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CELLULAR TRANSPORT MECHANISMS

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PERSPECTIVES AND SUMMARY

In this review the assumption is made that living cells are surrounded by lipid bilayer membranes that are inherently impermeable to most hydrophilic compounds. It is also assumed that hydrophilic molecules are transported into cells by specific transport systems that are present in the membrane and are made up of one or more proteins. These transport systems either equilibrate substrates across the membrane (facilitated diffusion) or use energy to concentrate substrates (active transport).

There have been two important recent technical advances in transport research. One is the development of methods for making closed membrane vesicles that retain the activity of certain transport systems (1, 2). In some cases it is possible to make vesicles that either have the overall orientation present in the original cell or that have an inverted orientation (3–5). Vesicles have the advantage that they are free of endogenous energy sources and do not metabolize most substrates. But they have the disadvantage that certain transport systems are not active in them, and some membrane proteins partially alter their orientation during vesicle formation (6). The second advance is the development of a number of procedures for incorporating membrane proteins into phospholipid vesicles so as to reconstitute transport activity (7). These techniques not only provide assays for transport proteins but also provide simple systems for the study of purified transport proteins.

There have also been two important conceptual advances in this field. One is a realization that transport models involving mobile or rotating carriers are not applicable to proteins containing exposed hydrophilic residues (8). It is likely that at least one component of each transport system spans the membrane—probably as an oligomer—to form a hydrophilic pore through the membrane. In addition, special mechanisms are probably required to insert transport proteins into the membrane with the correct orientation. The other advance is a realization that many active transport systems function as either proton or sodium symports (9); that is, the movement of substrate through the membrane is always accompanied by the parallel movement of the appropriate ion. In many cases these systems are electrogenic, so that energy for transport comes not only from the ion gradient, but also from the membrane potential.

Only work on bacterial and mammalian systems is discussed in this review. A detailed review of plant transport appeared in References 10–12,

and this subject is regularly discussed in the *Annual Review of Plant Physiology*. At the present time it appears that most bacterial systems carry out active transport and fall into one of three classes: group translocation systems, membrane-bound transport systems, and binding protein transport systems. Within the class of membrane-bound transport systems there appear to be some important differences between various systems. Mammalian transport systems appear to be fairly evenly divided between facilitative diffusion systems and active transport systems. Most mammalian active transport systems are either coupled directly to ATP hydrolysis or are sodium symports, although it is quite possible that other mechanisms will be found as more systems are thoroughly characterized.

INTRODUCTION

The purpose of this article is to review the information about the mechanisms by which molecules are moved through membranes and concentrated by living cells. It is impossible to give a complete account of all the work in this extensive field, so this article is limited to a discussion of general topics and a limited number of specific systems chosen to illustrate the different transport mechanisms. Despite their importance, proton transport systems will not be discussed, since work in this field is well reviewed (13–16). Even with this limitation, the literature is so extensive that no attempt is made to cite every paper. Instead, in many cases, recent reviews are cited that can be consulted for the references to specific articles.

General Topics

There is a fundamental controversy in the transport field. Most workers believe that living cells are surrounded by a membrane composed of a lipid bilayer containing proteins, some of which function to specifically transport hydrophilic molecules through the membrane. However, a small group maintains that the cell is permeable to most molecules and that the process called transport is due either to direct binding of molecules to cytoplasmic compounds or to altered properties of the cell water, which result from the high concentration of charged macromolecules in the cytoplasm (17, 18). Some of the arguments on both sides were reviewed in an article in *Science* (19) and in letters commenting on it (20).

Multiple Systems

A major difficulty that must be dealt with in transport studies is the fact that most substrates are transported by several systems. This phenomena is seen most strikingly in bacteria—in *Escherichia coli* at least five systems transport galactose (21)—but it is also observed in higher organisms (22).

The multiple K_m values seen for the uptake of a given substrate can be due either to several independent systems or to a single system with multiple binding sites. The leucine transport system provides a good illustration of the problem. A multiple site model has been proposed to explain the complex kinetics observed for leucine transport in *E. coli* (23). There are at least three K_m values for transport, so that a model with a single carrier having three binding sites was proposed and shown to fit the kinetic data slightly better than a model using two independent systems. However, a model containing three independent systems, which is the one proposed by other workers (24–26), would give the best fit to the kinetic data. Unfortunately, when there are more than two K_ms of uptake, it is probably not possible to decide the controversy by kinetic data alone because of the many degrees of freedom in the models and the errors of transport measurements. Some criteria distinguishing between independent systems and a single allosteric system have been discussed (27).

The fact that mutants can be isolated in separate genes that each inactivate a single system, without affecting the other systems, is evidence that favors three independent leucine transport systems (28). In addition, two of the systems are associated with periplasmic binding proteins and use ATP to drive active transport, whereas the third system is driven by the proton motive force (25). Finally, there is evidence showing differences in the regulation of the different systems (29). Thus, the data for leucine transport indicate that there are three distinct leucine transport systems, although there may be some interactions between them.

Glutamate transport in *E. coli* is another system showing complex kinetics in which variations of a single system model have been presented (30, 31). However, a recent study provides strong evidence that there are three different glutamate transport systems (32). These different systems were distinguished by the use of inhibitors and by the isolation both of mutations that inactivate certain systems and mutations that increase the level of certain systems significantly. The three systems are: a binding protein associated system, a sodium-stimulated membrane-bound system, and a nonsodium-stimulated membrane-bound system. Each system has a unique substrate specificity; moreover, the properties of each mutant are consistent with the systems being independent.

There are at least two systems where the evidence is consistent with an allosteric model. One is a potassium transport system in *Neurospora* (33), and the other is an aromatic amino acid transport system in *Bacillus subtilis* (34). Even for these systems the evidence is still indirect, so that further work is required to insure that a single carrier is responsible for the dual K_ms seen for these systems.

Properties of Membrane Proteins

The synthesis and properties of membrane proteins, with special emphasis on their significance for transport systems, are the subject of a recent article (8). While direct evidence for some of the conclusions of this article is still limited, the concepts appear very reasonable and should be considered by all workers in this field. The basic conclusion is that because almost all proteins contain both hydrophobic and hydrophilic regions, they cannot migrate through the membrane in their native state. Therefore, proteins that are either outside the plasma membrane, span the membrane, or are present on the side opposite to that on which protein biosynthesis occurs, will be synthesized on membrane-bound ribosomes. Many of these proteins will be inserted into or moved through the membrane as they are synthesized. A hydrophobic segment is present at the N terminus of some excreted proteins that is removed after the protein has passed through the membrane (35). Furthermore, bacterial periplasmic proteins and outer membrane proteins, which are moved through the inner membrane, are synthesized on membrane-bound ribosomes (36). Post-transcriptional processing such as glycosylation may also function to insert molecules into the membrane. However, there is no direct evidence for this process at the present time.

Multiple Mechanisms

A final complexity in the transport field is the existence of quite different mechanisms that are used by living cells to carry out active transport. Specific examples of the different classes of transport systems and their properties are discussed in the rest of this article.

BACTERIAL TRANSPORT SYSTEMS

Introduction

There are at least three distinct classes of active transport systems in bacteria (37). One class couples the transport of molecules across the cell membrane with chemical modification, a process called group translocation. A second class transports molecules against a concentration gradient with no change in the transported molecule. All of the proteins required by members of this class are firmly bound to the cell membrane and are retained in isolated membrane vesicles. Members of this class are called membrane-bound transport systems. About 40% of all known *E. coli* transport systems belong to this class, including transport system for sugars, ions, and amino acids. The third class also concentrates molecules without modification, but transport systems in this class require at least one component that is lost during the formation of membrane vesicles. This compo-

nent is a soluble protein that specifically binds the substrates of the transport system. Therefore, members of this class are called binding protein transport systems, and they include systems that transport ions, sugars, amino acids, and vitamins. Another 40% of the transport systems of *E. coli* are in this class.

