorylation,[175] and the initiation of DNA synthesis in mouse fibroblast cultures.[15,16] Although anion transport systems in erythrocytes[176,177] and mitochondria[178] have been characterized extensively, the mechanism and mode of energy coupling of phosphate ion transport across the plasma membrane of nucleated cells has been obscure until very recently. At least five distinct mechanisms for phosphate anion transport across various animal cell membranes have been proposed.

Phosphate transport in erythrocytes is a saturable, nonconcentrative process with properties expected of a facilitated diffusion mechanism governed by the Donnan relationship.[176] This pathway is inhibited by chemical reagents such as 4,4′-diisothiocyano-2,2′-stilbene disulfonate which react covalently with a 95,000 dalton glycoprotein implicated in anion permeability which spans the membrane.[176,177] Chloride, phosphate, sulfate, and other anions compete for translocation via this system. Cation gradients are apparently not directly coupled to this system. Wolosin et al.[177] have succeeded in reconstituting functional erythrocyte anion carrier in vesicles formed from erythrocyte lipids.

Mitochondrial phosphate ion transport occurs via two types of carriers present in the mitochondrial inner membrane: the P_i/OH-exchange carrier[179,180] and the P_i/dicarboxylic acid exchange carrier.[181,182]

In striking contrast to these mechanisms, studies using membrane vesicles have demonstrated that P_i translocation across the plasma membrane is directly coupled to Na^+ gradients in the case of mouse fibroblasts[183] and intestinal brush borders.[184] These examples extend to the inorganic phosphate anion transport system concepts previously stated for organic solutes in the Na^+ gradient hypothesis.

Mouse Fibroblast Cultures

Rapid increases in phosphate ion uptake accompany growth stimulation in mouse fibroblast cultures by serum, hormones, and growth factors.[15,16,149] Levinson[185] had shown that phosphate transport into Ehrlich ascites cells is mediated by a carrier which also recognizes arsenate. Basal and stimulated levels of phosphate uptake in fibroblasts were partially inhibited by ouabain.[149] This suggested that phosphate accumulation is coupled to Na^+ pump activity. Scholnick et al.[175] had observed that Na^+ was required for P_i transport in Ehrlich ascites cells, but did not pursue the implications of this observation.

The first evidence that energy utilized for active phosphate ion transport across the plasma membrane of mouse fibroblasts is derived from an electrochemical Na^+ gradient across the membrane was provided by studies using plasma membrane vesicles.[183] Carrier-mediated, active phosphate ion transport activity, which accompanied plasma membrane material during subcellular fractionation, could be demonstrated, with an apparent K_m of 0.1 m*M* at pH 6.5. No metabolic conversion of phosphate ion in vesicles was detectable. The central observation of these studies was that imposition of a Na^+ gradient (external > internal) across the membrane dramatically enhanced P_i accumulation in vesicles, as shown in Figure 13. An eightfold accumulation was observed. Accumulation was decreased after preincubation with Na^+ (Figure 13). This suggested that active P_i accumulation is energized by a Na^+ gradient across the membrane and P_i transport occurred by cotransport with Na^+.

Interestingly, coupling of P_i transport to Na^+ gradients was exquisitely sensitive to variation of external pH in the range 7 to 8, as illustrated in Figure 14. Na^+-independent P_i transport varied less than ± 15% in this pH range. At pH values above 7, stimulation of P_i transport by Na^+ was greatly decreased.

The property of stimulation by Na^+ differentiated plasma membrane transport from mitochondrial P_i carriers.[179-182] Furthermore, phosphate transport in plasma membrane vesicles was insensitive to succinate, malate, and citrate. By contrast, crude mitochondrial fractions showed marked stimulation of P_i uptake by succinate.[183] This observation distinguished P_i transport across the plasma membrane from the dicarboxylic acid-phosphate ion exchange carriers described for mitochondrial inner membrane.[181,182] The relative insensitivity to pH of P_i transport in the absence of Na^+[183] further differentiated this system from mitochondrial P_i/OH^- exchange carriers.[179,180] In addition, plasma membrane vesicle P_i transport was not affected by ATP, ADP, antimycin A, oligomycin, or ouabain.[183]

A tenfold proton gradient was artificially initiated across the membrane when vesicles containing Tris-HCl pH 7.5 were diluted to obtain an external medium buffered at pH 6.5. The possible contribution of such a pH gradient to energetics of P_i accumulation was investigated, using several ionophores expected to dissipate a pH gradient. As summarized in Table 3, monensin primarily dissipates Na^+ and H^+ gradients, whereas nigericin primarily dissipates K^+ and H^+ gradients, each without affecting electrical membrane potential. Table 11 shows that monensin reduced Na^+-stimulated P_i accumulation, but nigericin had much less effect. This is the result expected if Na^+ gradients provide the major driving force for P_i accumulation. Addition of monensin plus FCCP produced a further reduction to a level similar to that produced by Na^+ preincubation. This synergy presumably reflects the facilitation of Na^+ gradient dissipation due to proton entry mediated by FCCP. None of the ionophores reduced P_i accumulation to the 1.2-fold accumulation observed in the absence of Na^+. Whereas active mitochondrial P_i transport via P_i/OH^- exchange is coupled to pH gradients across the membrane and therefore short-circuited by proton ionophores, Na^+-independent P_i transport in plasma membrane vesicles was nonconcentrative and relatively insensitive to these ionophores. A chemical contribution of K^+ to P_i transport could not be demonstrated. The pH profile shown for Na^+-stimulated P_i tra[illegible]

