Transport Studies in Bacterial Membrane Vesicles

Cytoplasmic membrane vesicles devoid of soluble constituents catalyze the transport of many metabolites.

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Isolated bacterial cytoplasmic membrane vesicles have proved to be a particularly useful model system for studies of active transport. These vesicles are devoid of the cytoplasmic constituents of the intact cell, and their metabolic activities are restricted to those provided by the enzymes of the membrane itself. This constitutes a great advantage over intact cells in the study of transport mechanisms, since transport of various solutes by membrane vesicles is practically nil in the absence of the appropriate exogenous energy source. Thus the energy source for transport of a particular substrate can be determined by studying which substances stimulate solute accumulation. Moreover, metabolic conversion of the transport substrate and the energy source is minimal, allowing clear definition of the reactions involved in transport. With intact bacteria, transported solutes are usually rapidly accumulated by virtue of the cell's endogenous metabolism, and addition of an external energy source has little or no effect. Moreover, extreme measures are often required to deplete these endogenous energy reserves to the point where added energy sources stimulate transport. Even when this can be accomplished, the results may be difficult to interpret in view of the cells' ability to metabolize the energy source, the transported solute, or both. In this article some of the properties of bacterial membrane vesicles are described, and some recent experimental observations stemming from studies of this model system are reviewed. Certain topics have been reviewed by others (*1*, *2*).

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Preparation and Properties of Bacterial Membrane Vesicles

An electron micrograph of an intact *Escherichia coli* cell is shown in Fig. 1. The cell is rod-shaped, with two trilaminar, unit membrane structures bordering its exterior. The outer membrane, present in gram-negative bacteria only, is the lipopolysaccharide layer of the cell wall; the inner is the plasma membrane. Located between these membranes, in the periplasmic space, is the peptidoglycan layer of the cell wall; this structure, however, cannot be seen in Fig. 1. The rigid peptidoglycan layer is partially responsible for the shape of bacterial cells, allows concentration of solutes against large concentration gradients, and prevents the bacterium from bursting in hypotonic environments (*3*). Within the plasma membrane are the ribosomes, nucleoplasm, and most of the "soluble" components of the cell.

When a bacterium such as that shown in Fig. 1 is grown in the presence of penicillin or treated with enzymes that attack the rigid layer of the cell wall (that is, lysozyme with most bacteria or lysostaphin with *Staphylococci*), the rigid peptidoglycan layer is outgrown or degraded, respectively. As a result, the cell becomes sensitive to changes in osmolarity and will burst in sufficiently hypotonic media. This manipulation is the basis for the preparation of bacterial membrane vesicles (*4*).

The structures shown in Fig. 2 are membrane vesicles obtained from spheroplasts of *E. coli* ML strain that were lysed against a large osmotic gradient in the presence of ethylenediaminetetraacetic acid (EDTA), deoxyribonuclease, and ribonuclease, followed by extensive washing and differential centrifugation. The structures consist of intact unit membrane-bound sacs varying from about 0.5 to 1.0 micrometer in diameter. The sacs are empty and without apparent internal structure, and most are surrounded by a single 65- to 70-Å membrane. When other strains of *E. coli* or other gram-negative bacteria are subjected to these procedures, intact, empty membrane-bound sacs are also obtained, but the vesicles are more heterogeneous than those prepared from the ML strain. The diameters of the sacs vary from 0.1 to 1.5 μm, and they are surrounded by one to five or six membrane layers. The reason for this difference is probably related to the observation that ML vesicles contain negligible quantities of lipopolysaccharide, whereas vesicle preparations from other gram-negative cells contain significant quantities of this cell wall component. In any case, there are no apparent physiological differences between membrane vesicles prepared from the ML strain and those prepared from other strains of gram-negative bacteria, as judged by any of the functional activities studied thus far. Moreover, vesicles from gram-positive organisms that have no lipopolysaccharide also catalyze active transport.

The purity and homogeneity of the vesicles have been established by a variety of criteria (*1*, *4–7*). In summary, they retain only minute quantities of soluble cytoplasmic components, as well as at least 70 percent of the phospholipids of the cells from which they are prepared. Less than 10 percent of the diaminopimelic acid of the osmotically sensitized form is found in the vesicles, indicating that very little peptidoglycan is left in the preparations. Regarding lipopolysaccharide, ML vesicles have less than 3 percent (by dry weight), whereas vesicles from a number of other strains of *E. coli* and *Salmonella typhimurium* have from 7 to 17 percent.

Expressed as a function of dry weight, the vesicles are approximately 60 to 70 percent protein, 30 to 40 percent phospholipid, and about 1 percent carbohydrate.

An essential property of a system that is to be used to study transport is that it must have a continuous surface. Although the sectioned material presented in Fig. 2 suggests that the vesicles are closed structures, other techniques must be utilized to substantiate

this impression. The electron micrographs shown in Figs. 3 and 4 were obtained with positive staining (Fig. 3) or freeze etching (Fig. 4) so that the surface of the vesicles can be observed. The micrographs show typical vesicles prepared from *E. coli* ML 308-225. In both cases, there are no obvious defects in the surface of the vesicles. More convincing evidence for membrane continuity is the demonstration that the vesicles are osmotically intact. They shrink and swell appropriately when the osmolarity of the medium is altered, as shown by light scattering (*8*), or by measurement of the dextran-impermeable intramembranal space (*1*). Moreover, there is a diffusion barrier to phosphoenolpyruvate that can be overcome by osmotically shocking the vesicles in the presence of this highly charged phosphorylated compound (*1*).

A number of observations indicate that the vesicle membrane does not become inverted during lysis. The most direct evidence comes from freeze etch microscopy. Visualization of many vesicles such as those seen in Fig. 4 demonstrates that the "texture" of the convex and concave surfaces is always as shown. The reverse pattern is not observed unless the vesicles are disrupted by physical means. These findings have been confirmed with vesicles prepared from *Bacillus subtilis* and *E. coli* by Konings *et al.* (*9*) and Altendorf and Staehelen (*10*), respectively. Lysis can also be observed directly by phase contrast microscopy, and the cell membrane does not turn inside out during lysis (*11*). Finally, a high proportion of the vesicles catalyzes active transport (*12*).

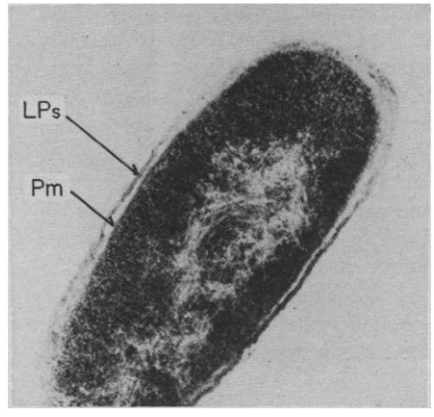

Fig. 1. Electron micrograph of a longitudinal section through an intact *E. coli* cell. LPs, lipopolysaccharide; Pm, plasma membrane. This micrograph was taken by Dr. Samuel Silverstein of the Rockefeller University. [Courtesy of Chemical Rubber Company]

Definitions

Several mechanisms by which substances cross membranes require definition since their distinction is important for the following discussion. These mechanisms, which have been described in detail (*13*), are as follows.

Passive diffusion. The solute crosses the membrane as the result of random molecular motion, and does not interact specifically with any molecular species in the membrane. Rates of passive diffusion may be altered by membrane charge, solvent drag (in which the penetrating solute is swept through aqueous pores in the membrane by bulk water flow), and by the degree of hydrophobicity of the diffusion barrier.