It is not clear what factors determine whether a given substrate is transported by a membrane-bound or a binding protein transport system. In fact, some substrates are transported by members of both classes. The one generalization that can be made is that binding protein systems appear to have somewhat lower K_m values than membrane-bound systems transporting the same or similar substrates.

There is strong evidence that each class utilizes a different mechanism to couple energy to the accumulation of substrates. The group translocation systems utilize the chemical energy of the modification reaction to accumulate substrates. The membrane-bound systems utilize the proton-motive force across the membrane to drive active transport, whereas the binding protein transport systems utilize ATP or a related compound to drive active transport.

In the case of the membrane-bound systems, the proton motive force can be used either directly by a proton symport mechanism or indirectly by symport or antiport with some other ion. The most commonly used indirect mechanism is sodium symport. The sodium gradients used by bacteria to drive transport appear to be produced by proton-driven sodium pumps (38). This description of the energy coupling process is a simplification of a very complex and controversial subject. Since there are a number of excellent reviews (9, 39–41) dealing with this question, it is not discussed further here. Neither are bacterial group translocation systems, as they have been well covered in recent reviews (42–45).

The uptake of nonmetabolizable substrates by all transport systems eventually reaches a steady state. The steady state for most bacterial transport systems belonging to either membrane-bound or binding protein classes results from a dynamic equilibrium between an entry reaction and an exit reaction. One exception is phosphate transport in *E. coli* (46), which is similar to amino acid transport in yeast in showing little or no exit, but instead the uptake reaction is inhibited by the pool of substrate in the cell (47).

Several reports suggest a relationship between membrane-bound and binding protein transport systems. Thus, in an ATPase mutant of *E. coli* where ATP levels are maintained by glycolysis colicin K inhibits the uptake of both glutamine, which is transported by a binding protein system, and proline, which is transported by a membrane-bound system (48). However, as glutamine transport is inhibited to a lesser extent and at a slower rate

than is proline transport, the two systems may be inhibited in different ways. In another study a mutation was reported to inactivate both classes of transport systems (49). However, no energy source was provided during the transport measurements, so that the mutation may have affected energy metabolism, not transport. In a more recent study, an energy source was provided in the transport assay, but only membrane-bound transport systems were tested (50). A mutant that resembles the one just discussed was shown to inactivate only membrane-bound transport systems when an energy source was provided during uptake measurements (51).

Finally, it was reported that the extent of stimulation of a large number of amino acid transport systems by an increase in the energy supply was directly proportional to the hydrophobicity of the amino acid (52). This relationship is true for members of both classes of transport systems. The basis for this phenomenon is not known, but the relationship is quite clear and was tested with five members of each class.

In summary, the latest evidence does not indicate a clear link between membrane bound and binding protein transport systems.

Membrane-Bound Transport Systems

LACTOSE PERMEASE *E. coli* lactose permease is the most extensively studied bacterial membrane-bound transport system, both because it was the first transport system to be identified and because it has a very high V_{max}, about 200 nmol min^{-1} mg^{-1} protein. In addition, nonmetabolizable substrates such as thiomethyl galactoside are available for studying this system. Only one protein is required for lactose transport, since every mutation that inactivates the transport system is found in the *Y* gene of the lactose operon (53). This is true also for mutants that are unable to concentrate lactose permease substrates, but that can move such substrates through the membrane at a normal rate (54, 55).

The product of the *Y* gene (the *M* protein) has been isolated in an inactive form by labeling it with radioactive N-ethylmaleimide after protecting it with substrate during a preincubation with unlabeled N-ethylmaleimide (56). It is a membrane protein with a molecular weight of 30,000 (57). Lactose permease is inducible, and the *M* protein appears to represent about 4% of the protein in the membrane fraction, or 0.3% of the total protein in induced cells (57–59). Lactose transport activity has been reconstituted into membrane vesicles prepared from a Y^- mutant by use of an aprotic solvent extract from induced wild-type membranes (60). However, the extract was quite unstable, and the protein responsible for transport activity was not purified.

Lactose permease is a proton symport system; that is, the movement of lactose through the membrane is coupled to the movement of one or more

protons. The movement of lactose into energy-deficient cells removes protons from the medium with a stoichiometry of 1 proton per lactose molecule (61), and the imposition of a proton gradient on whole cells drives lactose transport (62). In addition, the lactose-permease–dependent hydrolysis of *o*-nitrophenyl galactoside (ONPG) in starved cells is greatly stimulated by uncouplers that allow the protons moved into the cell by the lactose carrier to leak out, thus dissipating the membrane potential produced by their inward movement (63). Exit of lactose from the cell moves protons outside, and this proton movement can be used to drive the transport of proline (64). The lactose-permease–dependent hydrolysis of ONPG by thoroughly starved cells in the absence of uncouplers requires energy with an estimated stoichiometry of 1 proton per ONPG transported (65). However, the stoichiometry of lactose transport in membrane vesicles is pH-dependent, being 1 at pH 5.5 and 2.1 at pH 7.5. This result suggests that a group with a pK of 6.8 is responsible for this change (66). Finally, the energy requirements for forming and maintaining a lactose gradient have been determined in whole cells by microcalorimetry (67). These studies found a value that was consistent with a stoichiometry of 1 proton per lactose transported when the inward flux of lactose was less than 100 nmole/min/mg. In addition, the maintenance of the steady state required only a small amount of energy because most of the exiting lactose appeared to take a proton outside, and it could then be used to re-transport another lactose molecule. At higher rates of lactose uptake there was a 10-fold increase in the energy consumption. It is unlikely that this 10-fold increase represents energy required directly for transport. It seems more likely that either the increased energy consumption resulting from the higher rate of transport or the higher internal concentration of lactose present in these experiments activates some other energy-requiring process.

The complexity of lactose permease is shown by the following studies. First of all, there is trans-stimulation in which the presence of a substrate on one side of the membrane stimulates the movement of the same or other substrates across the membrane from the other side (68, 69). This phenomenon is not observed during active transport but can be observed in exit studies and counter-flow experiments (70). In addition, efflux of accumulated substrate can cause the transient accumulation of another substrate that is present outside the cell. This process, called counter flow, can occur at a rate close to the V_{max} of active transport in cells that are unable to carry out active transport because of the presence of energy poisons (70, 71). While some of the accumulation may result from the proton gradient produced by the exiting substrate, part of the accumulation results from some other process, since uncouplers cause only a partial inhibition of counter flow. A clear difference between counter flow and active transport

lies in their responses to the external pH. Counter flow is inhibited as the pH is raised from 5 to 8, while active transport has a broad pH optimum in this range (70).

Other observations that indicate that the system is complex come from studies on *E. coli* membrane vesicles that have the same overall membrane orientation as the whole cell, although the orientation of a number of proteins may be randomized during their preparation (6, 72, 73). A series of dansyl galactosides in which the dansyl group and the galactose residue are joined by from 0 to 6 methylene groups was used in this work. These molecules bind specifically to the lactose carrier and are not transported across the membrane (74). Their binding can be readily measured as their fluorescence is greatly altered upon binding. There is a low (about 10% of the maximum) but significant binding (measured with radioactive nitrophenyl galactoside) in the absence of any energy source (59). This binding is not inhibited by uncouplers and does not increase at low pH, which indicates that maximum binding requires more than the binding of a proton to the carrier. This binding is probably the same as that studied previously in a sonicated membrane preparation (75). Maximal binding of dansyl galactoside occurs when a respiratory substrate is added or a membrane potential is applied, and this binding is inhibited by uncouplers (58). Maximum binding also occurs when the vesicles are preloaded with substrates, and efflux occurs in the presence of dansyl galactoside. This binding is not inhibited by uncouplers and is probably the same phenomenon as the uncoupler-resistant counter flow mentioned previously (70). The K_d values for the energized and nonenergized binding are about the same. A study of thiodigalactoside binding to membrane vesicles found a high level of binding in the presence of azide, about 50% of the binding seen for energized vesicles (76). The ΔH of binding was very high, –21 kcal/mole (76). The reason for the high level of energy-independent binding seen in these experiments is not known.