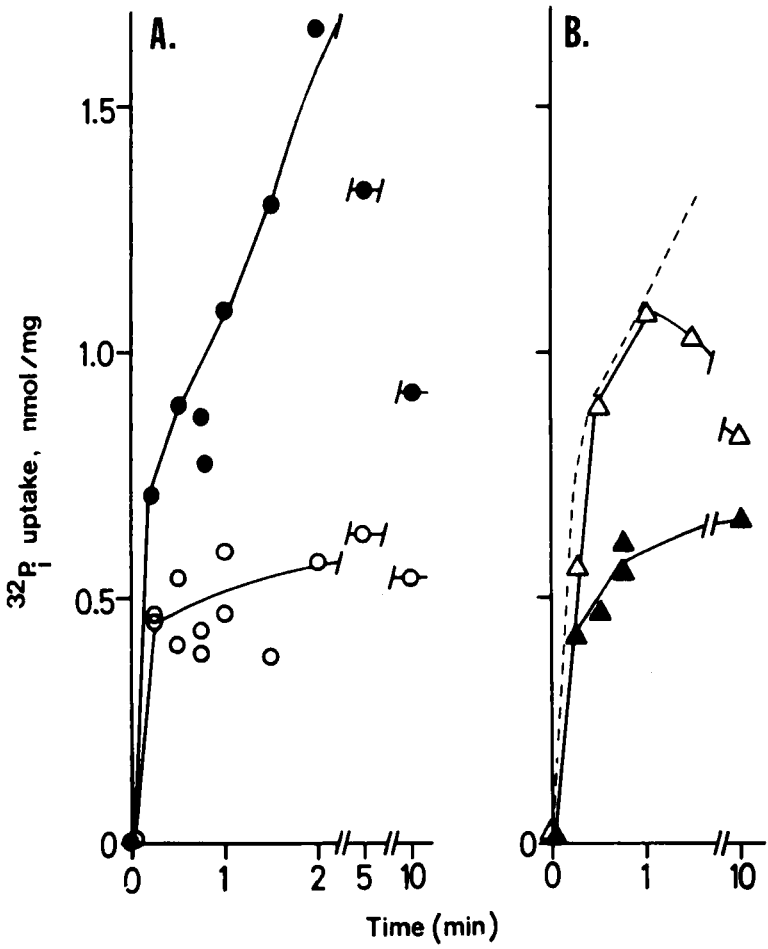

FIGURE 13. Na^+ gradient stimulation of phosphate transport at pH 6.5. At zero time, membrane vesicles (containing intravesicular 0.25 *M* sucrose, 10 m*M* Tris-HCl, pH 7.5) were diluted such that the external suspension medium contained 10 m*M* Tris-MES, pH 6.5, 0.1 m*M* Tris-$^{32}P_i$ and (A) either 50 m*M* NaCl (●) or 50 m*M* choline Cl (○). (B) Vesicles were preincubated with either 50 m*M* NaCl (▲) or 50 m*M* KCl (△) before transport assay as in (A). The dotted line shown in (B) indicates control uptake observed without salt preincubation or addition of ionophores, taken from filled circles in (A). (From Lever, J. E., *J. Biol. Chem.*, 253, 208, 1978. With permission.)

14 suggests that monovalent phosphate anion ($H_2PO_4^-$) is the predominantly translocated species cotransported with Na^+, since P_i transport activity closely paralleled the expected changes in the concentration of $H_2PO_4^-$ as a function of pH of the suspension medium. Stimulation of P_i transport by Na^+ was observed in the presence of proton-conducting ionophores. This observation indicates that Na^+/P_i cotransport can occur in the absence of a pH gradient across the membrane, although stimulation by Na^+ varies as a function of external pH. Proton translocation can accompany Na^+/P_i cotransport since $H_2PO_4^-$ accumulated in vesicles can dissociate at alkaline intravesicular pH values, yielding a proton internally. Since Na^+-dependent P_i transport showed a slight sensitivity to proton uncouplers, pH gradients may make an additive contribution to the total driving force. At present, regulation of cytoplasmic pH and pH gradients across the plasma membrane are not well understood. Thus it cannot be established whether regulation of cytoplasmic pH regulates Na^+/P_i cotransport.

The Na^+ stoichiometry and possible contribution of electrical forces to the total driving force acting on this system remain to be investigated. A 1:1 stoic[illegible]

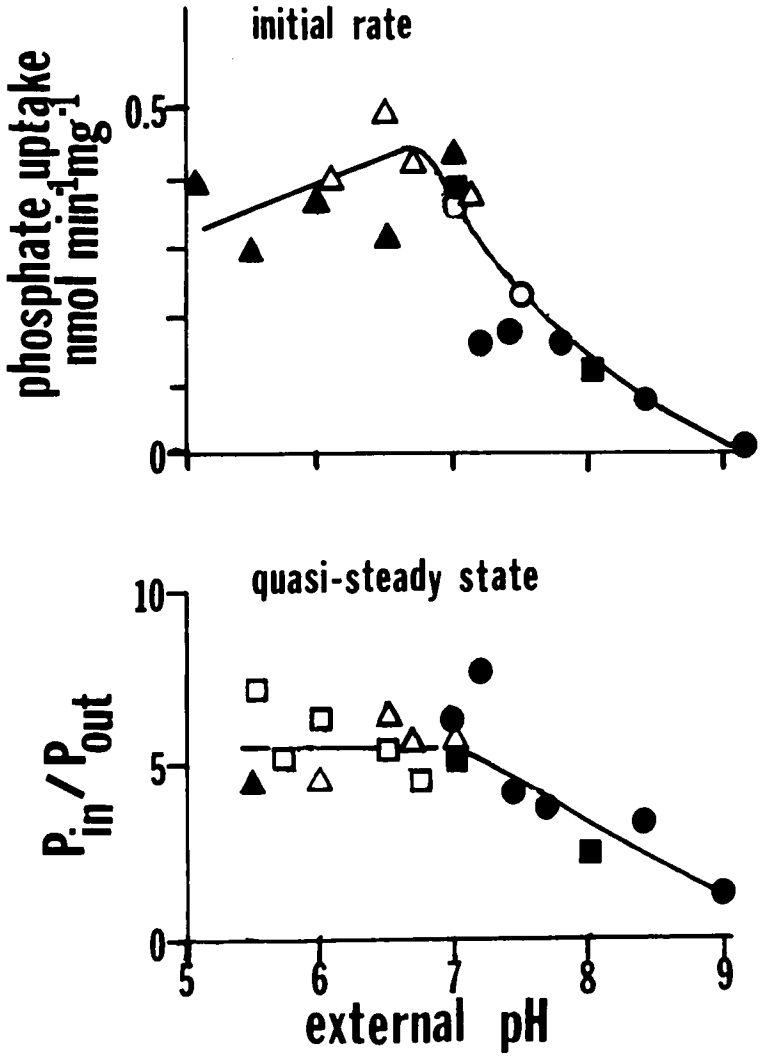

FIGURE 14. Effect of pH on initial rate and qausi-steady state of phosphate ion uptake. Incubation mixtures contained 50 m*M* NaCl, 0.1 m*M* Tris-^{32} P_i, and 50 m*M* of either Tris-HCl (●), sodium succinate (▲), N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid (○), N, N-Bis (2-hydroxyethyl) 2-amino ethane sulfonic acid (△), glycine (■), or Tris-MES (□) at the indicated pH. Vesicles contained intravesicular 10 m*M* Tris-HCl, pH 7.5. Uptake was assayed at 30 sec (initial rate) and 10 min (quasi-steady state). (From Lever, J. E., *J. Biol. Chem.*, 253, 2081, 1978. With permission.)

to PO_4^- would suggest an electroneutral transport mechanism. Alternately, transport of PO_4^- with more than one Na^+ could produce an electrogenic transport mechanism further stimulated by interior-negative membrane potential.

