Jacob, S. T., Sajdel, E. M., & Munro, H. N. (1970) *Biochem. Biophys. Res. Commun. 38,* 765–770.
Kirkegaard, L. H. (1973) *Biochemistry 12,* 3627–3632.
Kirkegaard, L. H. Johnson, T. J. A., & Bock, R. M. (1972) *Anal. Biochem. 50,* 122–127.
Lindell, T. J., Weinberg, F., Morris, P. W., Roeder, R. G., & Rutter, W. J. (1970) *Science 170,* 447–449.
Meilhac, M., Kedinger, C., Chambon, P., Faulstich, H., Govindan, M. V., & Wieland, T. (1970) *FEBS Lett. 9,* 258–260.
Morris, P. W., & Rutter, W. J. (1976) *Biochemistry 15,* 3106–3113.
Morris, P. W., Litman, R., & Passo, C. (1976) in *Molecular Mechanisms in the Control of Gene Expression* (Nierlich, D. P., & Rutter, W. J., Eds.) pp 255–260, Academic Press, New York, N.Y.
Morris, P. W., Venton, D. L., & Kelley, K. M. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36,* 882 (Abstract 3222).
Morrison, M., & Schonbaum, G. R. (1976) *Annu. Rev. Biochem. 45,* 861–888.
Roeder, R. G., & Rutter, W. J. (1969) *Nature (London) 224,* 234–237.
Schultz, L. D., & Hall, B. D. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 1029–1033.
Schwartz, L. B., Lawrence, C., Thach, R. E., & Roeder, R. G. (1974a) *J. Virol. 14,* 611–619.
Schwartz, L. B., Sklar, V. E. F., Jaehning, J. A., Weinman, R., & Roeder, R. G. (1974b) *J. Biol. Chem. 249,* 5889–5897.
Sober, H. A., Ed. (1970) *Handbook of Biochemistry,* 2nd ed, The Chemical Rubber Co., Cleveland, Ohio.
Somers, D. G., Pearson, M. L., & Ingles, C. J. (1975a) *J. Biol. Chem. 250,* 4825–4831.
Somers, D. G., Pearson, M. L., & Ingles, C. J. (1975b) *Nature (London) 253,* 372–374.
Valenzuela, P., Hager, G. L., Weinberg, F. & Rutter, W. J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 1024–1028.
Weil, P. A., & Blatti, S. P. (1975) *Biochemistry 14,* 1636–1642.
Weil, P. A., Sidikaro, J., Stancel, G. M., & Blatti, S. P. (1976) *J. Biol. Chem. 252,* 1092–1098.
Weinmann, R., & Roeder, R. G. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1790–1794.
Wieland, T. (1972) *Naturwissenschaften 59,* 225–231.
Wieland, T., & Fahrmeir, A. (1970) *Justus Liebigs Ann. Chem. 736,* 95–99.
Wieland, T., & Gebert, U. (1966) *Justus Liebigs Ann. Chem. 700,* 157–173.
Wieland, T., & Wieland O. (1972) *Microbiol. Toxins 8,* 249–280.

Sodium-Dependent Binding of *p*-Nitrophenyl α-D-Galactopyranoside to Membrane Vesicles Isolated from *Salmonella typhimurium*†

