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Functional *lac* Carrier Proteins in Cytoplasmic Membrane Vesicles Isolated from *Escherichia coli*. 1. Temperature Dependence of Dansyl Galactoside Binding and β-Galactoside Transport†

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ABSTRACT: Dansyl galactoside (6′-(*N*-dansyl)aminohexyl 1-thio-β-D-galactopyranoside) binds in an energy-dependent way to the *lac* carrier proteins of membrane vesicles isolated from *Escherichia coli* cells. The binding is not followed by transport but it is accompanied by a large increase in fluorescence and a shift of the emission maximum to a lower wavelength. These properties make it possible to titrate the amount of *lac* carrier proteins which become accessible upon energizing the membrane. The temperature dependence of the binding of dansyl galactoside has been determined in membrane vesicles of *E. coli* K 1059 and ML 308225. The number of binding sites of dansyl galactoside decreases with decreasing temperature. The range over which this occurs overlaps that at which the conformational order–disorder transition of the membrane lipids takes place. The dissociation constant is temperature independent. The initial rates of binding are graphed according to the Arrhenius equation. This is represented as nmol of dansyl galactoside bound per min either per mg of protein or per nmol of accessible *lac* carrier proteins. In the former case, the best fit to the data requires two lines. The line in the low-temperature range has a steeper slope. However, in the latter case, the data can be fit either by only one line (K 1059 membranes) or by two lines but with a much less pronounced difference in slope (ML 308225 membranes). There is a striking parallel between the temperature dependence of the initial rates of dansyl galactoside binding to a given membrane and the temperature dependence of the initial rates of β-galactoside transport by the same membrane. This parallelism supports the notion that the carriers which participate in the transport (functional carriers) are the ones which become accessible to the external medium upon energizing. It is concluded that the change in slope usually observed in the Arrhenius plot of transport in the temperature range extending over the order disorder transition is mainly a result of a change in the number of functional *lac* carriers.

Isolated cytoplasmic membrane vesicles have become a very useful tool for the study of active transport (Kaback, 1974). Kaback and co-workers (Schuldiner et al., 1976a) have carefully analyzed the mechanism by which the active transport of a variety of metabolites by cytoplasmic membranes of *E. coli* is coupled to the oxidation of D-lactate or to the artificial electron donor, phenazine methosulfate. A recent approach to the study of β-galactoside transport has been the use of reporter groups covalently linked to the sugar (Reeves et al., 1973; Schuldiner et al., 1975a; Rudnick et al., 1975). Among these, dansyl galactosides have been shown to bind specifically and in an energy-dependent way to the *lac* carrier proteins without being transported across the membrane (Schuldiner et al., 1975b,c, 1976b). Studies involving these dansyl galactosides have yielded new information on the mechanism of β-galactoside transport at the molecular level (Schuldiner et al., 1976a).

We have shown previously that, in cytoplasmic membrane vesicles isolated from *E. coli*, the active transport coupled to D-lactate oxidation responds to a change in conformation of the membrane lipids (Shechter et al., 1974, 1975). This change can be induced by temperature variations and involves a transition from a disordered fluid state of the lipids at high temperature to an ordered state of the lipids at low temperature (Ranck et al., 1974). At temperatures at or below those of the conformational transition, Arrhenius plots of active transport display a slope which is in general more pronounced than that at temperatures above the transition (Shechter et al., 1974, 1975; Overath and Träuble, 1973; Overath et al., 1976). The exact reason for this change in slope has been the subject of considerable study.

This report describes the use of dansyl galactoside in the investigation of the mechanism by which the disorder-to-order transition associated with the membrane lipids affects the active transport of β-galactosides. We show that the relationship between lipid conformation and transport is an indirect one and that it involves a change in the number of functional *lac* carrier proteins that participate in the transport process as the conformation of the lipids changes.

Materials and Methods

Growth of Bacteria and Preparation of Membrane Vesicles.