Intestinal Plasma Membrane Vesicles

Berner et al.[184] have demonstrated an electroneutral Na^+/P_i cotransport system in brush border membrane vesicles from rat small intestine. Several properties of this system show striking similarities to those described for mouse fibroblast plasma membranes.[183] In both the epithelial and fibroblast membranes, concentrative phosphate uptake was greatly stimulated by a Na^+ gradient (external > internal) across the membrane, decreased after Na^+ gradient dissipation by Na^+ ionophores or preincubation, and the degree of Na^+ stimulation closely paralleled external pH in a profile corresponding to the expected variation in $H_2PO_4^-$ concentration as a function of pH. Re-

TABLE 11

Effect of Ionophores on Na^+-Stimulated Phosphate Transport

Addition	Phosphate transport activity (pmol P_i/mg protein)	
	NaCl	Choline Cl
Control	1100 (6.1)	220 (1.2)
Nigericin	880 (4.9)	190 (1.1)
FCCP	910 (5.1)	200 (1.1)
Monensin	720 (4.0)	210 (1.2)
Monensin + FCCP	520 (2.9)	180 (1.0)

Note: Assay mixtures contained 0.1 m*M* Tris-[^{32}P] phosphate, 10 m*M* Tris-MES, pH 6.5, 50 m*M* of either NaCl or choline Cl and 1% (V/V) Me_2SO. Ionophore concentrations were: monensin, 14 μM; nigericin, 27 μM; FCCP, 200 μM. Maximal accumulation was estimated ± 15% using several time points in the interval from 30 sec to 5 min. The internal medium of vesicles contained 10 m*M* Tris-HCl, pH 7.5. Numbers in parentheses refer to the ratio of the apparent intravesicular P_i concentration to its external concentration, calculated using an intravesicular volume of 1.8 μl/mg protein. Total transport in the presence of NaCl is shown without subtraction of Na^+-independent uptake.

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sults in both cases suggest that P_i uptake appears to operate via $Na^+/H_2PO_4^-$ cotransport, although additional evidence will be required to establish Na^+ stoichiometry and P_i valency. Both systems show a similar apparent K_m of 0.1 m*M* and competitive inhibition by arsenate. By contrast, the relationship between pH and cation selectivity differs somewhat between fibroblast and intestinal membranes. In intestinal brush border vesicles, P_i transport at pH 6 showed decreased stimulation by Na^+ compared with choline Cl than observed at pH 7.4, and cation specificity was decreased. Rb^+, K^+, Cs^+, and Li^+ were almost as stimulatory as Na^+ at pH 6. At pH 7.4, Li^+ and Na^+ caused twice the stimulation of P_i uptake as K^+, Rb^+, or Cs^+.[184] These properties differ sharply from those of fibroblast P_i uptake in which stimulation by Na^+ is greatly decreased at pH 7.4, compared with pH 6.5, and specific for Na^+ at pH 6.5.[183]

It was demonstrated[184] that P_i transport into brush border vesicles at pH 7.4 was not influenced by electrical differences across the membrane created with valinomycin and K^+ gradients. At pH 6.0, P_i transport in intestinal vesicles was stimulated by conditions expected to create an interior-positive membrane potential. These observations were interpreted in favor of an electroneutral translocation of P_i and Na^+ across brush border membranes at pH 7.4. At pH 6.0, a Na^+-independent translocation of negatively charged phosphate anion becomes a major pathway for P_i entry. It is not clear whether the same carrier mediates both Na^+-dependent and Na^+-independent pathways.

The identification of a Na^+/H^+ antiport system in these membranes complicates the interpretation of these results. This system would catalyze proton expulsion from vesicles upon addition of external Na^+, creating a transiently alkaline vesicle interior and

a possible additional driving force for phosphate uptake by P_i/H^+ symport, as discussed above for the mitochondrial and fibroblast systems. However, nigericin plus external K^+ did not cause further stimulation of P_i uptake, although this condition would also be expected to produce a transient alkaline intravesicular space.[184]

Transfer of P_i as an electroneutral complex with Na^+ across the brush border membrane presents an interesting solution to the problem of anion accumulation in a cell with an interior-negative membrane potential. Coupling to Na^+ flux cancels the opposing electrical forces. These authors[184] speculate that membrane potential can act as an additional driving force in the Na^+-independent expulsion of negatively charged phosphate anion at the serosal membrane. Estimates of a 32-fold increased P_i accumulation across the epithelium (serosal > mucosal) might be explained by the cumulative contributions of such mechanism.[184]

GLUCOSE TRANSPORT IN MEMBRANE VESICLES

Summary — Whereas glucose enters most cell types by passive facilitated diffusion, brush borders of kidney and small intestine catalyze secondary active transport of glucose by an electrogenic Na^+ symport mechanism, stimulated by interior-negative membrane potentials.

Mouse Fibroblasts

A considerable increase in the capacity for metabolism of glucose from the medium is usually apparent in tumor cells and in fibroblast cultures after transformation by oncogenic viruses, compared with their normal counterparts.[15,16] This characteristic has been proposed as a necessary requirement for malignant growth in vivo[186] and used as a diagnostic for expression of the transformed cell phenotype in vitro. Similarly, reinitiation of DNA synthesis by serum or hormones in resting nontransformed mouse fibroblasts is accompanied by a striking increase in glucose uptake.[15,16] Attempts to establish the point of control of increased cellular uptake of hexoses have generally concluded that it results from specific increases in the number or activity of functional glucose transport sites in the plasma membrane rather than an enhanced intracellular sugar phosphorylation.[15,16,187,188] However, observations to the contrary have been reported,[189,190] and it has proven difficult to directly demonstrate this point using intact cells.

Membrane vesicles with functional transport carriers permit the assay of the membrane transport step separated from intracellular metabolism and subcellular compartments. By this means, changes in glucose uptake activity may be localized and characterized as an alteration in the permeability of the surface membrane.

The earliest studies of glucose transport in animal cell membrane vesicles used relatively impure plasma membrane preparations and were concerned mainly with differentiating substrate binding from facilitated diffusion. Thus, Carter and Martin[31] and Illinao and Cuatrecasas[29] provided the first reports of glucose uptake in plasma membrane vesicles from rat adipocytes. In rapid succession followed reports by Jung et al.,[191] Carter et al.,[192] and Taverna and Langdon[193] of glucose transport in vesicles prepared from erythrocyte ghosts. Subsequently, Na^+-dependent glucose transport was described by Busse et al.[194] in vesicles from renal tubule membranes and by Hopfer et al.[26] in vesicles from intestinal brush border membranes.

