Equilibrium between Two Forms of the *lac* Carrier Protein in Energized and Nonenergized Membrane Vesicles from *Escherichia coli*†

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ABSTRACT: *p*-Nitrophenyl α-D-galactopyranoside is a competitive inhibitor of lactose transport in membrane vesicles prepared from *Escherichia coli* ML 308-225 ($K_i \simeq 6.6$ μM) but is not accumulated by the vesicles. Binding of *p*-nitrophenyl α-D-[6-^{3}H]galactopyranoside to membrane vesicles has been measured by flow dialysis. In the presence of D-lactate, ligand binds to the vesicles with a K_D of about 6 μM, and a total of 2.3 nmol per mg of membrane protein is bound at saturation. In the absence of D-lactate, a small amount of binding can be detected (approximately 0.2 nmol per mg of membrane protein) with a similar affinity constant ($K_D \simeq 9$ μM). Binding in the presence or absence of D-lactate is dependent upon a functional *lac y* gene product and upon the structural integrity of the vesicle membrane and is reversed by *p*-hydroxymercuribenzenesulfonate. Agents such as 2,4-dinitrophenol, carbonyl cyanide *m*-chlorophenylhydrazone, and valinomycin, alone or in combination, abolish D-lactate-dependent binding, but do not affect binding in the absence of electron donors. The results confirm previous observations that the bulk of the *lac* carrier protein is unable to bind ligand unless the membrane is energized, and they also corroborate observations that a small amount of binding occurs in the absence of energy coupling. The findings are discussed in terms of a model in which the *lac* carrier protein exists in a state of dynamic equilibrium between two forms: (i) a low affinity, cryptic form which predominates in the absence of energy coupling; and (ii) a high affinity form, accessible from the external surface of the membrane, which predominates in the presence of an electrochemical gradient of protons (interior negative and alkaline).

Studies with (*N*-dansyl)aminoalkyl β-D-galactopyranosides (Reeves et al., 1973; Schuldiner et al., 1975a–c, 1976a) and 2-nitro-4-azidophenyl β-D-galactopyranosides (Rudnick et al., 1975a,b) indicate that the *lac* carrier protein in membrane vesicles isolated from *Escherichia coli* does not bind ligand significantly unless the vesicles are "energized" (see Schuldiner et al., 1976b, for a review). As a result of D-lactate or reduced phenazine methosulfate oxidation, an electrochemical gradient of protons develops across the vesicle membrane [interior negative and alkaline (Ramos et al., 1976)], and active transport, an increase in dansyl galactoside binding, and azidophenyl galactoside-dependent photoinactivation are observed. Many of these effects are mimicked by artificially induced ion gradients of appropriate polarity (Schuldiner et al., 1975a; Rudnick et al., 1975b), but dilution-induced carrier-mediated lactose efflux also causes increased dansylgalactoside binding in a manner which is apparently independent of the electrochemical potential across the membrane (Schuldiner et al., 1975a). In any case, based on these observations, it has been postulated that a membrane potential (interior negative) causes the *lac* carrier protein to become accessible to the external medium, to increase its affinity for ligand, or both, and it has been suggested that the *lac* carrier protein or part of it may be negatively charged (Schuldiner et al., 1975a, 1976b).

The validity of these postulates rests on the argument that the observations with the dansyl and azidophenyl galactosides reflect binding specifically and are not due to a subsequent translocational event. This aspect of the problem has been approached therefore in numerous ways. The dansyl galactosides are not transported to a demonstrable extent (Reeves et al., 1973; Schuldiner et al., 1975a,b, 1976b), and *p*-hydroxymercuribenzenesulfonate (*p*-HMBS[1]; Schuldiner et al., 1975b, 1976a,b) and *N*-ethylmaleimide (NEM; Reeves et al., 1973) cause reversal of D-lactate-induced dansyl galactoside binding but do not cause efflux of lactose from the intravesicular pool (Kaback and Barnes, 1971; Schuldiner et al., 1975b). Azidophenyl galactoside dependent photoinactivation of lactose transport is dependent upon energization of the vesicles but independent of the ability of the vesicles to transport the photoreactive probes (Rudnick et al., 1975a,b). There is a marked increase in fluorescence anisotropy of 2′-(*N*-dansyl)aminoethyl 1-thio-β-D-galactopyranoside (Dns^2-Gal) on addition of D-lactate to membrane vesicles containing the *lac* carrier protein, and the lifetime of the excited-state is increased when the molecule is bound. Concurrently, the rotational diffusion of Dns^2-Gal is dramatically decreased (Schuldiner et al., 1975c). Finally, [^{3}H]Dns^6-Gal has been synthesized, and binding has been measured fluorimetrically and directly by flow dialysis (Schuldiner et al., 1976a,b). These studies provide direct confirmation of the results described above. Binding is dependent upon the oxidation of D-lactate or reduced phenazine methosulfate, and there is excellent agreement between fluorescence and flow dialysis measurements with regard to the binding constant and the number of binding sites.