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Cells of *E. coli* ML 308225 ($i^- z^- y^+ a^+$), a generous gift from Dr. H. R. Kaback, were grown in minimal medium A containing 1% succinate as the sole carbon source. *E. coli* K 1059 ($i^+ z^+ y^+ a^+$) is an unsaturated fatty acid auxotroph (Overath et al., 1970). These cells were grown in Cohen-Rickenberg mineral salt medium (Anraku, 1967) supplemented with 1% succinate, 0.3% casamino acids, 2% brij 35, and 0.02% oleic acid (*cis*-$\Delta^9C_{18:1}$) as the exogenous unsaturated fatty acid. *E. coli* K 1059 cells were induced for β-galactoside transport using 10^{-4} M isopropyl β-D-thiogalactopyranoside. All cells were grown at 37 °C in 2-L Fernbach flasks in a New Brunswick rotary incubator and harvested at the end of the logarithmic phase. Membrane vesicles were prepared as described by Kaback (1971) and will be called ML 308225 and "oleate" membrane vesicles, respectively.

Fluorescence Measurements. Fluorescence was measured at 90° angle with an Aminco Bowman spectrofluorimeter with 1 cm^2 cuvettes. The sample chamber was thermostated with a circulating water bath and the temperature was measured inside the cuvette. Spectra were taken in the ratio mode and were not further corrected for variations in either intensity of the light source or response of the photomultiplier with wavelength. Additions to the cuvette were made with Hamilton microsyringes and the samples were mixed within 1 or 2 s without opening the sample chamber.

β-Galactosides Uptake. [1-^{14}C]Lactose (20 mCi/mmol) uptake by ML 308225 membrane vesicles was assayed as described by Kaback (1971). "Oleate" membrane vesicles are contaminated by trace amount of cytoplasmic β-galactosidase. Therefore, uptake was determined using TMG[1] (1-^{14}C-labeled, 35 mCi/mmol), a nonmetabolized analogue of lactose. Initial rates were measured over the linear portion of the uptake curves.

Reagents and Miscellaneous Analytical Methods. The synthesis of DG_6 has been described by Schuldiner et al. (1975a). All other chemicals were of reagent grade and were obtained from commercial sources. Protein was determined by the method of Lowry et al. (1951).

Results

Fluorescence Changes of DG_6 Induced by D-*Lactate.* The fluorescence changes of dansyl galactosides induced by the addition of D-lactate in the presence of ML 308225 membrane vesicles have been described previously (Reeves et al., 1973; Schuldiner et al., 1975a). Qualitatively we observe similar changes in the presence of "oleate" membrane vesicles: the emission maximum of DG_6 fluorescence in the absence of D-lactate is 540 nm (exciting wavelength: 340 nm); upon addition of D-lactate, an increase in fluorescence is observed; the difference between the emission spectra with and without D-lactate displays a maximum at 490 nm; the increase in fluorescence is not observed for "oleate" membrane vesicles prepared from uninduced cells; it is completely reversed or prevented by addition of lactose (see Figure 1). As demonstrated previously (Reeves et al., 1973; Schuldiner et al., 1975a,b, 1976b), the increase in DG_6 fluorescence observed on addition of D-lactate is due specifically to binding to the *lac* carrier proteins, and definitive evidence indicates that the dansyl galactosides are not transported to any extent by the isolated membrane vesicles.

The fluorescence properties of dansyl galactosides are polarity dependent. With decreasing polarity, the quantum yield of fluorescence increases and the emission spectrum is displaced to lower wavelength (Reeves et al., 1973). The increase in fluorescence observed on the addition of D-lactate results from the transfer of the dansyl moiety of the galactoside from the polar water environment (emission maximum: 540 nm) to the less polar environment of the membrane (observed emission maximum: 490 nm). Analysis of the emission spectra of DG_6 in various mixtures of water and nonpolar organic solvents shows that a shift in the emission maximum from 540 nm (water) to 490 nm (bound DG_6) results in an increase in fluorescence by a factor of 39 (data not shown). This value, together with the steady-state level of fluorescence increase observed on addition of D-lactate (Figure 1), can be used to calculate the amount of DG_6 bound to the membrane.