Hajime Tokuda and H. Ronald Kaback*

ABSTRACT: *p*-Nitrophenyl α-D-galactopyranoside is a competitive inhibitor of sodium-dependent methyl 1-thio-β-D-galactopyranoside transport in whole cells and membrane vesicles prepared from *Salmonella typhimurium* G-30 grown on melibiose ($K_i \simeq 7$ μM in whole cells and 0.4 μM in membrane vesicles). However, the compound is not transported to a discernible extent by either intact cells or membrane vesicles. Binding of *p*-nitrophenyl α-D-[6-^{3}H]galactopyranoside to membrane vesicles has been measured by flow dialysis under various conditions. When D-lactate is added to vesicles at low sodium concentrations, ligand binds with a $K_D \simeq 3.2$ μM, and a total of about 0.2 nmol is bound per mg of membrane protein at saturating concentrations of ligand. With optimal sodium concentrations in addition to D-lactate, both the affinity and the number of binding sites observed at saturation increase ($K_D \simeq 0.6$ μM and about 0.8 nmol per mg of membrane protein). Furthermore, ligand binding can be induced by imposition of a potassium diffusion gradient ($K^+_{in} > K^+_{out}$) in the presence of valinomycin and sodium ion. Binding studies as a function of pH and titration studies with valinomycin, nigericin, and monensin indicate that binding varies with the electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$) with a bias toward the electrical component ($\Delta\Psi$). Moreover, when the effect of these ionophores on the kinetics of binding is investigated, it is clear that $\Delta\Psi$ and the sodium gradient (ΔpNa) function in different capacities with respect to ligand binding. $\Delta\Psi$ (interior negative) appears to perturb the porter directly, altering its interaction with sodium so as to allow the cation to effect an increase in binding affinity, while ΔpNa ($Na^+_{out} > Na^+_{in}$) increases the number of binding sites independent of $\Delta\bar{\mu}_{H^+}$. It is concluded that sodium binding precedes solute binding and translocation.

The use of impermeant galactoside analogues has yielded unique information regarding the dynamics of the solute-specific component of the β-galactoside transport system in membrane vesicles isolated from *Escherichia coli* (for reviews, cf. Schuldiner et al., 1976b; Schuldiner & Kaback, 1977). These studies indicate that 90% or more of the *lac* carrier protein (i.e., M protein) is cryptic to ligand unless the membrane is "energized". Oxidation of electron donors which drive transport in the vesicles leads to the generation of an electrochemical gradient of protons ($\Delta\bar{\mu}_{H^+}$) across the vesicle membrane [interior negative and alkaline] (Ramos et al., 1976; Ramos & Kaback, 1977a–c) and a dramatic increase in the number of binding sites for (*N*-dansyl)aminoalkyl 1-thio-β-D-galactopyranosides (Reeves et al., 1973; Schuldiner et al., 1975a–c, 1976a, 1977) and *p*-nitrophenyl α-D-galactopyran-

† From the Laboratory of Membrane Biochemistry, The Roche Institute of Molecular Biology, Nutley, New Jersey 07110. *Received August 16, 1977.*

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oside [NPG][1] (Rudnick et al., 1976). Similarly, azidophenyl galactoside dependent photoinactivation of lactose transport requires generation of $\Delta\bar{\mu}_{H^+}$ (Rudnick et al., 1975a,b). Many of these effects are mimicked by artificially induced ion diffusion potentials of appropriate polarity (Schuldiner et al., 1975a; Rudnick et al., 1975b), but dilution-induced carrier-mediated lactose efflux also causes increased dansyl galactoside binding in a manner which is apparently independent of $\Delta\bar{\mu}_{H^+}$ (Schuldiner et al., 1975a). In any event, based on these observations and others (Ramos & Kaback, 1977c), it has been suggested that the *lac* carrier protein or part of it may be negatively charged. As emphasized, however, it is not possible to determine whether the effect of energization is due to an increase in the accessibility of the carrier, an increase in its affinity for ligand, or both (Schuldiner et al., 1975a; Rudnick et al., 1976). One reason for this difficulty is that proton movements are probably responsible for the generation of $\Delta\bar{\mu}_{H^+}$ as well as the recognition and symport of substrate (Mitchell, 1973; Harold, 1972; Kaback, 1976). For this and other reasons, we focused attention on the sodium-dependent α-galactoside transport system in *Salmonella typhimurium* [TMG permease II] (Prestidge & Pardee, 1965; Stock & Roseman, 1971; Tokuda & Kaback, 1977).

