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Lactose Carrier Protein of *Escherichia coli*. Transport and Binding of 2′-(*N*-Dansyl)aminoethyl β-D-Thiogalactopyranoside and *p*-Nitrophenyl α-D-Galactopyranoside†

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ABSTRACT: The elevated level of lactose carrier protein present in cytoplasmic membranes derived from *Escherichia coli* strain T31RT, which carries the Y gene of the *lac* operon on a plasmid vector (Teather, R. M., et al. (1978) *Mol. Gen. Genet. 159*, 239–248), has allowed the detection of a complex between the carrier and the fluorescent substrate 2′-(*N*-dansyl)-aminoethyl β-D-thiogalactopyranoside (Dns^2-S-Gal). Binding is accompanied by a 50-nm blue shift in the emission maximum of the dansyl residue. The complex (dissociation constant, K_D = 30 μM) rapidly dissociates upon addition of competing substrates such as β-D-galactopyranosyl 1-thio-β-D-galactopyranoside or upon reaction with the thiol reagent *p*-chloromercuribenzenesulfonate. Binding of both Dns^2-S-Gal and *p*-nitrophenyl α-D-galactopyranoside (α-NPG) occurs spontaneously in the absence of an electrochemical potential gradient across the membrane. Comparison of equilibrium binding experiments using Dns^2-S-Gal or α-NPG and differential labeling of the carrier with radioactive amino acids shows that the carrier binds 1 mol of substrate per mol of polypeptide (molecular weight 30000). In addition to specific binding to the lactose carrier, Dns^2-S-Gal binds unspecifically to lipid vesicles or membranes, as described by a partition coefficient, κ = 60, resulting in a 25-nm blue shift in the emission maximum of the dansyl group. Both Dns^2-S-Gal and α-NPG are not only bound by the lactose carrier but also transported across the membrane by this transport protein in cells and membrane vesicles. The fluorescence changes observed with dansylated galactosides in membrane vesicles in the presence of an electrochemical gradient (Schuldiner et al. (1975) *J. Biol. Chem. 250*, 1361–1370)) are interpreted as an increase in unspecific binding after translocation.

Recently, the Y gene of the *lac* operon has been cloned in a plasmid vector in *Escherichia coli* (Teather et al., 1978). Upon induction of the *lac* operon, this plasmid leads to elevated levels of lactose carrier protein (lactose permease, "M protein") in the bacterial cytoplasmic membrane. The carrier could thus be readily detected on polyacrylamide gels as a prominently stained band of molecular weight 30000 and could also be identified by previously described methods using the thiol reagent *N*-ethylmaleimide and by double-labeling experiments with radioactive amino acids (Fox & Kennedy, 1965; Fox et al., 1967; Jones & Kennedy, 1969).

The amount of lactose carrier in membranes of *E. coli* has been determined in several laboratories. A comparison of these data is difficult because of the use of different strains, the conditions of cell growth, and the type of membrane preparation. On the basis of amino acid double label experiments, Jones & Kennedy (1969; compare also Kennedy, 1970) estimated that the lactose carrier comprises 3% of the total protein in cell envelopes of strain ML30 grown on succinate as sole carbon source. This corresponds to 1 nmol of carrier/mg of protein. A reinvestigation by the same technique showed a carrier level of <1% of the cytoplasmic membrane protein, i.e., <0.3 nmol/mg of protein, in strain ML30 grown on glycerol as carbon source (Teather et al., 1978). Alternatively, several investigators have determined the amount of carrier by substrate binding experiments. Kennedy et al. (1974) showed that cell envelopes bind 0.11 nmol of β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG)[1]/mg of protein. A similar figure, 0.2 nmol/mg of protein, has been found by Rudnick et al. (1976) by binding studies using *p*-nitrophenyl α-D-galactopyranoside (α-NPG) and cytoplasmic membrane vesicles of strain ML308-225, grown on succinate, provided binding was estimated in the absence of an electrochemical gradient across the membrane. A considerably

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[1] Abbreviations used: Dns, 5-dimethylaminonaphthalene-1-sulfonic acid; Dns^2-S-Gal, 2′-(*N*-dansyl)aminoethyl 1-thio-β-D-galactopyranoside; Dns^2-O-Gal, 2′-(*N*-dansyl)aminoethyl β-D-galactopyranoside; Dns^6-S-Gal, 6′-(*N*-dansyl)aminohexyl 1-thio-β-D-galactopyranoside; Dns^6-O-Gal, 6′-(*N*-dansyl)aminohexyl β-D-galactopyranoside; IPTG, isopropyl 1-thio-β-D-galactopyranoside; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside; β-NPG, *o*-nitrophenyl β-D-galactopyranoside; α-NPG, *p*-nitrophenyl α-D-galactopyranoside; p-CMBS, *p*-chloromercuribenzenesulfonate; β-galactosidase (EC 3.2.1.23); transacetylase, acetyl-CoA:galactoside 6-*O*-acetyltransferase (EC 2.3.1.18). For genetic symbols, see Bachmann et al. (1976).

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higher estimate, 0.6–0.9 nmol/mg of protein (Belaich et al., 1976, 1978) was recently obtained for the same type of membrane preparation by equilibrium binding studies using TDG as substrate. Since in none of these studies the amount of carrier as determined by double labeling with radioactive amino acids was directly correlated to the amount of substrate binding, conclusions as to the stoichiometry of the substrate–carrier interaction are doubtful.

The apparent discrepancy between the amount of substrate binding in the absence of an electrochemical gradient (Rudnick et al., 1976) and the amount of carrier protein present in membranes (Jones & Kennedy, 1969) was rationalized by the recent proposal (Reeves et al., 1973; Schuldiner et al., 1975a,b; 1976b, 1977; Rudnick et al., 1976; see also reviews by Schuldiner et al., 1976a; Kaback, 1977; Schuldiner & Kaback, 1977) that binding of various dansylated β-galactosides or α-NPG to about 90% of the carrier molecules present in membrane vesicles of strain ML308-225 depends on the presence of an electrochemical gradient. It was suggested that such a gradient either exposes the binding site of the carrier to the outside surface of the vesicle membrane and/or causes an increase in its affinity for solute molecules. This concept has been largely formulated on the basis of two lines of experimental evidence. Firstly, a series of arguments has been put forward that these substances are not transported; their energy-dependent association with membrane vesicles is therefore interpreted as stoichiometric binding to the lactose carrier. Secondly, values for the amount of carrier molecules calculated from these experiments range from 1.1 to 2.3 nmol/mg of protein and are in reasonable agreement with the estimate of Jones & Kennedy (1969).

In this paper the binding of 2′-(*N*-dansyl)aminoethyl β-D-galactopyranoside (Dns^2-S-Gal) and α-NPG to the carrier protein is correlated to the amount of Y-gene product present in the membrane as determined by double labeling with radioactive amino acids. This comparison leads to the conclusion that the carrier shows stoichiometric binding of 1 mol of substrate in the absence of an electrochemical gradient. Furthermore, evidence is presented that the carrier mediates the translocation of 2′-(*N*-dansyl)aminoethyl β-D-galactopyranoside (Dns^2-O-Gal) and α-NPG in cells and catalyses the active uptake of Dns^2-S-Gal and α-NPG in membrane vesicles.

Materials and Methods

Chemicals. We are most grateful to Dr. H. R. Kaback for sending us samples of Dns^2-S-Gal and Dns^6-S-Gal. Dns^2-S-Gal and Dns^6-S-Gal were also prepared from 2′-aminoethyl 1-thio-β-D-galactopyranoside hydrobromide (kindly provided by Dr. R. Weil) and 6′-aminohexyl 1-thio-β-D-galactopyranoside (a gift from Dr. S. Roseman), respectively, according to Schuldiner et al. (1975a). Dns^2-O-Gal was synthesized as described by Schuldiner et al. (1975a), and Dns^6-O-Gal was obtained by dansylation of 6′-aminohexyl β-D-galactopyranoside (a gift from Dr. S. Roseman).

Bacterial Strains. Prototrophic strains ML308-225 (*lac* $I^+O^+Z^+Y^+$) and K131 (*lac* $I^-O^+Z^-Y^+$, carries a polar *lac* Z mutation, phenotype Lac $I^-O^+Z^-Y^-$; derived from strain ML308 (*lac* $I^-O^+Z^+Y^+$)) have been described before (Devor et al., 1976). Strain T44RT (*lac* $I^+O^+Z^+Y^-(A^+)$/$F'I^+O^+\Delta(Z)Y^+A^+$) and the plasmid-containing strain T31RT (*lac* $I^+O^+Z^+Y^-(A^+)/F'I^qO^+Z^+Y^-(A^+)$, carries *lac* $I^+O^+\Delta(Z)Y^+A^+$ on the plasmid pC7) have been described (Teather et al., 1978; see also for complete list of additional markers). Strains K168 (*lac* $I^-O^+Z^+Y^+$), K169 (*lac* $I^-O^+Z^-Y^+$), and K170 (*lac* $I^-O^+Z^+Y^-$) are derivatives of the prototrophic K12-strain W3102 (*gal*$^-$, *recA*, *rpsL*). Strains DS338 (*lac* $I^+O^+Z^+Y^-$, *mel* C1, *gal* K$^-$, *met* B$^-$, *raf* $R^-O^+A^+B^-D^-$) and its *lac*$^+$-derivative strain DS338-2 (*lac* $I^+O^+Z^+Y^+$) were kindly provided by Dr. R. Schmitt. Both strains are pleiotropically negative (i.e., *melC*) for α-galactosidase and thiomethyl β-D-galactoside permease II coded for by the *mel* operon. They contain part of a plasmid derived *raf* operon (i.e., *raf* $R^-O^+A^+B^-D^-$; cf. Schmid & Schmitt, 1976) on the chromosome and show the constitutive synthesis of an active α-galactosidase coded by the A gene.