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[1] Abbreviations used: *p*-HMBS, *p*-hydroxymercuribenzenesulfonate; NEM, *N*-ethylmaleimide; dansyl, 5-dimethylaminonaphthalene-1-sulfonic acid; Dns^2-Gal, 2′-(*N*-dansyl)aminoethyl 1-thio-β-D-galactopyranoside; Dns^6-Gal, 6′-(*N*-dansyl)aminohexyl 1-thio-β-D-galactopyranoside; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside; NPG, *p*-nitrophenyl α-D-galactopyranoside; APG_0, 2-nitro-4-azidophenyl 1-thio-β-D-galactopyranoside; APG_2, 2′-*N*-(2-nitro-4-azidophenyl)aminoethyl 1-thio-β-D-galactopyranoside; DNP, 2,4-dinitrophenol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; M, membrane.

Although it seems clear from these observations that binding of ligand by the *lac* carrier protein is dependent upon energy coupling, the findings of Kennedy et al. (1974) represent an apparent discrepancy (Schuldiner et al., 1976b). These workers demonstrated binding of β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG) and *p*-nitrophenyl α-D-galactopyranoside (NPG) to membrane particles prepared by ultrasonic disruption in the absence of exogenous energy sources which was not inhibited by sodium azide.

We have now synthesized high specific activity [6-^{3}H]NPG by the method of Kennedy et al. (1974) and measured its binding to membrane vesicles by flow dialysis (Schuldiner et al., 1976a). The results confirm the observations of Kennedy et al. (1974), as well as the previous findings from this laboratory. There is a small amount of NPG binding to vesicles containing the *lac* carrier protein in the absence of D-lactate, and the number of binding sites increases by a factor of 10 or more in the presence of the electron donor.

Experimental Procedure

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* ML 308-225 ($i^-z^-y^+a^+$), ML 30 ($i^+z^+y^+a^+$), and ML 35 ($i^+z^+y^-a^+$) were grown on minimal medium A with 1.0% disodium succinate (hexahydrate) as sole carbon source (Kaback, 1971). With ML 35, isopropyl 1-thio-β-D-galactopyranoside was added to the growth medium at a final concentration of 5×10^{-4} M. Membrane vesicles were prepared as described previously (Kaback, 1971; Short et al., 1975).

Transport Assays. Uptake of [1-^{14}C]lactose (22 mCi/mmol) and [6-^{3}H]NPG (238 and 21.3 mCi/mmol) was determined as described previously (Kaback, 1971, 1974a) at given final concentrations. In the case of NPG assays, the filters were dissolved in 10 ml of Instabray counting fluid (Yorktown Research) and counted in a Beckman LS-100 liquid scintillation spectrometer at approximately 25% efficiency.

Binding Measurements. Binding of [6-^{3}H]NPG was measured by flow dialysis as described by Colowick and Womack (1969) and modified by Schuldiner et al. (1976a). The flow rate was 6.0 ml per min and fractions of 1.5 ml were collected.

Protein Determinations. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Materials. [6-^{3}H]NPG was synthesized as described by Kennedy et al. (1974) using galactose oxidase (Worthington), followed by reductive tritiation with sodium [^{3}H]borohydride (New England Nuclear). The product was purified by silica gel chromatography using chloroform–methanol–diethylamine (4:2:1, v/v/v) and chloroform–methanol (9:1, v/v) as eluents. The final product had a specific activity of 2380 Ci/mol and was diluted appropriately with authentic NPG (Pierce Chemical Co.) as indicated. All other materials were of reagent grade and obtained from commercial sources.

Results

NPG Is an Impermeant Inhibitor of Lactose Transport. NPG is a potent competitive inhibitor of lactose transport in *E. coli* ML 308-225 membrane vesicles, exhibiting an apparent K_i of 6.6 μM (data not shown). However, the compound itself is not taken up to an appreciable extent by the vesicles. The data presented in Table I show that, although there is a small amount of NPG associated with the vesicles in the presence of reduced phenazine methosulfate (about 2 nmol per mg of membrane protein), this is but a small fraction of the lactose accumulated under the same conditions. Moreover, the amount of NPG "uptake" observed under these conditions approximates the number of NPG binding sites as determined by flow dialysis under similar conditions (see below).