To explain the existence of energy-dependent and energy-independent binding, it was proposed that the carrier can exist in two states in the absence of energy: one with high affinity, and one with very low or no affinity for substrates. In addition, it was proposed that these two states are in equilibrium and that energization shifts the equilibrium to the high-affinity form (73). However, the data yield an equilibrium constant of 10 for the interconversion of these two forms which requires only a small energy difference between the two forms, whereas direct measurements indicate that large amounts of energy are required to give maximal binding (58, 77). Therefore, although there are two types of carriers in the membrane, they probably are not in equilibrium. One species of carrier shows high-affinity binding at all times, whereas the other form binds with high affinity only

when the appropriate gradient is present. Since only one gene product is required for lactose permease, the two species of the carrier must result either from a chemical modification of part of the carrier or from different carrier molecules interacting with two types of membrane lipids or proteins.

There is evidence in whole cells for a carrier species that promotes facilitated diffusion in the absence of energy as long as an uncoupler is present to dissipate the potential produced by the protons carried in by the carrier (63). The energized carrier has a V_{max} of 180 μmol g^{-1} min^{-1} and a K_m of 0.7 mM for ONPG transport, while the deenergized system shows two types of transport, one that has a K_m of about 1 mM and a V_{max} of 40 μmol/g/min and a second that shows a linear increase with increasing ONPG concentration. The saturable component probably represents activity of the carrier species responsible for the non–energy-requiring binding observed in vesicles. This activity represents 23% of the total activity, compared with 10% found by the binding experiments. The many differences between the two experiments may explain this discrepancy, or, alternatively, the non–energy-requiring species may be preferentially inactivated during vesicle formation. Since no ONPG transport occurs in starved cells in the absence of uncoupler, both forms of the carrier must function as proton symporters, and therefore in energized cells both forms of the carrier would carry out active transport. The presence of the nonsaturable component seen in starved cells probably indicates that the energy-requiring component can bind substrate but with a low affinity. This would explain how a lactose gradient could convert the low-affinity form into the high-affinity form. If the energy-requiring state of the carrier did not bind lactose at all, it would be difficult to understand how it could respond to a lactose gradient.

Direct evidence from two different laboratories indicates that both species of the lactose carrier have the same binding properties on each side of the membrane and that active transport can occur in either direction, depending on the direction of the proton motive force (i.e. the carrier is symetrical). One study used *E. coli* membrane vesicles prepared by breaking the cells with a French pressure cell. Such vesicles have a membrane orientation opposite that of the intact cell (4). These vesicles can concentrate lactose in response to either a membrane potential (positive outside) or a pH gradient (alkaline inside). Efflux of accumulated lactose can be driven by ATP or by respiration, both of which produce a membrane potential and a pH gradient opposite to that required for uptake (78). Furthermore, the binding of dansyl galactoside to these vesicles (77) responds to a pH gradient, a membrane potential, or lactose efflux in the same way as do normal vesicles. The other study compared the efflux, counter flow, and uptake of lactose driven by an artifical potassium gradient in membrane vesicles that

have normal orientation and the same vesicles after brief sonication, which was shown to invert the membrane (79). In each case the two classes of vesicles gave identical results, except that the normal vesicle preparation appeared to contain some leaky vesicles.

A major study (80) concluded that energy did not effect the uptake of lactose but converted exit from a saturable reaction with a K_m close to the uptake reaction to a first order reaction. A later study (81) confirmed the last finding, but showed that the V_{max} of the uptake reaction was also increased by the presence of energy, as it was in the vesicle studies and the studies on ONPG transport discussed previously. The fact that energization of the membrane lowers the affinity of the exit reaction indicates that the presence of a membrane potential, or a proton gradient across the membrane, converts the site of the high-affinity form of the carrier present on the inside surface of the membrane to a low-affinity form.

Dansyl galactosides of varying chain length also have been used to try to determine the distance of the lactose binding site from the external water phase by measuring the ability of a hydrophilic quencher to inhibit fluorescence of the bound galactosides as well as the changes in their fluorescence (82). These experiments suggest that the immediate environment of the binding site is hydrophobic and that the external aqueous phase is about 6 or 7 Å from the binding site on the carrier.

In conclusion, almost all studies on this system are consistent with a model in which the lactose carrier is present in two different forms. One, representing about 20% of the carrier molecules, has a high affinity for substrates in the absence of energy; the other has a low affinity for substrates unless it is activated by either a pH gradient, a membrane potential, or a lactose gradient. In the absence of energy both forms of the carrier are symmetric in that they have identical properties on each side of the membrane. The presence of either a pH gradient or a membrane potential converts both forms into an asymmetric carrier having a high-affinity site on one side of the membrane and a low-affinity site on the other. This asymmetry can occur in either direction, depending on the direction of the proton motive force across the membrane. Transport of lactose through the membrane by either form of the carrier is always associated with the movement of one or of two protons across the membrane, depending on the external pH.

THE *E. COLI* DICARBOXYLIC ACID TRANSPORT SYSTEM The dicarboxylic acid transport system of *E. coli* is another well-studied membrane-bound transport system. This system transports succinate, malate, and fumarate and is inactivated by mutations in three different genes. One of these genes codes for a protein that binds lactate in addition to the above

three compounds. This protein has been purified to homogeneity and appears to function to transport the compounds that it binds through the outer membrane (83). The evidence for this is as follows. Mutants that lack this protein do not transport these compounds, whereas membrane vesicles prepared from the mutant strain transport them normally. In addition, N-ethylmaleimide inhibits transport in whole cells but not in membrane vesicles, and this compound inhibits the binding activity of the outer membrane protein.

Proteins with a similar function apparently are required for glycerol phosphate transport (84) and glucose-6-phosphate transport (85) in whole cells. This suggests that the outer membrane may be impermeable to large, negatively charged ions. Although these proteins can be released by osmotic shock, they are clearly not the same as the periplasmic binding proteins associated with binding protein transport systems. They have higher K_ds and, more importantly, the transport systems with which they are associated function normally in membrane vesicles that lack these proteins, whereas the true binding protein transport systems are inactive in membrane vesicles.

The products of the other two genes that are required for succinate transport in both whole cells and vesicles have been isolated and purified to apparent homogeneity from a lubrol extract of washed membranes by affinity chromatography (86). These proteins bind succinate, malate, and fumarate, but do not bind lactate. Labeling and crosslinking studies indicate that both these proteins appear to span the membrane and are close to each other in the membrane. Furthermore, it appears that the binding site of protein SBP_2, which has a K_d for succinate of 2 μM, is present on the external side of the inner membrane, whereas the site of SB_1, which has a K_d for succinate of 23 μM, is on the inside surface of the inner membrane. Vesicles treated with a crosslinking reagent under conditions in which 90% of the two membrane proteins are coupled together still show 70% of the rate of uptake seen in uncrosslinked vesicles. Treatment with phospholipase D, which specifically hydrolyses phosphoserine, inhibits 80% of the succinate uptake in the vesicles while causing only a small inhibition of proline transport (87).