Growth Conditions and Membrane Isolation. Strains ML308-225, K131, T44RT, and T31RT were grown as previously described (Devor et al., 1976; Teather et al., 1978). Strains K168, K169, K170, DS338, and DS338-2 were grown in Cohen–Rickenberg (CR) mineral salts medium (Anraku, 1967) containing 0.5% glycerol and 0.2% casamino acids. For induction of the *lac* operon, 0.5 mM isopropyl 1-thio-β-D-galactopyranoside was included in the medium for growth of strains DS338 and DS338-2. Cells were harvested in mid-exponential phase and washed with CR salts medium.

Membrane vesicles from strains ML308-225 and K131 were prepared according to Kaback (1971). The same procedure was used for strain T31RT in the experiment shown in Figure 8. For all other experiments with cytoplasmic membranes from strains T31RT and T44RT, a combined $L_1 + L_2$ fraction was prepared according to Osborn et al. (1972).

Fluorescence Measurements. A Hitachi Perkin-Elmer Model MPF-3 spectrofluorimeter with a cuvette holder equipped with a magnetic stirrer was used. The fluorescence signal was either directly recorded on an X–Y recorder or averaged over a given time and recorded on punch tape using a PCS interface unit. Uncorrected emission spectra are presented.

Dns^2-S-Gal Binding Experiments. Specific, unspecific, and transport-dependent unspecific binding of Dns^2-S-Gal (cf. Results for definitions) can be differentiated by the spectral changes which occur upon binding (cf. Figures 4 and 9). The validity of such an analysis depends on an adequate correction for unspecific fluorescence, light scattering, and inner filter effects. This spectral procedure was only used for unspecific and transport-dependent unspecific binding to ML308-225 vesicles and unspecific binding to lipid vesicles. For determination of specific and unspecific binding to membranes derived from strain T31RT and K131, we have used a more direct centrifugation assay.

Unspecific and transport-dependent unspecific binding to membrane vesicles from strain ML308-225 was determined by performing experiments such as the one shown in Figure 9 (cf. Results) at various Dns^2-S-Gal concentrations. The spectra were corrected for unspecific fluorescence, integrated by cutting and weight determination, and corrected for inner filter effects. Unspecific binding was estimated from the difference between the intensity before addition of D-lactate and the intensity of water-dissolved Dns^2-S-Gal using a ratio for the quantum yield of unspecifically bound to water-dissolved Dns^2-S-Gal of 17 (cf. Table I). The transport-dependent unspecific binding component is similarly obtained from the difference between the intensity at the steady state of uptake (cf. Figure 8) and the intensity of the unspecifically bound compound. The relative change in quantum yield upon transport-dependent unspecific binding is 27. The same procedure has been used to estimate the unspecific binding to *E. coli* lipid vesicles.

Binding to membranes from strain T31RT was determined in the following way (cf. Figure 3). Membranes (59.2 mg of

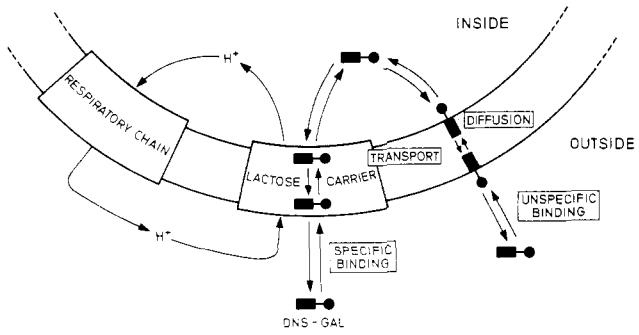

FIGURE 1: Modes of association of Dns-Gal with cytoplasmic membranes of *E. coli*. See text for explanation.

protein) were suspended in a total volume of 11.8 mL of 50 mM potassium phosphate, 10 mM magnesium sulfate, pH 6.6, containing 5.5×10^5 cpm of tritiated water. A second sample contained, in addition, p-CMBS at a final concentration of 0.2 mM. Both samples were divided into 0.5-mL aliquots, and Dns^2-S-Gal was added at various concentrations. All samples were centrifuged at 20 °C for 1 h at 150000*g*. An aliquot of the supernatant was taken for the determination of the specific radioactivity. The supernatant was removed and the pellet resuspended in 1 mL of 50% ethanol. After centrifugation, aliquots of the supernatant were counted or diluted with 50% ethanol. The Dns^2-S-Gal in the ethanolic solution was determined fluorimetrically by comparison with suitable standards in 50% ethanol. The amount of Dns^2-S-Gal in the pellet was corrected both for water-dissolved substrate using the amount of pellet-associated ^3H_2O and for other fluorescing compounds extracted from the membranes by inclusion of samples without Dns^2-S-Gal. Samples without p-CMBS give the sum of specific and unspecific binding, samples with p-CMBS give only the unspecific binding component.

Equilibrium Binding of α-NPG. Cytoplasmic membranes from strain T31RT (77 mg of protein) were suspended to a total volume of 4.1 mL in 50 mM potassium phosphate, pH 6.6, containing 10 mM $MgSO_4$, 10 mM NaN_3, and 10 mM 2-mercaptoethanol. Aliquots (0.1 mL) were pipetted into one side of multicavity microdialysis cells. The other side was provided with 0.1 mL of 4–100 μM [^{3}H]-α-NPG (0.03 μCi/sample; for preparation of [^{3}H]-α-NPG, see Kennedy et al., 1974) dissolved in the same buffer. Dialysis was performed for 6–9 h at room temperature with slow rotation. Aliquots of 50 μL were counted in 5 mL of Tritosol (Fricke, 1975).

Results

Modes of Association of Dansyl Galactosides with Cytoplasmic Membranes. The conceptual framework within which this work was conducted is depicted in Figure 1. Dns-Gal stands for a number of dansyl β-galactosides (Schuldiner et al., 1975a), in our work in particular for Dns^2-S-Gal. Three means of association of Dns^2-S-Gal with cytoplasmic membranes must be differentiated. First, Dns^2-S-Gal binds stoichiometrically to the lactose carrier *in the absence of an electrochemical gradient*. This type of binding is called *specific binding*. Second, Dns^2-S-Gal binds to the lipid bilayer and, possibly, to various membrane proteins by way of its hydrophobic dansylaminoethyl residue. This type of binding is termed *unspecific binding*. Since Dns^2-S-Gal rapidly equilibrates across the membrane both by passive diffusion and by transport via the carrier, unspecific binding occurs on both sides of the membrane. Thus the amount of membrane associated Dns^2-S-Gal in the absence of an electrochemical gradient is the sum of the specific and unspecific binding components. Third, the creation of an electrochemical gradient across the membrane by addition of an oxidizable substrate like D-lactate leads to active transport of Dns^2-S-Gal (H^+/Dns^2-S-Gal symport) by way of the lactose carrier. Because of its marked tendency to partition into the membrane, most of the transported Dns^2-S-Gal will be bound to the membrane. Only a minor fraction will be in solution in the aqueous lumen of the vesicles. Thus, active transport leads to an increase in the unspecific binding component. This type of binding will be termed *transport-dependent unspecific binding*. Under energized conditions, the amount of membrane-associated Dns^2-S-Gal is the sum of specific, unspecific, and transport-dependent unspecific binding components. It might be noted that, while it seems most likely that the transported substrate is released into the aqueous lumen as shown in Figure 1, it is possible that release could occur in the lipid bilayer.