TABLE I: Transport of Lactose and NPG by *E. coli* ML 308-225 Membrane Vesicles.[a]

	NPG		Lactose
	15 μM	400 μM	400 μM
Initial rate of uptake[b]	1.60	1.50	38.7
Steady-state uptake[c]	0.81	2.00	63.2

[a] Measurements of [6-^{3}H]NPG and [1-^{14}C]lactose uptake were made at various times from 15 s to 5 min as described previously (Kaback, 1971, 1974a). Lithium ascorbate and phenazine methosulfate were added to final concentrations of 20 and 0.1 mM, respectively, before addition of [6-^{3}H]NPG or [1-^{14}C]lactose. When NPG uptake was assayed at 15 μM, [6-^{3}H]NPG at a specific activity of 238 mCi/mmol was used. When NPG uptake was assayed at 400 μM, [6-^{3}H]NPG at a specific activity of 21.3 mCi/mmol was used. [1-^{14}C]Lactose was used at a specific activity of 22 mCi/mmol. The data have been corrected for uptake observed in the absence of electron donors. [b] In nmol (mg of membrane protein)$^{-1}$ min^{-1}. [c] In nmol (mg of membrane protein)$^{-1}$ (5 min)$^{-1}$.

The ability of certain substrates of the lactose transport system to affect dilution-induced, carrier-mediated lactose efflux has been used to differentiate binding of substrate per se from binding followed by translocation (Schuldiner et al., 1975b). Compounds such as Dns^2-Gal, Dns^6-Gal, and 2′-*N*-(2-nitro-4-azidophenyl)aminoethyl 1-thio-β-D-galactopyranoside (APG_2)[2] (Rudnick et al., 1975b) inhibit lactose efflux presumably by binding to the carrier on the exterior surface of the membrane. Conversely, substrates such as lactose or 2-nitro-4-azidophenyl 1-thio-β-D-galactopyranoside (APG_0)[2] which are bound and translocated accelerate efflux by exchanging with intravesicular lactose. As shown by the data presented in Figure 1, NPG inhibits the rate of lactose efflux from preloaded vesicles by approximately fourfold, providing further evidence that it is not translocated. As shown previously (Schuldiner et al., 1975b), lactose increases the rate of efflux by approximately fivefold (Figure 1).

Determination of NPG Binding by Flow Dialysis. The data presented in Figure 2 were derived from flow dialysis experiments using [6-^{3}H]NPG and ML 308-225 and ML 30 membrane vesicles. At the inception of the experiments, [6-^{3}H]NPG (6.7 μM, final concentration) is added to the upper chamber containing membrane vesicles and, within 30 s, radioactivity appears in the dialysate pumped from the lower chamber. Subsequently, the concentration of [6-^{3}H]NPG in the dialysate increases linearly for about a minute, reaching a maximum which then decreases at a slow rate. When D-lactate is added to the upper chamber containing ML 308-225 vesicles (closed symbols), the concentration of [6-^{3}H]NPG appearing in the dialysate decreases dramatically, indicating that the vesicles bind the galactoside and remove it from solution. After a new equilibrium is reached in the presence of D-lactate, stepwise addition of nonradioactive NPG to the upper chamber establishes a series of new equilibria in which an increased fraction of the total ligand becomes diffusible with each addition of NPG. When sufficient NPG is added, (i.e., 240 μM and above), the concentration of [6-^{3}H]NPG in the

[2] Rudnick, G., Weil, R., and Kaback, H. R., unpublished results.

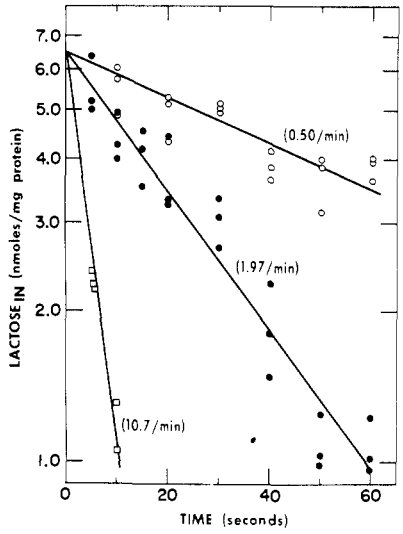

FIGURE 1: Effect of NPG on dilution-induced, carrier-mediated lactose efflux. *E. coli* ML 308-225 membrane vesicles (40 mg of membrane protein per ml) suspended in 0.1 M potassium phosphate (pH 6.6) were incubated with [1-^{14}C]lactose (22 mCi/mmol) at a final concentration of 4 mM for 4 h at 4 °C (Schuldiner et al., 1975b) Efflux was then induced by diluting aliquots of the suspension 400-fold into vessels containing 2.0 ml of 0.1 M potassium phosphate (pH 6.6) and 0.01 M magnesium sulfate (●). Where indicated, NPG (○) or lactose (□) was also present at a final concentration of 0.3 or 2.5 mM, respectively. At the times shown, the reactions were terminated and the samples assayed as described previously (Schuldiner et al., 1975b). The values given in parentheses are first-order rate constants given in reciprocal minutes.