The dicarboxylic acid transport system is energized by a proton gradient (88, 89). Unlike other membrane-bound transport systems, succinate uptake was reported to be inhibited by an ATPase mutation (87). But other workers (90) did not see this effect, so it may result from some secondary property of the particular ATPase mutant used in the first study.

Since lactose permease requires only one membrane protein whereas this system requires two membrane proteins, we may conclude that even two systems using the same energy source show differences in their transport

machinery. In summary, the dicarboxylic acid system is currently the most thoroughly characterized membrane-bound transport system, despite the fact that no successful reconstitution of succinate transport has been reported.

THE HALOBIUM GLUTAMATE TRANSPORT SYSTEM Another membrane-bound system that appears to differ in some ways from lactose permease and the dicarboxylic acid transport system is the glutamate transport system of *Halobacterium halobium.* This organism has been the subject of many recent studies because it contains a novel light-driven proton pump, bacterial rhodopsin, in its membrane (16). This organism lives in a high salt environment and contains a proton, sodium antiport system (91); thus it has a large sodium gradient across the membrane. Membrane vesicles having a normal orientation can be prepared that retain an active light-driven proton pump and most transport activities. The transport of amino acids by these vesicles appears to be driven by both the sodium gradient and the membrane potential; however, the transport of glutamate, which has been studied most extensively, appears to be driven by a sodium gradient alone. Efflux of all amino acids can be driven by an inverted sodium gradient and it appears that sodium is cotransported during the exit reaction (92).

A protein that binds glutamate has been isolated by cholate extraction of *Halobacterium* membranes (93). It has a molecular weight of about 50,000 and has been purified about 15-fold. When this protein is incorporated into soybean phospholipid vesicles it catalyzes equilibration of glutamate across the membrane but does not carry out active transport. Surprisingly, the equilibration does not require sodium. This result indicates either that another component is required for active transport or that the protein has been partially modified during isolation.

A number of other bacterial uptake systems have been shown to be driven by sodium gradients: melibose (94) and glutamate (95–97) in *E. coli,* proline in *Mycobacterium phlei* (98), and melibose in *Salmonella* (99, 100).

THE PS3 ALANINE TRANSPORT SYSTEM Another unusual organism that has been used in transport studies is the thermophilic organism, PS3. Alanine transport was successfully reconstituted into phospholipid vesicles by use of a cholate extract of PS3 membranes (101). The reconstituted vesicles could carry out active transport of alanine driven by a membrane potential created by the valinomycin-catalyzed efflux of potassium. The alanine carrier was purified about 13-fold, which suggests that a single protein may be responsible for the transport activity, but until a pure preparation of the carrier protein is obtained, this conclusion remains tentative.

PROLINE TRANSPORT IN E. COLI AND M. PHLEI A proline transport protein has been extracted from *E. coli* membranes with acidic *n*-butanol and then chromatographed on Sephadex LH-20, which gave a four-fold purification (102). The initial extract contained only 2% of the membrane protein. Both the extract and the LH-20 material were reconstituted with *E. coli* phospholipids, and these vesicles catalyzed the transient accumulation of proline driven by valinomycin-induced potassium efflux. The vesicles reconstituted with the butanol extract were reported to transport glutamate and cysteine, in addition to proline. These isolated proteins were quite unstable, so that further purification may be difficult.

An *M. phlei* protein that binds proline has been extracted from membranes by use of cholate and Triton X-100 (103). This protein was purified by several steps, including isoelectric focusing. There were two peaks of binding activity: one that bound several amino acids and one that bound only proline. The purified fractions were tested for transport activity by reconstitution into detergent-extracted *M. phlei* vesicles and into liposomes. In each case there was no activity for the peak showing multiple binding activity, but the other peak did give activity that was specific for proline.

VITAMIN TRANSPORT IN *LACTOBACILLUS CASEI* *L. casei* carries out the active transport of folate compounds apparently by a single system. A membrane protein that binds folate has been extracted from *L. casei* membranes with 5% Triton X-100 and purified 100-fold (104). The purified protein, which moves as a single band of mol wt 25,000 on gel electrophoresis in the presence of SDS, binds 0.85 mole of folate per mole of protein. The amino acid composition indicates that it is an extremely hydrophobic protein. Several mutants have been isolated that are deficient in folate transport; in these strains the binding activity is altered (105). In addition, the specificity and pH optimum of the binding activity and transport reaction are similar. These studies indicate that the isolated protein is the carrier for the folate transport system.

A similar *L. casei* protein that binds thiamine has been isolated and purified to homogeneity (105). This protein is also extremely hydrophobic, and the properties of the binding reaction and the transport system are similar. Even though there is no genetic evidence that this protein functions in the transport of thiamine, the strong indirect evidence mentioned above makes it likely that it is the thiamine carrier.

Binding Protein Transport Systems

INTRODUCTION Binding proteins are present in the periplasmic space, the area between the inner and outer membrane of the cell (106). Proteins in this region can be selectively removed from the cell by a gentle procedure

called osmotic shock (85). Despite the fact that the specificity and binding constants for each binding protein are nearly identical to the specificity and K_ms for the associated transport system, the binding proteins do not appear to be the membrane carriers for these systems, since they are hydrophilic proteins that do not interact strongly with membranes. In addition, strains with mutations in the structural gene for galactose-binding protein still retain the ability to transport substrates of the associated transport system with the same V_{max} as wild-type cells but with a greatly increased K_m (about 1000-fold) (107, 108).

Mutations in the binding protein drastically reduce the ability of the systems to concentrate substrates which indicates that binding proteins are essential for the physiological function of all the associated transport systems (109–113). It is unlikely that the binding proteins function simply to allow substrates to pass through the outer membrane, since the properties of proteins with this function are quite different from those of the binding proteins discussed here. One class of outer membrane proteins that function to move large molecules through the outer membrane (proteins required for maltose, vitamin B_{12}, and ferrichrome-mediated iron transport) are lipophilic molecules that are tightly bound in the outer membrane (114). A second class that appear to move charged molecules through the outer membrane (proteins associated with succinate, α-glycerol phosphate and possibly glucose-6-phosphate transport) were discussed in the section on succinate transport.

There have been many studies of binding proteins as they are readily isolated and purified, and these have been recently reviewed (13, 115). The complete amino acid sequence of the arabinose-binding protein has been determined (116) as well as its three-dimensional structure at a resolution of 2.8 Å (117). The structure of this protein can be described as an ellipsoid with dimensions 70 Å by 35 Å by 35 Å, and it contains two globular domains separated by a cleft. The galactose-binding protein is structurally related to the arabinose-binding protein, since the two molecules show immunological cross reactivity (118).

A number of reports over the past ten years claim to successfully reconstitute binding-protein transport by the addition of purified binding protein to shocked cells or vesicles. However, none of these studies so far has been successfully repeated, and in many cases the reported activity probably resulted from the binding of substrate to the added binding protein.

For all systems studied so far, the product of at least one gene in addition to the binding protein is required for a functional transport system (109, 111–119). This gene product has not been identified for any system, despite extensive efforts. Strong indirect evidence that the *Salmonella* histidine binding protein interacts directly with the product of the second gene,

required for the high-affinity histidine transport system, has been provided by genetic experiments (120). Certain mutations in the histidine binding protein structural gene were found to be suppressed by mutations in the second required gene and vice versa. This finding provides important additional evidence that the binding protein participates directly in the transport process rather than playing an auxiliary role.

THE *E. COLI* β-METHYL GALACTOSIDE TRANSPORT SYSTEM The high-affinity galactose transport system, called the β-methyl galactoside transport system, which is associated with the galactose binding protein has been extensively studied. Although galactose itself is transported by at least four other transport systems, β-methyl galactoside and β glycerol galactoside, are transported only by the β-methyl galactoside transport system in cells that lack lactose permease and TMG II permease.