Properties of Bacterial Strains. The expression of the *lac* operon products in the strains used in this study should be briefly described (see Teather et al., 1978, for details). The constitutive strain ML308 (*lac* $I^-O^+Z^+Y^+A^+$) gives rise to an activity of 0.28 μmol/(min·mg of protein) for in vivo β-NPG hydrolysis, a measure of Y-gene expression, and 0.003 μmol/(min·mg of protein) for transacetylase, the A-gene product. These values are representative for strains carrying a single chromosomal *lac* operon. Strain T44RT, which carries active Y and A genes only on an F′ factor, forms about twice as much of both products, i.e., 0.5 and 0.006 μmol/(min·mg of protein) for β-NPG transport and transacetylase, respectively. The plasmid-carrying strain T31RT shows activities of 0.5–1.7 μmol/(min·mg of protein) for in vivo β-NPG hydrolysis and 0.012–0.024 μmol/(min·mg of protein) for transacetylase after 1–1.5 generations of induction. The reason for the variability of this strain is probably related to the rapid loss of the plasmid from the cells upon induction. Since in vivo β-NPG hydrolysis is not an adequate measure for high levels of carrier protein in intact cells (cf. Teather et al., 1978), the strains are best compared in terms of their transacetylase activity. The ratio of transacetylase activity in cells from strains ML308 (or its derivative ML308-225), T44RT, and T31RT is 1:2:(4–8). The same ratio can be expected for the amount of carrier protein incorporated into the cytoplasmic membrane.

Estimation of Lactose Carrier Content in Cytoplasmic Membranes. The amount of lactose carrier in cytoplasmic membranes is determined by double labeling with radioactive amino acids and by equilibrium binding of the high affinity substrates α-NPG and Dns^2-S-Gal. Figures 2A and 2B show the radioactivity profile of polyacrylamide gels from strain T44RT and T31RT derived membranes double labeled with radioactive amino acids. The profiles show an excess of ^{3}H label at the position of the lactose carrier protein (molecular weight 30000) of 1.4% for strain T44RT and 12.5% for strain T31RT. These values correspond to 0.47 nmol of carrier/mg of newly made protein for strain T44RT and 4.2 nmol/mg of newly made protein for strain T31RT. Since strain T31RT has normally been induced for only one generation, the membranes contain 2.1 nmol of carrier/mg of total membrane protein. Similar experiments using strain ML30 (*lac* $I^+O^+Z^+Y^+A^+$) showed that this strain forms less than 0.3 nmol of carrier/mg of membrane protein (Teather et al., 1978). By giving the level in strain T44RT an arbitrary value of two, strains ML30, T44RT, and T31RT contain a relative amount of carrier <1.3:2:8.9.

The amount of lactose carrier has also been determined by equilibrium binding of [^{3}H]-α-NPG to membranes from strains T44RT and T31RT. Membranes from the latter strain

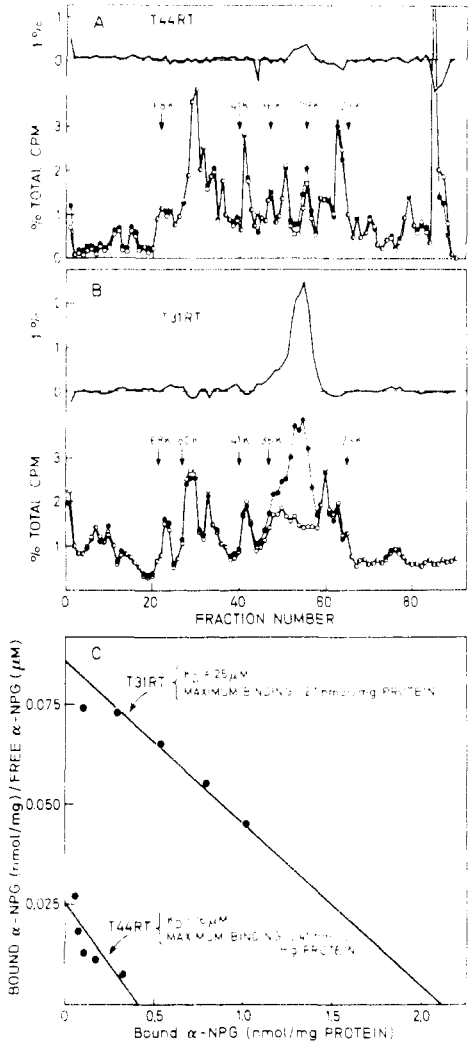

FIGURE 2. Quantitation of lactose carrier by double labeling with radioactive amino acids and equilibrium binding of [^{3}H]-α-NPG. (A and B) Cultures (40 mL) of strains T44RT (part A) and T31RT (part B) were grown in minimal medium to OD_{420} 0.5 (conversion factor OD'_{420} / = 0.107 mg of protein/mL). ^{14}C-labeled amino acids (25 μCi, Amersham mixture CFB.104) or ^{3}H-labeled amino acids (125 μCi, Amersham mixture TRK.440) plus IPTG (final concentration 0.5 mM) were then added, and growth continued to OD_{420} 1.2. The cells were harvested and mixed, and cytoplasmic membrane was isolated as described by Osborn et al. (1972). Membrane samples (approximately 50 μg of total protein) were dissolved in sample buffer without heating and applied to a 10% acrylamide gel as described by Laemmli & Favre (1973). The gels were fixed and stained in methanol:acetic acid:water (5:1:5) containing 0.1% Coomassie Brilliant Blue R250 and destained in 5% acetic acid, 7% methanol overnight at 37 °C. After photographing the gel, the strips were cut from the slab and cut into 1-mm sections. The sections were treated with 0.5 mL of 90% Protosol (NEN Chemicals), 10% H_2O for 2 h at 65 °C. Then 5 mL of Tritosol (Fricke, 1975) was added; the samples were shaken at 37 °C for 30 min, held at room temperature overnight, and counted in a Packard Tri-Carb liquid scintillation spectrometer. Counts per slice have been normalized to 100%. In the case of T31RT, the ^{3}H profile has been normalized to 114% so that the two profiles coincide except in the area of ^{3}H excess corresponding to the lactose carrier protein (molecular weight 30000). Molecular weight standards given are from left to right: bovine serum albumin (68000), catalase (60000), alcohol dehydrogenase (41000), lactate dehydrogenase (36000), carbonic anhydrase (29000), and trypsin (23000). (C) Scatchard plots of [^{3}H]-α-NPG binding to cytoplasmic membranes from strains T31RT and T44RT. Membranes from strain T31RT were derived from the same culture which had been induced with IPTG under the same conditions as that used for double labeling with radioactive amino acids (cf. part B). For strain T44RT a continuously induced culture was used for membrane preparation. Data for T44RT have been corrected for unspecific binding of [^{3}H]-α-NPG.

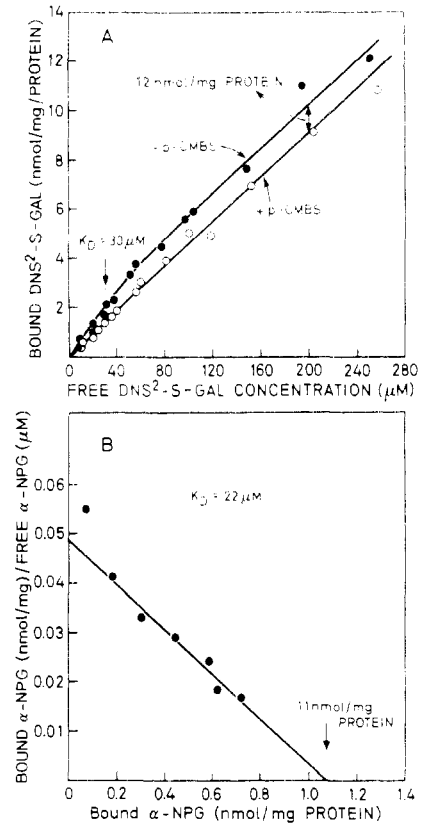

FIGURE 3: Binding of Dns^2-S-Gal and α-NPG to cytoplasmic membranes from strain T31RT. Binding experiments of Dns^2-S-Gal (A) and α-NPG (B) were performed as described in Materials and Methods, using the same preparation of strain T31RT derived membranes. Part A shows the unspecific binding (O—O) and the sum of unspecific and specific binding (●—●). An estimate for the K_D of specific binding of Dns^2-S-Gal to the lactose carrier is obtained at the concentration of half-maximum specific binding. See text for further explanations.

have been derived from the same culture as used for the experiment shown in Figure 2B. At saturating substrate concentrations, membranes from strain T44RT bind 0.41 nmol/mg of protein. Strain T31RT derived membranes bind 2.1 nmol/mg of protein. These values are in good agreement with the values obtained by the double-labeling experiments and establish that 1 mol of carrier proteins binds 1 mol of substrate. Taking the value of 0.2 nmol/mg of protein as determined by [^{3}H]-α-NPG binding for membranes from strain ML308-225 (Rudnick et al., 1976), strains ML308-225, T44RT, and T31RT contain relative carrier levels of 1:2:10.5. Our estimate for the dissociation constant, K_D = 21 μM (average from six experiments), is three times higher than in previous reports (Kennedy et al., 1974; Rudnick et al., 1976). It should be added that [^{3}H]-α-NPG binding to carrier-deficient membranes derived from uninduced strain T31RT shows a linear concentration dependence up to 50 μM. At this concentration the membranes bind 0.09 nmol/mg of protein. Therefore, unspecific binding of this substrate is low.