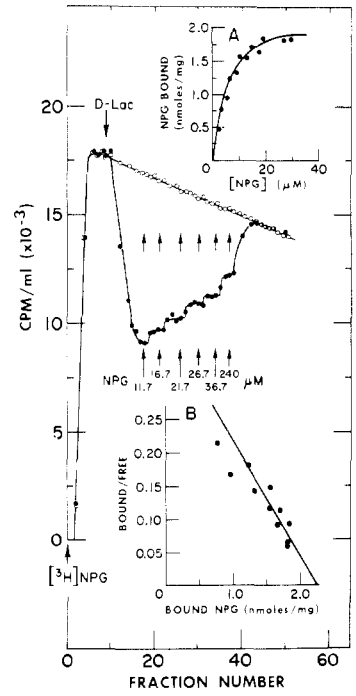

FIGURE 2: Binding of [6-^{3}H]NPG by membrane vesicles prepared from *E. coli* ML 308-225 (●) and uninduced ML 30 (○). Binding of [6-^{3}H]NPG was assayed by flow dialysis as described previously (Schuldiner et al., 1976a) and in Experimental Procedure. The upper chamber contained 0.05 M potassium phosphate (pH 6.6), 0.01 M magnesium sulfate, and membrane vesicles (4 mg of protein per ml) in a total volume of 0.8 ml, and 0.05 M potassium phosphate (pH 6.6) was pumped from the lower chamber. The experiment was initiated by addition of [6-^{3}H]NPG (2380 mCi/mmol) to the upper chamber to a final concentration of 6.7 μM. Where indicated, lithium D-lactate was added to the upper chamber to give a final concentration of 20 mM. Additions of nonradioactive NPG were made to the upper chamber as indicated using solutions sufficiently concentrated such that the volume of the upper chamber was increased by less than 3%. Fractions of 1.5 ml were collected, and 1.0-ml aliquots were assayed for radioactivity by addition of 10.0 ml of Instabray (Yorktown Research) and counting in a liquid scintillation spectrometer (Beckman LS-250). Inset A: The data shown in the body of Figure 2 and others were treated as described by Colowick and Womack (1969) to determine the amount of NPG bound per mg of membrane protein at each NPG concentration. Inset B: Data plotted according to Scatchard (1949).

dialysate approximates the value observed in the absence of D-lactate. Using these data, the amount of NPG bound at each NPG concentration can be calculated (Colowick and Womack, 1969; Schuldiner et al., 1976a), and the binding constant (K_D) of the *lac* carrier protein for NPG and the number of NPG binding sites can be determined (Figure 2, insets). Clearly, D-lactate-induced NPG binding is a saturable function of the NPG concentration (Figure 2A). The apparent K_D calculated from the Scatchard plot (Figure 2B) is approximately 6 μM, and the amount of NPG bound at saturation is about 2.3 nmol per mg of membrane protein. Within experimental error, the latter value is indistinguishable from that obtained with Dns^2-Gal and Dns^6-Gal (Reeves et al., 1973; Schuldiner et al., 1975, 1976a) and from the value reported by Jones and Kennedy (1969) for the total amount of M protein (i.e., *lac* carrier protein) in the membrane. It is also apparent from the data presented in the body of Figure 2 that addition of neither D-lactate nor NPG to uninduced ML 30 vesicles causes a change in the concentration of [6-^{3}H]NPG in the dialysate (open symbols), indicating that binding of NPG is dependent upon the presence of a functional *lac y* gene product.

Addition of the proton conductors, 2,4-dinitrophenol (DNP) or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (not shown) to ML 308-225 vesicles incubated in the presence of [6-^{3}H]NPG and D-lactate causes rapid release of bound ligand (Figure 3A), demonstrating that the ability of the vesicles to bind NPG in the presence of D-lactate depends upon the generation of an electrochemical proton gradient. Moreover, as shown in Figure 3B, addition of *p*-HMBS to ML 308-225 vesicles incubated in the presence of [6-^{3}H]NPG and D-lactate also results in rapid, quantitative release of bound ligand. Thus, NPG, like Dns^2-Gal, Dns^6-Gal, and APG$_2$, is bound to the *lac* carrier protein on the surface of the vesicle membrane but is not transported into the intravesicular space.