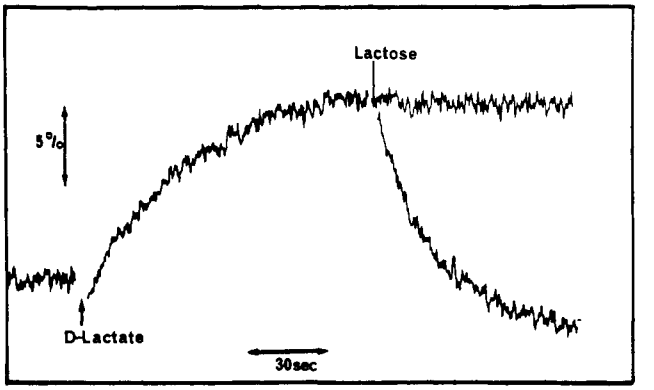

FIGURE 1: Time course of changes in DG_6 fluorescence dependent on D-lactate at 25 °C. Lithium D-lactate (20 mM) was added to a cuvette containing DG_6 (10 μM), 50 mM potassium phosphate (pH 6.6), 10 mM magnesium sulfate, and membrane vesicles from induced *E. coli* K 1059 (oleate membrane, 0.4 mg of protein per mL) in a total volume of 1.5 mL. The fluorescence at 500 nm was recorded as a function of time (exciting wavelength: 340 nm). At the time shown, 0.1 M lactose was added at a final concentration of 1 mM (arrow). Typically, at 25 °C the half-time of fluorescence increase was 10 to 15 s. Qualitatively, similar increases in fluorescence are observed upon addition of succinate rather than D-lactate as the electron donor (data not shown). Similar experiments conducted with membrane vesicles from uninduced *E. coli* K 1059 cells displayed no increase in fluorescence on addition of D-lactate (data not shown).

Temperature Dependence of the Number of DG_6 Binding Sites. Figure 2 shows the amount of DG_6 bound to "oleate" membrane vesicles as a function of external DG_6 concentration and at three different temperatures. At each temperature, the steady-state fluorescence increase induced by the addition of D-lactate is a saturable function of external DG_6 concentration. From each saturation curve, the amount of DG_6 bound at infinite DG_6 concentration (number of binding sites) and the dissociation constant K_D can be calculated (see inset in Figure 2).

These parameters have been calculated as a function of temperature between 5 and 40 °C for both ML 308225 and "oleate" membrane vesicles. The results are shown in Figure 3.

The number of binding sites is strongly temperature dependent. For ML 308225 membrane vesicles, it increases from 0.62 nmol/mg of protein at 5 °C to a constant value of 1.3 nmol/mg of protein at temperatures above 22 °C. For the same membrane vesicles, Schuldiner et al. (1975a) report a value of 2 nmol/mg of protein at 25 °C. The discrepancy stems from their use of a different factor for the increase in fluorescence associated with the transfer of the dansyl moiety of DG_6 (25 instead of our value of 39). For "oleate" membrane vesicles, the temperature dependence of the number of DG_6 binding sites is even more pronounced. It increases from 0.12 nmol/mg

[1] Abbreviations used: TMG, methyl β-D-thiogalactopyranoside; DG_6, 6′-(*N*-dansyl)aminohexyl 1-thio-β-D-galactopyranoside; SEM, standard error of the mean.

