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Energetics and Molecular Biology of Active Transport in Bacterial Membrane Vesicles

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Bacterial membrane vesicles retain the same sidedness as the membrane in the intact cell and catalyze active transport of many solutes by a respiration-dependent mechanism that does not involve the generation of utilization of ATP or other high-energy phosphate compounds. In E. coli vesicles, most of these transport systems are coupled to an electrochemical gradient of protons ($\Delta\bar{\mu}_H+$, interior negative and alkaline) generated primarily by the oxidation of D-lactate or reduced phenazine methosulfate via a membrane-bound respiratory chain. Oxygen or, under appropriate conditions, fumarate or nitrate can function as terminal electron acceptors, and the site at which $\Delta\bar{\mu}_H+$ is generated is located before cytochrome b_1 in the respiratory chain.

Certain (N-dansyl)aminoalkyl-β-D-galactopyranosides (Dns-gal) and N(2-nitro-4-azidophenyl)aminoalkyl 1-thio-β-D-galactopyranosides (APG) are competitive inhibitors of lactose transport but are not transported themselves. Various fluorescence techniques, direct binding assays, and photoinactivation studies demonstrate that the great bulk of the *lac* carrier protein (ca. 95%) does not bind ligand in the absence of energy-coupling. Upon generation of a $\Delta\bar{\mu}_H+$ (interior negative and alkaline), binding of Dns-gal and APG-dependent photoinactivation are observed. The data indicate that energy is coupled to the initial step in the transport process, and suggest that the *lac* carrier protein may be negatively charged.

Key words: bioenergetics, membrane sidedness, electrochemical proton gradient, D-lactate dehydrogenase, dansylgalactosides, azidophenylgalactosides

Over the past decade or so, it has become increasingly apparent that membrane vesicles isolated from bacteria as well as eucaryotes (1) provide a unique and useful system for the study of certain aspects 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, constituting a considerable advantage over intact cells. Since transport by membrane vesicles per se is practically nil, the driving force for transport of a particular substrate can be determined by studying which compounds or experimental manipulations stimulate its accumulation. In addition, metabolic conversion of the transport substrate and the energy source is minimal, allowing clear definition of the reactions involved. Finally, removal or disruption of the cell wall allows

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the use of certain probes, inhibitors, and ionophores which are normally inaccessible to the plasma membrane.

Since many aspects of the bacterial membrane vesicle system have been reviewed (2–6), this contribution is intended as more of a progress report than a review. Thus, various aspects of the work will be summarized, but emphasis is placed upon more recent observations dealing with respiration-linked active transport. In a general sense, however, it should be stressed that studies with this system are relevant not only to active transport, but to the general problem of energy transduction and possibly other membrane-related phenomena (7–12). As opposed to mitochondria and chloroplasts where respiratory energy and light are converted to another form of chemical energy (i.e., ATP), in this experimental system respiratory energy is converted primarily into work in the form of solute concentration against an electrochemical or osmotic gradient.

GENERAL ASPECTS OF VESICULAR TRANSPORT

Transport assays with membrane vesicles are performed in various ways (3, 4). The most widely used method is a filtration assay in which vesicles are incubated with a radioactive transport substrate in the presence of an appropriate energy source, and at a given time, the reaction mixtures are diluted to terminate the uptake reaction. The vesicles are then immediately separated from the medium by means of rapid membrane filtration. Although this method is rapid and convenient, it suffers from the disadvantage that the reaction mixtures must be diluted prior to filtration, and in many instances, this operation results in significant losses of certain solutes (13). The vesicles can also be separated from the reaction mixtures by centrifugation, but in this case, samples cannot be assayed rapidly, corrections must be made for radioactive solute trapped in the pellet, and the reaction mixtures become anaerobic even during rapid centrifugation (13). Very recently, the flow dialysis technique devised by Colowick and Womack (14) has been adapted to measure transport (13, 15, 16), and it is presently clear that this is the method of choice in many ways. By this means, changes in the external concentration of solute are determined continuously, accurately, and highly reproducibly under conditions which require no manipulation of the experimental system.

Evidence was presented as early as 1960 (17) which indicated that bacterial membrane vesicles would provide a useful model system for the study of active transport. Subsequent studies demonstrated that the vesicles catalyze the transport of a plethora of metabolities in the presence of appropriate energy sources (5), and that initial rates of transport of many of these metabolites are comparable to those of the intact cell (18, 19). Moreover, the vesicles accumulate many solutes to concentrations markedly in excess of those in the external medium. Initial progress with the system was slow primarily because of preconceived ideas regarding the physical nature of the vesicles and the energetics of active transport. It was generally thought that transport would be driven by ATP or other nucleoside triphosphates, and considerable effort was expended in an effort to provide evidence for this notion. However, as it turns out, the energy source for transport in isolated membrane vesicles varies with the organism, with the substance transported, and ATP or other nucleoside triphosphates do not drive transport in the vesicle system (see Ref. 5 for a review of the evidence).

In general, the transport systems elucidated in the vesicle system fall into 3 categories: i) group translocation mechanisms in which a covalent change is exerted upon the trans-

ported molecule so that the reaction itself results in the passage of the molecule through the diffusion barrier (2,5), ii) active transport in which solute is accumulated against an electrochemical or osmotic gradient, and iii) passive diffusion of certain weak acids and lipophilic ions, followed by equilibration with the pH gradient and the electrical potential, respectively, across the membrane.

Transport of many sugars, amino acids, organic acids, and ions by E. coli and S. typhimurium membrane vesicles occurs by active transport. These transport systems are coupled primarily to the oxidation of D-lactate to pyruvate catalyzed by a flavin adenine dinucleotide-linked, membrane-bound D-lactate dehydrogenase (D-LDH) which has been purified to homogeneity (20, 27). Electrons derived from D-lactate are passed to oxygen via a membrane-bound respiratory chain, and during this process, respiratory energy is converted into work in the form of active transport (18, 22–25). Although other oxidizable substrates stimulate transport to some extent, they are not nearly as effective as D-lactate unless ubiquinone-1 (CoQ_1) is added to the vesicles (25). It should be emphasized, however, that generation of NADH from inside the vesicles stimulates transport in the absence of exogenous CoQ_1 (26) and that D-lactate is not an effective electron donor for active transport in all bacterial membrane systems (5). Active transport in the vesicles is also driven very dramatically by the nonphysiologic electron donors reduced phenazine methosulfate [PMS] (27) or pyocyanine (25), both of which donate electrons to the respiratory chain at a site prior to the cytochromes. The use of these nonphysiologic electron donors has allowed the generalization of the vesicle system to many bacteria (5, 27).