It was suggested that this system used proton motive force to drive active transport (121), but more recent studies have shown that, like all other binding-protein transport systems that have been studied, the system uses ATP to drive active transport (122). This conclusion is supported by the absence of any proton influx associated with β-methyl galactoside transport in whole cells (123, 124).

All of the known mutations that inactivate this system map in a region called the *mgl* locus (119, 125, 126). This locus contains three complementation groups, one of which—the *mgl* B cistron—is the structural gene for the galactose-binding protein (110, 127). There is some evidence that the two non–binding-protein cistrons, *mgl* A and *mgl* C, are actually a single cistron showing intra-cistronic complementation. In the first place, eight out of thirty-two mutants in the A or C loci do not complement any other A or C mutation (127). This seems a very high proportion of double mutations, but is a reasonable proportion of noncomplementing mutants. In the second place the phenotypes of A and C mutants are identical in a number of studies (108, 127, 128). If the two cistrons are really one, then the A, C product must be at least a dimer in its functional state to allow the observed complementation.

There have been many studies of the galactose-binding protein (129–131), some of which have produced conflicting results. The most recent experiments (132) confirm the original studies (129, 130) and show that this protein has a single substrate binding site like all other binding proteins that have been studied. In addition to its role in transport, the galactose-binding protein is the receptor for galactose chemotaxis (133).

The properties of transport and chemotaxis in a number of binding-protein mutants were examined, and all mutants that altered the K_m of transport also altered the K_m of galactose chemotaxis (127). Some mutants were found that had normal transport but had lost galactose chemotaxis,

and one mutant was found that had lost the β-methyl galactoside transport system but had normal chemotaxis. These results indicate that chemotaxis and transport utilize the same substrate binding site, but that both require other regions of the molecule. Furthermore, the regions required for chemotaxis and transport cannot be exactly the same. The galactose-binding protein probably interacts with the product of the *trg* gene, signaling the galactose concentration to the cell, since mutants in the *trg* gene do not show galactose or ribose chemotaxis but do respond normally to other chemotaxis substrates (127).

A functional difference between strains carrying *mgl* B mutations and those carrying *mgl* A or C mutations has been observed (107, 108). The *mgl* B mutant strains can grow on high concentrations of β-methyl galactoside and retain a β-methyl galactoside transport activity with the same V_{max} as the wild-type strain but with a 1000-fold higher K_m, while the *mgl* A, C mutant strains do not grow on β-methyl galactoside and have no transport activity. It was suggested that the *mgl* A, C gene codes for the membrane carrier of the system and that the binding protein functions to reduce the apparent K_m of the carrier for substrates. However, a study of the β-methyl galactoside exit reaction suggests that the system may require a third component (128). Uptake of the nonmetabolizable substrate β-methyl galactoside reaches a plateau that was shown to result from a dynamic equilibrium between the entry reaction and an exit reaction. The exit reaction followed first order kinetics even when the internal substrate concentration was 0.1 M. Exit was greatly stimulated in energized cells. This stimulation required ATP and did not occur with proton motive force alone. The exit reaction did not require the product of any *mgl* cistron. Equilibration of β-methyl galactoside into cells that lack the *mgl* products either because of mutations or from growth on the repressing substrate, glucose, occurred readily and was stimulated by an energy source. Furthermore, the equilibration showed specificity, since two substrate analogues, thio-methyl galactoside and β-ethyl galactoside, which are not substrates of the β-methyl galactoside transport system did not equilibrate into the cells. Since equilibration occurs normally in *mgl* A, C mutants, it seems possible that the carrier involved in the exit reaction also functions in the entry reaction. If so, the uptake reaction, studied in *mgl* B mutant strains, would probably result from the residual activity of the mutant binding proteins, since none of the *mgl* B cistron mutants contain deletions or chain-termination mutations (108). If these suggestions are correct, the function of the *mgl* A, C product could be to catalyze the energy-coupling reaction.

A possible model that integrates these results is given below. The binding protein that carries substrate would bind to a site on the inner membrane that includes the exit carrier and the *mgl* AC gene product. ATP would

be utilized in a reaction catalyzed by the *mgl* AC product to cause a conformational change in the binding protein so that it loses its affinity for substrate. The released substrate would pass through the inner membrane into the cell via the exit carrier, while the binding protein would dissociate and return to its normal conformation ready to repeat the cycle.

The use of energy to alter the affinity of the binding protein for its substrates appears to be required in any model of binding-protein transport systems, because the affinity of these proteins for their substrates is so high. This means that a carrier that could accept substrates from a binding protein would have an extremely high affinity and so would be detected by binding studies with whole membranes. Such a protein has not been found for any binding-protein system. Furthermore, the calculated rate of dissociation of substrate from the amount of glutamine-binding protein present in *E. coli* is only about 10% of the rate of glutamine transport (134). So little is currently known about the membrane proteins involved in binding-protein transport systems that any model is highly speculative. It is clear that the mechanism of these systems will differ in many ways from that of the class of membrane-bound systems.

Anomalous Transport Systems

There are two transport systems in *E. coli* that do not fall clearly into any one of the three classes. These are the cystine-specific (135) and the lysine-specific transport systems (136). These two systems are not inhibited by osmotic shock when the cells are grown in minimal medium, but are inhibited when the cells are grown in a medium containing yeast extract and bactotryptone. Furthermore, shock fluid from the cells grown in rich medium binds lysine and cystine; however, these activities are unstable and could not be purified further, so they are not well characterized. Both these amino acids are transported by vesicles (137), but the relationship of the systems in the vesicles to those seen in the whole cell has not been clearly established.

In the *E. coli* maltose transport system, which is clearly a binding-protein system, some results are not completely consistent with ATP being the energy source (138). However, in the cited study no controls were presented to show that arsenate had inhibited the ATP pool, so the possibility remains that ATP is the energy source as it is for all other binding-protein transport systems that have been studied.

The nature of the energy source for three potassium transport systems of *E. coli* has been studied (139). One system associated with a binding protein used ATP. The second was a membrane-bound system driven by proton motive force. The third system appeared to require both ATP and proton motive force; since it was not inhibited by osmotic shock and was

not active in membrane vesicles it may represent a new class of transport system. The dual energy requirement may result from use of one type of energy to drive active transport, and another to regulate transport. This system, which is the major potassium transport system in *E. coli,* clearly warrants more study.

MAMMALIAN TRANSPORT SYSTEMS

Red Cell Systems

INTRODUCTION Human red cells have been used to study a class of transport systems that move substrates through the membrane but do not concentrate them. Because of their simplicity these systems have been the subject of extensive kinetic studies in an attempt to see if they can be explained by a simple carrier model (140). A mechanism has been proposed that involves the binding of substrate to a single site followed by its movement across the membrane. The substrate then dissociates from a second site on the other side, and the carrier is altered so that the original site is available for another cycle. A thorough kinetic study of uridine transport indicates that this system does fit the model for a simple asymmetric carrier (141).

HEXOSE TRANSPORT SYSTEM The hexose transport system of red cells has been the subject of many studies, some of which are not consistent with a simple carrier model (142–144). A recent study concludes that there are two carriers in the membrane. The major one is responsible for about 85% of the transport and appears to satisfy the kinetic criteria for a simple asymmetric carrier (145). Because of its relatively low activity, kinetic properties of the second carrier have not been determined accurately.

Both kinetic data and studies with a number of inhibitors indicate that the carrier has different properties on the two sides of the membrane (146). Moreover, use of modified sugars showed that compounds with large substituents at the 4 or 6 position bind and inhibit at the outside surface of the membrane, but not on the inside, while molecules with large substituents at the 1 position given the opposite result (147).