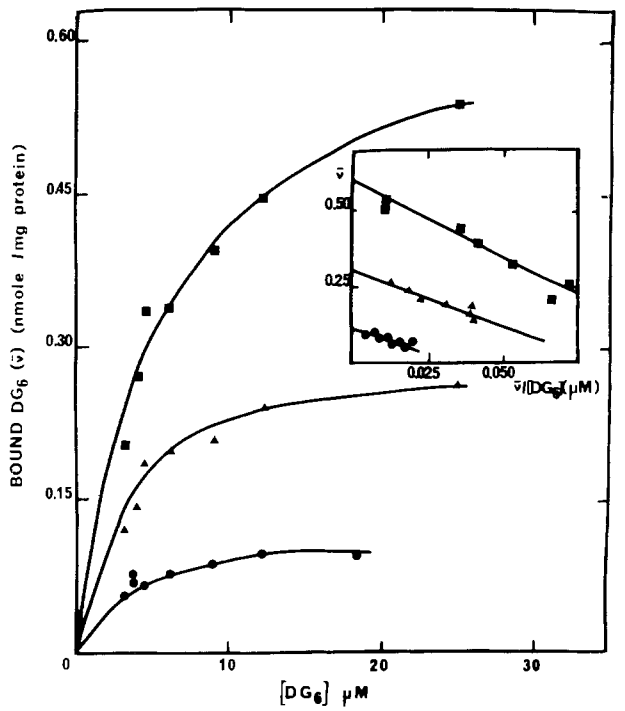

FIGURE 2: Effect of DG_6 concentration on the D-lactate fluorescence changes (steady state) for "oleate" membranes and at three different temperatures (5 °C (●); 14 °C (▲); 25 °C (■)). At each temperature, the percentage increase in DG_6 fluorescence on D-lactate addition, corrected by subtracting the intensity of light scattered by the membranes, was determined at each DG_6 concentration (excitation, 340 nm; emission, 500 nm). The number of bound DG_6 at each concentration was calculated assuming that each bound DG_6 increased its fluorescence by a factor of 39. Inset: data plotted according to the Scatchard representation. The regression lines were calculated by the method of least squares. $K_D \pm$ SEM for the fluorescence increase is 2.5 ± 0.7, 4.0 ± 0.6, and 4.9 ± 0.6 μM at 5, 14, and 25 °C, respectively. The number of binding sites is 0.11 ± 0.01, 0.31 ± 0.01, and 0.59 ± 0.01 nmol per mg of protein at 5, 14, and 25 °C, respectively.

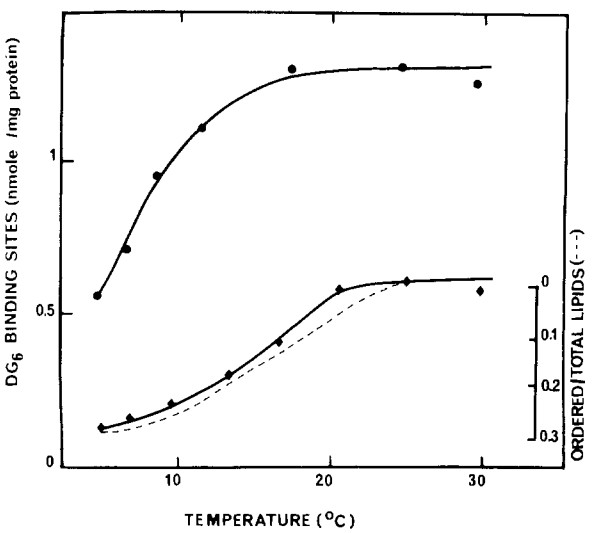

FIGURE 3: Temperature dependence of the number of binding sites for DG_6 induced by the addition of D-lactate. (♦) "Oleate" membrane vesicles; (●) ML 308225 membrane vesicles. For "oleate" membrane vesicles, the order-disorder transition associated with the membrane lipids as a function of temperature and as determined by high-angle x-ray diffraction (Shechter et al., 1974) is shown for comparative purposes (- - -). Within experimental errors the changes in the number of binding sites parallel the changes in the amount of ordered membrane lipids. Qualitatively, a similar temperature dependence of the number of binding sites for DG_6 is observed on addition of succinate rather than D-lactate as the electron donor (data not shown).

of protein at 5 °C to a constant value of 0.60 nmol/mg of protein at temperatures above 20 °C.

In contrast, the dissociation constant remains relatively constant with temperature (data not shown) and is equal to 5 ± 2 μM for both "oleate" and ML 308225 membrane vesicles. This value agrees well with that reported by Schuldiner et al. (1975a) for ML 308225 membrane vesicles at 25 °C.

Relation between Number of Binding Sites and Conformation of the Membrane Lipids. Both ML 308225 and "oleate" membrane vesicles display a conformational transition of their lipids as a function of temperature from an ordered state to a disordered state (order-disorder transition) (Shechter et al., 1974, 1975; Dupont et al., 1972). The temperature range over which these transitions extend overlaps the temperature range over which the number of DG_6 binding sites changes. This is particularly striking in the case of "oleate" membrane vesicles (see Figure 3).

The reason for the difference between the number of binding sites at high temperature for ML 308225 (1.3 nmol/mg of protein) and for "oleate" membrane vesicles (0.60 nmol/mg of protein) is not clear. At the higher temperatures all the membrane lipids are disordered and it should be possible to titrate all the DG_6 binding sites. A possible explanation is that *E. coli* ML 308225 is constitutive for the *lac* carrier protein, while *E. coli* K 1059 is inducible. The amount of carriers synthesized and incorporated into the membrane may depend on the conditions of induction.