E. coli membrane vesicles also catalyze active transport in the absence of oxygen when the appropriate anaerobic electron transfer systems are present (28–30). Lactose and amino acid transport under anaerobic conditions can be coupled to the oxidation of α-glycerol-P with fumarate as an acceptor or to the oxidation of formate utilizing nitrate as electron acceptor. Both of these anaerobic electron transfer systems are induced by growth of the organism under appropriate conditions, and components of both systems are loosely bound to the membrane.

Finally, active transport can be driven by artificially induced potassium gradients of appropriate polarity (31, 32). When membrane vesicles prepared in potassium-containing buffers are diluted into solutions lacking this cation, and the ionophore valinomycin is added, the diffusion of potassium out of the vesicles creates an electrical potential across the vesicles membrane (interior negative), and uptake of certain substrates is observed. Although the extent of transport observed under these conditions is considerably less than that observed with D-lactate and reduced PMS, the finding that solute accumulation occurs under these conditions has great implications with respect to the energetics of respiration-linked active transport (see below).

SIDEDNESS OF MEMBRANE VESICLES AND SPECIFICITY OF D-LACTATE AS AN ELECTRON DONOR FOR ACTIVE TRANSPORT

One of the most striking and therefore controversial aspects of the vesicle system is the degree of specificity of the physiologic electron donors which drive active transport. In E. coli vesicles, of a large number of potential energy sources tested, very few replace D-lactate to any extent whatsoever, and none is as effective although many are oxidized at least as rapidly (18, 22, 23, 33, 34). It should also be emphasized in passing that incubation of the vesicles with radioactive D-lactate, L-lactate, succinate, or α-glycerol-P results in

stoichiometric conversion of these compounds to pyruvate, fumarate, or dihydroxyacetone-P, respectively (33–35). Thus, in each case, the ability of these compounds to drive transport is related to a clearly defined enzymatic reaction.

Since each electron donor reduces the same membrane-bound cytochromes, both qualitatively and quantitatively (22, 35), it was suggested that the energy-coupling site for active transport in E. coli and Staphylococcus aureus vesicles is located in a relatively specific segment of the respiratory chain between D-LDH and cytochrome b_1, the first cytochrome in the common portion of the respiratory chain. Some critics have argued, however, that a significant number of vesicles become inverted during preparation, and that these inverted vesicles oxidize NADH and other electron donors but do not catalyze active transport (36–38). This is an extremely important consideration, but there is now a large body of evidence demonstrating that it is extremely unlikely. That is, the membrane of each vesicle retains the same orientation as the membrane in the intact cell. Another explanation that has been proposed for the inability of certain electron donors to drive transport is dislocation of dehydrogenases from the inner to the outer surface of the vesicle membrane during preparation (39–41). In the one instance in which this possibility has been studied extensively (i.e., D-LDH, see below), it is clearly not the case (42–44). Some of the evidence supporting these contentions are as follows:

i) Initial rates of transport in the vesicles are, in many cases, similar to those observed in whole cells (18, 19). Moreover, in most instances, the steady-state level of accumulation of transport substrates is comparable to that observed in the intact cell, and the electrochemical proton gradient generated by E. coli vesicles (see below) is at least as great as that of the intact cells (compare the data presented in Refs. 13, 15, 16 with that in Ref. 45).

ii) Freeze fracture studies of membrane vesicles in at least 3 different laboratories (3, 5, 39, 46) demonstrate that the "texture" of the convex surface of the vesicles is distinctly different than that of the concave surface and that the vesicles are homogeneous in this respect. Moreover, the texture observed on the respective surfaces is exactly the same as that observed in the intact cell.

iii) As mentioned previously, all of the electron donors which are oxidized by the vesicles reduce the same cytochromes both qualitatively and quantitatively (22, 35). If a percentage of the vesicles were inverted, and only these inverted vesicles oxidized NADH, it is difficult to understand how NADH could reduce all of the cytochrome in the preparations.

iv) Although NADH is generally a poor electron donor for transport in E. coli vesicles, it is the best physiologic electron donor for transport in B. subtilis vesicles which are prepared in a similar manner (47). Moreover, recent experiments carried out in this laboratory (25) demonstrate that addition of ubiquinone (CoQ_1) to E. coli ML 308–225 vesicles in the presence of NADH results in rates and extents of lactose and amino acid transport which are comparable to those observed with D-lactate. Since this effect of CoQ_1 is not observed in the presence of NADPH nor in vesicles lacking NADH dehydrogenase activity, it seems apparent that CoQ_1 is able to shunt electrons from NADH dehydrogenase to an energy-coupling site which is not located in that portion of the respiratory chain between NADH dehydrogenase and the cytochromes. As such, these observations provide direct evidence for specific localization of the energy-coupling site.

v) Studies by Reeves et al. (48) demonstrate that fluorescence of 1-anilino-8-naphthalene sulfonate (ANS) is dramatically quenched upon addition of D-lactate to E. coli ML 308-225 membrane vesicles, an observation similar to that observed in energized

mitochondria and ethylenediaminotetraacetic acid-treated intact E. coli. In chloroplasts and submitochondrial particles, in which the polarity of the membrane is opposite to that of intact mitochondria, ANS fluorescence is enhanced upon energization. It follows that any inverted membrane vesicles in the preparations would exhibit enhanced ANS fluorescence in the presence of D-lactate. Thus, if 50% of the vesicles were inverted, no net change in ANS fluorescence should have been observed by Reeves et al. because half of the vesicles would exhibit quenching and half would exhibit enhancement.