Facilitated diffusion. Solute combines reversibly with a specific carrier molecule in the membrane, and the carrier-solute complex oscillates between the inner and outer surfaces of the membrane, releasing and binding solute on either side. In view of the short distances covered, thermal energy or molecular deformation (or both) resulting from binding and release of solute can account for the small amount of motion required.

Neither of the above two mechanisms requires metabolic energy, nor do they lead to concentration of solute against an electrochemical or osmotic gradient.

Active transport. Solute is accumulated against an electrochemical or osmotic gradient at the expense of metabolic energy. This mechanism, like facilitated diffusion, requires a specific membrane carrier molecule. Most models for this mechanism postulate that the penetrating species combines with a carrier and that the carrier or the carrier-solute complex is then subjected to modification in the membrane. The carrier-solute complex formed on the outside surface of the membrane is modified in such a way that the carrier has a lower affinity for solute. Solute is released into the interior of the cell, the high affinity form of the carrier is regenerated, and the cycle is repeated.

Group translocation. A covalent change is exerted upon the transported molecule so that the reaction itself results in the passage of the molecule through the diffusion barrier. This is not a "classic" active transport mechanism since the solute is modified chemically.

Transport in Bacterial Membrane Vesicles

Transport studies with membrane vesicles (*14*) are carried out by incubating vesicles with a radioactive trans-

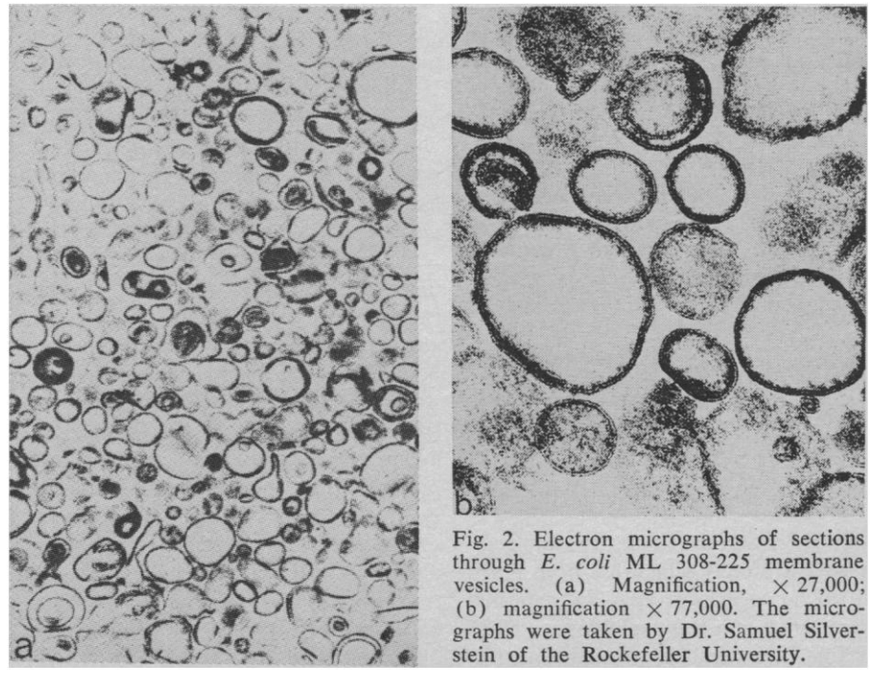

Fig. 2. Electron micrographs of sections through *E. coli* ML 308-225 membrane vesicles. (a) Magnification, × 27,000; (b) magnification × 77,000. The micrographs were taken by Dr. Samuel Silverstein of the Rockefeller University.

port substrate in the presence of a specific energy source. At a given time, the reaction mixtures are diluted to terminate the uptake reaction, and the vesicles are separated from the medium by means of rapid filtration. Alternatively, the vesicles can be separated from the reaction mixture by centrifugation, but, in this case, samples cannot be assayed very rapidly and corrections must be made for radioactive solute trapped in the pellet.

As early as 1960 (*15*), evidence was presented which suggested that bacterial membrane vesicles might provide a useful model system for the study of active transport and other membrane-related phenomena. Subsequent studies have demonstrated that the vesicles catalyze the active transport of a wide variety of metabolites in the presence of appropriate energy sources (*1, 5*). Initial rates of transport of many of these substances are comparable to those of the intact cell (*16, 17*), and the vesicles accumulate these solutes to concentrations many times in excess of that in the external medium. Isolated membrane vesicles have also provided an interesting system for studying certain aspects of phospholipid biosynthesis (*18*).

What are the energy sources for transport in isolated membrane vesicles? The answer varies with the organism and the substance transported. In *E. coli* membrane vesicles, the uptake of glucose, fructose, and mannose occurs by vectorial phosphorylation via the phosphoenolpyruvate-phosphotransferase system [a group translocation mechanism] (*1, 2, 19, 20*). This phosphotransferase system, which was described initially in 1964 (*21*), catalyzes the transfer of phosphate from phosphoenolpyruvate to certain carbohydrates according to the following overall reaction:

$$\text{Sugar} + \text{phosphoenolpyruvate} \longrightarrow \text{sugar phosphate} + \text{pyruvate}$$

The individual reactions involved are as follows:

$$\text{Phosphoenolpyruvate} + \text{HPr} \xrightleftharpoons{\text{enzyme I, } Mg^{2+}} \text{pyruvate} + \text{phospho-HPr} \quad (1)$$

$$\text{Phospho-HPr} + \text{sugar} \xrightarrow[\text{(factor III)}]{\text{enzyme II, } Mg^{2+}} \text{sugar phosphate} + \text{HPr} \quad (2)$$

Both HPr, a heat stable, low-molecular-weight protein which has been purified to homogeneity (*22*), and enzyme I are predominantly soluble proteins; enzyme II is membrane bound. Enzyme II is responsible for specificity with respect to the various sugars studied, and it has been solubilized and partially purified (*23, 24*). At least two protein fractions (one of which is sugar specific) and phosphatidylglycerol are required for *E. coli* enzyme II activity (*23, 25*). There is also evidence in some systems for the involvement of a fourth protein (factor III) whose function is unknown (*26*).

Uptake and phosphorylation of α-methylglucoside, glucose, fructose, or mannose by membrane vesicles from *E. coli* (*1, 5, 19*) are almost totally dependent on the presence of phosphoenolpyruvate and only phosphoenolpyruvate. There is little or no dephosphorylation or subsequent metabolism of the sugar phosphates formed, and membrane vesicles prepared from various HPr or enzyme I mutants are not able to take up or phosphorylate α-methylglucoside or glucose with or without phosphoenolpyruvate. Thus, the effect of phosphoenolpyruvate is mediated by the phosphoenolpyruvate-phosphotransferase system. In addition, there is a stoichiometric relationship between ^{32}P-labeled phosphoenolpyruvate disappearance and the appearance of ^{32}P in α-methylglucoside phosphate, suggesting that phosphoenolpyruvate provides energy for the simultaneous uptake and phosphorylation of these sugars.

Direct evidence for vectorial phosphorylation is derived from double isotope experiments (*19*) in which the intravesicular pool is first loaded with [^{14}C]glucose under conditions in which there is no phosphorylation. After removal of the external isotope, the loaded vesicles are exposed to [^{3}H]glucose in the presence of phosphoenolpyruvate. If glucose passes through an internal pool before phosphorylation, the [^{14}C]-glucose already present in the internal pool should be phosphorylated before external [^{3}H]glucose. Conversely, if glucose does not pass through an internal pool prior to phosphorylation, the rate of phosphorylation of [^{3}H]glucose might exceed that of [^{14}C]glucose already present in the intravesicular pool. The experimental results demonstrate that the vesicles phosphorylate [^{3}H]glucose in the external medium much more rapidly than [^{14}C]glucose present in the intravesicular pool. As such, the experiment provides strong evidence that the phosphoenolpyruvate-phosphotransferase system catalyzes the translocation of sugars, as well as their concentration within the vesicles as phosphorylated derivatives.