Temperature Dependence of the Initial Rates of DG_6 Binding. The initial rates of DG_6 binding are calculated from the linear portion of the fluorescence increase observed on addition of D-lactate (see Figure 1). These are temperature dependent. They were determined between 5 and 35 °C using a single concentration of DG_6 (10 μM or approximately $2K_D$). The data for "oleate" and ML 308225 membrane vesicles are shown in Figures 4 and 5, respectively, and are plotted according to the Arrhenius equation. When the initial rates are calculated as nmol of DG_6 bound per mg of protein per min (left ordinate of the figures), the best fit to the experimental points requires two straight lines having different slopes. A different best fit to the experimental points is obtained if the initial rates are calculated as nmol of DG_6 bound per nmol of DG_6 binding sites per min (right ordinate of the figures). The nature of the difference is discussed below.

Temperature Dependence of the Initial Rates of TMG and Lactose Uptake by "Oleate" and ML 308225 Membrane Vesicles, Respectively. The initial rates of uptake of TMG by "oleate" membrane vesicles and of lactose by ML 308225 membrane vesicles are temperature dependent. Figures 4 and 5 show the data for "oleate" and ML 308225 membrane vesicles, respectively, plotted according to the Arrhenius equation. The left ordinate corresponds to initial rates calculated as nmol of substrate transported per mg of protein per min. The right ordinate corresponds to initial rates calculated as nmol of substrate transported per nmol of functional carriers (equivalent to DG_6 binding sites (see Discussion)) per min. The behavior is similar, if not identical, to that described above for the temperature dependence of the initial rates of DG_6 binding.

Discussion

The Arrhenius representation of transport in bacterial membranes is in general biphasic with a more pronounced slope at lower temperatures (Schairer and Overath, 1969; Wilson et al., 1970; Shechter et al., 1974). The temperature at which the change in slope occurs correlates fairly well with the tem-

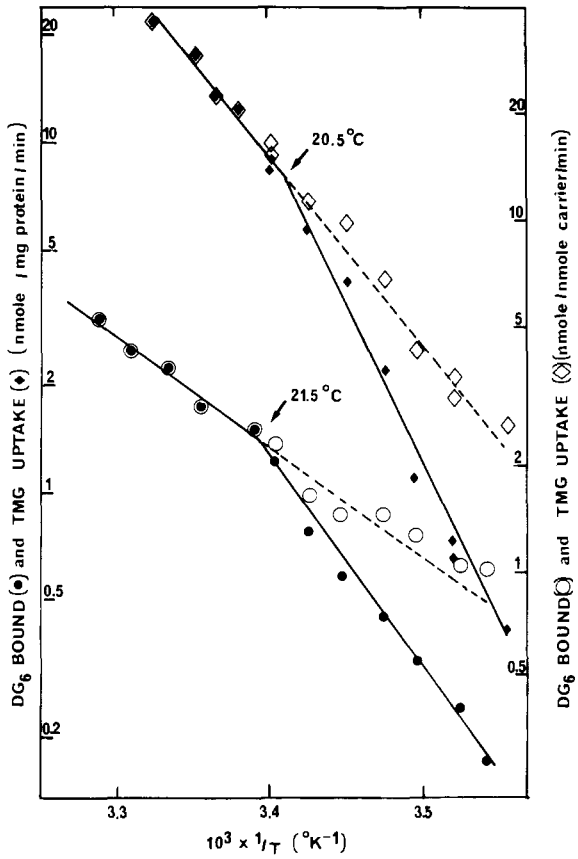

FIGURE 4: Temperature dependence of the initial rates of binding of DG_6 and of uptake of TMG by "oleate" membrane vesicles. The initial rates of binding were calculated from the linear portion of the fluorescence increase induced by the addition of D-lactate (see Figure 1). The initial rates of uptake were measured over the linear portion of the uptake curves. The data are plotted according to the Arrhenius equation. Left ordinate: initial rate of binding (●) and uptake (♦) expressed as nmol bound or transported per mg of protein per min. Right ordinate: initial rate of binding (○) and uptake (◇) expressed as nmol bound or transported per nmol of carrier per min. The carriers correspond to functional ones and are equivalent to DG_6 binding sites (see Discussion). The number of binding sites is taken from Figure 3. At temperatures above 22 °C, the number of binding sites is constant (0.6 nmol) and the two representations (per mg of protein or per nmol of carrier) lead to parallel curves displaced by a constant quantity. The two ordinates have been displaced by this quantity (log 0.6) and, thus, the experimental points above 22 °C are common to the two representations.