This system has been the subject of a number of studies designed to determine which of the membrane proteins identified by gel electrophoresis (146) functions in sugar transport. These studies were of two types: In one, membranes were reacted with labeled inhibitors and the labeled protein was identified; in the other, membranes were extracted to remove as many proteins as possible while retaining glucose transport. Most of these studies were interpreted to indicate that a protein present in band 3 functioned in glucose transport. However, one of these studies, which used an imper-

meant maleimide, indicated that a protein present in zone 4.5 had this function (148). Another method used to identify the glucose carrier was the reconstitution of glucose transport into phospholipid vesicles by Triton extracts of red cell membranes (149). This assay was then used to purify the protein responsible for glucose transport, and it was shown to be present in zone 4.5 (150). The reconstituted transport was stereospecific and was inhibited by several inhibitors of red cell glucose transport. The purified protein had a specific activity 15-fold higher than the initial Triton extract and a mol wt of 55,000. It is a glycoprotein and was estimated to represent about 2% of the protein in the red cell membrane. The purified carrier moved as a very broad but symmetrical band during SDS gel electrophoresis, while zone 4.5 is broad and irregular, which suggests that it contains several components. Another group also used reconstitution to show that the glucose carrier is present in zone 4.5 (151). The turnover number of the glucose carrier is almost 500 sec^{-1}, which is about average for transport systems. A study on the transport of polyols of different sizes by this system has been interpreted as supporting a gated pore type model (152), which will be discussed further in the section on the mitochondrial adenine nucleotide transporter. This seems to be a reasonable mechanism for this type of carrier.

ANION TRANSPORT SYSTEM A third extensively studied red cell system is the anion transport system. Its properties have been recently reviewed (153), and only the work on the nature of the carrier itself is discussed here. This system has a very high turnover number, 7×10^5 ions $site^{-1}$ sec^{-1} (154). A number of amino reagents inhibit anion transport; the most specific reagents are the disulfonated stilbene derivatives, SITS and DIDS. Use of labeled derivatives showed that more than 90% of the label present in membrane proteins was in band 3 (155). This protein is one of the major red cell membrane proteins and has been shown to span the membrane (156). There has been a study of the position of different parts of the band 3 protein within the membrane (157). The C terminus is on the outside surface, and the N terminus is on the inside surface. Carbohydrate is attached toward the C-terminal end of this protein. When the outside surface of the membrane is treated with trypsin, a single cut produces a 63,000 mol wt fragment containing the original N terminus and a 30,000 mol wt fragment. Extensive digestion of the inside surface leaves only a 17,000 mol wt fragment of the protein in the membrane.

A number of other irreversible inhibitors also bind to to this same protein (158–160). The photo affinity compound N-(4-azido-2-nitrophenyl)-2-amino ethyl sulfonate, which is a substrate of the anion carrier, irreversibly inactivates this system in the light and is mainly bound to the band 3 protein (161).

Further evidence that band 3 is the anion carrier is the fact that red cell membranes depleted of most proteins except band 3, retain anion transport. The anion transport in these membranes has the same properties as that present in intact red cells (162). Finally band 3 has been incorporated into phospholipid vesicles that then were able to transport anions. Vesicle transport was not inhibited by several compounds that normally inhibit the anion transport system. However, when band 3 was isolated from membranes in which anion transport had been inhibited by an irreversible inhibitor, it did not reconstitute anion transport when it was incorporated into vesicles (162). Although the reconstitution results alone are not definitive, the results of all the different experiments indicate that band 3 functions in anion transport.

Two-dimensional gel electrophoresis of purified band 3 gave several components. The major protein represented 75% of the total protein and appears to be the anion carrier (163, 164). This protein probably is present in the membrane as a dimer (165, 166). Furthermore, reaction of one polypeptide per dimer pair with DIDS inhibits anion transport completely.

Mitochondrial Transport Systems

ADENINE NUCLEOTIDE CARRIER The movement of ADP into mitochondria and of ATP out of mitochondria is catalyzed by an electrogenic exchange carrier. This system appears to be the rate-limiting step in the overall process of mitochondrial ATP production, despite the fact that the carrier makes up 6% of the mitochondrial membrane protein (167). This system has been extensively studied, and there is strong evidence that it functions by a two-state gated pore mechanism. In this mechanism ADP binds to a form of the carrier present on the outside surface, and the carrier then undergoes a conformational change that allows the ADP to pass through the pore into the mitochondria. In the new conformation the binding site is now accessible from the inside and binds ATP. The carrier now reverts to its original conformation, allowing the ATP to pass through the pore to the cytoplasm and altering the binding site so it opens to the outside.

The studies that support this model have been reviewed in two articles (168, 169) that will be summarized. These studies were made possible by the existence of two compounds, atractyloside and bongkrekate, which bind tightly to the carrier, causing competitive inhibition. Atractyloside appears to bind predominantly to the form of the carrier present on the outside surface of the mitochondria; bongkrekate is a lipid soluble molecule that appears to bind predominantly to the site present on the inside surface of the mitochondria. Studies of ADP and ATP binding to whole mitochondria and to submitochondrial particles in which the membrane is inverted indicate that the carrier binding sites present on the two sides of the membrane are quite different. The outside site has a high affinity for ADP and a low

affinity for ATP; the inside site has the opposite specificity. This same conclusion has been reached in studies of the binding of spin-labeled inhibitors of the exchange carrier to mitochondria and submitochondrial particles (170, 171). These studies were interpreted to show that the carrier molecule does not span the membrane, at least when the inhibitors are bound; however, the evidence presented does not provide any strong support for this conclusion, and it is inconsistent with the other data for this system.

The carrier has been isolated and purified to homogeneity using the binding of labeled atractyloside or bongkrekate as an assay. The same molecule was isolated in each case, but the properties of the two carrier preparations were very different. The purified carrier has a subunit mol wt of 29,000 and appears to be present as a dimer in the membrane and in the detergent extract. Antibodies prepared against the two forms of the carrier did not cross-react, but the two forms could be interconverted by the addition of the appropriate inhibitor or adenine nucleotide. The binding of nucleotides to the carrier changes the shape of the mitochondrial membrane to such an extent that the change can be observed by light scattering or electron microscopy. This provides further evidence that binding of nucleotides to the carrier causes a large conformational change.

Another assay that has been used to isolate the carrier is the reconstitution of activity into phospholipid vesicles (172). The carrier was purified about 50-fold; the largest protein present in the preparation had a mol wt of 29,000. The 50-fold purification was higher than would be predicted if 6% of the protein is the carrier but may result from partial inactivation during the extraction step. The properties of the reconstituted system were similar to those found in whole mitochondria.

PHOSPHATE TRANSPORTER Mitochondria have two different pathways for transporting phosphate. One exchanges phosphate for dicarboxylic acids and has a V_{max} of 15 nmole/min/mg; the other pathway exchanges phosphate for OH and has a V_{max} of 200 nmole/min/mg. Both systems have K_m values for phosphate near 1.5 mM (173). The carrier for the latter system has been extracted from mitochondria with octylglucoside and reconstituted into phospholipid vesicles (174). The reconstitution of activity was used as an assay to obtain about a six-fold purification of the carrier. The reconstituted phosphate/OH^- exchange reaction was shown to be electrogenic. The orientation of the reconstituted carrier resembed that of mitochondria. It is inhibited by both N-ethylmaleimide and N-benzylmaleimide, in contrast to the carrier in submitochondrial particles, which is inhibited only by N-benzylmaleimide.