Similarly, Rosen and McClees (49) have demonstrated that inverted membrane preparations catalyze calcium accumulation but do not catalyze D-lactate dependent proline transport. In contrast, vesicles prepared by osmotic lysis (3) do not exhibit calcium transport but accumulate proline effectively in the presence of D-lactate.

vi) Although D-LDH mutants exhibit normal transport and vesicles prepared from these mutants do not exhibit D-lactate-dependent transport, addition of succinate to these vesicles drives transport to the same extent as D-lactate in wild-type vesicles (50). Since succinate oxidation by both wild-type and mutant vesicles is similar, it seems apparent that the coupling between succinate dehydrogenase and transport is increased in the mutant vesicles. In vesicles prepared from double mutants defective in both D-LDH and succinate dehydrogenase, the coupling between L-lactate dehydrogenase and transport is increased, and L-lactate is the best physiologic electron donor for transport (F. Grau, J.-S. Hong, and H. R. Kaback, unpublished information). Moreover, in vesicles prepared from a triple mutant defective in D-LDH, succinate dehydrogenase, and L-lactate dehydrogenase, the coupling between NADH dehydrogenase and transport is markedly increased, and NADH drives transport as well as D-lactate in wild-type vesicles (F. Grau, J.-S. Hong, H. R. Kaback, unpublished information). In addition, it is noteworthy that vesicles prepared from galactose-grown E. coli exhibit high rates and extents of lactose transport in the presence of NADH. These observations indicate that the coupling between a particular dehydrogenase and the energy-coupling site for transport is subject to regulation, and that it may be difficult, if not impossible, to demonstrate specificity of energy-coupling in the intact cell. In some bacteria, however, evidence in favor of this hypothesis has been presented with intact cells. In Arthrobacter pyridinolis (51), hexose transport in both intact cells and membrane vesicles is coupled to malate dehydrogenase; and in a marine pseudomonas (52), it has been shown that amino acid transport in whole cells and membrane vesicles is coupled to alcohol dehydrogenase.

vii) As will be discussed below (see section on Reconstitution), studies with antibodies against D-LDH demonstrate that this membrane-bound enzyme is present exclusively on the inner surface of the vesicle membrane (42–44). Moreover, D-lactate oxidation drives transport normally in dld^- membrane vesicles reconstituted with D-LDH, and in this system, the enzyme is located exclusively on the outer surface of the vesicle membrane. Thus, none of the wild-type vesicles can be inverted or sufficiently damaged to allow access of antibody to the interior surface of the membrane, and D-LDH can drive transport normally even when it is present on the wrong side of the membrane.

viii) 2-Hydroxy-3-butenoic acid (vinylglycolate) is an analogue of lactate which is actively transported by the lactate transport system and oxidized by D- and L-LDHs. As opposed to normal substrates, however, oxidation of this compound yields a reactive electrophile (2-keto-3-butenoate) which is attacked by many sulfhydryl-containing proteins on the membrane. Although there is considerable evidence supporting these conclusions (53–56) only 2 points are critical for this discussion: a) Vinylglycolate transport is the limiting step for labeling the membrane proteins, and b) almost all of the vinylglyco-

late taken up is covalently bound to the vesicles. In experimental terms, the rate of covalent binding of vinylglycolate is stimulated at least tenfold by ascorbate-phenazine methosulfate (ascorbate-PMS); and stimulation is completely abolished by uncoupling agents or phospholipase treatment, neither of which affect vinylglycolate oxidation.

Using extremely high specific activity [^{3}H] vinylglycolate, vesicles have been labeled for an appropriate time in the presence of ascorbate-PMS and examined by radioautography in the electron microscope (53). Each vesicle that takes up vinylglycolate is overlaid with exposed silver grains, and examination of the preparations reveals that 85–90% 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. Thus most, if not all, of the vesicles in the preparations catalyze active transport.

ENERGETICS OF ACTIVE TRANSPORT

An initial model proposed by Kaback and Barnes (23) 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 concomittant decrease in the affinity of the carrier for substrate. The model was posed as a tentative working hypothesis that could provide a role for sulfhydryl groups in translocation and at the same time, account for the observation that only certain electron transfer inhibitors cause efflux of accumulated solutes. A very different hypothesis, one that emphasizes the positioning of respiratory chain components within the matrix of the membrane giving rise to an electrochemical gradient of protons as the immediate driving force for active transport, was proposed by Peter Mitchell (36, 37, 57–62), and over the past few years, it has become eminently clear that Mitchell's so-called "chemiosomotic hypothesis" provides the best explanation for active transport to date.

As visualized by Mitchell, oxidation of electron donors via the membrane-bound respiratory chain or hydrolysis of ATP catalyzed by the membranous Ca^{2+}, Mg^{2+}-stimulated ATPase complex is accompanied by the expulsion of protons into the external medium, leading to an electrochemical gradient of protons ($\Delta\bar{\mu}_H+$) which is composed of an electric and a chemical parameter according to the following relationship:

$$\Delta\bar{\mu}_H+ = \Delta\Psi - \frac{2.3RT}{F}\Delta pH \qquad (1)$$

where $\Delta\Psi$ represents the electric potential across the membrane, and ΔpH is the chemical difference in proton concentrations across the membrane (2.3RT/F is equal to 58.8 mV at room temperature). According to this hypothesis, it is the electrochemical gradient of protons or one of its components which is the immediate driving force for the inward movement of transport substrates. Transport of organic acids is postulated to be dependent upon the pH gradient (ΔpH) (i.e., the undissociated acid is transported through the membrane and is presumed to accumulate in the ionized form due to the relative alkalinity of the internal milieu), while the transport of positively charged compounds such as lysine

or potassium is purportedly coupled to the electric component ($\Delta\Psi$), and the uptake of neutral substrates such as lactose or proline is thought to be coupled to $\Delta\bar{\mu}_{H}+$ and to occur via symport with protons.

Using lipophilic cations and rubidium (in the presence of valinomycin), it has been demonstrated that E. coli membrane vesicles generate a $\Delta\Psi$ (interior negative) of approximately −75 mV in the presence of reduced PMS or D-lactate (31, 63, 64). Furthermore, the potential causes the appearance of high affinity binding sites for dansylgalactosides, azidophenylgalactosides, and p-nitrophenyl-α-D-galactopyranoside on the surface of the vesicles membrane (65, 66) and is partially dissipated as a result of lactose accumulation (64). Although these findings lend strong support to the chemiosmotic hypothesis, it was apparent that $\Delta\Psi$ in itself is insufficient to account for the magnitude of solute accumulation by the vesicles if it is assumed that the stoichiometry between protons and solute is 1:1 (36). This deficiency, in addition to the apparent absence of a transmembrane pH gradient, left reasonable doubt as to the quantitative relationship between $\Delta\bar{\mu}_{H}+$ and solute accumulation (64). Recent experiments from this laboratory (13, 15, 16) have resolved this problem to a large extent.