The possibility that the phosphoenolpyruvate-phosphotransferase system phosphorylates sugars that enter an intramembranal pool by facilitated or passive diffusion is also inconsistent with a number of other observations (*1, 5, 19*), and recent studies with intact *Aerobacter aerogenes* cells (*27*) provide independent support for this interpretation.

Evidence for other group translocation reactions has also been presented. Uptake of adenine by *E. coli* membrane vesicles is accompanied by its conversion to adenosine monophosphate (AMP) and is stimulated by 5-phosphoribosyl-1-pyrophosphate (*28*). The enzyme mediating this activity—adenine phosphoribosyltransferase—is required for uptake, and variations in enzyme activity are reflected by changes in adenine transport. Acetyl coenzyme A : butyryl coenzyme A transferase appears to catalyze the translocation of butyrate in membrane vesicles prepared from a strain of *E. coli* that is depressed for acetoacetate degradation (*29*). The vectorial role of the transferase reaction in butyrate uptake is supported by experiments demonstrating that butyrate uptake is stimulated by acetyl coenzyme A, and that butyrate is accumulated in the vesicles as butyryl coenzyme A. A number of kinetic observations are also consistent with this conclusion. Testosterone uptake in membrane vesicles from *Pseudomonas testosteroni* is dependent upon nicotinamide adenine dinucleotide (NAD), and testosterone is converted initially to 4-androstenedione (*30*). 17β-Hydroxy-steroid dehydrogenase is apparently essential for testosterone uptake since 4-androstenedione is not accumulated when added to the vesicles.

As opposed to the group translocation mechanisms, uptake of β-galactosides (*31–33*), galactose (*34*), arabinose (*33*), glucuronic acid (*33, 35*), gluconic acid (*36*), hexose phosphates (*33, 37*), amino acids (nine independent systems) (*15, 38*), peptides (*39*), hydroxy- (*40*), keto- (*40*), and dicarboxylic acids (*40, 41*), and nucleosides (*42*) by *E. coli* membrane vesicles occurs by active transport. Potassium or rubidium transport in the presence of valinomycin (*43, 44*) is also included in this rapidly expanding list. These transport systems are coupled primarily to the oxidation of D-lactate to pyruvate, catalyzed by a flavin-linked, membrane-bound D-lactate dehydrogenase (D-LDH). Electrons derived from D-lactate are passed to oxygen via a mem-

brane-bound respiratory chain, and in the sequence of reactions between the primary dehydrogenase and cytochrome b_1 (*5, 32*), which is the first cytochrome in the *E. coli* respiratory chain, respiratory energy is converted into work in the form of active transport.

Although other oxidizable substrates such as L-lactate, succinate, or reduced NAD (NADH) also stimulate transport to some extent, they are not nearly as effective as D-lactate. It should be emphasized that D-lactate is not the electron donor for active transport in all bacterial membrane vesicles. In membrane vesicles from *Staphylococcus aureus*, for instance, amino acid transport is coupled to the oxidation of either L-α-glycerol phosphate or L-lactate, depending on the conditions of growth of the cells used to prepare the vesicles (*17, 45, 46*). For *Bacillus subtilis* vesicles, oxidation of L-α-glycerol phosphate, L-lactate, or NADH provide the driving force for amino acid transport (*47, 48*). Nonphysiological electron donors such as reduced phenazine methosulfate (*49*) or pyocyanine (*50*) will also drive transport in membrane vesicles from many different bacteria. The observations summarized in Table 1 represent a tabulation of electron donors that effectively drive transport in the vesicle systems studied to date.

It is important to recognize that active transport in these systems does not involve either the generation or hydrolysis of adenosine triphosphate (ATP). This contention is supported by several lines of evidence (*5*): (i) ATP does not stimulate transport of any substrate under any of the conditions tested (*51*), although it can be shown that ATP has access to reactive sites within the vesicles (*18*); (ii) the vesicles are not able to catalyze oxidative phosphorylation (*48, 52*), and transport occurs in the presence of high concentrations of arsenate (*25, 31, 34, 35, 53*); (iii) very little ATP can be detected in the membrane preparations whether or not oxidizable substrates are present (*45, 48*); (iv) conditions that stimulate or inhibit adenosine triphosphatase have no effect on transport in the vesicles (*53, 54*); and (v) transport occurs in a completely normal fashion in membrane vesicles prepared from *uncA* mutants of *E. coli* (*54*). These mutants are deficient in Ca^{2+}, Mg^{2+}-stimulated adenosine triphosphatase and uncoupled for oxidative phosphorylation (*55*).

Transport Activity of Individual Membrane Vesicles

Do all of the membrane vesicles in a preparation catalyze transport or is the activity observed a property of only a small percentage of the population? Although the transport activity of isolated membrane vesicles is comparable to that of the parent whole cells in many cases (*16, 17*), quantitative comparisons between vesicles and whole cells are difficult to interpret, especially when the activity manifested by whole cells may be a composite result of more than one uptake system or when one component of a transport system is not tightly bound to the membrane. The experiments described below have allowed a direct approach to this problem with *E. coli* membrane vesicles.

The acetylenic hydroxy acid, 2-hydroxy-3-butynoic acid (*56*), is an irreversible inactivator of D- and L-LDH's and D-lactate–dependent active transport in isolated membrane vesicles from *E. coli* (*57, 58*). The compound is a substrate for the membrane-bound, flavin-linked D-LDH, which undergoes 15 to 30 turnovers prior to inactivation. Inactivation is due to covalent attachment of a reactive intermediate to flavin adenine dinucleotide (FAD) at the active site of the enzyme. The proposed reaction sequence is shown in Fig. 5 (reaction II). Both the hydroxy function and the alkyne linkage in 2-hydroxy-3-butynoate are critical for inactivation. Appropriately, 3-butynoate has no effect on the enzyme; and vinylglycolate (2-hydroxy-3-butenoate) serves as a substrate for D-LDH, and is thus an effective electron donor for transport.

Inactivation of D- and L-LDH and

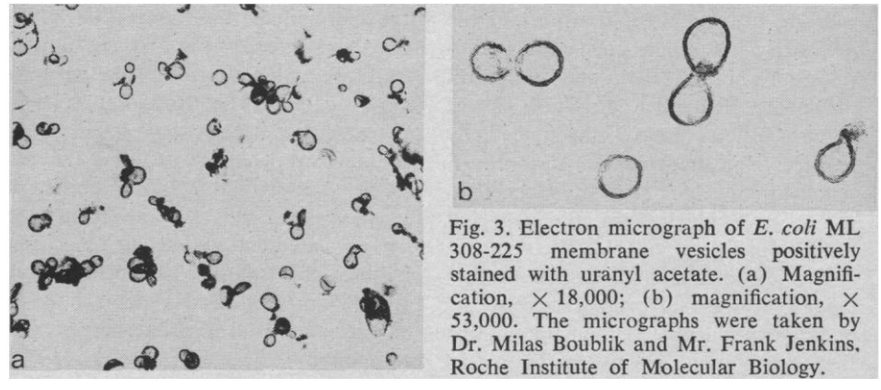

Fig. 3. Electron micrograph of *E. coli* ML 308-225 membrane vesicles positively stained with uranyl acetate. (a) Magnification, × 18,000; (b) magnification, × 53,000. The micrographs were taken by Dr. Milas Boublik and Mr. Frank Jenkins, Roche Institute of Molecular Biology.