Membrane vesicles isolated from *S. typhimurium* G-30 grown in the presence of melibiose catalyze active transport of methyl 1-thio-β-D-galactopyranoside (TMG) in the presence of D-lactate and sodium or lithium (Tokuda & Kaback, 1977). Since accumulation of TMG is apparently dependent upon sodium or lithium gradients and TMG-dependent sodium uptake is also observed under certain conditions, it is clear that this system exhibits properties which are typical of many sodium-dependent cotransport systems in eucaryotic cells (Schultz & Curran, 1970; Crane, 1978). On the other hand, cation gradient-dependent TMG accumulation in this system is not dependent upon sodium, potassium-stimulated ATPase activity, but varies with the $\Delta\bar{\mu}_{H^+}$ generated as a result of D-lactate oxidation. Moreover, the vesicles catalyze D-lactate-driven sodium efflux in a manner which is consistent with the operation of a proton/sodium (lithium) antiport mechanism (Tokuda & Kaback, 1977). The results suggest a sequence of coupled reactions in which sodium (lithium)/TMG symport (i.e., cotransport) is driven by $\Delta\bar{\mu}_{H^+}$ through the mediation of a proton/sodium (lithium) antiport mechanism which serves to maintain low intravesicular sodium or lithium concentrations. Thus, in this system, the movements of protons and sodium function in different capacities—proton movements give rise to $\Delta\bar{\mu}_{H^+}$, while sodium is involved in the recognition and symport of solute.

Although *o*-nitrophenyl β-D-galactopyranoside is widely used to assay the activity of the β-galactoside transport system in *E. coli*, the results presented here demonstrate surprisingly that NPG is not accumulated significantly by the sodium-dependent α-galactoside transport system in *S. typhimurium*. However, NPG is a potent competitive inhibitor of cation-dependent TMG accumulation, and binding studies utilizing high specific activity [6-^{3}H]NPG demonstrate that it binds to TMG permease II under appropriate conditions.

[1] Abbreviations used: NPG, *p*-nitrophenyl α-D-galactopyranoside; TMG, methyl 1-thio-β-D-galactopyranoside; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $TPMP^+$, triphenylmethylphosphonium (bromide salt); $\Delta\bar{\mu}_{H^+}$, the electrochemical gradient of protons; $\Delta\Psi$, the electrical potential across the membrane; ΔpH, the pH gradient across the membrane; ΔpNa, the sodium gradient across the membrane; P, phospho.

Experimental Section

Methods

Growth of Cells and Preparation of Membrane Vesicles. *S. typhimurium* G-30 (*Gal* E$^-$) was grown in the presence and absence of melibiose, and membrane vesicles were prepared as described previously (Tokuda & Kaback, 1977).

Transport Assays. Uptake of [^{14}C]methyl 1-thio-β-D-galactopyranoside (TMG) (7.1 mCi/mmol) and [6-^{3}H]NPG (121 mCi/mmol) was determined by rapid filtration using Millipore Cellotate filters (Tokuda & Kaback, 1977). The filters were dissolved in 10 mL of Instabray counting fluid (Yorktown Research) and counted in a Beckman LS-100 liquid scintillation spectrometer.

Binding Measurements. Binding of [6-^{3}H]NPG was measured by flow dialysis as described by Colowick & Womack (1969) and modified by Schuldiner et al. (1976a; Rudnick et al., 1976). The flow rate was 6.0 mL per min and fractions of 1.7 mL were collected.

Measurement of ΔpH and $\Delta\Psi$. ΔpH and $\Delta\Psi$ were determined by assaying the accumulation of [1,2-^{14}C]acetate (54 mCi/mmol) and [^{3}H]triphenylmethylphosphonium ($TPMP^+$) [bromide salt] (4.36 Ci/mmol), respectively, using flow dialysis (Ramos et al., 1976; Ramos & Kaback, 1977a–c; Tokuda & Kaback, 1977).