Utilizing flow dialysis, a technique uniquely suited to the measurement of ΔpH across isolated membrane vesicles, it has been shown that membrane vesicles isolated from E. coli grown under various conditions generate a transmembrane pH gradient of about 2 units (interior alkaline) under appropriate conditions. Using the distribution of weak acids (i.e., acetate, propionate, butyrate, and 5,5-dimethyloxazolidine-2, 4-dione) to measure ΔpH and the distribution of the lipophilic cation triphenylmethylphosphonium to measure $\Delta\Psi$, the vesicles are demonstrated to develop a $\Delta\mu_{H}+$ of almost −200 mV (interior negative and alkaline) at pH 5.5 in the presence of reduced PMS or D-lactate, the major component of which is a ΔpH of about −120 mV. As external pH is increased, ΔpH decreases, reaching 0 at about pH 7.5 and above, while $\Delta\Psi$ remains at about −75 mv and internal pH remains at pH 7.5–7.8. To some extent, these variations in ΔpH are probably caused by changes in the oxidation of reduced PMS or D-lactate, both of which vary with external pH in a manner similar to that described for ΔpH. However, it should also be mentioned that recent experiments (66a; S. Ramos, H. Rottenberg, and H. R. Kaback, unpublished information) suggest the operation of a mechanism which catalyzes the exchange of external protons for intravesicular sodium or potassium at relatively alkaline pH. Finally, and importantly, ΔpH and $\Delta\Psi$ can be varied reciprocally in the presence of valinomycin and nigericin with little change in $\Delta\bar{\mu}_{H}+$ and no apparent change in respiratory activity. In addition to providing direct support for some of the general predictions of the chemiosmotic hypothesis, these results provide a powerful experimental framework within which to test the relationship between $\Delta\bar{\mu}_{H}+$, ΔpH, and $\Delta\Psi$ and the accumulation of specific transport substrates.

Addition of lactose or glucose-6-P to membrane vesicles containing the appropriate transport systems results in partial collapse of ΔpH, demonstrating that respiratory energy can drive active transport via the pH gradient across the membrane. Titration studies with valinomycin and nigericin lead to the conclusion that at pH 5.5, there are 2 general classes of transport systems: Those that are coupled primarily to $\Delta\bar{\mu}_{H}+$ (lactose, proline, serine, glycine, tyrosine, glutamate, leucine, lysine, cysteine, and succinate) and those that are coupled primarily to ΔpH (glucose-6-P, lactate glucuronate, and gluconate). Strikingly, however, it is eminently clear that at pH 7.5, all of the transport systems are driven by

$\Delta\Psi$ which comprises the only component of $\Delta\bar{\mu}_{H}+$ at this external pH. In addition, when the effect of external pH on the steady-state level of accumulation of various transport substrates is examined, none of the pH profiles corresponds to those observed for $\Delta\bar{\mu}_{H}+$, $\Delta\Psi$, or ΔpH, and at external pH values exceeding 6.0–6.5, $\Delta\bar{\mu}_{H}+$ is insufficient to account for the concentration gradients observed for most of the substrates. This finding and the observation that the accumulation of organic acids is coupled to $\Delta\Psi$ at relatively high external pH values indicate that the stoichiometry between protons and transport substrates may vary as a function of external pH, exhibiting a value of 1 at relatively low external pH and increasing to 2 or more as external pH is increased. Experimental evidence which provides direct support for this conclusion has been presented (66b).

One attractive conceptual aspect of the chemiosmotic hypothesis for bacterial active transport is its analogy to the mechanism suggested for sugar and amino acid transport in many eucaryotic cells (67). In these systems, an electrochemical gradient of sodium rather than protons in generated through the action of the membranous sodium, potassium-dependent ATPase, and accumulation of sugars and amino acids occurs via coupled movements with sodium (this process is referred to traditionally as cotransport rather than symport).

Although it is almost certain that many bacterial transport systems catalyze proton/substrate symport, several instances have been reported in which the transport of a specific solute is dependent upon the presence of sodium or lithium ion (see Ref. 68 for a review). Moreover, some of these studies, in particular those of Stock and Roseman (69) and Lanyi et al. (70), indicate that symport or cotransport mechanisms may be operative. Since the basic energy-yielding process in bacteria is thought to be proton extrustion and bacteria apparently do not possess a sodium, potassium-dependent ATPase or a primary sodium pump, the existence of such transport systems presents certain obvious problems, among which are: i) the relationship between the proton electrochemical gradient and these transport systems, ii) the mechanism by which the internal sodium concentration is maintained at a low level.

Tokuda and Kaback (68) have recently shown that membrane vesicles isolated from Salmonella typhimurium G-30 grown in the presence of melibiose catalyze methyl-1-thio-β-D-galactopyranoside (TMG) transport in the presence of sodium or lithium as shown initially with intact cells by Stock and Roseman (69). TMG-Dependent sodium uptake is also observed, but only when a potassium diffusion potential (interior negative) is induced across the vesicle membrane. Cation-dependent TMG accumulation varies with the electrochemical gradient of protons generated as a result of D-lactate oxidation, and the vesicles catalyze D-lactate-dependent sodium efflux in a manner which is consistent with the operation of a proton/sodium exchange mechanism. Although the stoichiometry between sodium and TMG appears to be 1:1 when transport is induced by a potassium diffusion potential, evidence is presented which indicates that the relationship may exceed unity under certain conditions. The results are consistent with a model in which TMG/sodium (lithium) symport is driven by an electrochemical gradient of protons which functions to maintain a low intravesicular sodium or lithium concentration through proton/sodium (lithium) antiport. A similar mechanism has been suggested for light-dependent glutamate transport in vesicles from Halobacterium halobium (70).

RECONSTITUTION OF D-LACTATE DEHYDROGENASE-DEPENDENT FUNCTIONS IN D-LACTATE DEHYDROGENASE MUTANTS

The membrane-bound D-LDH of E. coli has been solubilized and purified to homogeneity (20, 21). The enzyme has a molecular weight of 75,000 ± 7%, contains approximately 1 mole of flavin adenine dinucleotide per mole of enzyme, and exhibits low activity towards L-lactate. Oxidized diphosphopyridine nucleotide (NAD) has no effect on the catalytic conversion of D-lactate to pyruvate. Finally, recent work carried out in collaboration with Drs. John Salerno and Tomoko Ohnishi of the Johnson Foundation of The University of Pennsylvania indicates that the enzyme probably does not contain a nonheme iron center (P. Stroobant, J. Salerno, H. R. Kaback, and T. Ohnishi, unpublished information).