When both the purified phosphate carrier and the adenine nucleotide carrier were reconstituted into the same vesicles, phosphate stimulated ADP/ATP exchange (175). This result supports the proposal that these two

electrogenic carriers move charges in opposite directions in order to maintain electroneutrality during oxidative phosphorylation in mitochondria. Evidence that this system may function by a gated pore type mechanism is the observation that the treatment of mitochondria, loaded with phosphate, with an SH reagent that inhibits the phosphate carrier causes the loss of an amount of phosphate that is approximately equal to the number of phosphate carrier molecules present in the mitochondria (176). A suggested explanation for this finding is that the inhibitor binds to the carrier site only on the outside of the mitochondria, and normally the carrier is present on the inside, bound to phosphate; thus when the carrier moves a phosphate across the membrane it becomes inhibited, releasing the phosphate bound to it into the medium.

Sodium-Potassium ATPase

The sodium-potassium ATPase creates and maintains the sodium and potassium gradients present in the cells of most higher organisms and also maintains osmotic equilibrium. The gradients that it produces, higher potassium and lower sodium inside the cell, are used for excitation by nerve and muscle cells and to drive active transport of certain compounds in many cells. Many articles review the properties of this enzyme. Some of the more recent ones are listed in References 177–181.

The enzyme has been purified to homogeneity by quite different procedures, and in each case the purified enzyme contained two polypeptides. One has a mol wt of 95,000 and contains both an aspartic residue that becomes phosphorylated during the transport reaction and the ouabain binding site. A tripeptide containing the phosphorylation site has been isolated by pronase digestion of ^{32}P-labeled enzyme and has the sequence $\binom{\text{thr}}{\text{ser}}$-asp-lys. The same labeled tripeptide was isolated after pronase digestion of the Ca^{2+} ATPase of the sarcoplasmic recticulum (182).

The second polypeptide has a mol wt of 50,000 and is a glycoprotein. Antibodies prepared against this subunit partially inhibit the ATPase activity of the native enzyme. Crosslinking studies on purified ATPase show that the large and small subunit are adjacent and that the large subunit is present as a dimer (183). Other workers who studied the enzyme both in intact membranes and in its purified form found evidence for dimer formation with both the large and small subunits in both forms of the enzyme but did not find crosslinking between the large and the small subunit (184).

There is some disagreement about the ratio of small to large subunits in the enzyme, with values of 2, 1, and 1/2 being reported (179, 183, 185). The last value is the most recent and gives a mol wt of 250,000 for the native enzyme, which agrees with the value reported from radiation inactivation studies. There appears to be one ouabain binding site and either one or two phosphorylation sites per 250,000 mol wt (186). These values are consistent

with kinetic studies that indicate that the enzyme shows 1/2 site reactivity. About 2 moles of sodium are bound with high affinity per 250,000 grams of protein and this binding is inhibited by ouabain (187). Since the ouabain binding site is present on the outer surface of the membrane, and the phosphorylation site is present on the inside surface, the large subunit must span the membrane.

The purified enzyme has been reconstituted into phospholipid vesicles, which then carry out sodium and potassium transport. The properties of the transport reaction in the reconstituted system are quite similar to those of the native system (179, 188).

The basic reaction catalyzed by this enzyme is the movement of $3Na^+$ from the inside of the cell to the external medium and the movement of $2K^+$ in the opposite direction. During this reaction a molecule of ATP is split to ADP and inorganic phosphate, providing the energy to move the ions against concentration gradients. The reaction is reversible if the ion gradients are large. There is evidence that the reaction occurs in a number of steps. In one proposed mechanism the first step is the binding of $3Na^+$ and ATP to the enzyme, which is followed by the hydrolysis of ATP to form phosphorylated enzyme and ADP. The next step is a conformational change in the enzyme that allows the Na^+ to pass through the membrane. Then, $2K^+$ ions bind to the enzyme and the phosphorylated group is hydrolyzed to give phosphate. In the final step the enzyme returns to its original conformation, which allows the K^+ to enter the cell. Mg^{2+} is required for the overall reaction. The last step may require the binding of ATP to a second low-affinity site on the enzyme (189). Kinetic arguments have been raised against a sequential model, but the direct evidence in favor of it seems much stronger (190).

The K_m for Na^+ on the inside surface of the membrane is about 1/100 the K_m for Na^+ on the outside surface, whereas the K_m values for K^+ on the two surfaces show the opposite relationship. There is also evidence that the Na^+-binding sites are different from the K^+-binding sites (180). Tryptic digestion of the small subunit produced Na^+-dependent ionophoric activity as tested by a black lipid membrane, but this finding could not always be reproduced (191). Potassium-dependent ionophoric activity was present in the peptides produced by cyanogen bromide cleavage of the whole enzyme. Other indirect evidence that Na^+ and K^+ binding is associated with the small subunit is the fact that Na^+ and K^+ partially protect this subunit against tryptic digestion (192).

In different experiments, which used much lower levels of trypsin, digestion of the intact enzyme in the presence of Na^+ gave a different set of products than when K^+ was present during digestion. This suggests that the two ions are bound to different conformational states of the enzyme (193). Additional evidence for two conformational states is the formation of two

classes of antibody against the pure enzyme that appear to react with different conformational states, although the evidence for this is indirect (194).

The binding of Mg^{2+} and P_i to the enzyme was studied by microcalorimetry. The ΔH values for binding of these compounds were –49 kcal mol^{-1} and –42 kcal/mole respectively (195). These large values indicate that the binding of either substrate results in a conformational change in the enzyme. The binding of either molecule may cause the same conformational change, since the binding of both molecules gave a ΔH of –41 kcal $mole^{-1}$. The binding of these compounds altered the reactivity of the enzyme to both sodium borohydride and to 7-chloro-4-nitrobenzo-oxa-1-diazole, although the changes were not large. Finally it has been shown that cardiac glycosides bind 100 times more tightly to the phosphorylated form of the enzyme than to the nonphosphorylated form (183).

Kinetic studies indicate that in the presence of K^+, ATP, and free Mg^{2+} the enzyme can undergo a conformational change to an inactive form and that this change may occur in vivo (196). The overall properties of this enzyme are consistent with the two-state gated pore model described for the adenine nucleotide carrier, with the additional feature that the interconversion of the two forms is driven by ATP hydrolysis so that active transport can occur.

Sarcoplasmic Reticulum Ca^{2+} ATPase

The sarcoplasmic reticulum is a vesicular membranous component of muscle cells that controls muscle contraction by changing the internal Ca^{2+} concentration. In the resting state, Ca^{2+} is inside the reticulum vesicles and stimulation causes its release. The cell is returned to the resting state by the transport of Ca^{2+} into the vesicle. About 65% of the protein in this membrane is a single polypeptide, with a mol wt of 100,000, which catalyzes the ATP-driven movement of Ca^{2+} across the membrane. The properties of this enzyme have been the subject of several recent reviews (197–199).

The stoichiometry of the reaction is clearly $2Ca^{2+}$-transported per ATP hydrolyzed and probably $1Mg^{2+}$ moved in the opposite direction, although this is not as well defined. The enzyme is composed of the 100,000 mol wt polypeptide and variable amounts of a low molecular weight proteolipid. The enzyme can be phosphorylated by ATP in the presence of Ca^{2+}. About 1 mole of phosphate is incorporated per mole of enzyme. The β carboxyl group of an aspartic residue accepts the phosphate group, and a peptide containing this residue has been sequenced (200). The enzyme can also be phosphorylated by inorganic phosphate. If a calcium gradient is present, this phosphate residue can be donated to ADP in a reversal of the normal reaction. Under these conditions only one mole of phosphate per two enzyme molecules is incorporated when inorganic phosphate is the donor.