Protein Determinations. Protein was measured as described by Lowry et al. (1951) using bovine serum albumin as a standard.

Sodium Determinations. A 1.0-mL sample of membrane vesicles suspended in 0.05 M potassium phosphate (pH 6.6) at about 2 mg of protein per mL was boiled for 15 min. Denatured material was removed by centrifugation, and the sodium content of the supernatant was determined by ion chromatography using a Dionex Corp. instrument (Model P-14) (Small et al., 1975).

Materials

[6-^{3}H]NPG was prepared as described (Kennedy et al., 1974; Rudnick et al., 1976). [^{3}H]$TPMP^+$ was prepared by the Isotope Synthesis Group at Hoffmann-La Roche, Inc., under the direction of Dr. Arnold Liebman (Schuldiner & Kaback, 1975). [^{14}C]TMG and [1,2-^{14}C]acetate were purchased from New England Nuclear. Valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Calbiochem. Nigericin and monensin were donated by Dr. J. Berger of Hoffmann-La Roche, Inc. All other materials were of reagent grade and obtained from commercial sources.

Results

NPG is not accumulated but inhibits TMG uptake competitively. As shown previously (Stock & Roseman, 1971; Tokuda & Kaback, 1977) and in Figure 1, intact cells grown in the presence of melibiose (A) and membrane vesicles prepared from these cells (B) catalyze active transport of TMG in the presence of lithium [or sodium (not shown)]. Under identical conditions, little or no NPG is taken up in either case, and there is no stimulation by sodium or lithium. It is also noteworthy that intact cells hydrolyze NPG at very low rates as judged by the release of the chromogen *p*-nitrophenol.[2] Moreover, when the cells are treated with toluene or sonicated, these low rates of NPG hydrolysis are completely abolished

[2] *S. typhimurium* G-30 grown on melibiose as described in Methods hydrolyze NPG at a rate of 2.5 nmol per mg of dry weight per min. In comparison, *E. coli* ML 30 grown on lactose hydrolyze *o*-nitrophenyl β-D-galactopyranoside and *p*-nitrophenyl β-D-galactopyranoside at a rate of 35 nmol per mg of dry weight per min.

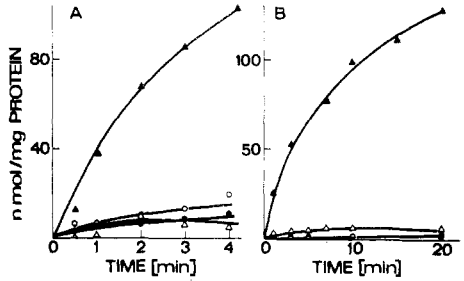

FIGURE 1: Transport of TMG and NPG by *S. typhimurium* G-30 cells (A) and membrane vesicles (B). (A) Cells grown on melibiose (Tokuda & Kaback, 1977) were washed and resuspended in 0.1 M potassium phosphate (pH 6.6) to a final concentration of 1.1 mg of protein per mL. Aliquots (25 μL) were diluted to a final volume of 50 μL containing, in final concentrations, 0.05 M potassium phosphate (pH 6.6) and 0.01 M magnesium sulfate. After 30 s to 1 min preincubation at 25 °C in the presence (●, ▲) or absence (○, △) of 20 mM lithium chloride, [^{14}C]TMG (7.1 mCi/mmol) [▲, △] or [6-^{3}H]NPG (121 mCi/mmol) [●, ○] was added at final concentrations of 1.3 mM and 0.4 mM, respectively. Incubations were continued at 25 °C for the times indicated, and the reactions were terminated and assayed as described (Tokuda & Kaback, 1977). (B) Membrane vesicles were prepared from melibiose-grown cells (Tokuda & Kaback, 1977) and suspended in 0.1 M potassium phosphate (pH 6.6) to a final concentration of about 4 mg of protein per mL. Transport was assayed in the presence of 20 mM potassium D-lactate with (●, ▲) and without (○, △) 20 mM lithium chloride as described (Tokuda & Kaback, 1977). Concentrations of [^{14}C]TMG (▲, △) and [6-^{3}H]NPG (●, △) are given above.