While much of this work was in progress, Reeves et al. (71) demonstrated that guanidine·HCl extracts from 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 (*dld*$^-$). These studies have been confirmed and extended by Short et al. (72) using the homogeneous preparation of D-LDH described above, and Futai (73) has independently confirmed many of the observations.

Reconsitituted *dld*$^-$ vesicles carry out D-lactate oxidation and catalyze the transport of a number of substrates when supplied with D-lactate. D-Lactate is not oxidized, and will not support transport of any of these substrates in unreconstituted *dld*$^-$ membranes. 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 which is 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 upon D-lactate oxidation, and this fluorescent probe can be utilized to quantitate the number of *lac* carrier proteins in the membrane vesicles (see subsequent discussion). When *dld-3* membrane vesicles are reconstituted with increasing amounts of D-LDH, there is a corresponding increase in the binding of DG_2. Assuming that 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 mg membrane protein (based on the specific activity of the homogeneous enzyme preparation), about 1.1 nmoles of *lac* carrier protein per mg membrane protein, yielding a ratio of about 15 for *lac* carrier protein relative to D-LDH.

Although the rate and extent of transport decreases dramatically with reconstitution, the rate and extent of labeling of *dld*$^-$ vesicles with radioactive vinylglycolate remains constant. As discussed above, this compound is transported via the lactate transport system, and oxidized to a reactive product by D- and L-LDHs on the inner surface of

the vesicle membrane. The observation that reconstituted *d1d*$^-$ 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 vesicle membrane, the rate of labeling would be expected to increase with reconstitution, since the reactive product should accululate within the vesicles to higher effective concentrations.

The suggestion that D-LDH is localized on the outer surface of reconstituted *d1d*$^-$ membrane vesicles, as opposed to the inner surface of native ML 308-225 vesicles has received strong support from recent experiments with antibody against D-LDH (42–44). Incubation of ML 308-225 membrane vesicles with anti-D-LDH does not inhibit D-LDH activity (assayed by tetrazolium dye reduction, oxygen uptake, and/or D-lactate-dependent transport) unless the vesicles are disrupted physically or spheroplasts are lysed in the presence of antibody. In contrast, treatment of reconstituted *d1d*$^-$ vesicles with anti-D-LDH results in marked inhibition of D-LDH activity. The titration curves obtained with reconstituted *d1d-3* membrane vesicles are almost identical quantitatively to that obtained with the homogeneous preparation of D-LDH. The conclusion that D-LDH is able to drive transport from the outer surface of the membrane is also consistent with recent experiments of Konings (74) and Short et al. (43) demonstrating that reduced 5-N-methylphenazonium-3-sulfonate, an impermeable electron carrier, drives transport as well as reduced PMS, its lipophilic analogue. In addition to providing information about the localization of D-LDH in native and reconstituted vesicles, the results with the native vesicles are consistent with other experiments which demonstrate that essentially all of the vesicles catalyze active transport (cf. above) and therefore cannot be inverted or sufficiently damaged to allow access of antibody to D-LDH. Given these conclusions and the suggested mechanism for generation of an electrochemical proton gradient (36, 37), it is amazing that oxidation of D-lactate by reconstituted *d1d*$^-$ vesicles leads to an electrochemical proton gradient which is indistinguishable in polarity and magnitude from that observed in wild-type vesicles where the enzyme is on the inner surface of the vesicle membrane (S. Ramos, R. Schuldiner, and H. R. Kaback, unpublished information).

The flavin moiety of the holoenzyme appears to be critically involved in binding D-LDH to the membrane (72). Treatment with [1-^{14}C] hydroxybutynoate leads to inactivation of D-LDH by modification of the flavin adenine dinucleotide coenzyme bound to the enzyme. Enzyme labeled in this manner does not bind to *d1d*$^-$ 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. 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 which are defective in the flavin binding site should exhibit soluble material which cross-reacts immunologically with native D-LDH.

MOLECULAR ASPECTS OF CARRIER FUNCTION

It seems apparent from the foregoing discussion that although many of the details are not yet completely clear, a unifying concept which explains the energetics of respiration-

dependent active transport in bacterial systems is emerging. However, it should be emphasized that this is only the beginning, that ultimately, carrier molecules must be solubilized, purified, and reconstituted so that this concept can be studied on a molecular level. Initial promising developments in this respect have already been reported (75, 76). In any case, within the past few years, certain important insights into carrier function at a more refined level of resolution have been achieved. These studies (see Refs. 65, 77 for reviews) have involved a collaborative effort between the authors' laboratory and that of Dr. Rudolf Weil of Sandoz Forschungsinstitut in Vienna, Austria.

In addition to the wealth of information available with regard to the β-galactoside transport system, and the ability to manipulate it genetically, this transport system has another notable property which makes it particularly advantageous for study. As opposed to most other bacterial transport systems, the β-galactoside system is relatively nonspecific with respect to the nongalactosyl moiety of the disaccharide. Thus, reporter groups or chemically reactive species can be incorporated into this class of sugars without abolishing their affinity for the *lac* carrier protein.

Substituted Galactopyranosides

The structures of the compounds to be discussed are shown in Figs. 1 and 2. As shown, they fall into 2 general categories (N-dansyl)aminoalkyl-β-D-galactopyranosides [dansylgalactosides] (78, 83) and (2-nitro-4-azidophenyl)-β-D-galactopyranosides [azidophenylgalactosides] (84, 85). In both cases, a β-D-galactopyranoside (usually 1-thio-β-D-galactopyranoside) is linked directly or through an alkyl chain of varying length to the appropriate probe. In one instance, a fluorescent moiety, commonly referred to as a dansyl fluorophore is sensitive to solvent polarity, exhibiting an increase in quantum yield and a blue shift in the emission spectrum as the solvent becomes less polar. Thus, the dansyl moiety is useful as a reporter group, yielding information about the polarity of its environment.