ATP synthesis also occurs with purified enzyme that is not present in sealed vesicles (201). In these experiments the binding of Ca^{2+} ions probably provides the energy for the synthesis of ATP from ADP and P_i. Only about 0.14 mole of ATP were formed per mole of enzyme in these experiments. The purified enzyme binds Ca^{2+}, but the exact amount and the affinities of the sites vary between different studies. Rapid kinetic experiments indicate that phosphorylation of the enzyme causes after a short lag, the release of bound Ca^{2+} (202).

Isoelectric focusing separates the pure enzyme into 6 species, all of which have the original 100,000 mol wt (203). This separation is probably the result of either chemical modification or differential binding of charged molecules to a single class of polypeptide chains, since all the ATPase molecules can be phosphorylated during the transport reaction. In addition, limited tryptic digestion of sarcoplasmic reticulum vesicles cleaves all the enzyme molecules to give species of 55,000 and 45,000 mol wt. This cleavage does not inhibit either ATPase or transport activity. The larger fragment, which contains the phosphorylation site, is mostly present on the outside surface of the membrane, since it becomes heavily labeled during iodination of sarcoplasmic reticulum vesicles. The small fragment appears to be buried inside the membrane and has a considerably more hydrophobic amino acid composition than the larger fragment. More extensive digestion cleaves the 55,000 fragment to species of 30,000 and 20,000 mol wt. The site of phosphorylation is in the 30,000 mol wt fragment.

The enzyme has Ca^{2+}-dependent ionophoric activity when it is either succinylated or digested with trypsin (191). The ionophoric activity of a limited tryptic digest was associated with a 20,000 mol wt fragment that was derived from the 55,000 mol wt fragment discussed before. When the 20,000 mol wt fragment was cleaved with cyanogen bromide, the products retained ionophoric activity. The 45,000 mol wt fragment produced by a single cut in the enzyme also had ionophoric activity, but it did not show any ion dependence. The proteolipid fraction also has a nonspecific ionophoric activity.

A direct role for the proteolipid in Ca^{2+} transport was proposed, based on reconstitution experiments. Preparations of the ATPase deficient in the proteolipid gave good ATPase activity after reconstitution, but had low Ca^{2+} transport (204) activity. The ratio of Ca^{2+} transport to ATPase activity was restored to its normal value by the addition of purified proteolipid. Furthermore, when the proteolipid was incorporated into phospholipid vesicles, they became permeable to Ca^{2+}. It is not known whether the proteolipid functions directly to allow Ca^{2+} to pass through the membrane or indirectly by changing the membrane in a nonspecific fashion. The proteolipid was characterized in an earlier study, but no further chemical study has been reported, in contrast to the proteolipid associated with

proton-translocating ATPase, which has been thoroughly characterized (205).

There have been extensive studies on the phospholipid requirements of the Ca^{2+} ATPase (206). The minimum amount of phospholipid that still preserves activity is 30 molecules per ATPase molecule. The specificity of the lipid requirement for ATPase activity is different from the specificity for Ca^{2+} transport.

The Ca^{2+} ATPase also appears to function in Ca^{2+} efflux. This efflux is extremely rapid and does not appear to be a reversal of the uptake reaction. It has been proposed that the efflux occurs through a pore formed by the aggregation of 4 ATPase molecules (207). A number of results suggest that ATPase molecules do aggregate, but this model is not yet proven.

Sodium Symport Systems

There is considerable evidence that many mammalian active transport systems are electrogenic sodium symports. Two such systems are the neutral amino acid system (208–210) and a sugar transport system present in the brush border membrane of intestinal and kidney cells (211, 212). The properties of the sugar transport systems of kidney cells have been reviewed recently (213). Some of the strongest evidence for a sodium symport mechanism comes from studies using membrane vesicles (211, 214, 215). There is still controversy about this mechanism, especially in the case of the neutral amino acid transport system for which it has been suggested that transport may also be directly coupled to the oxidation of DPNH (216). Much of the controversy arises because of the difficulties involved in determining the membrane potentials present in the cell under different conditions. Overall, the recent data strongly supports a sodium symport model. An amino acid binding protein that functions in amino acid transport has been extracted from ascites cell membranes and partially purified (217). This protein can reconstitute amino acid uptake into phospholipid vesicles. The reconstituted transport requires a sodium gradient and has essentially the same specificity as does the neutral amino acid transport system of whole cells.

Sodium-dependent glucose transport has also been reconstituted into phospholipid vesicles by use of a Triton extract of brush border membranes (218). The protein or proteins responsible for this have not yet been purified to homogeneity, but work is continuing on this project.

Binding Proteins

Proteins that bind calcium and are linked by indirect evidence to calcium transport have been isolated from the intestinal mucosa of a number of species (for review see 219, 220). These proteins are not hydrophobic, and probably do not function as membrane carriers. If they function at the

external surface of the membrane, as has been suggested, some mechanism is required to keep them from diffusing away from the cell. If they are intracellular proteins, a nontransport role would seem more probable, and the fact that the primary structure of one of these proteins is similar to a large number of intracellular Ca^{2+}-binding proteins that do not function in transport makes this a possibility (221).

Two proteins that function in vitamin B_{12} transport, transcobalamine II and intrinsic factor, are clearly present outside the cell and function to bring vitamin B_{12} to the membrane (222, 223). The available evidence, although still indirect, suggests that the B_{12} protein complex is moved into the cell by pinocytosis. Mutants altering each protein are known and appear to inactivate B_{12} transport.

Intestinal Hydrolyases

The mammalian intestinal brush border membranes contain a number of hydrolytic enzymes and transport systems. These function in the digestion and uptake of metabolites from the lumen. Most of each hydrolyase molecule is external to the membrane, but a small segment of about 10,000 mol wt at the N terminal end of each molecule is integrated into the membrane (224, 225). Studies on an intestinal amino peptidase have shown that part of the hydrophobic tail is present on the inside surface of the membrane, which indicates that this molecule spans the membrane (226).

It has been suggested that sucrase-isomaltase not only catalyzes the hydrolysis of its substrates but, in addition, transports some of the products across the membrane (227, 228). Most of the hydrolysis products are transported by the Na^+-dependent transport systems present in the membrane; however, some enter the cell by another mechanism. This transport only occurs with products resulting from the activity of the enzyme. There was an early report that purified sucrase-isomaltase, reconstituted into black lipid membranes, transported some of the glucose derived from sucrose (229). However, this work utilized enzyme that lacked the hydrophobic segment and has not been repeated. In more recent studies that used the intact enzyme and liposomes, there was no transport by the reconstituted enzyme (230). It appears at present that while there is evidence suggesting a limited transport by intestinal hydrolyases, it is not of major physiological significance. The process is complex and still not understood.

The γ-Glutamyl Cycle

γ-Glutamyl transferase is present in the membrane fraction of most cells and catalyzes the reaction:

$$\text{Amino acid} + \text{Glutathione} \rightarrow \gamma\text{-Glutamyl-Amino acid} + \text{Cys-Gly}.$$

It has been proposed that this and several other enzymes function in the transport of amino acids (231) by a cyclic set of reactions. Evidence for this proposal is the fact that the transferase is bound to the membrane and is particularly active in tissues that transport amino acids at a high rate. The symptoms of patients with a defect in the enzyme γ-glutamyl-cysteine synthetase, which catalyzes one of the proposed set of reactions required for transport by γ-glutamyl transferase, are consistent with this model but do not prove it. However, a fibroblast cell line has been isolated that has less than 0.5% of the wild-type activity of this enzyme, and amino acid transport in this strain is indistinguishable from that in the wild-type strain (232). This result indicates that if γ-glutamyl transferase functions in amino acid transport, it must be responsible for only a minor component of the total transport by fibroblasts.

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