Binding experiments of Dns^2-S-Gal and [^{3}H]-α-NPG using the same preparation of T31RT-derived membranes are presented in Figures 3A and 3B. Judged by the amount of [^{3}H]-α-NPG bound at saturating concentrations, these membranes contained 1.1 nmol of carrier/mg of protein. Binding of Dns^2-S-Gal has been determined in the presence and absence of the SH reagent *p*-chloromercuribenzenesulfonate (p-CMBS). It is well established that the lactose carrier contains a thiol group in the substrate binding site which is blocked by SH reagents (Fox & Kennedy, 1965; Fox

Table I: Binding Characteristics of Dansyl Galactosides

mode of binding	type of membrane	dansyl galactoside	partition coeff[a]	emission max[e] (nm)	rel quantum yield[f]	dielectric constant
unspecific	*E. coli* lipids	Dns^2-S-Gal	49[b]	518	20	12
	ML308-225	Dns^2-S-Gal	113[b]	521 ± 9[g]	17	16
	K131	Dns^2-O-Gal	60[c] (900 μM)[d]	nd	nd	nd
	T31RT	Dns^2-S-Gal	59[c] (260 μM)[d]	514 ± 6	22	9
	K131	Dns^6-O-Gal	100[c] (850 μM)[d]	nd	nd	nd
transport-dependent	ML308-225	Dns^2-S-Gal		507 ± 6	27	5
unspecific	T31RT	Dns^2-S-Gal		520	18	15
specific	T31RT	Dns^2-S-Gal		493 ± 6	34	3

[a] Partition coefficients are calculated on the basis of the lipid content taking a density of 1. Representative samples of membranes from strain K131 prepared according to Kaback (1971) contained 0.31 mg of lipid/mg of protein. Membranes from strain T31RT prepared according to Osborn et al. (1972) contained 0.71 mg of lipid/mg of protein. [b] Values determined by analysis of fluorescence spectra as described in Materials and Methods. [c] Values determined by the centrifugation assay as described in Materials and Methods. [d] Numbers refer to the free substrate concentration up to which unspecific binding has been found to be a linear function of the substrate concentration; i.e., the partition law has been verified. [e] Emission maxima have been derived in the way described in Figures 4 and 9. The excitation wavelength was 340 nm in all cases. [f] Values refer to the quantum yield relative to water. A standard curve was prepared by integrating the fluorescence spectra of 16.3 μM Dns^2-S-Gal in dioxane/water mixtures. This yields a relation between the relative quantum yield (cf. also Schuldiner et al., 1977), the emission maximum, and the dielectric constant of dioxane/water mixtures. [g] Mean standard deviation.

et al., 1967; Rudnick et al., 1976). In the absence of p-CMBS Dns^2-S-Gal binds specifically to the carrier protein and unspecifically to lipids and perhaps to some other membrane-associated proteins. In the presence of p-CMBS only unspecific binding is observed. It is clear from Figure 3A that the unspecific component dominates the binding behavior. At saturating concentrations about 1.2 nmol/mg of protein are specifically bound to the lactose carrier, in agreement with the amount of [^{3}H]-α-NPG binding depicted in Figure 3B. Specific binding of Dns^2-S-Gal is half-maximal at a concentration, K_D = 30 μM. This value is in agreement with the apparent affinity of Dns^2-S-Gal for the carrier (K_i = 32 μM) inferred from the inhibition of lactose transport in vesicles from strain ML308-225 (Reeves et al., 1973). Since specific binding of Dns^2-S-Gal is superimposed on a high background of unspecific binding, determination of the lower carrier levels in membranes from strains T44RT or ML308-225 by binding of Dns^2-S-Gal has not been attempted.

In summary, the data presented in this section demonstrate that in the absence of an electrochemical gradient there is a 1:1 stoichiometry for the binding of substrates to the lactose carrier protein. The absolute carrier levels in different strains determined either by double labeling with radioactive amino acids or by substrate binding show good agreement and the relative levels agree with the levels expected on the basis of transacetylase activities, assuming coordinate expression of the *lac* Y and A genes.

Fluorescence Spectrum of the Dns^2-S-Gal–Carrier Complex. The high carrier level in membranes from strain T31RT enables the direct demonstration of the carrier–substrate complex by fluorimetry. Addition of Dns^2-S-Gal to the membrane preparation results in the spectrum labeled "membranes + Dns^2-S-Gal" shown in Figure 4A. The addition of p-CMBS to the cuvette leads to a fluorescence decrease in the 440–560-nm region since the Dns^2-S-Gal bound specifically to the lactose carrier is released. The difference spectrum (cf. Figure 4C, specific binding) has a maximum at 499 nm (range 490–500, average 493 ± 6 nm; see Table I), corresponding to the highly hydrophobic environment of a solvent dielectric constant, ε = 3. Figure 4C also shows that the unspecifically bound Dns^2-S-Gal reveals a slightly higher emission maximum at 507 nm (range 506–520, average 514 ± 6 nm). Since small differences in the emission maximum are difficult to ascertain, a possibly significant difference between the fluorescence properties of specifically and unspecifically bound compound requires further investigation.

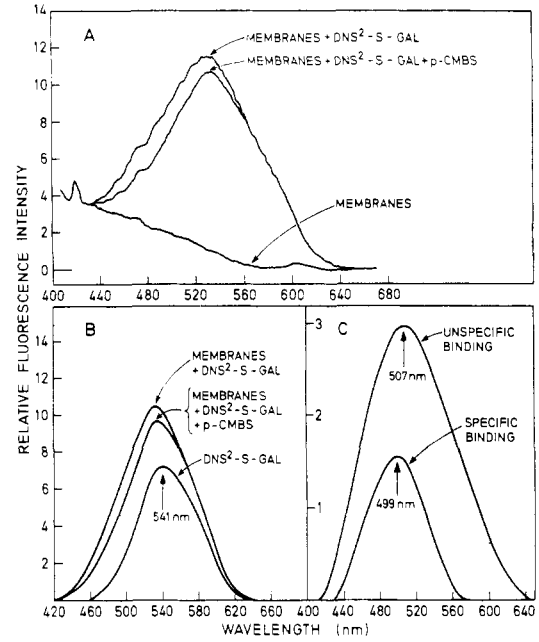

FIGURE 4: Fluorescence emission spectra of specifically and unspecifically bound Dns^2-S-Gal to cytoplasmic membranes of strain T31RT. (A) The trace labeled "membranes" shows the unspecific fluorescence of cytoplasmic membranes (1.53 mg of protein) in 2 mL of 50 mM potassium phosphate buffer, pH 6.6, 10 mM magnesium sulfate. Addition of 32.5 μM Dns^2-S-Gal results in the spectrum labeled "membranes + Dns^2-S-Gal". The spectrum obtained after the further addition of 0.1 mM p-CMBS is labeled "membranes + Dns^2-S-Gal + p-CMBS". The exciting light had a wavelength of 340 nm. (B) The two upper spectra are those corresponding to scans "membranes + Dns^2-S-Gal" and "membranes + Dns^2-S-Gal + p-CMBS" shown in part A after subtraction of unspecific fluorescence. The spectrum labeled "Dns^2-S-Gal" shows the fluorescence of a 32.5 μM solution of Dns^2-S-Gal in 2 mL of 50 mM potassium phosphate buffer, pH 6.6, 10 mM magnesium sulfate. (C) The spectrum labeled "unspecific binding" is obtained by subtracting the "Dns^2-S-Gal" spectrum from the spectrum "membranes + Dns^2-S-Gal + p-CMBS". The spectrum labeled "specific binding" is the difference spectrum between spectra "membranes + Dns^2-S-Gal" and "membranes + Dns^2-S-Gal + p-CMBS".