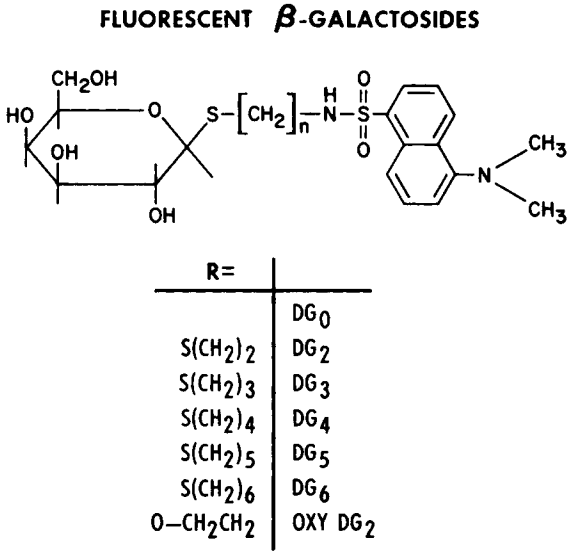
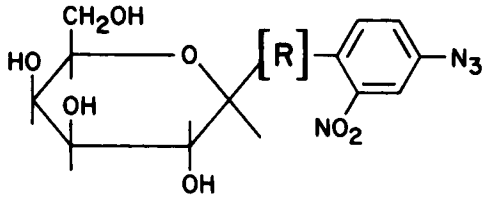


Fig. 1. Structural formulae of various dansylgalactosides.

PHOTOREACTIVE β-GALACTOSIDES



R=	
S	APG_0
SCH_2CH_2-N-H	APG_2

Fig. 2. Structural formulae of axidophenylgalactosides.

The second group of compounds contains an arylazide moiety linked to the galatosyl portion of the molecule (Fig. 2). The rationale behind the use of this class of compounds is that irradiation with visible light causes photolysis of the azido group to form molecular nitrogen and a highly reactive nitrene which then reacts covalently with the macromolecule to which the nitrene-containing ligand is bound, resulting in irreversible inactivation.

Dansylgalactosides Are Not Transported.

Each of the dansylgalactosides shown in Fig. 1 is a competitive inhibitor of lactose uptake in membrane vesicles prepared from strains of E. coli which contain a functional *lac* carrier protein (K_i values for Dns 0,2,3,4,5, and 6-Gal are approximately 550, 30, 12, 6, 3, and 5 μM, respectively). Strikingly, however, an exhaustive number of experiments indicates that the dansylgalactosides are not transported to a significant extent (78–80).

Binding Is Energy Dependent

The fluorescent properties of the dansylgalactosides in aqueous solution are similar to those of other dansyl derivatives, exhibiting emission maxima at approximately 540–550 nm and excitation maxima at approximately 340 nm. Significantly, no change in these parameters is observed in the presence of membrane vesicles. On addition of D-lactate, however, there is a marked increase in dansylgalactoside fluorescence which is absolutely dependent upon the presence of functional *lac* carrier protein in the vesicles. Moreover, no change in the fluorescence of 2′-(N-dansyl)aminoethyl-1-thio-β-D-glucopyranoside (dansylglucoside) is observed, indicating that the effects observed are specific for the galactosyl configuration of the ligand. It is also highly significant that the effect of D-lactate can be mimicked by the imposition of an ionic diffusion potential (interior negative) or by a lactose diffusion gradient (inside ——→outside).

The fluorescence increase observed with each of the dansylgalactosides under the conditions described exhibits an emission maxima at 500 nm and excitation maxima at

345 and 292 nm. The blue shift in the emission maximum is equivalent to that observed when the dansylgalactosides are dissolved in 85% dioxane, indicating that the affected molecules find themselves in a relatively hydrophobic environment. The appearance of the new peak in the excitation spectrum at 292 nm indicates moreover that the bound dansylgalactoside molecules are excited by energy transfer from tryptophanyl residues in the membrane proteins.

The increase in dansylgalactoside fluorescence induced by D-lactate is blocked or rapidly reversed by addition of β-galactosides, sulfhydryl reagents, inhibitors of D-lactate oxidation, and proton conductors or other reagents which collapse the membrane potential. On the other hand, the fluorescence increase induced by imposition of ionic diffusion potentials is blocked by β-galactosides, sulfhydryl reagents, and proton conductors, but not by respiratory poisons. Moreover, the degree to which dansylgalactoside fluorescence is increased under these conditions is dependent upon the magnitude of the applied ionic diffusion potential. Since D-lactate oxidation leads to the generation of a membrane potential (interior negative), it seems clear that the increase in dansylgalactoside fluorescence observed with D-lactate or artificially imposed ion gradients occurs by a similar mechanism. Finally, the increase in dansylgalactoside fluorescence observed with a lactose diffusion gradient is inhibited by β-galactosides and sulfhydryl reagents, both of which block the binding site of the carrier, but not by respiratory poisons or reagents which collapse the membrane potential. Thus, it seems clear that the fluorescence changes induced under these conditions occur by a mechanism which is independent of the membrane potential.

Titration of vesicles containing the *lac* carrier protein with each dansylgalactoside demonstrates that the vesicles bind 1–2 nmol of each dansylgalactoside per mg of membrane protein, a value almost identical to that obtained from direct binding measurements with [^{3}H] DG_6 (82, 83). Assuming that one dansylgalactoside molecule is bound per molecule of *lac* carrier protein and that the molecular weight of the *lac* carrier protein is approximately 30,000, 1–2 nmol/mg membrane protein is equivalent to 3–6% of the membrane protein. This value is very similar to that reported by Jones and Kennedy (85) who used a completely independent method. In addition, these titration studies indicate that the affinity of the *lac* carrier for ligand is directly related to the length of the aklyl chain between the galactosyl and dansyl moieties of the dansylgalactosides. There is excellent agreement, moreover, between the affinity constants of the various dansylgalactosides as determined by fluorimetric titration or flow dialysis (78, 79, 82, 83) and their apparent K_i values for lactose transport as given above.