Figure 15 summarizes some characteristics of an assay system developed for glucose carrier activity in plasma membrane vesicles from mouse fibroblasts.[48] Exchange diffusion and osmotic sensitivity of accumulated glucose provided evidence for carrier-mediated uptake into an intravesicular space. Glucose accumulated in vesicles without metabolic conversion to glucose-6-phosphate. Uptake into vesicles was stereospecific, time dependent, and showed a temperature dependence such that influx and efflux

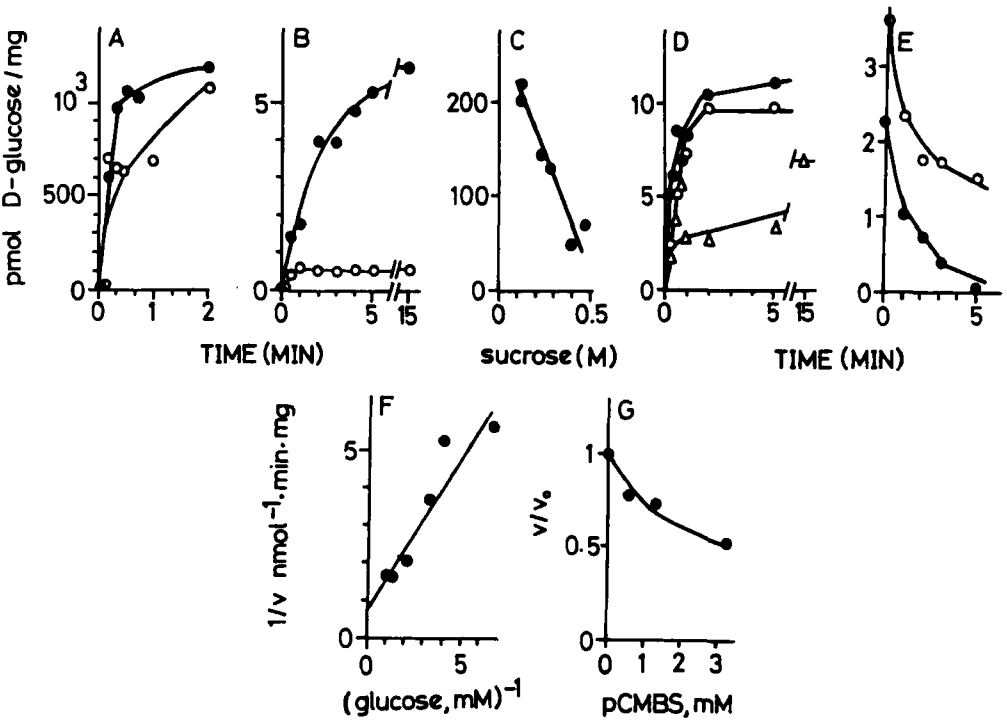

FIGURE 15. Glucose transport in plasma membrane vesicles from mouse fibroblasts. (A) Exchange diffusion. Vesicles were incubated 5 min with (●) or without (O) 5 m*M* D-glucose and then diluted to achieve equal specific activities of external 0.7 m*M* D-[1-^{3}H] glucose. Uptake was measured at the indicated times, (B) stereospecificity. Vesicles were incubated with 50 m*M* NaCl and either 4 μM D-[1-^{3}H] glucose (●) or 0.84 m*M* L-[U-^{14}C] glucose (O), (C) osmotic sensitivity. Vesicles containing 0.25 *M* sucrose and previously incubated 5 min with 0.2 m*M* D-[1-^{3}H] glucose were diluted to obtain the indicated external sucrose concentrations. Intravesicular glucose was determined after 5 min; (D) temperature dependence. Vesicles were incubated with 4 μM D-[1-^{3}H] glucose at 37°C (●), 21°C (O) or 2° (Δ); (E) phloretin inhibition of glucose efflux. (●), 1 m*M* phloretin in 1% Me_2SO; (O), 1% Me_2SO; (F) concentration dependence. Inverse reciprocal plot of the rate of glucose uptake into membrane vesicles as a function of external glucose concentration. Uptake was determined after 15 sec. Glucose concentrations from 0.15 to 1 m*M* D-[1-^{3}H] glucose were used; (G) inhibition by *p*-chloromercuriphenyl sulfonate. V/V_o refers to glucose transport activity at 15 sec relative to that of untreated controls. Vesicles were pretreated 15 min with inhibitor at the indicated concentration.

were accelerated as temperature increased from 2° to 37°C. Transport activity was mediated by components of the surface membrane because glucose transport activity closely paralleled the subcellular distribution of 5′-nucleotidase, a marker for plasma membrane.[224] Glucose transport was independent of Na^+ and membrane potential.

Evidence that glucose uptake into vesicles was mediated by components which accomplish glucose uptake into cells was obtained. Phloretin,[195] a specific inhibitor of glucose uptake in intact cells, inhibited glucose efflux from vesicles (Figure 15). Phloretin stimulated glucose uptake in vesicles, a reflection of its inhibition of glucose efflux. Similar observations were reported by Benes et al.[196] who suggested that phloretin is a nonpenetrating inhibitor which inhibits sugar flux from the side of the membrane *trans* to the side which interacts with this inhibitor. Phlorizin was without appreciable effect. D-Glucose transport into vesicles showed an apparent $K_m = 1.4 \pm 0.2$ m*M* with $V_{max} = 1.4$ nmol glucose/min · mg (Figure 15). An apparent $K_m = 5$ m*M* was measured for 3-*O*-methylglucose uptake into vesicles. The same value has been reported for cellular uptake of this nonmetabolizable glucose analogue.[190]

Interestingly, glucose transport into vesicles was insensitive to cytochalasin B, despite the presence of high-affinity binding sites for this drug in these membrane prep-

arations. Paradoxically, cytochalasin B was an extremely potent inhibitor of glucose uptake when tested in the intact cells used for vesicle preparations.[224] These findings suggest that cytochalasin B inhibits glucose uptake in fibroblasts by an interaction which becomes uncoupled from glucose transport activity after vesicle isolation.

An additional dissimilarity in properties was noted in comparing behavior of glucose transport activity in vesicles with that of intact cells. No appreciable variation in glucose transport specific activity was noted among vesicle preparations from growing and resting nontransformed mouse fibroblasts or their SV40-transformed counterparts.[48] In striking contrast, rates of 2-deoxyglucose uptake in the same intact cells used for vesicle preparation conformed with the expected fluctuations in activity:[15,16] a tenfold increase in activity in SV40-transformed cells compared with nontransformed cells and markedly reduced activity in nontransformed fibroblasts which had become quiescent. Estimates of variation in yield of transport-competent plasma membrane material would not be large enough to obscure the expected large differences in glucose transport activity. This demonstration that changes in cellular glucose uptake activity do not persist after vesicle isolation leaves unanswered the question of how uptake regulation is accomplished. Rates of glycolysis may serve as the major factor regulating rates of sugar transport, as suggested by Romano and Colby,[189] and in a recent reevaluation of this problem.[197] Alternately, control may be exerted by interaction of the glucose carrier with a factor which is dissociated after membrane isolation. The intriguing discovery by Lee and Lipmann[198] of a factor that binds glucose and stimulates its transport in normal and transformed chicken fibroblasts supports the latter hypothesis.