Determination of Relative Carrier Levels by Fluorimetry. The change in the fluorescence spectrum in the 500-nm region provides a convenient way to compare the amount of carrier present in different membrane preparations. This is dem-

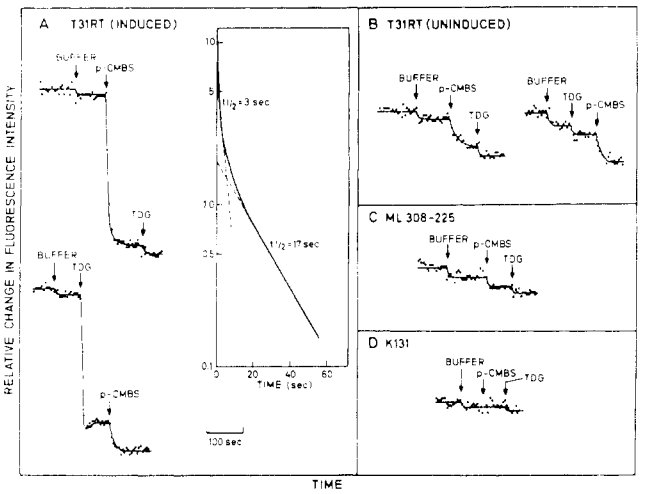

FIGURE 5: Specificity of interaction of Dns^2-S-Gal with the lactose carrier. All samples contained membranes corresponding to 1 mg of protein, 32 μM Dns^2-S-Gal, 50 mM potassium phosphate, pH 6.6, and 10 mM magnesium sulfate in a final volume of 2 mL (T = 25 °C). All experiments were run under constant instrumental conditions (excitation 340 nm; emission 500 nm). The changes in fluorescence in parts A–D are therefore directly comparable. (A) Membranes from strain T31RT induced for the synthesis of lactose carrier. The upper part shows the fluorescence change upon successive addition of 20 μL of buffer, 10 μL of p-CMBS (20 mM), and 20 μL of TDG (0.2 M). The inset shows the fluorescence change upon addition of p-CMBS plotted semilogarithmically on an extended time scale. In the lower part the order of addition of p-CMBS and TDG has been reversed. (B) Same as A for strain T31RT derived membranes grown without inducer. (C and D) Same as A for membranes from strain ML308-225 and K131.

onstrated in Figure 5 for membranes from induced and uninduced strain T31RT and for vesicles derived from strain ML308-225 and the carrier-deficient strain K131. The changes in fluorescence intensity are directly comparable since the amount of membrane and the instrumental conditions were the same. Addition of p-CMBS or TDG to membranes from induced strain T31RT causes a large and comparable decrease in fluorescence (cf experiments in the left of Figure 5A). The subsequent addition of TDG or p-CMBS, respectively, results in further small changes in fluorescence which are also observed in the control membranes (Figure 5B). Comparison of parts C and D reveals that p-CMBS causes a barely significant fluorescence decrease in vesicles from strain ML308-225. Therefore, a carrier level in the order of 10–15% of that present in membranes from strain T31RT is difficult to detect.

The change in fluorescence intensity induced by p-CMBS has been studied as a function of Dns^2-S-Gal concentration using membranes from strain T31RT. The apparent affinity of Dns^2-S-Gal for the carrier obtained in this way, K_D = 22 μM, is in reasonable agreement with the estimates given before. At constant Dns^2-S-Gal concentration (33 μM) the change in fluorescence intensity upon addition of p-CMBS is a linear function of the amount of membrane up to a concentration of 0.75 mg of protein/mL. Thus, although an exact quantitation of fluorescence changes in these highly scattering membrane samples is difficult, this assay provides a convenient semiquantitative estimate of relative carrier levels.

Kinetics of Dns^2-S-Gal Release from the Carrier. The kinetics of the fluorescence decrease upon addition of p-CMBS is biphasic as shown in the inset to Figure 5A. Some 80% of the change occurs with a half-life, $t_{1/2}$ = 3 s, while the remaining 20% has a much slower half-life of $t_{1/2}$ = 17 s. The most likely interpretation of this observation is that only the initial fast component is related to the rate of reaction of p-CMBS with the carrier because a minor slow component is also observed with control membranes (cf. Figure 5B). The displacement of Dns^2-S-Gal from the carrier upon addition of TDG is also very rapid (half-life $t_{1/2}$ < 3 s, cf. lower part of Figure 5A). The fluorescence decrease shows a small but highly characteristic overshoot which is more pronounced when TDG is replaced by lactose (5 mM final concentration, data not shown). We interpret this effect as a TDG or lactose driven counterflow of Dns^2-S-Gal from inside to outside via the lactose carrier which causes a transient decrease of unspecifically membrane bound molecules. The important conclusion for the subsequent discussion is that both p-CMBS and a competing substrate like TDG cause the rapid release of Dns^2-S-Gal from the lactose carrier.

Unspecific Binding of Dansylated Galactosides to Lipids and Membranes. The unspecific binding properties of Dns^2-S-Gal and its analogues require a closer examination because they provide the basis for the interpretation of the transport dependent fluorescence changes discussed below. These data are summarized in Table I. Dansyl galactosides bind both to lipid vesicles and membranes. For all preparations the emission maximum, in the 514–521-nm region, is the same within the experimental error. Binding has been investigated as a function of Dns-Gal concentration (see, for example, Figure 3A). Within the concentration range investigated (see numbers in brackets in Table I), binding was found to be a linear function of the Dns-Gal concentration. Therefore, for this concentration range we can approximate the binding behavior by defining a partition coefficient

$$\kappa = C_L / C_W$$

where C refers to the concentration of the compound in the lipid phase of the membrane (L) and the aqueous phase (W), respectively. This definition implies that dansyl galactosides preferably associate with the lipid phase of the membrane. A direct centrifugation assay shows that dansylaminoethyl galactosides bind unspecifically to membranes from the carrier-deficient strain K131 (an ML strain) or to p-CMBS-treated membranes from strain T31RT (a K12 strain) with a similar partition coefficient, κ = 60, although these membranes differ in their lipid content. This value is close to the partition coefficient, κ = 49, obtained for *E. coli* lipid vesicles by analysis of the fluorescence spectra. For membranes from strain ML308-225, this latter, indirect method yields a higher value of κ = 113 which is probably due to an overestimate of the emission intensity in these highly scattering samples. The more hydrophobic Dns^6-O-Gal binds unspecifically to membranes from strain K131 with a value of κ = 100. A similar value, κ = 85, can be calculated from the binding data of Dns^6-S-Gal to membranes of the closely related strain ML30 reported by Schuldiner et al. (1976b).

In summary, dansyl galactosides partition into the membrane over a wide concentration range. This result has the important consequence that, if Dns^2-S-Gal or its analogues are actively accumulated in membrane vesicles, most of the molecules transported will be associated with the membrane rather than dissolved in the aqueous lumen.

Transport of Dns^2-O-Gal and α-NPG by the Lactose Carrier in Cells. Transport of the Dns^2-S-Gal analogue, Dns^2-O-Gal, and of α-NPG can be readily demonstrated in cells, provided an intracellular hydrolase splits the substrates after translocation across the cytoplasmic membrane. A typical experiment using Dns^2-O-Gal is shown in Figure 6. Formation of dansylaminoethanol from Dns^2-O-Gal is clearly dependent on the presence of β-galactosidase since no reaction occurs in the mutant K169 (*lac* I$^-$Z$^-$Y$^+$). In strain K168 (*lac* I$^-$Z$^+$Y$^+$),

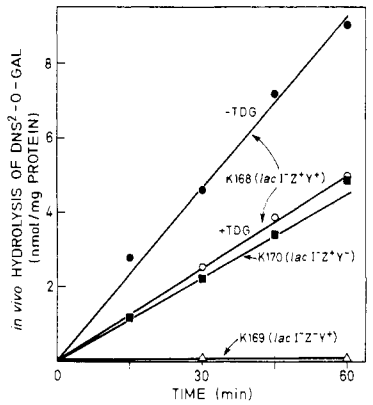

FIGURE 6: Transport of Dns²-O-Gal by the lactose transport system in *E. coli* cells. Incubation mixtures contained freshly harvested cells corresponding to 1.8 mg of protein in Cohen–Rickenberg salts medium, 50 μM Dns²-O-Gal and, when present, 1 mM TDG in a final volume of 1 mL. Reactions were allowed to proceed at 20 °C, and aliquots (200 μL) were removed at appropriate times and added to an equal volume of absolute ethanol which had been preheated to 60–70 °C. After centrifugation, 20-μL aliquots of the supernatant solution were spotted on silica gel thin-layer plates and chromatographed using benzene:methanol (4:1) as a solvent system. The amount of dansylaminoethanol formed during the incubation was determined by scanning the fluorescence directly on the plate using a Vitatron Model TLD 100. Standard curves were constructed by chromatography and scanning of known amounts of dansylaminoethanol under the same conditions as used for experimental samples.

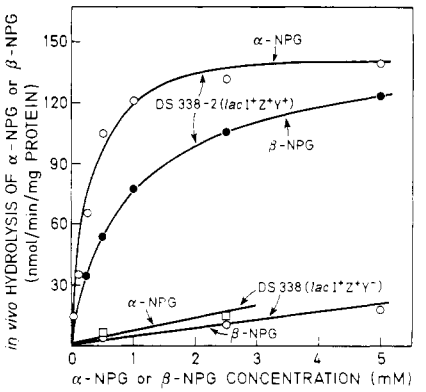

FIGURE 7: Transport of α-NPG or β-NPG in cells of strains DS338 and DS338-2. Cells were grown in the presence of the inducer IPTG as described in Materials and Methods. The rate of formation of *p*-nitrophenol or *o*-nitrophenol was followed as described before (Overath et al., 1971; Teather et al., 1978) in a double beam spectrophotometer at 420 nm (T = 28 °C). The sample cuvette contained cells (0.1–0.2 mg of protein) in Cohen–Rickenberg buffer (Anraku, 1967) and the indicated concentrations of substrate. The reference cuvette contained only cells and buffer. Values for strain DS338-2 are uncorrected for passive diffusion of α-NPG or β-NPG. The rate of passive diffusion is indicated by the two lower curves obtained for cells without the Y-gene product.