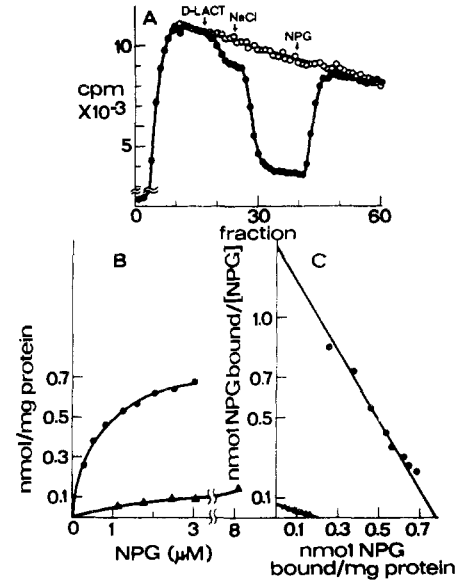

FIGURE 2: D-Lactate-induced, sodium-dependent binding of [6-^{3}H]NPG by *S. typhimurium* G-30 membrane vesicles. (A) NPG binding by vesicles prepared from cells grown in the presence (●) and absence (○) of melibiose was measured by flow dialysis as described previously (Schuldiner et al., 1976a, 1977; Rudnick et al., 1976) 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 oxygenated 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 at a final concentration of 4 μM. Where indicated, potassium D-lactate, sodium chloride, and nonradioactive NPG were added to the upper chamber to yield final concentrations of 20 mM, 20 mM, and 0.94 mM, respectively. The stock solutions were sufficiently concentrated such that the volume of the upper chamber was increased by less than 3%. Fractions of 1.7 mL were collected and assayed for radioactivity by liquid scintillation spectrometry. (B) The effect of NPG concentration on NPG binding in the presence (●) and absence (▲) of added sodium ion was measured by flow dialysis as described in A, except that 20 mM sodium and potassium D-lactate were used, respectively. Each experimental point represents the results of an individual flow dialysis determination in which binding was measured at a given concentration of [6-^{3}H]NPG (2380 mCi/mmol). The quantity of NPG bound was calculated as described (Schuldiner et al., 1976a). (C) Data shown in B plotted according to Scatchard (1949). (●) Sodium D-lactate, 20 mM; (▲) 20 mM potassium D-lactate.

for unknown reasons.[3] Further evidence indicating that NPG is not transported will be presented below.

Although it is not accumulated, kinetic data provide a strong indication that NPG binds to the α-galactoside porter (data not shown). When the initial rate of TMG uptake is measured as a function of TMG concentration in the absence and presence of various concentrations of NPG, the compound causes an increase in apparent K_m without significant alteration in V_{max}, and the apparent K_i can be calculated to be approximately 0.4 μM. Similar data were obtained with intact cells, except that the apparent K_i is approximately 7 μM. Possibly, the decreased sensitivity of the transport system in whole cells to inhibition by NPG is related to the partial inability of NPG to penetrate the outer membrane.

NPG binding is dependent upon $\Delta\bar{\mu}_{H^+}$ and sodium or lithium ion. When D-lactate is added to the upper chamber of a flow dialysis apparatus containing induced G-30 vesicles (Figure 2A, closed symbols), the concentration of [6-^{3}H]NPG in the dialysate decreases slightly, indicating that the vesicles bind a small amount of the galactoside without added sodium[4] or lithium. After equilibrium is achieved in the presence of D-lactate, addition of 20 mM sodium chloride or lithium chloride (not shown) causes a dramatic decrease in the dialyzable concentration of NPG, and over 50% of the ligand is bound.[5] When excess unlabeled NPG is added, the concentration of [6-^{3}H]NPG in the dialysate increases markedly and approximates the level observed in the absence of D-lactate and sodium chloride. Although not shown, potassium or choline chloride are completely ineffective when added in place of sodium or lithium chloride, and addition of CCCP (10 μM, final concentration) instead of unlabeled NPG also causes release of most of the bound ligand (cf. Figure 3). Finally, D-lactate and sodium or lithium chloride elicit no change in the dialyzable concentration of NPG when added to uninduced G-30 vesicles (Figure 2A, open symbols), demonstrating that binding is dependent upon the presence of TMG permease II.