NPG Binding in the Absence of Electron Donors. Since small amounts of nonspecific dansyl galactoside binding were detected by fluorescence anisotropy measurements (Schuldiner et al., 1975c), it is possible that a minute amount of carrier-dependent binding could have been obscured. Because of the importance of this point, binding of [6-^{3}H]NPG was determined by flow dialysis under conditions designed to maximize binding in the absence of D-lactate (Figure 4). When excess nonradioactive NPG is added to a concentrated suspension of ML 308-225 vesicles (10 mg of protein per ml) which were previously equilibrated with [6-^{3}H]NPG in the absence of exogenous electron donors, there is a small but significant increase in the concentration of [6-^{3}H]NPG in the dialysate, suggesting that a small amount of ligand is bound. Moreover, binding observed under these conditions is not altered when the vesicles are subjected to ultrasonic sound treatment (Figure 4A). This effect is not observed with vesicles prepared from induced ML 35 ($i^+z^+y^-a^+$) (Figure 4B), indicating that binding of NPG is dependent upon the presence of the *lac* carrier protein and cannot be due to a small amount of catalytically inactive, membrane-bound β-galactosidase that might be present in ML 308-225 vesicles. Addition of *p*-HMBS to ML 308-225 vesicles causes rapid release of bound

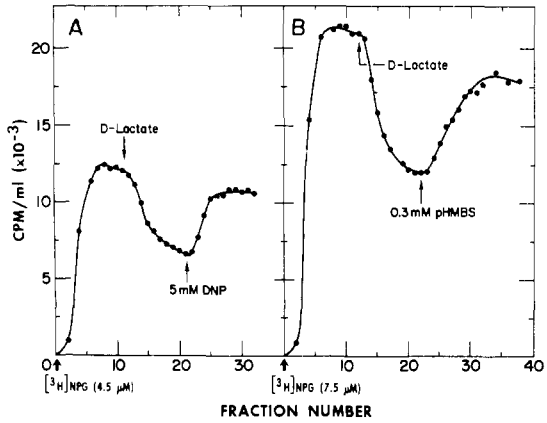

FIGURE 3: (A) Effect of DNP on D-lactate-induced NPG binding by ML 308-225 membrane vesicles. NPG binding was assayed by flow dialysis as described in Figure 3 using 4.5 μM [6-^{3}H]NPG (2380 mCi/mmol) and 20 mM lithium D-lactate. Where indicated, DNP was added to the upper chamber to a final concentration of 5 mM. (B) Effect of *p*-HMBS on D-lactate-induced NPG binding by ML 308-225 membrane vesicles. NPG binding was assayed by flow dialysis as described in Figure 3 using 7.5 μM [6-^{3}H]NPG (2380 mCi/mmol) and 20 mM lithium D-lactate. Where indicated, *p*-HMBS was added to the upper chamber to a final concentration of 0.3 mM.