Dns²-O-Gal is hydrolyzed at a rate of 0.15 nmol/(min·mg of protein). About 50% of the uptake occurs by way of the lactose transport system, the other half by passive diffusion as shown by the extent of inhibition using the competing substrate TDG and by the control rate in the transport-deficient strain K170 (*lac* I⁻Z⁺Y⁻). While this experiment shows that Dns²-O-Gal is transported by the lactose carrier, albeit at the rather slow rate of 0.08 nmol/(min·mg of protein), it also shows the rapid passive permeability of this compound. In fact, a similar experiment with the more hydrophobic substrate Dns⁶-O-Gal did not reveal a significant difference of the rate of in vivo hydrolysis in the presence or absence of TDG in strain K168. However, specific transport of this substrate could be shown with strain T31RT, which contains an increased level of carrier activity.

The demonstration of α-NPG transport by the lactose transport system was simplified by the availability of *E. coli* strains (DS338-2 and its *lac* Y derivative DS338; cf. Figure 7) which synthesize an active α-galactosidase. In these strains the rate of in vivo hydrolysis of α-NPG and β-NPG could be directly compared in the same culture. It is evident from Figure 7 that both substrates are transported because control rates in the lactose-carrier deficient strain are low and presumably due to passive leakage into the cells. Transport of both substrates was competitively inhibited by substrates of the lactose transport system including TDG, IPTG, lactose, or melibiose. Reciprocal plots of $1/V$ vs. $1/S$ reveal a similar rate at saturating substrate concentrations, V_{max} = 0.2 μmol/(min·mg of protein) for both α-NPG and β-NPG. The apparent affinity, K_M = 50–200 μM, as well as the V_{max}, for α-NPG transport is somewhat uncertain because the α-galactosidase (K_M = 0.14 mM; Schmid & Schmitt, 1976) may be rate limiting.

Transport of Dns²-S-Gal in Membrane Vesicles. This subject is best introduced by describing the basic experiment performed by Reeves et al. (1973). As shown in Figure 8, experiment 1, Dns²-S-Gal is first equilibrated with a vesicle suspension of strain ML308-225. Upon subsequent addition

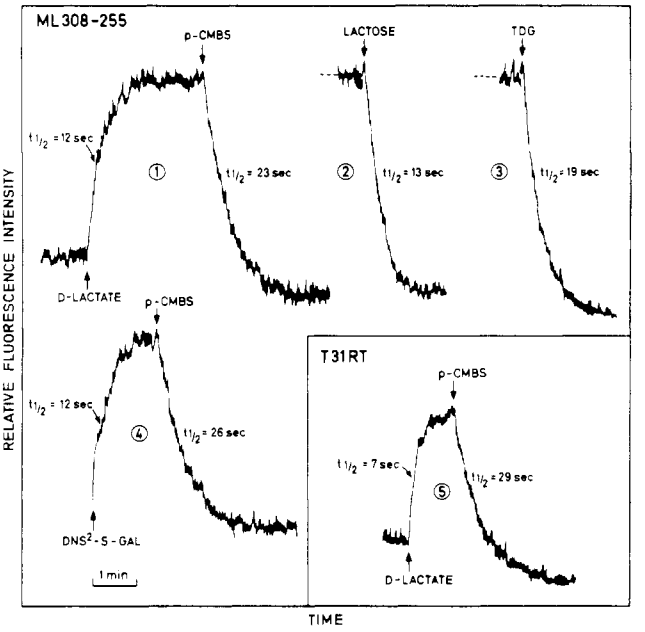

FIGURE 8: Active uptake and efflux of Dns²-S-Gal in membrane vesicles from strains ML308-225 and T31RT. Membranes from both strains were prepared as described by Kaback (1971). All assays were performed at 25 °C and a constant setting of the fluorimeter (excitation 340 nm; emission 500 nm). Assays no. 1–3 and 5 contained the indicated membrane preparation (0.6 mg of protein) and 32.5 μM Dns²-S-Gal in 2.0 mL of oxygen saturated 50 mM potassium phosphate/10 mM magnesium sulfate, pH 6.6. Active uptake was induced by addition of 20 mM D-lactate. After reaching the steady state, efflux was induced by addition of 0.1 mM p-CMBS (experiments 1 and 5), 5 mM lactose (experiment 2) or 2 mM TDG (experiment 3). Experiment no. 4 shows a similar assay where D-lactate was added first followed by Dns²-S-Gal after 1 min. $t_{1/2}$ refers to the half-time required for reaching the steady state or to the half-time of efflux taken from a semilogarithmic plot.

of D-lactate, a respiratory chain substrate creating an electrochemical potential across the membrane (see reviews by Harold (1977) and Kaback (1977)), one observes an increase in fluorescence intensity to a new steady state. This fluorescence change is dependent on the presence of the lactose carrier and shows all properties of an active, energy-requiring

process (Reeves et al., 1973; Schuldiner et al., 1975a). We propose the following interpretation for this effect. Dns^2-S-Gal is actively taken up by the vesicles. The accumulated Dns^2-S-Gal partitions into the membrane according to the partition coefficient, κ, resulting in a fluorescence increase. The fluorescence of these membrane-associated molecules can be used to measure influx or efflux rates as well as the steady state, where these opposing fluxes are equal. In the case of dansyl galactosides, the passive efflux rate is high and limits the steady-state level of accumulation. This interpretation is based on the following arguments. First, on a molar basis, the amount of vesicle associated Dns^2-S-Gal in the steady state greatly exceeds the carrier level. Second, the fluorescence spectrum of the Dns^2-S-Gal molecules which become newly associated with the membrane in the presence of an electrochemical gradient is characteristic for unspecifically bound molecules. Third, the kinetics of the observed fluorescence changes are consistent with carrier mediated transport of Dns^2-S-Gal. We will discuss these arguments sequentially.

(i) As shown above, stoichiometric binding of Dns^2-S-Gal to the carrier occurs spontaneously in the absence of an electrochemical potential. Therefore, Dns^2-S-Gal is already in equilibrium with the carrier before D-lactate is added to the membrane suspension. The amount of lactose carrier present in vesicles from strain ML308-225 allows for the binding of about 0.2 nmol of ligand/mg of protein at saturating concentrations. In contrast, the fluorescence change upon addition of D-lactate corresponds to 1.3 nmol/mg of protein, in agreement with the value of 1.4 nmol/mg of protein reported by Schuldiner et al. (1975a). These estimates exceed the carrier level by a factor of seven. Furthermore, the steady state of the D-lactate induced fluorescence change depends on factors such as the quality of the vesicle preparation, while the specific binding to the lactose carrier does not. With the membrane preparations shown in Figure 8, the relative ratio of specific binding is 1:4 for strains ML308-225 and T31RT, while on the same scale D-lactate induces a relative change of 20:10 for the same vesicle types, respectively. Therefore, in spite of the higher carrier content, membranes from strain T31RT show a smaller increase in Dns^2-S-Gal binding in the presence of an electrochemical potential than membranes from strain ML308-225.

(ii) The fluorescence spectrum of Dns^2-S-Gal molecules which become associated with the membrane in the presence of an electrochemical gradient has a maximum at 505 nm (cf. transport-dependent unspecific binding in Figure 9; range 500–515 nm; average 507 ± 6 nm; cf. Table I; compare also Reeves et al., 1973) for strain ML308-225 and 520 nm for strain T31RT. These values are characteristic for the emission maximum of unspecifically bound ligand. Thus, the spectra are consistent with the view that Dns^2-S-Gal molecules partition into the membrane after translocation.

(iii) The kinetic properties of the fluorescence change observed with vesicles are readily explained in terms of carrier mediated transport. The half-time, required to complete the fluorescence increase after addition of D-lactate, is $t_{1/2}$ = 12 s (cf. Figure 8, experiment 1), in agreement with previous results (Reeves et al., 1973). The time course of the fluorescence change is similar when the membranes are first incubated with D-lactate followed by the addition of Dns^2-S-Gal (cf. Figure 8, experiment 4); i.e., the kinetics of the fluorescence change cannot be explained by the rate of formation of the electrochemical gradient. Therefore, the initial rate of the fluorescence increase appears to be limited by the activity of the lactose carrier. The initial rate of the