There are at least 3 possible mechanisms by which energy might lead to dansylgalactoside binding to the *lac* carrier protein: i) The carrier is accessible to the external medium and binding occurs spontaneously. In this case, energy coupling results in partial translocation of the bound ligand, resulting in its exposure to the hydrophobic interior of the membrane, and thus to the fluorescence changes observed. This possibility seems unlikely because no changes in the emission or excitation spectra of the dansylgalactosides are observed in the absence of D-lactate and for reasons to be discussed below. ii) The carrier is accessible to the external medium in the absence of energy coupling, but its affinity is increased when energy is supplied. iii) The carrier is inaccessible to the external medium, and energy coupling casues a conformational change in the carrier such that high affinity binding sites appear on the external surface of the membrane. If it is postulated moreover that the *lac* carrier protein (or part of it) has a negative charge, the appearance of binding sites on the exterior surface of the membrane can be more easily

conceptualized. Imposition of a membrane potential (interior negative) would cause "movement" of the negatively charged carrier to the external surface of the membrane and binding of dansylgalactosides. It should be emphasized that the last 2 possibilities are not mutually exclusive and that energy-coupling may well increase the accessibility of the carrier and its affinity for ligand simultaneously. In any case, the data suggest that energy is coupled to one of the initial steps in transport, and that facilitated diffusion therefore cannot represent the first step in the active transport of β-galactosides.

Further evidence for the proposition that the fluorescence changes observed upon "energization" of the membrane are due to binding of the dansylgalactosides per se rather than binding followed by translocation into the hydrophobic interior of the vesicle membrane has been provided by the use of 2 other independent techniques. Anisotropy of fluorescence can be used to assess binding specifically since changes in this parameter reflect alterations in the rotation of molecules in solution. Studies with Dns^2-Gal and Dns^6-Gal (81) demonstrate that there is a marked increase in fluorescence anisotropy in vesicles containing the *lac* carrier protein on addition of D-lactate. In the absence of this electron donor, anisotropy values are minimal and identical in vesicles with or without the *lac* carrier protein, and no increase in anisotropy is observed in vesicles devoid of the *lac* carrier protein when D-lactate is added. In addition to these changes in anisotropy, fluorescence lifetime studies with Dns^2-Gal and Dns^6-Gal are also consistent with the proposition that changes in dansylgalactoside fluorescence observed on "energization" of the appropriate vesicles reflect binding of the fluorescent probes to the *lac* carrier protein (81). Furthermore, it can be calculated from the anisotropy and lifetime values that the rotational relaxation time of Dns^2-Gal increases dramatically when the probe is bound to the *lac* carrier protein.

Finally, high specific activity [^{3}H] Dns^6-Gal has been synthesized, and its binding to membrane vesicles has been studied directly by flow dialysis (82, 83). With vesicles containing the *lac* carrier protein, little, if any, binding is detected in the absence of D-lactate or reduced PMS. In the presence of these electron donors, binding is observed and the binding constant and number of binding sites are approximately 5 μM and 1.5 nmol/mg membrane protein, respectively. Both values are in excellent agreement with those obtained by fluorescence titration. These results demonstrate almost unequivocally that the changes in dansylgalactoside fluorescence observed on energization of membrane vesicles containing the β-galactoside transport system reflect binding of the probe to the *lac* carrier protein.

ENERGY DEPENDENT PHOTOINACTIVATION

Studies with photoreactive azidophenylgalactosides (Fig. 2) have provided completely independent support for the conclusions derived from the dansylgalactoside experiments. 2-Nitro-4-azidophenyl-1-thio-β-D-galactopyranoside (APG_0) is a competitive inhibitor of lactose transport in ML 308-225 membrane vesicles, exhibiting an apparent K_i of 75 μM (84). The initial rate and steady-state level of [^{3}H] APG_0 accumulation are markedly stimulated by the addition of D-lactate to vesicles containing the β-galactoside transport system, and kinetic studies reveal an apparent K_m of 75 μM. Vesicles devoid of the β-galactoside transport system do not take up significant amounts of APG_0 in the presence or absence of D-lactate. When exposed to visible light in the presence of D-lactate APG_0 irreversibly inactivates the β-galactoside transport system. Strikingly, APG_0-dependent photoinactivation is not observed in the absence of D-lactate. Kinetic studies

of the inactivation process yield a K_D of 77 μM, a value which is almost identical to the K_m and K_i values obtained with this compound. Moreover, lactose protects against APG_0 photoinactivation and significant inactivation of amino acid transport is not observed with APG_0. Thus, it is clear that these effects are specific for the *lac* carrier protein.

Analogous studies carried out with 2′-N(2-nitro-4-azidophenyl)aminoethyl-1-thio-β-D-galactopyranoside demonstrate that this compound behaves similarly with respect to photoinactivation of the β-galactoside transport system with 2 important exceptions (85). Like its analogue Dns[2]-Gal, APG_2 is not accumulated by the vesicles in the presence of D-lactate or ascorbate-PMS and it exhibits a higher affinity for the *lac* carrier protein than APG_0 (i.e., the K_I for competitive inhibition of lactose transport and the K_D for photoinactivation in the presence of D-lactate are 35 μM). In addition, it has been demonstrated that an artificially imposed membrane potential (interior negative) also leads to APG_2-dependent photoinactivation of the *lac* carrier protein.

An Apparent Discrepancy

As discussed above, it seems clear from studies with dansyl- and azidophenyl-galactosides that binding of these ligands by the *lac* carrier protein is energy dependent. On the other hand, Kennedy et al. (87) have reported that binding of β-D-galactopyranosyl-1-thio-β-D-galactopyranoside (TDG) and p-nitrophenyl-α-D-galactopyranoside (NPG) to membrane particles prepared by ultrasonic disruption is independent of the presence of an energy source and is not inhibited by sodium azide. However, assuming that the purity of these membrane particles is comparable to that of isolated membrane vesicles,[1] that binding rather than trapping of ligand within an internal space was measured,[2] and that TMG permease II (88) was not present in the particles, the amount of binding observed by Kennedy et al. (87) is considerably less than the total amount of *lac* carrier protein (i.e., M protein) present in the membrane as determined by titration studies with the dansylgalactosides and as determined by Jones and Kennedy (85). In any case, a small but significant amount of nonspecific binding of dansylgalactosides is detected by fluorescence anisotropy and lifetime studies, allowing the possibility that the techniques described thus far are not sufficiently sensitive to detect less than 10% of the binding observed under energized conditions. For this reason, high specific activity [^{3}H]NPG was synthesized and its binding to membrane vesicles investigated by means of flow dialysis (66). These studies corroborate the findings of Kennedy et al. (87), but they also confirm the observations discussed above. There is a small amount of NPG binding by vesicles containing the *lac y* gene product (about 0.2 nmol per mg membrane protein at saturation) which is abolished and reversed by p-chloromercuribenzenesulfonate (p-CMBS) but not by proton conductors, and this binding is not dependent upon the presence of D-lactate. On addition of D-lactate, however, approximately 2.3 nmol NPG are bound per mg membrane protein (i.e., an amount similar to that observed with the dansylgalactosides), and all of the bound ligand is displaced by p-CMBS.