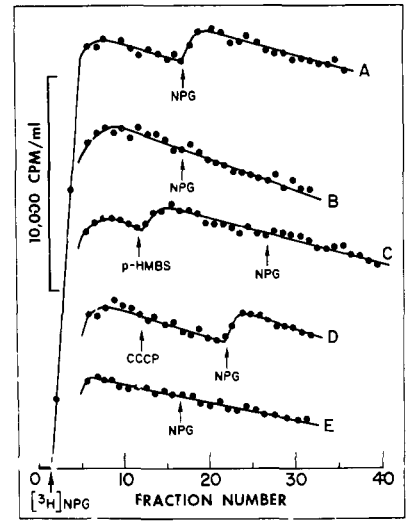

FIGURE 4: Binding of NPG to membrane vesicles in the absence of exogenous electron donors. The data shown were obtained from flow dialysis experiments performed as described in Figure 3, except that membrane vesicles were used at a concentration of 10 mg of protein per ml in the upper chamber, [6-^{3}H]NPG (2380 mCi/mmol) was used at a final concentration of 7.5 μM, and D-lactate was not added. (A) Binding to ML 308-225 vesicles. Where indicated, nonradioactive NPG was added to the upper chamber at a final concentration of 200 μM. Identical results were obtained when the vesicles were subjected to ultrasonic sound for 10 min at 0 °C in a bath-type sonicator (Laboratory Supplies Co., Inc., Hicksville, N.Y., Model T-80-80-1 RS). (B) Binding to membrane vesicles prepared from *E. coli* ML 35 ($i^+z^+y^-a^+$) grown in the presence of isopropyl 1-thio-β-D-galactopyranoside (see Experimental Procedure). Where indicated, nonradioactive NPG was added to the upper chamber at a final concentration of 200 μM. The same results were obtained with vesicles prepared from uninduced *E. coli* ML 30 ($i^+z^+y^+a^+$). (C) Effect of *p*-HMBS on NPG binding to ML 308-225 vesicles in the absence of electron donors. Where indicated, *p*-HMBS and nonradioactive NPG were added sequentially to the upper chamber at final concentrations of 0.3 mM and 200 μM, respectively. (D) Effect of CCCP on NPG binding to ML 308-225 membrane vesicles in the absence of electron donors. Where indicated, CCCP and nonradioactive NPG were added sequentially to the upper chamber at final concentrations of 10 and 200 μM, respectively. Identical results (not shown) were obtained with 80 μM CCCP in the presence or absence of 25 μM valinomycin. (E) Effect of bee venom phospholipase A and various detergents on NPG binding to ML 308-225 membrane vesicles in the absence of electron donors. The results shown were the same with vesicles treated in the following manner: 20-min incubation with bee venom phospholipase A (Sigma) at a final concentration of 100 μg per ml, 10 min incubation with sodium cholate (2%), 10-min incubation with Lubrol PX (2%), 10-min incubation with Lubrol WX (2%), or 10-min incubation with Triton X-100 (2%). In each case, the vesicles were incubated with the phospholipase or detergent for the time given in the upper chamber of the flow dialysis apparatus prior to the addition of [6-^{3}H]NPG. Where indicated, nonradioactive NPG was added to the upper chamber to a final concentration of 200 μM. Although curves A through E are displayed in decreasing order, the levels of radioactivity obtained initially in the dialysate (fraction 1 to fraction 10) were the same in each case (i.e., curves A through E should be superimposed).

[6-^{3}H]NPG, and subsequent addition of NPG does not displace more radioactive ligand (Figure 5C). Thus, binding of NPG to the *lac* carrier protein under these conditions responds to the sulfhydryl reagent in a manner similar to that observed in the presence of D-lactate (Figure 3B). Since low rates of endogenous respiration in these concentrated membrane suspensions could give rise to a small membrane potential and lead to binding of NPG, it is noteworthy that addition of CCCP does not release [6-^{3}H]NPG bound under these conditions nor does it interfere with the displacement of [6-^{3}H]NPG by nonradioactive ligand (Figure 4D). Although not shown, similar results were obtained with DNP, valinomycin, and valinomycin in combination with CCCP. Finally, NPG binding under these conditions is dependent upon the structural integrity of the membrane (Kennedy et al., 1974). When vesicles are treated with bee venom phospholipase A or a number of detergents and then incubated with [6-^{3}H]NPG, addition of excess NPG causes no increase in the concentration of radioactive ligand in the dialysate, indicating that binding has been abolished (Figure 4E).

Binding of NPG by ML 308-225 membrane vesicles in the absence of electron donors can be quantitated as described in Figure 5. The data presented in the body of the figure demonstrate that NPG binding observed under nonenergized conditions is a saturable function of NPG concentration. Moreover, when the data are treated according to Scatchard (1949) (Figure 5, inset), it can be determined graphically that the K_D is about 9 μM and there are approximately 0.2 nmol of NPG bound per mg of membrane protein at saturation. Thus, the K_d under these conditions is very similar to that observed in the presence of D-lactate, while the number of binding sites is approximately tenfold less than that observed in the presence of the electron donor (compare Figure 5 with Figure 2).

Discussion

These studies resolve an important apparent conflict regarding the binding properties of the *lac* carrier protein or M protein (Schuldiner et al., 1976b). Previous work utilizing dansyl and azidophenyl galactosides (Schuldiner et al., 1976b) provides strong evidence that the *lac* carrier protein is largely unable to bind ligand unless the vesicles are energized, while Kennedy et al. (1974) observed binding of certain galactosides to membrane particles in the absence of energy. Superficially, the results appear to be mutually exclusive, until the data are examined quantitatively. It then becomes apparent that the number of binding sites observed by Kennedy et al. (1974) is approximately 10 to 20 times less than the total amount of *lac y* gene product present in the membrane as determined by Jones and Kennedy (1969) and by titration studies with the dansyl galactosides (Reeves et al., 1973; Schuldiner et al., 1975a, 1976a). Both sets of observations are corroborated in this paper. Using NPG, an impermeant α-galactoside which