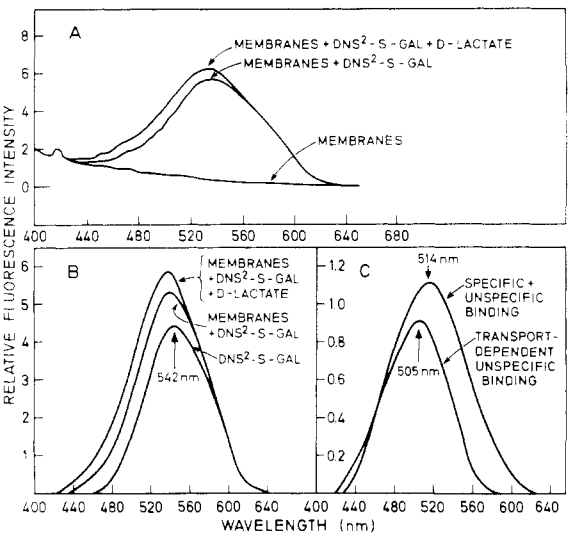

FIGURE 9: Spectral changes upon transport of Dns^2-S-Gal by cytoplasmic membranes of strain ML308-225. (A) The trace labeled "membranes" shows the unspecific fluorescence of cytoplasmic membranes (0.6 mg of protein) in 2 mL of 50 mM potassium phosphate buffer, pH 6.6, 10 mM magnesium sulfate saturated with oxygen. Addition of 32.5 μM Dns^2-S-Gal results in the trace labeled "membranes + Dns^2-S-Gal". 20 mM lithium D-lactate was added to the cuvette and the increase in fluorescence was monitored at 500 nm (excitation at 340 nm). Maximal increase was observed after 2 min. The spectrum labeled "membranes + Dns^2-S-Gal + D-lactate" was recorded within the next 2 min. (B) The two upper spectra correspond to the scans "membranes + Dns^2-S-Gal" and "membranes + Dns^2-S-Gal + D-lactate" after subtraction of unspecific fluorescence. The spectrum labeled "Dns^2-S-Gal" shows the fluorescence of a 32.5 μM solution of Dns^2-S-Gal in 2 mL of buffer. (C) The spectrum "specific + unspecific binding" is obtained by subtracting the "Dns^2-S-Gal" spectrum from the "membranes + Dns^2-S-Gal" spectrum. Note that the contribution of the specific binding to the lactose carrier to the spectrum is negligible. Transport-dependent binding is obtained by subtracting the spectra "membranes + Dns^2-S-Gal" from "membranes + Dns^2-S-Gal + D-lactate".

fluorescence change expressed as nmol/(min·mg of protein) can be plotted as a function of Dns^2-S-Gal concentration. The maximum rate is V_{max} = 5 nmol/(min·mg of protein); the apparent Michaelis constant K_M = 33 mM. This value is the same as that obtained for the K_D of Dns^2-S-Gal binding to the lactose carrier (Figure 3). Moreover, a similar estimate for the apparent affinity is also obtained by analysis of the steady state of the fluorescence change as a function of Dns^2-S-Gal concentration (Reeves et al., 1973). The fact that both the initial rate and steady state of uptake show the same K_M is typical for active solute uptake by the lactose transport system (Rickenberg et al., 1956) and is consistent with the proposal of Winkler & Wilson (1966) that energy coupling changes the affinity of the carrier for the substrate on the inside of the membrane.

Further arguments for the translocation of Dns^2-S-Gal by the lactose carrier have been obtained by studying the rate of efflux from the vesicles. Addition of p-CMBS causes a rapid decrease of the D-lactate induced fluorescence level which can be described by the half-time, $t_{1/2}$, of a first-order process (cf. Figure 8, experiments 1, 4, and 5, and Schuldiner et al., 1975b). Average values for the rate of efflux of Dns^2-S-Gal from "energized" vesicles as well as for the efflux of Dns^2-S-Gal from passively loaded vesicles are shown in Table II. The rate of efflux from "energized" vesicles from strain ML308-225 ($t_{1/2}$ = 23 s) and strain T31RT ($t_{1/2}$ 31 s; compare also Figure 8) upon addition of p-CMBS is similar to the rate of efflux from passively loaded p-CMBS-treated ML308-225 vesicles ($t_{1/2}$ = 35 s) or lactose carrier deficient vesicles from

Table II: Efflux of Dns²-S-Gal from Membrane Vesicles

	addition	half-time[d] (s)
efflux from passively loaded vesicles		
ML308-225[a]		17
	lactose	15
	p-CMBS	35
K131[a]		34
	lactose	32
	p-CMBS	37
E. coli lipid vesicles[b]		32
efflux from steady state of active uptake[c]		
ML308-225	p-CMBS	23
	lactose	11
T31RT	p-CMBS	31
	lactose	9

[a] Vesicles (4.5 mg of protein) were incubated for 30 min at 0 °C in 1 mL of 50 mM potassium phosphate/10 mM magnesium sulfate, pH 6.6, containing 0.65 mM Dns²-S-Gal. The samples were heated for 5 min at 25 °C and a 0.1-mL aliquot was rapidly diluted into 2 mL of buffer with or without 5 mM lactose. The time course of efflux was followed fluorimetrically at the emission wavelength of 500 nm. For the determination of non-carrier-mediated efflux, vesicles were first treated with 0.1 mM p-CMBS, collected by centrifugation, resuspended in Dns²-S-Gal-containing buffer, and diluted as above. [b] Total phospholipids of *E. coli* (5 mg/mL) were sonicated in buffer for 3 min at room temperature and subsequently incubated with 0.33 mM Dns²-S-Gal at 0 °C for 30 min. After heating for 5 min to 25 °C an aliquot of 0.2 mL was diluted tenfold with buffer and the fluorescence decrease recorded as described above. [c] The data refer to the efflux experiments shown in Figure 8. [d] The values given are the average from several experiments. Half-times for efflux were taken from semilogarithmic plots of fluorescence decrease vs. time. All plots indicated a monophasic exponential decrease of vesicle associated Dns²-S-Gal.

strain K131 ($t_{1/2}$ = 34 s). A similar rate ($t_{1/2}$ = 32 s) is observed for efflux from vesicles of total lipid extracts of *E. coli*. These rates are about an order of magnitude slower than the rate of release of Dns²-S-Gal from the carrier upon addition of p-CMBS (cf. Figure 5A). Therefore, the half-time, $t_{1/2} \approx$ 30 s, is characteristic for the rate of passive diffusion of Dns²-S-Gal through the lipid bilayer of the membrane. It should be noted that upon dilution of Dns²-S-Gal loaded vesicles equilibration of compound bound to the outer surface of the vesicles with the external aqueous phase is expected to be fast so that the rate of fluorescence decrease observed upon dilution is determined by the rate of efflux of molecules bound to the inner face of the vesicles.

In spite of the high passive permeability, carrier-catalyzed efflux or counterflux can be demonstrated for Dns²-S-Gal as for other more hydrophilic galactosides. First, efflux from ML308-225 vesicles ($t_{1/2}$ = 17 s) is twice as fast as in control vesicles from strain K131; i.e., the carrier catalyzes Dns²-S-Gal efflux. Second, while addition of high lactose concentrations causes only a small increase in the rate of carrier-mediated efflux from passively loaded ML308-225 vesicles ($t_{1/2}$ = 15 s vs. $t_{1/2}$ = 17 s; cf. Table II), this substrate leads to a significant increase in the rate of Dns²-S-Gal efflux from "energized" vesicles (cf. Figure 8, experiment 2, and lower part of Table II; see also Reeves et al. (1973) and Schuldiner et al. (1975a)). This effect is readily explained by facilitated exchange diffusion. Third, when vesicles containing a high internal lactose concentration are diluted into a buffer containing Dns⁶-S-Gal, a transient increase in fluorescence is observed (Schuldiner et al., 1975a) which finds a straightforward explanation if interpreted as Dns⁶-S-Gal counterflux driven by lactose efflux.

In summary, the data presented in this section show that the lactose carrier catalyzes the transport of Dns²-S-Gal in membrane vesicles. In fact, this fluorescent compound and its analogues are most convenient tools for studying the kinetics of β-galactoside transport.

Transport of α-NPG by Membrane Vesicles. In the presence of D-lactate, membrane vesicles from strain ML308-225 rapidly take up α-NPG to a final level of 2 nmol/mg of protein (data not shown; compare also Rudnick et al., 1976), which corresponds to an inside vs. outside concentration gradient of 45. In the absence of D-lactate less than one-tenth as much solute becomes associated with the vesicles. This small amount is most likely due to a combination of carrier mediated and passive diffusion of solute into the vesicles, since the inclusion of p-CMBS in the wash buffer assured the inactivation of the carrier and release of specifically bound solute in a few seconds. Addition of p-CMBS causes efflux with a half-life of 40 s while addition of lactose causes a ten times more rapid exchange diffusion of the accumulated α-NPG. In taking into account the arguments for the rate of reaction of p-CMBS with the carrier and the actual carrier level in these membranes (see above), it is clear that the lactose carrier catalyzes active uptake of α-NPG in membrane vesicles.

Discussion

The lactose carrier binds one molecule of Dns²-S-Gal or α-NPG per polypeptide of molecular weight 30 000 in the absence of an electrochemical gradient. Both solutes are transported by the carrier in cells. In membrane vesicles, the carrier catalyzes both the translocation of Dns²-S-Gal and, in the presence of an electrochemical gradient, the active uptake of Dns²-S-Gal and α-NPG. These conclusions are at variance with the proposal of Schuldiner et al. (1976a) that these solutes are not transported by membrane vesicles and bind to the carrier only in the presence of an electrochemical gradient. Our reinterpretation of the fluorescence changes observed upon transport of Dns²-S-Gal and its homologues in cytoplasmic vesicles is based on the passive permeability and unspecific binding properties of these amphipathic molecules which cause a distinctly different behavior compared with a hydrophilic substrate such as lactose.