Intestine

Studies using separate populations of purified brush border and basolateral membranes permitted for the first time the selective evaluation of the contribution of each membrane face to the net concentrative transport of glucose across the epithelium of the small intestine. The first convincing demonstration of Na^+ gradient-stimulated, carrier-mediated accumulation of an organic solute in purified plasma membrane vesicles from a nucleated animal cell was the report by Hopfer et al.[26] of glucose transport in intestinal brush border vesicles. Subsequent investigations[163] established that glucose was actively accumulated in brush border vesicles in response to an imposed Na^+ gradient and Na^+-stimulated glucose transport is an electrogenic process further stimulated by conditions expected to generate an interior-negative membrane potential.

By contrast, glucose transport in basolateral membrane vesicles was largely independent of Na^+.[167] A further difference in glucose transport properties of the two types of membranes was their sensitivity to inhibitors of glucose transport. Glucose transport of brush border membrane vesicles was much more sensitive to phlorizin than that of basolateral vesicles.[167] Phloretin inhibited glucose uptake into basolateral vesicles, but had minimal effects on glucose uptake in brush border vesicles.[167] Cytochalasin B had no effect on glucose transport in brush border membranes, but strongly inhibited transport in basolateral vesicles.[167] The effects of membrane potential or gradients of other cations on basal-lateral glucose transport were not demonstrated.

These differential responses to Na^+ and inhibitors suggest that different types of glucose carriers reside on opposite faces of the intestinal cell. Whereas studies with intact epithelium did not permit unequivocal separate evaluation of the transport properties of the basolateral segment of the membrane, the assessment of the separate contributions of each membrane using vesicles led to direct evidence for the following scheme for transepithelial sugar absorption: Na^+/glucose cotransport at the brush border face driven by an electrochemical Na^+ gradient maintained by the Na^+, K^+, ATPase located at the basal-lateral membrane, followed by facilitated diffusion of glucose down its concentration gradient at the basal-lateral surface. A similar mechanism ac-

complishes transepithelial movement of amino acids as described in the previous section. Whereas interactions between amino acid and sugar absorption in the intestine had been interpreted in terms of a polyfunctional carrier which functioned for both types of nutrients, Murer et al.[199] provided evidence, using brush border vesicles, that this competitive interaction could be explained on the basis that both classes of nonelectrolytes were cotransported with Na^+ and were coupled electrically.

Hopfer[77] has drawn attention to the importance of determining kinetic parameters of Na^+-organic solute cotransport systems under conditions where the driving forces due to the Na^+ gradient and membrane potential are constant. In most measurements of Na^+-dependent carriers in vesicles, these driving forces imposed across the membrane tend to dissipate and vary as a function of time. Also, Na^+ and electrical gradients are expected to vary as a function of concentration of cotransported substrate, since fluxes of both species are coupled. Using the equilibrium exchange method, in which vesicles are preequilibrated with unlabeled solute and Na^+, Hopfer[77] determined kinetic parameters using isotopic flux in the absence of net substrate flux, at a constant Na^+ gradient. Interestingly, the K_m for glucose transport in brush border vesicles under these conditions was independent of Na^+ in contrast to results obtained in other Na^+-coupled systems[22,54,204] without preequilibration. V_{max} for glucose entry showed marked variation with Na^+ concentration. These results were those expected if the translocation step is the Na^+-sensitive step, such that the Na^+/glucose carrier complex moves much faster across the membrane than the glucose carrier complex in the absence of Na^+. However, the possibility exists that the equilibrium exchange protocol may bias measurements to minimize the contribution of the unloaded carrier. These parameters were not evaluated at different flux conditions for comparison. Furthermore, these measurements were carried out at zero membrane potential and therefore do not reveal the kinetic contribution of electrical effects. Crane[109] has reviewed various kinetic models proposed for Na^+-coupled transport systems.

The use of vesicles to investigate mechanisms of transport regulation was innovatively demonstrated by Hopfer's study[200] of the effects of alloxan-induced acute diabetes on glucose transport in intestinal microvillus membranes. The ability of diabetic membranes to maintain higher glucose concentrations in the presence of a standard imposed Na^+ gradient, together with the elimination of this difference in the presence of ionophores which accelerate the dissipation of a Na^+ gradient, suggested that intestinal membranes from diabetic animals exhibit lowered Na^+ conductance compared with that of healthy animals. Pleiotypic effects of a Na^+ permeability defect would explain the elevation of a Na^+-dependent but not Na^+-independent transport systems which has been observed in diabetics.

Recently, Crane et al.[201] and Ferguson and Burton[202] have described the reconstitution in phospholipid vesicles of a Na^+-dependent glucose transport system from small intestine. In both cases, Na^+-dependent, stereospecific glucose transport into liposomes could be demonstrated. Na^+-stimulated uptake was sensitive to phlorizin, a potent competitive inhibitor of this system in intestinal cells. While this approach offers great promise for molecular studies of Na^+-coupled carriers, other attempts at reconstitution have illustrated difficulties inherent in this approach.[129] Racker[203] has recently surveyed sources of artifact and technical problems in reconstitution experiments.

Kidney

Glucose transport in the renal proximal tubule shows striking similarities with the intestinal system. Unlike most cell types in which accumulated glucose is destined for intracellular metabolism, these epithelial tissues are specialized to permit net concentrative glucose flux through the cell from the luminal to the serosal face without appreciable shunting to the glycolytic pathway. Studies with perfused isolated proximal tubules[13,14] defined the apical brush border as the site of Na^+-dependent, phlorizin sensitive

uphill glucose transport. The model system provided by purified brush border and basal-lateral plasma membrane vesicles derived from renal proximal tubule offers an experimental approach directly analagous to that provided by vesicles from the small intestine.

Initial reports of Na^+-stimulated glucose uptake in isolated renal membranes[194] were followed by more extensive characterizations using highly purified renal brush border vesicles which do not metabolize glucose.[19,204,205] Aronson and Sacktor[204] provided compelling evidence for the direct contribution of Na^+ gradients in energizing the transient uphill glucose accumulation observed in brush border membrane. Beck and Sacktor[205] extended this thorough analysis to demonstrate that this process is electrogenic, stimulated by interior-negative membrane potentials generated either by ionophores or by anion diffusion potentials, as described in previous sections for amino acid transport. Together, these studies provided all the lines of evidence listed in Table 4 in support of the Na^+ gradient hypothesis. Both Na^+ gradients and electrical potential contribute additively to the total driving force. The mechanics of coupling of Na^+ to sugar flux were reflected as a decrease in K_m for glucose as a function of Na^+ concentration. Concentrative glucose accumulation in vesicles required conditions which would generate an electronegative vesicle interior. Thus, permeant anions such as Cl^-, NO_3^-, or SCN^- supported an overshoot of uphill glucose transport, but gradients of Na^+ salts, which dissipated across the membrane by an electroneutral process, did not generate the overshoot of active accumulation.