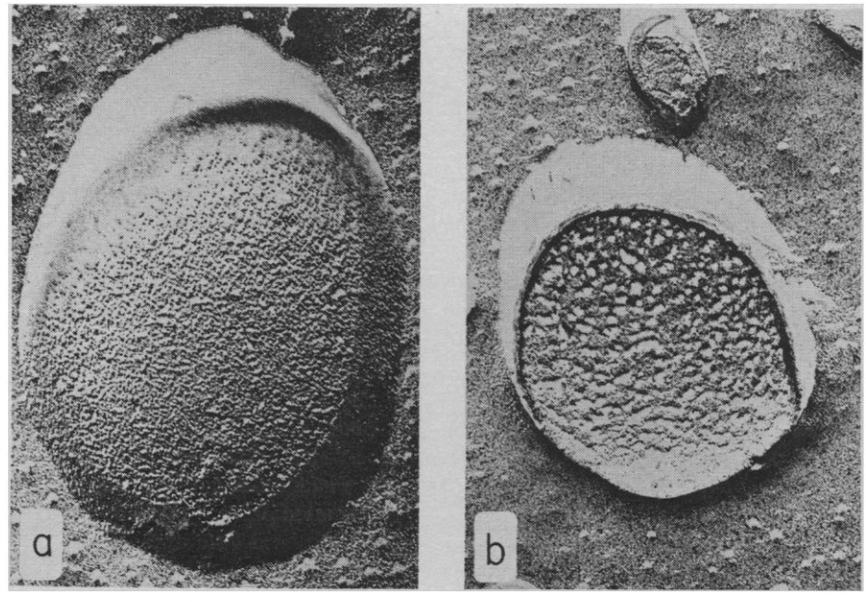

Fig. 4. Electron microscopy of freeze-etched membrane vesicles from *E. coli* ML 308-225. (a) Outer surface; (b) inner surface (magnification of both × 85,000). The micrographs were taken by Drs. Thomas Tillack and Vincent Marchesi at the National Institute of Arthritis and Metabolic Diseases [Courtesy of Chemical Rubber Company]

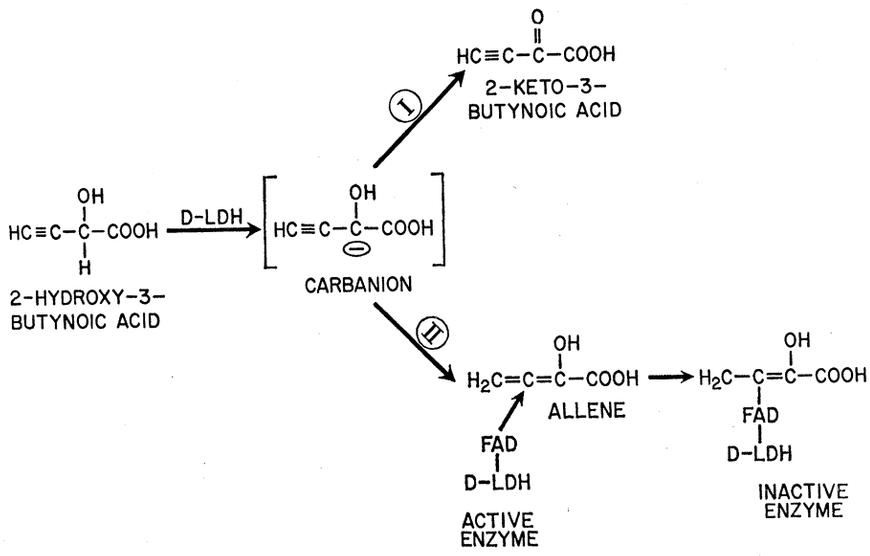

Fig. 5. Inactivation of D-LDH by 2-hydroxy-3-butynoic acid.

D-lactate-dependent transport by 2-hydroxy-3-butynoate is highly specific. Other membrane-bound dehydrogenases are not inhibited, transport in the presence of ascorbate-phenazine methosulfate is not affected, and α-glycerol phosphate dependent transport in *S. aureus* vesicles is not inactivated by the acetylenic hydroxy acid. Moreover, inactivation of D-lactate–dependent transport is blocked by D-lactate but not by succinate and NADH.

In view of the high degree of specificity, it was surprising when subsequent experiments demonstrated that the compound inactivates vectorial phosphorylation catalyzed by the phosphoenolpyruvate-P-transferase system, and that vinylglycolate, a substrate for D-LDH, is 50 to 100 times more potent. The key to the puzzle came with the realization that reaction I in Fig. 5, although inconsequential for D-LDH, leads to the formation of a highly reactive electrophile. Shortly thereafter, it was demonstrated that vinylglycolate inactivates enzyme I of the phosphotransferase system, and by this means blocks vectorial phosphorylation in whole cells and membrane vesicles of *E. coli* (*58, 59*). The relative lack of potency of hydroxybutynoate is due to inactivation of D- and L-LDH's by this compound. The generation of 2-keto-3-butynoate (Fig. 5) is limited therefore by inactivation of the enzymes which catalyze its formation. Vinylglycolate, on the other hand, is a noninactivating substrate, and the putative electrophile (2-keto-3-butenoic acid) is generated at a rapid rate and for an extended period of time. The synthesis of isotopically labeled vinylglycolate has allowed a detailed study of the biochemical properties of this compound.

Prior to inactivation of the phosphotransferase system, vinylglycolate is transported by the lactate transport system. Subsequently, it is oxidized by the membrane-bound D- and L-LDH's to yield a reactive electrophile (presumably 2-keto-3-butenoate), which then reacts with enzyme I and many other sulfhydryl-containing proteins on the membrane (Fig. 6). There is considerable evidence supporting these conclusions; however, only two points are critical for this discussion (*12*): (i) vinylglycolate transport is the limiting step for labeling the membrane proteins; and (ii) almost all of the vinylglycolate taken up is covalently bound to the vesicles. In experimental terms, the rate of covalent binding of vinylglycolate to the vesicles is stimulated at least tenfold by ascorbate-phenazine methosulfate; and stimulation is completely abolished by 2,4-dinitrophenol or phospholipase treatment, neither of which affect the rate of vinylglycolate oxidation.

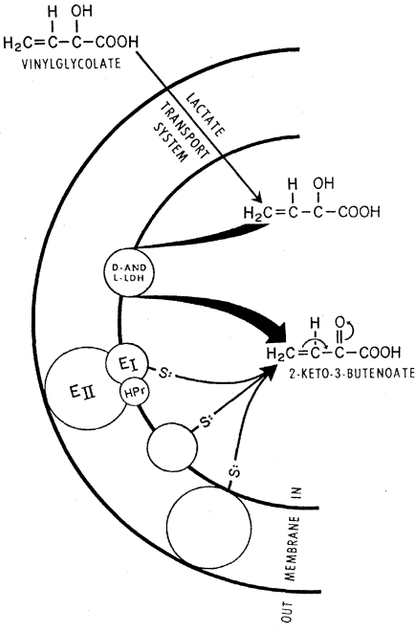

Fig. 6. Proposed sequence of events in uptake and covalent binding of vinylglycolate (2-hydroxy-3-butenoate) by *E. coli* membrane vesicles. E II, enzyme II of the phosphoenolpyruvate-P-transferase system; E I, enzyme I of the phosphotransferase system; HPr, histidine-containing protein; S, sulfhydryl groups contained in membrane proteins. Reaction of 2-keto-3-butenoate with sulfhydryl groups exclusively on the inner surface of the membrane is a presumption at the present time.