One argument of Kaback and his collaborators which clearly supported some kind of binding phenomenon is based on the observation that the amount of Dns⁶-S-Gal which becomes associated with vesicles from strain ML308-225 upon addition of D-lactate is the same (1.5 nmol/mg of protein) whether determined fluorimetrically or by flow dialysis using radioactive ligand (Schuldiner et al., 1976b). The fluorescence measurements are based on a spectral shift and can only monitor molecules that have changed their environment by association with the membrane. This method does not detect molecules which are dissolved in the aqueous lumen of the vesicles. On the other hand, flow dialysis determines all vesicle associated molecules whether they are bound to the membrane or in the lumen. The agreement between these two determinations can be accounted for in terms of preferential partitioning of the transported Dns⁶-S-Gal molecules into the membrane. First, there is a class of nonsaturable binding sites in the lipid phase of the membrane. The data summarized in Table I show that, up to a concentration of 1 mM, unspecific binding is a linear function of the Dns²-O-Gal or Dns⁶-O-Gal concentration and, therefore, can be described by a partitioning

between membrane and aqueous phase. Moreover, it is evident from flow dialysis experiments of Schuldiner et al. (1976b) that even at a concentration of 1 mM the unspecific binding sites are not saturated because nonradioactive Dns^6-S-Gal does not displace unspecifically bound radioactive ligand from the membrane. Thus, the flow dialysis experiments are consistent with the view that a partition law is applicable in this concentration range. Second, the partition coefficient, κ, causes a preferential association of the transported Dns^6-S-Gal molecules with the membrane. Taking an intravesicular volume of 2.2 μL/mg of protein (Kaback & Barnes, 1971), a membrane lipid volume of 0.31 μL/mg of protein (cf. Table I), and a value for κ = 100 (cf. value for Dns^6-O-Gal in Table I), it is calculated that 0.1 nmol/mg of protein, i.e., 7% of the total amount of 1.5 nmol/mg of protein, will be in the aqueous lumen. Therefore the fluorimetrically determined estimate is expected to be the same as that determined by flow dialysis, within the experimental error, because the fraction of Dns^6-S-Gal in the aqueous lumen of the vesicles is small. Although a similar comparison between flow dialysis and fluorimetry is not available for the more hydrophilic Dns^2-S-Gal, the partition coefficient for this compound (κ = 60) ensures that the radioactivity measurement using flow dialysis should exceed the fluorimetrically determined value by not more than 11%. Third, Dns^2-S-Gal molecules bound unspecifically in the presence or absence of an electrochemical potential are in a hydrophobic environment as shown by a comparable blue shift in the emission maximum. At present, it is not clear if the difference in the spectrum of the Dns^2-S-Gal molecules bound to the membrane in the absence (λ_{max} = 521 ± 9 nm) and the presence of an electrochemical gradient (λ_{max} = 507 ± 6 nm, cf. Figure 9 and Table I) is significant. The first value refers to an equilibrium situation at low intra- and extravesicular Dns^2-S-Gal concentration, while the latter value refers to a high internal and low external concentration. The difference in the fluorescence spectrum may indicate that the surroundings of the unspecific and transport-dependent unspecific binding components are not the same. Moreover, these two binding components differ in their rotational relaxation times (Schuldiner et al., 1975c).

The kinetic aspects of the fluorescence changes observed for membrane vesicles must take into account the high passive permeability of Dns^2-S-Gal and its homologues. This property explains the rapid decrease of the D-lactate induced fluorescence change upon addition of p-CMBS (Schuldiner et al., 1975b) and the inability to drive lactose uptake into vesicles by Dns^2-S-Gal efflux (Schuldiner et al., 1975a). A related observation, namely, that α-NPG and Dns^6-S-Gal at high concentrations (20–50 times above the K_D value) inhibit rather than accelerate lactose efflux (Schuldiner et al., 1975b; Rudnick et al., 1976), is explained by the rapid saturation of the carrier by these high affinity substrates on either side of the membrane. Both the work of Schuldiner et al. (1975a) and our results suggest that under appropriate conditions flux coupling between lactose and α-NPG or Dns^2-S-Gal via the carrier can be demonstrated.

Several points raised by the studies of Kaback et al. must be clarified by future studies. The observation (Schuldiner et al., 1977) that a homologous series of dansyl galactosides shows approximately the same steady-state level of accumulation must be treated in terms of a theory which takes into account the rates of both the carrier-mediated and passive fluxes. Furthermore, it remains unclear why dansyl galactosides do not serve as inducers of the *lac* operon in vivo, while they do so in vitro (Schuldiner et al., 1975b).

In addition to the work of Kaback and his collaborators, two groups have used Dns^6-S-Gal for studying further aspects of the β-galactoside carrier on the assumption that this compound only binds and is not transported. First, Lancaster & Hinkle (1977a) have shown that membrane vesicles from strain ML308-225 having an inverted orientation reveal fluorescence changes induced by an electrochemical gradient or by lactose efflux very similar to those observed in vesicles of native orientation. While these experiments with inverted vesicles provide evidence (see also Lancaster & Hinkle, 1977b) that the lactose carrier is functionally symmetrical, a conclusion independently reached by us (Teather et al., 1977), they can be interpreted as Dns^6-S-Gal transport rather than binding on the same grounds as discussed for vesicles of native orientation. Second, Therisod et al. (1977) have used Dns^6-S-Gal as a probe for studying the effect of an order ↔ disorder transition in the lipid phase on the lactose carrier protein (cf. Overath & Thilo, 1978, for background information on this topic). On the assumption that D-lactate induced changes in the fluorescence of Dns^6-S-Gal in membrane vesicles reflect binding of this substrate to the carrier, Therisod et al. propose that the lipid phase transition affects the effective carrier concentration rather than the rate constant via a change of the preexponential frequency factor of the Arrhenius equation (cf. Overath et al., 1976; Thilo et al., 1977). Their experiments reveal changes in the temperature characteristic of the steady state or the initial rate of the D-lactate induced fluorescence change. In view of the results presented in this paper, both parameters are a function of the transport rate of the lactose carrier. Therefore, the experiments of Therisod et al. (1977) cannot be used to distinguish whether the lipid phase transition causes a change in the effective carrier concentration or a change in the frequency factor.

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Reconstitution of Oxidative Phosphorylation by Chemically Modified Coupling Factor F_1: Differential Inhibition of Reactions Catalyzed by F_1 Labeled with 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole or 2,3-Butanedione†

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ABSTRACT: Energy coupling factor F_1 from beef heart mitochondria has been chemically modified by either 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) or 2,3-butanedione. Labeled F_1 was used for the reconstitution of oxidative phosphorylation in urea-washed submitochondrial particles (ASU particles). Inhibition of ATPase activity by NBD-Cl follows a simple exponential decay, reaction is first order with respect to NBD-Cl, and magnesium complexes of methylene analogues of ADP or ATP produce sevenfold reduction in the rate of inhibition. Spectral evidence indicates labeling of tyrosine, with a biphasic incorporation approaching 2 mol of NBD label per mol of F_1. Experiments on F_1 involving labeling by both NBD-Cl and 2,3-butanedione reveal no competition between the two labeling agents. When ASU particles are reconstituted with F_1 containing 1.35 to 1.65 mol of NBD label/mol of F_1, the activities for ATP $\rightleftharpoons$ P_i exchange, ATP-driven reverse electron transport, and membrane-bound ATPase are almost completely inhibited. However, the remaining activity for net synthesis of ATP is 35–65% of the initial value. For ASU particles reconstituted with 2,3-butanedione labeled F_1, the loss of activity for reverse electron transport occurs at a fivefold greater rate than loss of activity for net ATP synthesis, emphasizing a functional separation of these processes. These results are difficult to rationalize by a compulsory alternating site model but can be explained by the presence of catalytic sites specialized for ATP utilization and synthesis, respectively.

Coupling factor F_1, first isolated by Racker and co-workers (Pullman et al., 1960; Penefsky et al., 1960), is generally regarded as the terminal enzyme of oxidative phosphorylation. Its known properties have been recently reviewed and emphasis has been placed on the remarkable complexity of this enzyme (Pedersen, 1975; Panet & Sanadi, 1976). Studies by Senior (1975) and Wagenvoord et al. (1977) support a subunit stoichiometry of $\alpha_2\beta_2\gamma_2\delta_x\epsilon_2$, where x is presumably 1 or 2. Preliminary crystallographic studies have revealed a twofold axis of symmetry (Amzel & Pedersen, 1978).

It is clear from work in several laboratories that F_1 possesses several nucleotide binding sites, including 2 sites for tightly bound ADP and 0, 1, or 3 sites for tightly bound ATP

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