The stimulatory effect of sodium exhibits saturation kinetics when NPG binding is measured as a function of increasing sodium chloride concentrations, and the concentration of sodium chloride required to produce half-maximal stimulation of binding is achieved at about 1.1 mM (not shown). This value compares favorably with the sodium concentration required for half-maximal stimulation of TMG transport (Tokuda & Kaback, 1977).

The K_D for NPG binding and the number of binding sites were also investigated (Figure 2). In these experiments, binding was measured as a function of [6-^{3}H]NPG concentration in

[3] It is clear, however, that the lack of NPG hydrolysis by permeabilized cells is not due to the operation of a P-enolpyruvate-P-transferase-mediated transport system coupled to a P-α-galactosidase, as is the case for β-galactosides in *Staphylococcus aureus* (Kennedy & Scarborough, 1967; Hengstenberg et al., 1967). Supplementation of permeabilized cells or sonic extracts with P-enolpyruvate at various concentrations does not result in the release of *p*-nitrophenol from NPG.

[4] Analysis of the sodium content of vesicle suspensions as described in Methods demonstrates that they contain approximately 0.5 mM sodium. None of the values given for sodium in this paper have been corrected for this background contamination.

[5] Although lithium-dependent NPG binding is not shown, it is important that NPG bindings observed in the presence of sodium and lithium ion are essentially identical. Since lithium stimulates the steady-state level of TMG accumulation significantly better than sodium (Tokuda & Kaback, 1977), this observation supports the argument that NPG is not accumulated in the intravesicular pool.

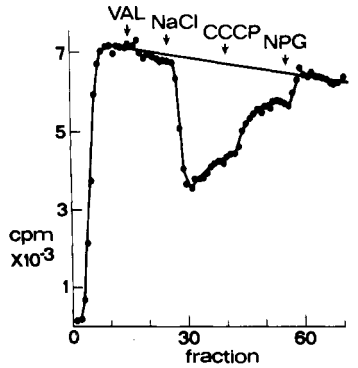

FIGURE 3: Effect of valinomycin-induced potassium efflux on NPG binding by induced G-30 vesicles. Membrane vesicles prepared as described in Experimental Procedure were centrifuged and resuspended in 0.1 M potassium phosphate (pH 6.6) to a concentration of 58 mg of protein per mL. An aliquot (0.17 mL) was added to the upper chamber of a flow dialysis apparatus which already contained 0.63 mL of 0.1 M choline phosphate (pH 6.6) and 0.01 M magnesium sulfate, and 0.1 M choline phosphate (pH 6.6) was pumped from the lower chamber for 5 min before the inception of the experiment. At this time, [6-^{3}H]NPG (2380 mCi/mmol) was added to the upper chamber at a final concentration of 3 μM, and, after equilibrium was achieved, valinomycin, sodium chloride, CCCP, and nonradioactive NPG were added sequentially at final concentrations of 12.5 μM, 20 mM, 12.5 μM, and 0.94 mM, respectively.