With this background, vinylglycolate can be used to estimate the transport activity of individual membrane vesicles on an all-or-none basis. With the use of [^{3}H]vinylglycolate of extremely high specific activity, vesicles have been labeled for an appropriate time in the presence of ascorbate–phenazine methosulfate, and examined by radioautography in the electron miscroscope (*12*). Each vesicle that has taken up vinylglycolate is overlaid with exposed silver grains. Examination of these preparations reveals that at least 85 to 95 percent of the vesicles are labeled. It should be emphasized that this is a minimal estimation. Virtually all of the large vesicles are labeled, while the size of the smaller vesicles is such that their proximity to individual silver grains in the emulsion may be limiting. Moreover, essentially identical radioautographic results are obtained with [^{3}H]-acetic anhydride, a reagent which reacts nonspecifically with the vesicles. These studies provide strong evidence that most, if not all, of the vesicles in the preparation catalyze active transport. This type of study is not possible with the usual transport substrates because they are not covalently bound by the vesicles, and are readily lost with even the most gentle manipulations.

Anaerobic Transport

One aspect of the respiration-dependent transport systems that has only begun to be studied is their relation to anaerobic transport. Obligate anaerobes

or facultative anaerobes growing under anaerobic conditions transport nutrients; moreover, mutants of *E. coli* requiring δ-aminolevulinic acid or heme do not manifest transport defects (*60*). Although ATP is not apparently involved in active transport under aerobic conditions (*45, 48, 53, 54, 61*), evidence has been presented which suggests that intact cells can utilize glycolytically generated ATP to drive transport under anaerobic conditions (*53, 60–62*). It is also possible, however, that cells under anaerobic conditions might use the same general type of transport mechanism as that used aerobically, with the exception that an alternative electron acceptor is used rather than the cytochrome chain and oxygen.

Recent experiments (*63*) have demonstrated that anaerobic lactose transport in whole cells and membrane vesicles from *E. coli* is coupled to the oxidation of α-glycerol phosphate or D-lactate with fumarate as an electron acceptor. Alternatively, anaerobic lactose transport may be coupled to the oxidation of formate utilizing nitrate as electron acceptor. Both anaerobic electron transfer systems are induced by growth of the organism under appropriate conditions. Components of both systems are loosely bound to the membrane, necessitating the use of a modified procedure for vesicle preparation in order to demonstrate anaerobic transport in the vesicle system. Inclusion of ATP or an ATP-generating system during lysis and the subsequent steps in the preparation of vesicles does not stimulate transport. The results support the conclusion that at least one type of anaerobic transport is coupled primarily to electron flow.

Dansylgalactosides, Fluorescent Probes of Active Transport

Fluorescent compounds that exhibit polarity-dependent fluorescence properties have been used to investigate the structure of biological membranes (*64*). Two such compounds, 1-anilino-8-naphthalene sulfonate (*65*) and dansyl phosphatidyl ethanolamine (*66*) have been used to study structural changes associated with transport in membrane vesicles of *E. coli*. However, the nonspecificity of these probes limits the type of information that can be obtained.

The fluorescent galactosides, 2-(*N*-dansyl)-aminoethyl-β-D-thiogalactoside [DG_2] (*67*) and 2-(*N*-dansyl)-aminohexyl-β-D-thiogalactoside [DG_6] (*68*), competitively inhibit lactose transport by membrane vesicles of *E. coli* but are not actively transported. An increase in the fluorescence of these dansylgalactosides is observed upon addition of D-lactate, imposition of a membrane potential (positive outside), or dilution-induced, carrier-mediated lactose efflux. The increase is not observed with 2-(*N*-dansyl)aminoethyl-β-D-thioglucoside nor with membrane vesicles lacking the β-galactoside transport system. Moreover, the D-lactate-induced fluorescence increase is blocked or rapidly reversed by addition of β-galactosides, sulfhydryl reagents, inhibitors of D-lactate oxidation, or uncoupling agents. The fluorescence increase observed with both DG_2 and DG_6 exhibits an emission maximum at 500 nm and excitation maxima at 345 nm and at 292 nm. The latter excitation maximum indicates that the bound dansylgalactoside molecules are excited by energy transfer (*69*) from the membrane proteins. Titration of vesicles

Table 1. Respiration-dependent transport systems in isolated bacterial membrane vesicles.

Source of vesicles	Electron donors*	Transport systems
Escherichia coli	D-Lactate; ASC-PMS; NADH-pyocyanine (*50*)	β-Galactosides (*31–34, 49*); galactose (*34*); arabinose (*33*); glucuronic acid (*33, 35*); gluconic acid (*36*); hexose-P (*33, 37*); nine amino acid transport systems (*14, 38, 49*); deoxycytidine (*42*); valinomycin-induced Rb^+ or K^+ uptake (*43, 44*)
	NADH, ASC-PMS	D- and L-lactate (*40*)
	ASC-PMS	Pyruvate (*40*)
Salmonella typhimurium	D-Lactate, ASC-PMS	Amino acids (*49, 81*); valinomycin-induced Rb^+ (*97*)
	ASC-PMS	Citrate (*50*)
Micrococcus denitrificans	D-Lactate, formate, ASC-PMS	Gly-Ala; Gln-Asn (*49, 98*); Val-induced Rb^+ uptake (*44*)
Azotobacter vinelandii	Malate (+ FAD), ASC-PMS, ASC-TMPD	Glucose (*92*)
Arthrobacter pyridinolis	Malate	Fructose, rhamnose, glucose (*99*); various amino acids (*100*)
Mycobacterium phlei	NADH, ASC-TMPD, ASC-PMS	Pro (*101*)
Pseudomonas aeruginosa	Malate (+ FAD), D-glucose, ASC-PMS	Gluconate (*94*)
Pseudomonas sp.	L-Lactate, ASC-PMS	Succinate (*40*)
	NADH, succinate, ASC-PMS	D- and L-lactate (*40*)
Marine *Pseudomonas* B-16	NADH, ASC-TMPD (*102*) ethanol (*104*)	Ala (*102, 103*), galactose (*104*)
Pseudomonas putida	ASC-PMS; D-Lactate	Pro (*49*); various amino acids (*102*)
Proteus mirabilis	ASC-PMS	Pro (*49*)
Staphylococcus aureus	α-Glycerol phosphate (vesicles from gluconate-grown cells); L-Lactate (vesicles from glucose-grown cells); ASC-PMS	Twelve amino acid transport systems (*17, 45, 46, 49*); valinomycin-induced Rb^+ uptake (*44*)
Bacillus subtilis	α-Glycerol phosphate (vesicles from glycerol-grown cells); NADH; ASC-PMS	Nine amino acid transport systems (*47, 48*)
	NADH, ASC-PMS	D- and L-lactate, succinate (*40*)
Bacillus megaterium	ASC-PMS	Pro (*49*)
Bacillus licheniformis	NADH (*104*), ASC-PMS (*105*)	Amino acids

* Abbreviations: ASC, ascorbate; PMS, phenazine methosulfate; NADH, reduced nicotinamide adenine dinucleotide; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride; FAD, flavin adenine dinucleotide; Gly, glycine; Ala, alanine; Gln, glutamine; Asn, asparagine; Pro, proline.

with DG_2 and DG_6 in the presence of D-lactate demonstrates that the β-galactoside carrier protein represents about 3 to 6 percent of the total membrane protein, a value which is in excellent agreement with that of Jones and Kennedy (*70*). Moreover, the affinity of the carrier increases as the length of the carbon chain between the galactose and dansyl moieties is increased—that is, the dissociation constants (K_D) for DG_0 [2-[*N*-dansyl)-amino-β-D-thiogalactoside], DG_2 and DG_6 are approximately 500 μM, 30 μM, and 5 μM, respectively.