The glucose transport system expressed in vesicles exhibited the same competitive interactions among sugars and high sensitivity to phlorizin which had been defined for perfused tubules. It has been proposed[13,14] that high-affinity binding sites for phlorizin on the brush border membrane represent its interaction with the D-glucose carrier and may be a useful marker for isolation of the glucose carrier. Binding of phlorizin to high-affinity sites requires the presence of Na^+ and is competitive with D-glucose.[13,14]

Slack et al.[168] demonstrated that basal-lateral vesicles of renal proximal tubule catalyze mainly Na^+-independent facilitated diffusion of D-glucose, although a small Na^+-stimulated component persisted, largely accountable by contamination with brush border membranes. Since the concentrative glucose uptake catalyzed by contaminating membranes greatly exceeds the Na^+-independent rates, more extensively purified basal-lateral membrane vesicles will be required to establish detailed characteristics of glucose flux at the basal-lateral membrane. Characterization of Na^+-independent amino acid transport in these basal-lateral preparations is also hindered by this contamination.

Results obtained thus far for glucose transport are consistent with the same general hypothesis proposed for sugar and amino acid flux in small intestine and for neutral amino acid transport in renal proximal tubules: electrogenic uphill Na^+ cotransport at the luminal brush border and Na^+-independent downhill exit at the basal-lateral surface. However, additional characterizations of the transport properties of more purified preparations of both kidney and intestinal basal-lateral membranes are needed in order to evaluate other possible mechanisms, such as a possible contribution of flux of other ions. Effects of membrane potential at the basal-lateral surface have not been described.

CALCIUM ION

Introduction

Despite the profound physiological significance of calcium ion, little is known about the regulation of calcium ion flux across the plasma membrane. A ubiquitous Ca^{++}, ATPase transport system of mitochondria[206] has been implicated in the control of free

Ca^{++} concentrations in the cytoplasm, in addition to its intimate role in respiratory energy transduction.[106,206] In addition, the intracellular sarcoplasmic reticulum or endoplasmic reticulum membrane systems of various muscle and nonmuscle tissues contain another Ca^{++}, ATPase which serves as a Ca^{++} pump to regulate cytosolic levels of this ion.[207] Ca^{++} levels regulate muscle contraction and are strongly implicated in the regulation of cell movement in certain nonmuscle cell types.[208] Ca^{++} transport activity has been demonstrated in plasma membrane vesicles from erythrocytes,[209] kidney, intestine,[14,15] and mouse fibroblasts.[210] Rasmussen and Tenenhouse[211] propose that Ca^{++} released from bound sites on the plasma membrane is the ultimate intracellular effector of cyclic AMP effects on cells.

Mouse Fibroblasts

Ca^{++} is the only nonserum component of growth medium which can trigger the reinitiation of DNA synthesis when added to resting mouse fibroblast cultures.[212] In addition to this implied role in growth regulation, Ca^{++} has been named as an effector of fibroblast motility.[210]

Moore and Pastan[210] have characterized Ca^{++} transport activity of microsomes (plasma membrane plus endoplasmic reticulum, contaminated with 2 to 5% of total cellular mitochondria as judged by marker enzymes) derived from nontransformed and transformed 3T3 mouse fibroblasts. Ca^{++} uptake by microsomal preparations specifically required ATP; other nucleoside triphosphates tested were much less effective. Coupling of Ca^{++} uptake to ATP hydrolysis was not demonstrated. Although exchange diffusion of Ca^{++} was not characterized, efflux of accumulated Ca^{++} after addition of the Ca^{++} ionophore A 23187 was used as criterion for transport rather than binding. Furthermore, stimulation of Ca^{++} accumulation by oxalate, phosphate, and pyrophosphate was interpreted as evidence for intravesicular accumulation, since these anions would form insoluble Ca^{++} salts trapped inside the vesicle. Whereas mitochondrial Ca^{++} transport was inhibited by azide, oligomycin, and antimycin A, microsomal membrane Ca^{++} uptake was insensitive to these compounds. Transport activity was routinely assayed in the presence of 100 m*M* KCl; substitution of 100 m*M* NaCl had no effect.

Ca^{++} transport activity of microsomes showed a dramatic increase as nontransformed cells reached confluency, and a moderate increase after addition of cyclic AMP derivatives to intact cells before vesicle isolation. Addition of cyclic AMP or protein kinases together or separately in vitro to isolated membranes had no effect. These authors[210] propose the hypothesis that increased activities of microsomal Ca^{++} transport may be causally related to a reduction in cell motility. Cyclic AMP, which remarkably alters morphology, adhesion, and motility of fibroblasts when its derivatives are added to the culture medium, may mediate these effects. The inability to demonstrate cyclic AMP and/or protein kinase-mediated effects on this transport system in vitro suggests that these phenomena are complex. Furthermore, the separate contributions of plasma membrane and endoplasmic reticulum, which comprise these mixed vesicle preparations, remain to be characterized.

Renal and Intestinal Membranes

A Ca^{++}-stimulated ATPase has been localized in the basal-lateral membrane of kidney.[213] This enzyme is implicated as a primary active transport system for Ca^{++}, distinct from mitochondrial Ca^{++}, ATPase,[206] and is postulated to pump Ca^{++} out of the cell into the interstitial fluid. Ca^{++} entry at the luminal brush border surface is presumed to occur by downhill influx, facilitated by an interior-negative membrane potential and a Ca^{++} concentration gradient.

In striking contrast, Martin et al.[214] and others[13,14] have localized intestinal Ca^{++}, ATPase at the brush border membrane. This system is inducible by vitamin D,[214] con-

current with the induction of alkaline phosphatase.[13,14] Presumably, this serves as an active Ca^{++} pump, mediating Ca^{++} absorption from the intestinal lumen across the brush border membrane. A Ca^{++}, ATPase stimulated by Na^+, sensitive to ethacrynic acid and resistant to ouabain, was also detected in basal-lateral preparations from intestine.[215] The relative contributions of these two systems to transepithelial Ca^{++} flux remains to be established.

Other Cell Types

McDonald et al.[216] presented a detailed kinetic analysis of calcium binding to adipocyte plasma membranes using both equilibrium dialysis and filtration assays. Two classes of binding sites with affinity constants, K_a, of $5.2 \times 10^4\ M^{-1}$ and $1.8 \times 10^3\ M^{-1}$, respectively, were detected. Na^+, K^+, and Mg^{++} at high concentrations were noncompetitive inhibitors of Ca^{++} binding. The experimental procedures used in this study would not rule out the possibility that Ca^{++} retained by membranes was due to transport into an intravesicular space rather than binding. Suitable control experiments to distinguish between these alternatives were not employed.