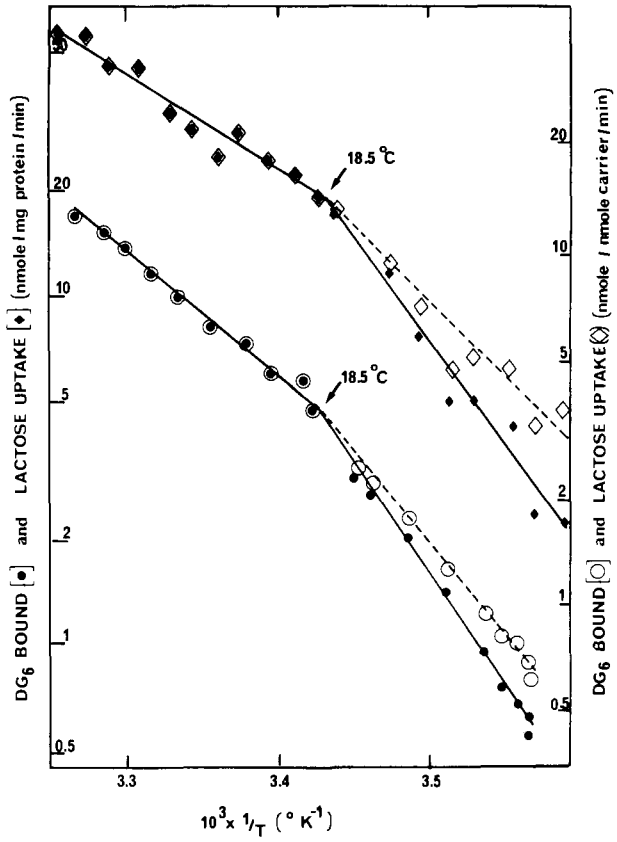

FIGURE 5: Temperature dependence of the initial rates of binding of DG_6 and of uptake of lactose by ML 308225 membrane vesicles. The data are plotted as in Figure 4, except that lactose replaces TMG. See Figure 4 for further explanations.

perature of the mid-order-disorder transition associated with the membrane lipids (Shechter et al., 1974, 1975). This apparently indicated a good correlation between transport and conformation of the membrane lipids. However, the mechanism by which the conformation of the lipids governed and influenced transport was not clear.

It has been proposed originally that the increased slope observed at lower temperatures reflects an increase in the energy of activation of transport as a certain fraction of the membrane lipids become ordered (Overath et al., 1970). Lately, more precise experiments in the field of β-galactoside transport by *E. coli* cells (Overath et al., 1976), extending over a wider temperature range and including in particular temperature regions well below those of the order-disorder transition of the membrane lipids, have revealed that the Arrhenius plot of transport is in fact triphasic. At temperatures below the transition, the slope assumes again a smaller value and becomes similar to that observed in regions above the transition temperature. Thus, the original proposal of high energy of activation of transport at low temperatures when the lipids are ordered is no longer applicable.

Based on these data, a mechanism by which the order-disorder transition influences transport has been advanced by Overath et al. (1976). It is proposed that the steep intermediate part of the Arrhenius plot, extending over the order-disorder transition temperature range, results from a change in the rate of transport due to the distribution of the carrier proteins between the membrane domains containing the ordered and the fluid lipids, respectively. Such a distribution does indeed exist, and we present experimental evidence for it in the accompanying paper (Letellier et al., 1977). However, the mechanism proposed by Overath et al. (1976) is based on a certain number of implicit assumptions. One of these stipulates that the lipid environment acts only on the rate constant and not on the effective carrier concentration. This assumption is not supported by the results documented in this article. We show indeed that the increase in the slope in the temperature range extending over the order-disorder transition of the membrane lipids is mainly, if not totally, the result of a decrease in the amount of the carrier accessible for DG_6 binding and probably for transport as well. (However, it cannot be ruled out that the transport mechanism is somewhat different in whole cells and in isolated membrane vesicles.)