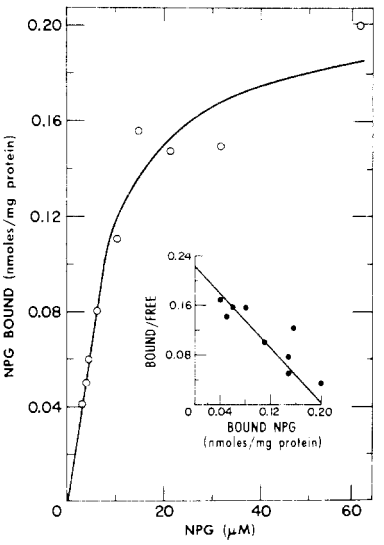

FIGURE 5: Titration of NPG binding sites in the absence of electron donors. For each experimental point shown, a separate flow dialysis experiment was carried out with ML 308-225 membrane vesicles as described in Figure 5A, except that [6-^{3}H]NPG was added to the upper chamber at the concentrations given on the abscissa. After equilibrium had been achieved (Figure 5A), 240 μM nonradioactive NPG was added to the upper chamber. The amount of NPG bound at each initial concentration of [6-^{3}H]NPG was then calculated from the increase in radioactivity in the dialysate (Colowick and Womack, 1969). Inset: Data plotted according to Scatchard (1949).

binds to the *lac* carrier protein, it is clear that a small amount of binding occurs in the absence of energy, and that the number of binding sites observed under these conditions agrees reasonably well with the results of Kennedy et al. (1974), as well as those of Fox et al. (1967). In the presence of D-lactate, however, the number of NPG binding sites increases by a factor of 10 or more, and approximates the total amount of *lac y* gene product in the membrane as reflected by the values reported by Jones and Kennedy (1969) and by titration studies with the dansyl galactosides (Reeves et al., 1973; Schuldiner et al., 1975a, 1976a).

The observation that a small but significant amount of NPG binding to membrane vesicles containing the *lac* carrier protein occurs in the absence of an electrochemical gradient of protons or artificially applied ion diffusion potentials suggests that the *lac* carrier protein may exist in two forms which are in a state of dynamic equilibrium: (i) a high affinity form which is accessible on the surface of the membrane; and (ii) a low-affinity, cryptic form which we are unable to detect. In the absence of D-lactate or reduced phenazine methosulfate, 90% or more of the carrier is in the low-affinity, cryptic form and only 10% or less is in the high-affinity, accessible form. Upon generation of an electrochemical proton gradient across the vesicle membrane (interior negative and alkaline), one or more negatively charged groups in the low-affinity, cryptic form of the protein might be influenced, resulting in a conformational change and a shift in the equilibrium. According to such a model, active transport would occur by binding of ligand to the high-affinity form of the carrier on the external surface of the membrane, followed by conversion of the carrier to the low-affinity, cryptic form and release of ligand from the inner surface of the membrane. However, since the carrier is presumed to be negatively charged, translocation of ligand would require neutralization of the high-affinity form of the carrier on the external surface of the membrane. This might be accomplished if ligand binding increased the pK_a of a negatively charged functional group(s) in the carrier resulting in protonation. The protein would then be uncharged and no longer under the influence of the electrochemical gradient. The protonated carrier–ligand complex would "relax" to the cryptic form and release proton(s) and ligand on the inner surface of the membrane, regenerating the charged form of the carrier, and the cycle could then be repeated. Clearly, this reaction sequence is consistent with the proposal of Mitchell (1973) that the uptake of β-galactosides occurs by cotransport with protons (i.e., symport), and recent experiments with the vesicle system (Ramos et al., 1976; Ramos and Kaback, submitted for publication), in addition to a number of earlier experiments with intact cells (West, 1970; West and Mitchell, 1972, 1973; West and Wilson, 1973; Kashket and Wilson, 1973), provide strong support for this contention. Under appropriate conditions, D-lactate or reduced phenazine methosulfate oxidation by *E. coli* vesicles leads to the generation of a large transmembrane pH gradient [interior alkaline] (Ramos et al., 1976), as well as an electrical potential, interior negative (Hirata et al., 1973; Altendorf et al., 1975; Schuldiner and Kaback, 1975), and both components of the electrochemical proton gradient are partially dissipated during lactose accumulation (Schuldiner and Kaback, 1975; Ramos and Kaback, submitted for publication). It is also noteworthy that, according to this model, the carrier would not translocate protons across the membrane in the absence of ligand, a stipulation which is necessary lest the carriers themselves dissipate the electrochemical gradient without performing work. It should also be emphasized that this formulation might account for low rates of facilitated diffusion without necessitating that this process represent the initial step in the active transport of β-galactosides (Kaback, 1974b; Schuldiner et al., 1976c).