Fluorescence lifetime studies corroborate the results discussed above (*71*). In the absence of D-lactate, DG_2 fluorescence exhibits a half-life of 3 nanoseconds. On addition of D-lactate to membrane vesicles containing the lac transport system, the bound DG_2 molecules exhibit a half-life of 18 nanoseconds.

The fluorescence parameters mentioned above do not distinguish between binding per se as opposed to binding followed by translocation of dansylgalactoside into the hydrophobic environment of the membrane. Polarization of fluorescence, on the other hand, can be used to assess binding specifically since changes in this parameter reflect alterations in the rotation of molecules in solution. Studies with DG_2 and DG_6 (*68*) demonstrate that there is a dramatic increase in fluorescence polarization in vesicles containing the lac transport system on addition of D-lactate. In the absence of D-lactate, polarization values are minimal and identical in vesicles with or without the lac carrier protein. No increase in polarization is observed in vesicles devoid of lac carrier protein. These studies provide a strong indication that the observed changes in DG_2 and DG_6 fluorescence observed on addition of D-lactate reflect binding of the fluorescent probes to the lac carrier protein.

Studies with a photoaffinity-labeled galactoside, 2-nitro-4-azidophenyl β-D-thiogalactoside, provide independent support for this conclusion (*72*). This compound is a competitive inhibitor of lactose transport, and in its presence, lactose transport is irreversibly inactivated by exposure to visible light. Addition of lactose protects against photoinactivation, and amino acid transport is only mildly inhibited, indicating that the site of inactivation is the lac carrier protein. Finally, the rate of inactivation of the lac transport system by azidophenylthiogalactoside is markedly enhanced by addition of D-lactate during exposure to light.

The data, taken as a whole, indicate that in the absence of energy, the lac carrier protein is unable to bind significant amounts of substrate. There are at least two possible explanations for these observations. Energy coupling could (i) increase the affinity of the lac carrier at the external surface of the membrane, or (ii) result in "movement" of the carrier from the inner to the outer surface of the membrane. A detailed discussion of these alternatives is omitted because the data do not allow a clear choice. Furthermore, the two possibilities are not mutually exclusive. In any case, the data suggest that energy is coupled to one of the initial steps in transport, and that facilitated diffusion, therefore, cannot be the limiting step for active transport of β-galactosides. Kinetic studies in *E. coli* (*96*) and *S. aureus* vesicles (*46*) are consistent with this conclusion.

Reconstitution of D-Lactate–Dependent Active Transport

The membrane-bound D-LDH of *E. coli* has been solubilized and purified to homogeneity (*74*). The enzyme has a molecular weight of 75,000 (± 7 percent), contains approximately 1 mole of FAD per mole of enzyme, and exhibits low activity toward L-lactate. NAD has no effect on the catalytic conversion of D-lactate to pyruvate.

While this work was in progress, Reeves *et al.* (*75*) demonstrated that guanidine hydrochloride extracts of wild-type membrane vesicles containing D-LDH activity are able to reconstitute D-lactate–dependent oxygen consumption and active transport in membrane vesicles from *E. coli* and *S. typhimurium* mutants defective in D-LDH (that is, *Dld*$^-$ mutants). These studies have been confirmed and extended by Short *et al.* (*76*), who used the homogeneous preparation of D-LDH described above. Futai (*77*) has independently confirmed many of the observations.

Reconstitution of D-lactate oxidation and D-lactate–dependent active transport is accomplished by diluting the enzyme preparation (dissolved in 0.1 percent Triton X-100 and 0.6*M* guanidine hydrochloride) 25-fold or more into a suspension of *Dld*$^-$ membrane vesicles. Presumably, dilution of the detergent and the guanidine hydrochloride decreases the solubility of the enzyme, causing it to bind to the vesicles. The reconstituted vesicles are centrifuged, resuspended in a small volume of buffer, and assayed for transport and dehydrogenase activities. Optimal reconstitution takes place in the presence of 0.6*M* guanidine hydrochloride, at 25° to 37°C and *p*H 6.6. The vesicles can be washed several times after reconstitution without loss of activity.

Reconstituted *Dld*$^-$ membranes carry out D-lactate oxidation and take up a number of transport substrates when supplied with D-lactate. D-Lactate is not oxidized and will not support transport of any of these substances in unreconstituted *Dld*$^-$ vesicles. Binding of enzyme to wild-type membranes produces an increase in D-lactate oxidation but has little or no effect on the ability of the membranes to catalyze active transport. Reconstitution of *Dld*$^-$ membranes with increasing amounts of D-LDH produces a corresponding increase in D-lactate oxidation, and transport approaches an upper limit similar to the specific transport activity of wild-type membrane vesicles. However, the quantity of enzyme required to achieve maximum initial rates of transport varies somewhat with different transport systems.

Binding of 2-(*N*-dansyl)aminoethyl-β-D-thiogalactoside (DG_2) to membrane vesicles containing the lac transport system is dependent on D-lactate oxidation, and this fluorescent probe can be utilized to quantify the number of lac carrier proteins in the membrane vesicles (see previous discussion). When *Dld*$^-$ membrane vesicles are reconstituted with increasing amounts of D-LDH, there is a corresponding increase in the binding of DG_2. If each lac carrier protein molecule binds one molecule of DG_2, it can be estimated that there is at least a seven- to eightfold excess of lac carrier protein relative to functional D-LDH in reconstituted *Dld*$^-$ vesicles. A similar determination can be made for wild-type vesicles. These vesicles contain approximately 0.07 nmole of D-LDH per milligram of membrane protein (based on the specific activity of the homogeneous enzyme preparation) and about 1.1 nmole of lac carrier protein per milligram of membrane protein, yielding a ratio of about 15 for lac carrier protein relative to D-LDH.

Although the rate and extent of transport of a number of substrates increase dramatically with reconstitution,

the rate and extent of labeling of *Dld*$^-$ vesicles with radioactive vinylglycolate remain constant. As discussed above, this compound is transported via the lactate transport system, and oxidized to a reactive product by D- and L-LDH's on the inner surface of the vesicle membrane. The observation that reconstituted *Dld*$^-$ membranes do not exhibit enhanced labeling by vinylglycolate suggests that bound D-LDH is present on the outer surface of the vesicles. In this case, the reactive product released from D-LDH would be diluted into the external medium, whereas, if the enzyme were on the inner surface of the vesicles membrane, the rate of labeling would be expected to increase with reconstitution since the reactive product should accumulate within the vesicles to higher effective concentrations. This conclusion is consistent with recent experiments of Konings (*78*) demonstrating that reduced 5-*N*-methylphenazonium-3-sulfonate (*79*), an impermeable electron carrier, drives transport as well as reduced phenazine methosulfate, its lipophylic analog.

The suggestion that D-LDH is localized on the outer surface of reconstituted *Dld*$^-$ membrane vesicles, as opposed to the inner surface of native ML 308-225 vesicles, has received strong support from recent experiments with antibody prepared against D-LDH (*80*). Incubation of ML 308-225 membrane vesicles with antibody to D-LDH does not inhibit D-LDH activity (assayed by tetrazolium dye reduction, oxygen uptake, and D-lactate–dependent transport) unless the vesicles are disrupted physically or spheroplasts are lysed in the presence of antibody. In striking contrast, treatment of reconstituted *Dld*$^-$ vesicles with antibody to D-LDH results in marked inhibition of D-LDH activity. The titration curves obtained with reconstituted *Dld*$^-$ membrane vesicles are almost identical quantitatively to that obtained with the homogeneous preparation of D-LDH. In addition to providing information about the localization of D-LDH in native and reconstituted vesicles, the results with the native vesicle preparations are consistent with other experiments which demonstrate that essentially all of the vesicles catalyze active transport (as discussed above) and therefore cannot be inverted or sufficiently damaged to allow access of antibody to D-LDH to the enzyme.