Jones et al.[217] discovered that cardiac sarcoplasmic reticulum preparations used to study Ca^{++}, ATPase-mediated Ca^{++} transport also contained considerable plasma membrane material. Mild detergent treatment revealed high latent ouabain-sensitive Na^+, K^+, ATPase activity. Both Ca^{++} uptake and Ca^{++}, ATPase (ouabain-insensitive) were increased by K^+. This draws attention to a pitfall in using K^+-dependent ATP hydrolysis to assay the Na^+, K^+, ATPase. Presumably this K^+-stimulated Ca^{++} transport system is localized in the sarcoplasmic reticulum. However, due to the extensive contamination of these preparations by plasma membrane material, Ca^{++} uptake into purified plasma membrane vesicles should be separately characterized.

HORMONE RECEPTOR-EFFECTOR COUPLING: ION FLUXES

Many actions of polypeptide hormones, catecholamines, and prostaglandins, which bind to receptors on the cell surface, appear to be mediated via regulation of intracellular cyclic AMP levels. Therefore, considerable attention has focused on molecular aspects of coupling between plasma membrane-localized hormone receptors and hormone-sensitive adenylate cyclase. Less emphasis has been placed on ionic fluxes which accompany these interactions.

The rationale of these investigations of the integration of receptor recognition with membrane functions is to define the initial stages in a comprehensive mechanism of hormone action. Such a model would have to explain discrimination between responses to different hormones and the causal relationship between a bewildering cascade of other cellular responses such as ion and nutrient fluxes, cytoskeletal changes, and triggering of the initiation of DNA and protein synthesis.

The transport studies in vesicles described in this review illustrate in detail a fundamental concept utilized by a diversity of biological membranes: the capacity of membranes to store and integrate metabolic energy in the form of ionic and electrical gradients across the membrane and the recognition and utilization of these gradients by integral membrane transport proteins. The perception of the importance of instantaneous and integrative ionic and electrical coupling for certain transport systems leads to the speculation that similar coupling mechanisms may operate for other membrane functions.

Earlier views that receptor and cyclase activities resided in a single molecule or were distinct entities physically coupled either via adjacent localization or via motile receptors[218] had to be discarded in the face of astounding evidence arguing against a direct interaction of receptor and cyclase. Certain polarized epithelial cell types contain hor-

mone receptors on the opposite, nonintermixing plasma membrane face from that which contains adenylate cyclase, creating a situation in which physical coupling is unlikely.[13,14] Orly and Schramm[219] reconstituted isoproterenol-sensitive adenylate cyclase within minutes after fusing receptor-inactivated erythrocytes with receptor-deficient Friend erythroleukemia cells. Ross and Gilman[220] incorporated solubilized adenylate cyclase from β-adrenergic receptor-deficient L cells into acceptor plasma membranes from lymphoma cell variants deficient in adenylate cyclase but containing receptor. This manipulation reconstituted hormone-sensitive adenylate cyclase.

The realization that physically and genetically separate receptor and enzymatic entities are functionally coupled led to a search for diffusible intracellular mediators. GTP may serve such a role.[221] However, this theory replaces one question with another — how is GTP activation regulated?

Grollman et al.[56] provided the first experimental evidence that a primary and direct effect of hormone binding to receptors on the surface membrane is to increase the electrical potential difference across the membrane. As shown in Figure 16C, addition of thyrotropin (TSH) to plasma membrane vesicles prepared from cultured thyroid cells evoked a threefold stimulation of triphenylmethylphosphonium ion ($TPMP^+$) accumulation in vesicles. As discussed in the section entitled Neutral Amino Acids, this lipophilic cation accumulates in quantitative response to interior-negative membrane potentials. Similar effects were observed with intact cells, as shown in Figure 16A. Hyperpolarization was not triggered by human chorionic gonadotropin (HCG), a structurally similar hormone not active on thyroid cells, and was abolished after receptor inactivation by trypsin or addition of the H^+ ionophore CCCP (which would dissipate membrane potential, as discussed in the section mentioned above). These electrical responses markedly preceded activation of adenylate cyclase assayed simultaneously.

The mechanism of hyperpolarization is unclear from these preliminary studies. From the composition of the vesicle suspension medium during $TPMP^+$ uptake, it may be deduced that $TPMP^+$ accumulation is driven by dissipation of a Cl^- gradient (external > internal) imposed across the membrane.

Figure 17 illustrates a possible scheme to explain hyperpolarization induced by ligand-receptor interaction. This is a receptor-ionophore, electrical coupling model of hormone action on membranes. The hormone receptor, R, is comprised of both ligand recognition and specific ion channel entities. Binding of hormone, H, to its receptor triggers a change in specific ion conductance via the channel. Either enhanced Cl^- electrogenic entry or blocked cation permeation could account for hyperpolarization in the in vitro experiments. Since enhanced $TPMP^+$ uptake was produced in the presence of either $Tris^+$, Na^+, or K^+ chloride salts, putative TSH effects on cation permeability would be relatively nonspecific, or mediated by H^+ flux.

The enhanced interior-negative membrane potential would then trigger simultaneous generalized effects, individually modulated according to the particular properties of each responsive system. Electrical asymmetry would induce conformational changes in individual intrinsic membrane enzymatic proteins, E, thus affecting their activity. Furthermore, membrane potential would serve as a driving force for cation influx, e.g., Ca^{++}, which would modulate intracellular targets. Electrogenic nutrient transport systems, T, would monitor hyperpolarization by increased activity.