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Properties and Subcellular Distribution of Guanylate Cyclase Activity in Rat Renal Medulla: Correlation with Tissue Content of Guanosine 3′,5′-Monophosphate†

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ABSTRACT: The properties of the guanylate cyclase systems of outer and inner medulla of rat kidney were examined and compared with those of the renal cortex. A gradation in steady-state cyclic guanosine 3′,5′-monophosphate (cGMP) levels was observed in incubated slices of these tissues (inner medulla > outer medulla > cortex). This correlated with the proportion of total guanylate cyclase activity in the 100 000*g* particulate fraction of each tissue, but was discordant with the relative activities of guanylate cyclase (highest in cortex) and of cGMP-phosphodiesterase (lowest in cortex) in whole tissue homogenates. Soluble guanylate cyclase of cortex and inner medulla exhibited typical Michaelis–Menten kinetics with an apparent K_m for MnGTP of 0.11 mM, while the particulate enzyme from inner medulla exhibited apparent positive cooperative behavior and a decreased dependence on Mn^{2+}. Thus, the particulate enzyme could play a key role in regulating cGMP levels in the intact cell where Mn^{2+} concentrations are low. The soluble and particulate enzymes from inner medulla were further distinguished by their responses to several test agents. The soluble enzyme was activated by Ca^{2+}, NaN_3, $NaNO_2$, and phenylhydrazine, whereas particulate activity was inhibited by Ca^{2+} and was unresponsive to the latter agents. In the presence of $NaNO_2$, Mn^{2+} requirement of the soluble enzyme was reduced and equivalent to that of the particulate preparation. Moreover, relative responsiveness of the soluble enzyme to $NaNO_2$ was potentiated when Mg^{2+} replaced Mn^{2+} as the sole divalent cation. These changes in metal requirements may be involved in the action of $NaNO_2$ to increase cGMP in intact kidney. Soluble guanylate cyclase of cortex was clearly more responsive to stimulation by NaN_3, $NaNO_2$, and phenylhydrazine than was soluble activity from either medullary tissue. The effectiveness of the agonists on soluble activity from outer and inner medulla could also be distinguished. Accordingly, regulation and properties of soluble guanylate cyclase, as well as subcellular enzyme distribution, are distinct in the three regions of the kidney.

Cyclic guanosine 3′,5′-monophosphate (cGMP[1]) has been implicated as an intracellular modulator of several important physiological processes (Berridge, 1975) and may be involved in the regulation of renal metabolic and excretory functions (Goodman et al., 1972; DeRubertis et al., 1976). Previous studies from our laboratory have described the properties of the guanylate cyclase–cGMP system of rat renal cortex. In this tissue, both Ca^{2+}-dependent (DeRubertis and Craven, 1976a) and independent mechanisms (DeRubertis and Craven, 1976b) exist for modulation of cGMP levels, and guanylate cyclase activity is found predominantly in the soluble fraction of cell homogenates. The inner medulla of kidney is known to be unique in several of its anatomical, functional, and metabolic characteristics (Rhodin, 1958; Kean et al., 1961, 1962; Bernanke and Epstein, 1965; Sternberg et al., 1956; Lee et al., 1962; Rennie et al., 1958; Aperia and Leebow, 1964; Cohen and Barac-Nieto, 1973). Preliminary observations (Craven and DeRubertis, 1976a) indicated that these differences extended to the properties of the guanylate cyclase–cGMP system of this tissue compared with cortex or outer medulla. Specifically, basal cGMP levels in incubated slices of inner medulla are consistently higher than those of cortex or outer medulla. Basal cGMP did not correlate with the relative activities of guanylate cyclase (highest in cortex) or cGMP-phosphodiesterase (lowest in cortex) found in whole homogenates of the three tissues. Data from other tissues have suggested that the particulate form of guanylate cyclase may be a key determinant of cGMP economy in the intact cell due to its distinct properties (Kimura and Murad, 1974, 1975a,b;

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[1] Abbreviations used are: cGMP, cyclic guanosine 3′,5′-monophosphate; cAMP, cyclic adenosine 3′,5′-monophosphate; SE, standard error; SEM, standard error of the mean; Tris, tris(hydroxymethyl)aminomethane; ip, intraperitoneally.