The flavin moiety of the holoenzyme appears to be critically involved in binding D-LDH to the membrane. Treatment with [1-^{14}C]hydroxybutynoate leads to inactivation of D-LDH by modification of the FAD coenzyme bound to the enzyme (see Fig. 5). Enzyme labeled in this manner does not bind to *Dld*$^-$ membrane vesicles. The findings suggest that the flavin coenzyme itself may mediate binding or alternatively, that covalent inactivation of the flavin may result in a conformational change that does not favor binding. In any case, it is tempting to speculate on the relevance of this finding to the synthesis of membrane-bound dehydrogenases in the intact cell. Possibly, the apoprotein moiety of D-LDH is synthesized on cytoplasmic ribosomes, but is not inserted into the membrane until coenzyme is bound. If this is so, D-LDH mutants that are defective in the flavin binding site should exhibit soluble material which cross reacts immunologically with native D-LDH.

Mechanism of Active Transport

It is not known how energy released by oxidation of D-lactate or other electron donors is coupled to transport. There are several contending theories that attempt to provide an answer to this question, but the experimental evidence to date does not allow a clear choice to be made between them. If anything, in my opinion, the available evidence reveals a necessity for bringing together aspects of theories which have been looked upon heretofore as mutually exclusive.

There are certain fundamental observations that any proposed mechanism for energy coupling must take into account. (i) There is no correlation between rates of oxidation of various electron donors and their relative effects on transport. Both succinate and NADH, for instance, are oxidized more rapidly than D-lactate by *E. coli* membrane vesicles, yet D-lactate is by far the most effective energy source for transport. (ii) The cytochrome chain of *E. coli* vesicles is completely reduced by D-lactate, L-lactate, succinate, or NADH. Since D-lactate stimulates transport so much more effectively than the other electron donors, the site at which energy is coupled to transport must lie within a segment of the electron transfer chain between D-LDH and the common cytochrome chain. This conclusion is supported by a number of other experimental approaches (*5*). (iii) D-Lactate–dependent transport is completely blocked by uncouplers such as carbonylcyanide *m*-chlorophenylhydrazone (CCCP) or 2,4-dinitrophenol (DNP), although these agents do not inhibit D-lactate oxidation. (iv) The Michaelis constant (K_m) for efflux is much higher than the K_m for uptake; the maximal rates (V_{max}) for influx and efflux are identical. (v) Although all inhibitors of D-lactate oxidation block uptake, only those that act after the site of energy coupling in the electron transfer chain cause efflux when added to preloaded vesicles. (vi) There is a large excess of carriers relative to D-LDH. (vii) Valinomycin-induced potassium efflux results in uptake of lactose and other transport substrates in the absence of D-lactate oxidation.

An initial model proposed by Kaback and Barnes (*33*) depicted the carriers as electron transfer intermediates in which a change from the oxidized to the reduced state results in translocation of the carrier-substrate complex to the inner surface of the membrane and a concomitant decrease in the affinity of the carrier for substrate. This model was intended merely to provide a working hypothesis that could account for much of the data available at that time within the framework of common biochemical concepts. Aside from its basis in experimental observation, the model did not require the membrane to exhibit any special characteristics other than its function as a diffusion barrier. Its major defects are that it fails to account adequately for the behavior of electron transfer coupling mutants (*81*) and other mutants (*82*, *83*) which exhibit normal electron transfer properties but are defective in D-lactate–dependent transport, for the inhibitory action of uncoupling agents, for the excess of carriers relative to D-LDH, and for certain carriers which are insensitive to sulfhydryl reagents (*11*).

A very different type of hypothesis, one that emphasizes the positioning of respiratory chain components within the matrix of the membrane, was proposed by Mitchell (*84*). As visualized by this chemiosmotic model, oxidation of electron donors is accompanied by expulsion of protons into the external medium, leading to a *p*H gradient or electrical potential (or both) across the membrane. This electrochemical gradient is postulated to be the driving force for inward movement of trans-

port substrates by way of passive diffusion in the case of lipophilic cations such as dibenzyldimethylammonium ion (*85*), by facilitated diffusion in the case of positively charged substrates such as lysine or potassium ions (in the presence of valinomycin), or by coupled movement of protons with a neutral substrate such as lactose or proline (that is, "symport"). In instances where sodium efflux is observed (*44*), the chemiosmotic model invokes the concept of sodium-proton "antiport," which is postulated to catalyze electroneutral exchange of internal sodium with external protons, and vice versa. Moreover, the inhibitory effects of uncoupling agents on transport are attributed to the ability of these compounds to conduct protons across the membrane, thus short-circuiting the "proton-motive force" that drives transport (*86*).

A review by Harold (*87*) lucidly discusses the chemiosmotic model and summarizes much of the experimental evidence that supports it. More recent experiments (*83, 88, 89*) have provided more direct support for this hypothesis, and some of these findings have been corroborated and extended in my laboratory. During D-lactate oxidation, lipophilic cations such as dimethyldibenzylamine (in the presence of tetraphenylboron) (*44, 73, 88*), safranine (*90*), and triphenylmethylphosphonium (*90*) are accumulated. These observations are consistent with the interpretation that D-lactate oxidation generates a membrane potential that is positive on the outside. Moreover, when potassium-loaded membrane vesicles are treated with valinomycin (a potassium-specific ionophore) and rapidly diluted into media lacking potassium, lactose and other transport substrates are taken up (*73, 88*), indicating that a positive membrane potential can drive lactose uptake in the absence of electron flow. Finally, binding of dansylgalactosides (DG_2 and DG_6) by vesicles containing the lac carrier protein can be induced by potassium or thiocyanate diffusion gradients in the absence of D-lactate oxidation (*68*), suggesting that the membrane potential may cause the binding site in the carrier to become accessible on the exterior surface of the membrane. Notwithstanding findings that make the interpretation of some of these observations less than clear-cut (*44, 73, 91*), there is reason to accept the contention that chemiosmotic phenomena play an important role in active transport.

Despite the attractive simplicity of the chemiosmotic hypothesis and the experiments which support aspects of the concept, a number of inconsistencies remain. Space limitations prevent a full discussion of this highly controversial issue, but there are a few experimental observations that should be emphasized, if only because their full import with regard to the chemiosmotic theory has seldom been appreciated.

First, the efficiencies of various electron donors relative to D-lactate in stimulating transport of different transport substrates in *E. coli* vesicles vary over a wide range (*16*). For example, succinate is nearly 70 percent as effective as D-lactate in stimulating lysine uptake but only 4 percent as effective in stimulating proline uptake. With *A. vinelandii* vesicles (*92*), Barnes has demonstrated that although malate (in the presence of FAD) is the best electron donor for glucose transport and D-lactate has very little effect, with calcium uptake in the same preparations, D-lactate is a much better electron donor (*93*). Similarly, Stinnett *et al.* (*94*) have shown that the gluconate transport system in *P. aeruginosa* vesicles is stimulated by FAD when it is driven by malate dehydrogenase, but not when it is driven by glucose dehydrogenase. The findings are reversed with the amino acid uptake systems (*95*). According to chemiosmotic theory, a particular oxidizable substrate should have approximately the same efficiency for all of the different transport systems, its efficiency reflecting its effectiveness in generating a proton-motive force. The results do not bear out this prediction, and the problem is not ameliorated by invoking either threshold effects or variations in the efficiency of coupling at the level of the individual transport systems because these considerations still do not account for variations in the pattern of relative efficiencies.