The initial rate of transport of β-galactosides is proportional to the concentration of the *lac* carrier proteins effectively participating in transport, i.e., functional carriers. It has generally been assumed that the concentration of functional carriers remains constant with temperature and it is only under these conditions that the slope of the Arrhenius plot can be equated with the energy of activation of the overall transport process. This relationship is not tenable anymore if the concentration of the functional carriers involved in the transport process varies with temperature.

We have shown that the number of sites available for DG_6 binding following the addition of D-lactate is temperature dependent. On the other hand, it has been demonstrated that these binding sites represent *lac* carrier proteins which become accessible to the external medium on energizing (Reeves et al., 1973; Schuldiner et al., 1975a; Rudnick et al., 1975; Schuldiner et al., 1975b,c, 1976b). It is reasonable to assume that only these carriers are functional ones in the transport of β-galactosides. The temperature dependence of the functional carriers concentration can be taken into consideration by expressing the initial rates of DG_6 binding or TMG or lactose transport as nmol bound or transported per nmol of functional carrier per min rather than per mg of protein per min. The functional carrier concentrations at different temperatures are taken from Figure 3.

If the initial rates of DG_6 binding to "oleate" and ML 30225 membrane vesicles are calculated as nmol bound per mg of protein per min (left ordinates of Figures 4 and 5, respectively), the best fit to the experimental data requires two straight lines with a break and a higher slope in the order-disorder transition temperature range. If the initial rates of DG_6 binding to "oleate" membrane vesicles are calculated as nmol bound per nmol of DG_6 binding sites per min (right ordinate of Figure 4), the best fit to the experimental data requires only one line whose slope is practically identical with that observed in the high temperature range in the previous representation. If the initial rates of DG_6 binding to ML 308225 membrane vesicles are calculated as nmol bound per nmol of DG_6 binding sites per min (right ordinate of Figure 5), the best fit to the experimental data still requires two straight lines with a break. The difference in slope, however, is less pronounced than in the first representation.

There is a striking parallel between the temperature dependence of the initial rates of DG_6 binding to a given membrane and the temperature dependence of the initial rates of transport of TMG ("oleate" membrane vesicles) or lactose (ML 308225 membrane vesicles) by the same membrane (see Figures 4 and 5). This parallelism supports the assumption that the functional carriers which effectively participate in transport are those which become available for DG_6 binding on energizing of the membrane. Thus, these data demonstrate that the apparent increase in the energy of activation of transport as one goes through the disorder-to-order transition is mainly (ML 308225 membrane vesicles) if not totally ("oleate" membrane vesicles) a consequence of the change in the number of functional *lac* carrier proteins taking part in transport.

E. coli membrane vesicles catalyze the active transport of a variety of metabolites coupled to the oxidation of D-lactate through a series of specific membrane protein carriers (Kaback, 1974). We have presented previously the temperature dependence of the initial rates of proline transport by "oleate" membrane vesicles (Shechter et al., 1974). The initial rates were expressed as nmol of proline taken up per mg of protein per min and the Arrhenius plot was similar to that reported here for TMG uptake by the same membrane and using the same representation (a straight line in the low temperature range with a slope twice that of the straight line in the high temperature range; a break at 21 °C). Thus, if one assumes the same behavior for both proline and β-galactoside carriers, i.e., the same relative change with temperature of the amount of functional carriers, the Arrhenius plot of proline transport expressed as nmol of proline taken up per nmol functional carrier per min will be fitted with a single straight line. This could be a behavior shared by more carriers involved in the active transport of metabolites coupled to the oxidation of D-lactate in the case of *E. coli* membrane vesicles.

In the accompanying paper (Letellier et al., 1977), we demonstrate the existence of a segregation of the β-galactoside carriers between different membrane domains during the transition of the membrane lipids from the disordered to the ordered state. This segregation may explain some of the results presented here, in particular the change with temperature in the number of functional *lac* carrier proteins.

Acknowledgments

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