[1]This assumption may not be justified as membrane particles prepared as described by Kennedy et al. (87) apparently contain as much as 30–40% of the total cellular protein (C. F. Fox, personal communication).

[2]Kennedy et al. (87) utilized the distribution of inorganic phosphate to correct for ligand which may have been trapped in the internal space of these particles. The use of phosphate in this respect is somewhat unorthodox, as this anion is generally felt to be impermeant.

Moreover, the K_D for binding under energized and nonenergized conditions is very similar (i.e., 6–9 μM). Thus, although there is a small amount of binding in the nonenergized state, it seems quite evident that the great bulk of the *lac* carrier protein is unable to bind ligand unless the membrane is energized.

Translocation and Accumulation of β-Galactosides

The observation that a small but significant amount of NPG binding to membrane vesicles containing the *lac* carrier protein occurs in the absence of an electrochemical gradient of protons or artificially applied ion diffusion potentials suggests that the *lac* carrier protein may exist in two forms which are in a state of dynamic equilibrium: i) a high affinity form which is accessible on the external surface of the membrane, and ii) a low affinity, cryptic form. In the absence of D-lactate or reduced PMS, 90% or more of the carrier is in the low affinity, cryptic form and only 10% or less is in the high affinity, accessible form. Upon generation of an electrochemical proton gradient across the vesicle membrane (interior negative and alkaline), one or more negatively charged groups in the low affinity, cryptic form of the protein might be influenced, resulting in a conformational change and a shift in the equilibrium. According to such a model, active transport would occur by binding of ligand to the high affinity form of the carrier on the external surface of the membrane, followed by conversion of the carrier to the low affinity, cryptic form and release of ligand from the inner surface of the membrane. However, since the carrier may be negatively charged, translocation of ligand would require neutralization of the high affinity form of the carrier on the external surface of the membrane. This might be accomplished if ligand binding increased the pK_a of a negatively charged functional group(s) in the carrier. The protein would then be uncharged and no longer under the influence of the electrochemical gradient. The protonated carrier-ligand complex would "relax" to the cryptic form and release proton(s) and ligand on the inner surface of the membrane, regenerating the charged form of the carrier, and the cycle could then be repeated. Clearly, this reaction sequence is consistent with many of the observations discussed above. It is also noteworthy that according to this model, the carrier would not translocate protons across the membrane in the absence of ligand, a stipulation that is necessary lest the carriers themselves dissipate the electrochemical gradient without performing work. It should also be emphasized that this formulation might account for low rates of facilitated diffusion without necessitating that the process represent the initial step in the active transport of β-galactosides.

Distance Measurements With the *lac* Carrier Protein

It should be clear from the foregoing discussion that the fluorescence changes observed with the dansylgalactosides are due specifically to binding of these ligands to a site in the *lac* carrier protein and not to a subsequent translocation event. Since the spectral properties of the dansyl group are sensitive to solvent polarity, varying the distance between the galactosyl and dansyl moieties of the dansylgalactosides might be informative with respect to the environment in the immediate vicinity of the binding site in the *lac* carrier protein.

Recently (83), each of the dansylgalactoside homologues shown in Fig. 1 was synthesized in radioactive form and binding to membrane vesicles containing the *lac* carrier protein measured directly by flow dialysis in the presence of D-lactate. The results were then compared with the D-lactate-induced fluorescence enhancement observed with each dansylgalactoside and with the ability of N-methylpicolinium perchlorate (89) to quench

the fluorescence of the bound homologues. The following observations have been clearly documented (83): i) The binding affinity of the *lac* carrier protein is directly related to the length of the alkyl chain linking the galactosyl and dansyl ends of the molecules. ii) The maximum number of binding sites observed for each homologue is essentially identical. iii) The increase in fluorescence observed when the probes are bound to the *lac* carrier protein changes markedly when the distance between the galactosyl and dansyl moieties is varied. As the linkage is lengthened from 2 to 4 carbons, fluorescence decreases by a factor of 10 or more and then increases dramatically and progressively with Dns^5-Gal and Dns^6-Gal. These effects vary inversely with the ability of N-methylpicolinium perchlorate to quench the fluorescence of the bound probes.

In view of the specificity of dansylgalactoside binding and the fluorescence properties of the dansyl group, it seems apparent that the galactosyl end of these molecules is anchored at the binding site of the *lac* carrier protein, while the dansyl end proceeds from a hydrophobic environment to an aqueous environment to a hydrophobic environment as the alkyl linkage is lengthened. Possible interpretations of this behavior depend upon assumptions regarding the flexibility and hydrophobicity of the alkyl linkage in the molecules. If it is assumed simplistically that the linkage merely maintains a linear configuration, the variations in fluorescence might reflect differences in the polarity of the microenvironment within the membrane or on the surface in the vicinity of the *lac* carrier protein. An alternative interpretation is that the alkyl linkage is both flexible and hydrophobic, in which case, both parameters would vary directly with chain length. Under these circumstances, it seems reasonable to suggest that the dansyl moiety in Dns^2-Gal reflects a hydrophobic environment in the binding site of the *lac* carrier protein, and as the dansyl end of the molecule is removed 3 and then 4 carbons from the binding site, it becomes accessible to the aqueous solvent at the membrane interface. When the alkyl linkage is then elongated to 5 and subsequently 6 carbons, however, the molecules might become sufficiently flexible and hydrophobic such that the dansyl moiety and part of the alkyl chain adsorb to a hydrophobic site on the surface of the membrane which may or may not comprise part of the *lac* carrier protein. Although it is impossible to distinguish absolutely between these 2 alternatives at the present time, it should be apparent that the latter interpretation is favored by the results of the quenching studies with N-methylpicolinium perchlorate. Moreover, if the latter interpretation is correct, the binding site in the *lac* carrier protein is probably about 5–6 Å from the aqueous solvent at the surface of the membrane.

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