The second type of observation involves the effect of adding inhibitors of D-lactate oxidation to *E. coli* vesicles that have been preloaded with transport substrates by prior incubation with D-lactate. The results for lactose (*33*), amino acids (*16*), rubidium (*44*), and triphenylmethylphosphonium (*90*) are all qualitatively the same: addition of inhibitors that act after the site of energy coupling in the electron transfer chain causes efflux of preloaded substrates, whereas equally effective inhibitors that act before the site of energy coupling do not. The results are even more striking in *S. aureus* vesicles prepared so that L-lactate oxidation provides the driving force for transport (*46*). In these vesicles, oxalate, a competitive inhibitor of L-LDH, completely blocks amino acid uptake when added at concentrations that block L-lactate oxidation by more than 99 percent. Yet these concentrations of oxalate do not cause efflux when added to preloaded vesicles. Of particular interest with regard to the chemiosmotic theory is the finding that oxalate blocks efflux induced by other electron transfer inhibitors and reduces the initial rate of DNP-induced efflux by a factor of 5.

These results are extremely difficult to explain in chemiosmotic terms since there is no apparent reason why one class of electron transfer inhibitors should dissipate the membrane potential while another should not. Simplistic explanations for these phenomena are made even more difficult by observations demonstrating that efflux of solutes from reconstituted *Dld*$^-$ vesicles (where D-LDH is apparently on the outside of the membrane) is induced by inhibitors that block electron transfer both before and after the energy coupling site (*75, 76*).

Third, facilitated diffusion cannot be the initial step in active transport. This statement is supported by a number of observations, the strongest of which are those concerned with the properties of dansylgalactosides (as discussed earlier). Koch (*96*) has reached a similar conclusion as a result of his studies with intact cells. As implied in the chemiosmotic hypothesis, facilitated diffusion should be limiting for active transport because binding and release of protons and solutes by the carriers should be equal on both sides of the membrane in the absence of a proton gradient (*84, 87*).

Hopefully, within the near future, these apparently conflicting observations will be accounted for within one conceptual framework. At the present time, however, this is not possible without ignoring one important observation or another.

Summary

The use of bacterial membrane vesicles as an experimental system for the study of active transport has been discussed. Vesicles are prepared from osmotically sensitized bacteria, and consist of osmotically intact, membrane-bound sacs without internal structure.

They retain little or no cytoplasm. Under appropriate conditions, these vesicles catalyze the transport of a variety of solutes at rates which are comparable, in many cases, to those of intact cells. Two general types of transport systems have been elucidated in the vesicle system: (i) group translocation systems which catalyze vectorial covalent reactions; and (ii) respiration-linked transport systems that catalyze the active transport of a whole range of metabolites against an electrochemical or osmotic gradient.

In *E. coli* membrane vesicles, the respiration-linked transport systems are coupled primarily to the oxidation of D-lactate to pyruvate, catalyzed by a flavin-linked, membrane-bound D-lactate dehydrogenase which has been purified to homogeneity. Electrons derived from D-lactate or certain artificial electron donors are transferred to oxygen by means of a membrane-bound respiratory chain, and respiration is coupled to active transport within a segment of the respiratory chain between the primary dehydrogenase and cytochrome. b_1. The great majority of the individual membrane vesicles in the population catalyze active transport, and the generation or hydrolysis of ATP is not involved. Under anaerobic conditions, fumarate or nitrate can be utilized in place of oxygen as terminal electron acceptors. With the exception that D-lactate is not always the most effective electron donor for active transport, vesicles prepared from a number of other organisms catalyze transport in a similar manner.

Fluorescent dansylgalactosides are useful molecular probes of active transport in the vesicle system. These compounds are competitive inhibitors of β-galactoside transport, but are not transported themselves. Fluorescence studies indicate that the lac carrier protein constitutes approximately 3 to 6 percent of the total membrane protein, and that it is not accessible to the external medium unless the membrane is "energized." Thus, energy is coupled to one of the initial steps in the transport process. Studies with a photoaffinity-labeled galactoside provide independent support for this conclusion.

When membrane vesicles prepared from a D-lactate dehydrogenase mutant of *E. coli* are treated with D-lactate dehydrogenase, the enzyme binds to the vesicles and they regain the capacity to catalyze D-lactate oxidation and D-lactate–dependent active transport. The maximal specific transport activity obtained in the reconstituted system is similar in magnitude to that of wild-type vesicles. Titration studies with dansylgalactoside demonstrate that there is at least a seven- to eightfold excess of lac carrier protein relative to D-lactate dehydrogenase. Evidence is presented indicating that the enzyme is bound to the inner surface of native membrane vesicles and to the outer surface of reconstituted vesicles, and that the flavin coenzyme moiety is critically involved in binding.

Possible mechanisms of respiration-linked active transport are discussed.

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Size and Scaling in Human Evolution

Homo sapiens is a peculiar large primate; however, all australopithecines are versions of the "same" animal.

David Pilbeam and Stephen Jay Gould

Human paleontology shares a peculiar trait with such disparate subjects as theology and extraterrestrial biology: it contains more practitioners than objects for study. This abundance of specialists has assured the careful scrutiny of every bump on every bone. In this context, it is remarkable that the most general character of all—body size (the difference in absolute size among fossil hominids, and the clear phyletic trend toward larger bodies)—has been rather widely neglected.

Increase in body size has played an especially important role in evolution for two reasons.

1) It is so common. Several evolutionary phenomena are encountered so frequently that their canonization as "law" has been widely accepted. "Cope's law" of phyletic size increase is the best known and most widely touted of these statistical generalizations (*1*).

2) It has such important and ineluctable consequences. Galileo (*2*) recognized that a large organism must change its shape in order to function in the same way as a smaller prototype. The primary law of size and shape involves unequal scaling of surfaces and volumes (*3*), but other differential increases have their potent effect as well (*4*). As a terrestrial vertebrate evolves to larger size, its limb bones become relatively thicker, the ratio of brain weight to body weight decreases, and digestive and respiratory surfaces become more complex.

We cannot begin to assess the nature of adaptation in lineages obeying Cope's rule until we establish "criteria of subtraction" for recognizing the changes in shape that larger size requires. A simple description of changing shape will not suffice, for some changes merely compensate for increased size and reproduce the "same" animal at a larger scale, while others represent special adaptations for particular conditions. Yet such a separation is rarely attempted.

We make such an attempt in this article and use it to argue a simple thesis about human evolution. We try to demonstrate that the three generally accepted species of australopithecines (*5*) represent the "same" animal expressed over a wide range of size. In other words, size increase may be the only independent adaptation of these animals, changes in shape simply preserving the function of the smaller prototype at larger sizes. In evolving toward modern man, on the other hand, hominids also increased steadily in size, but they developed adaptations of brain and dentition that cannot be attributed to the mechanical requirements of larger bodies. In other words, the extinct branch of australopithecines did little more than increase in size during its evolution; the thriving branch of hominids increased in size and developed special adaptations as well.

Hominid Phylogeny

The hominids we discuss, known informally as Plio-Pleistocene hominids, come from African deposits ranging in age from a little less than 6 million to perhaps less than 1 million years (*5*). The first early hominid from South

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