The experiments of Grollman et al.[56] acquire added significance from indications that a common mechanism underlies the action of a wide variety of glycoprotein hormones including TSH, the antiviral agent interferon, and cholera and tetanus toxins. In particular, these ligands share a region of startling amino acid sequence homology.[56] Furthermore, electrical effects are rapidly triggered by all these agents: rapid activation of electrogenic Na^+, K^+, ATPase activity is stimulated by insulin and prostaglandin

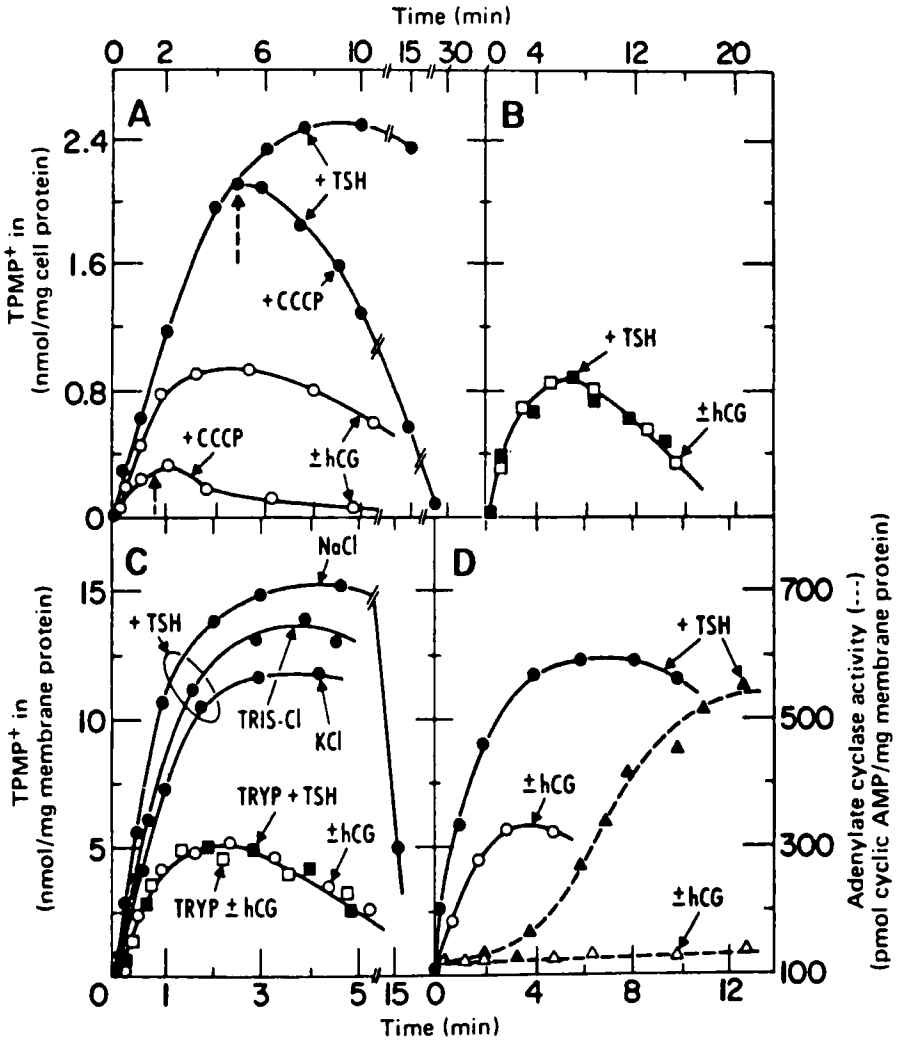

FIGURE 16. TSH-stimulated TPMP⁺ uptake by intact bovine thyroid cells (A), trypsinized bovine thyroid cells (B), plasma membrane vesicles prepared from bovine thyroid cells (C), and TSH-stimulated TPMP⁺ uptake in bovine thyroid vesicles relative to TSH-stimulated adenylate cyclase activity in the same vesicles (D). (From Grollman, E. F., Lee, G., Ambesi-Impiombato, F. S., Meldolesi, M. F., Aloj, S. M., Coon, H. G., Kaback, H. R., and Kohn, L. D., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 2352, 1977. With permission.)

$F_{2\alpha}$;[149] intestinal water and electrolyte flux are triggered by cholera toxin; tetanus toxin has immediate effects on neuronal transmission.[56] Lever et al.[149] showed that activation of the Na^+ pump of resting mouse fibroblasts by insulin or prostaglandin $F_{2\alpha}$ occurred by a direct mechanism independent of and temporally preceeding protein synthesis or cyclic nucleotide fluctuation.

Studies using membrane vesicles and the techniques for selective manipulation of ion gradients and membrane potential outlined in previous sections should clarify the precise events which give rise to hyperpolarization triggered by hormone binding. Analogies with two examples of ligand-membrane interaction may be pertinant. Catterall et al.[222] demonstrated that depolarization of neuroblastoma cells inhibited the binding of scorpion toxin and its stimulation of the action potential Na^+ ionophore. These results suggested that membrane potential induced conformational changes in

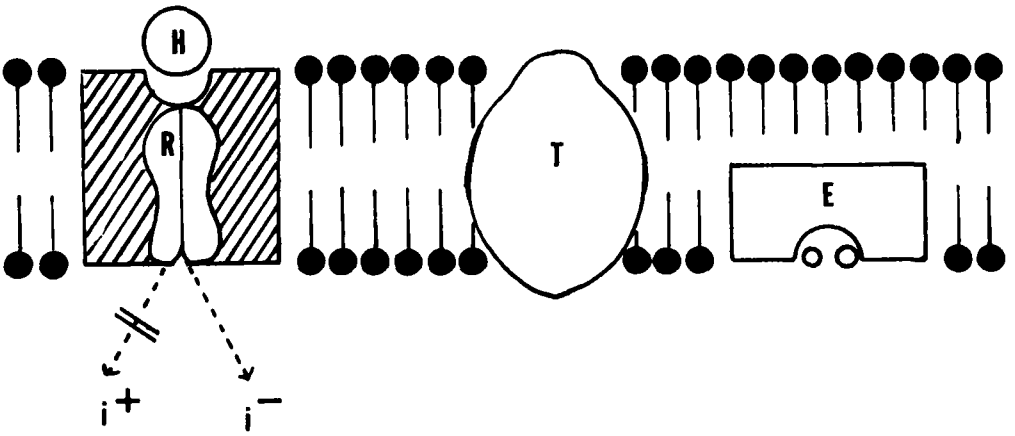

FIGURE 17. A receptor-ionophore electrical coupling model for hormone action at the cell surface.

this ionophore which serves as toxin receptor. Bacterial membrane vesicles[223] provided an observation which may serve as an additional model for electrical effects coupled directly to binding of an impermeant ligand at the cell surface. *p*-Nitrophenyl-α-D-galactopyranoside is a competitive inhibitor of Na^+-dependent methyl-1-thio-α-D-galactopyranoside transport, but is not transported across the membrane. A Na^+ gradient plus D-lactate induced an increased binding affinity and number of binding sites for this analogue on membrane vesicles. Binding enhancement varied with the electrochemical proton gradient across the membrane, mainly the electrical component. In the presence of Na^+, artificial creation of an interior-negative membrane potential using K^+ gradients and valinomycin also induced binding of this ligand.

CONCLUSION

This discussion of concepts and experimental data obtained using membrane vesicles has emphasized the diverse role of electrical and ionic gradients across the plasma membrane of animal cells. Many avenues of speculation have been presented, particularly with regard to membrane regulatory phenomena, balanced by an emphasis on experimental approaches. These membrane functions have yet to be described in precise terms at the molecular level, but the prospects of the membrane vesicle system have not yet been exhausted.

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