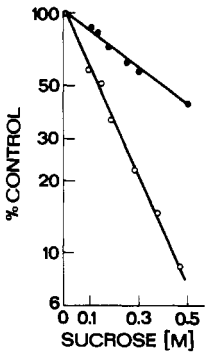

FIGURE 4: Effect of osmolarity on TMG accumulation and NPG binding. Steady-state levels of [^{14}C]TMG accumulation (○) by induced G-30 vesicles were determined at 10, 15, 20, 30, and 40 min as described previously (Tokuda & Kaback, 1977) and in Figure 1B, except that 20 mM sodium D-lactate was used rather than potassium D-lactate and sodium chloride, and sucrose was added in given concentrations. In the absence of sucrose, the vesicles accumulated 52 nmol of TMG per mg of membrane protein. NPG binding (●) was measured by flow dialysis as described in Figure 3A, except that 20 mM sodium D-lactate was used rather than potassium D-lactate and sodium chloride, sucrose was added in given concentrations, and [6-^{3}H]NPG (2380 mCi/mmol) was used at a final concentration of 6.7 μM. In addition, 0.1 M potassium phosphate (pH 6.6) containing sucrose at the same concentration as that present in the upper chamber of the flow dialysis apparatus was pumped from the lower chamber. In the absence of sucrose, 0.7 nmol of NPG was bound per mg of membrane protein. Data are presented as a percentage of control samples incubated in the absence of sucrose.

the presence of potassium D-lactate and sodium D-lactate (Figure 2B). The results are also plotted as described by Scatchard (1949) [Figure 2C]. In the absence of added sodium[4] or lithium, the K_D is approximately 3.2 μM and the number of binding sites observed at saturating concentrations of NPG is about 0.2 nmol per mg of membrane protein. In contrast, when sodium D-lactate is added, the K_D is approximately 0.6 μM and the maximum number of binding sites is 0.8 nmol per mg of membrane protein. Clearly the K_D value obtained in the presence of D-lactate and sodium ion is similar to the K_i obtained with respect to competitive inhibition of TMG uptake. In addition, although data will not be presented, it should be emphasized that TMG competitively inhibits NPG binding with a K_i of about 1 mM. Since the K_D for NPG is similar to its K_i with respect to TMG transport and the K_i for TMG with respect to NPG binding is similar to the K_m of the transport system for TMG (Tokuda & Kaback, 1977), it is likely that NPG binds to the same site in TMG permease II that catalyzes the translocation of TMG.

Previous studies on the *lac* carrier protein in *E. coli* membrane vesicles demonstrate that imposition of a potassium diffusion gradient across the membrane in such a manner that a $\Delta\Psi$ (interior negative) is established results in the appearance of high affinity binding sites for dansyl galactosides (Schuldiner et al., 1975a) and 2′-*N*-(2-nitro-4-azidophenyl)aminoethyl 1-thio-β-D-galactopyranoside dependent photoinactivation (Rudnick et al., 1975b). Generation of $\Delta\Psi$ in this manner causes binding of NPG in this system as well provided sodium is present (Figure 3). In the experiment shown, induced G-30 vesicles prepared in potassium phosphate were placed in the upper chamber of the flow dialysis apparatus and choline phosphate was pumped through the lower chamber. [6-^{3}H]-NPG was then added, and, after equilibrium was achieved, valinomycin and subsequently, sodium chloride, CCCP, and unlabeled NPG were added sequentially to the vesicles. On addition of valinomycin, a small amount of binding is observed which is markedly enhanced by addition of sodium chloride. The binding equilibrium achieved under these conditions is considerably more unstable than that observed in the presence of D-lactate (Figure 2), an effect which presumably results from the transient nature of the ion diffusion gradients (Schuldiner & Kaback, 1975). In any event, CCCP accelerates the release of ligand, although a small but significant fraction remains bound and is released only after addition of unlabeled NPG.

Effect of Osmolarity on NPG Binding Relative to TMG Transport. The effect of osmolarity on the steady-state level of solute accumulation has been used as a means of differentiating transport from binding (Hopfer & Sigrist-Nelson, 1974; Lever, 1976). Since the absolute level of solute accumulation is dependent upon the volume of the intravesicular space, solute accumulation should be more sensitive to changes in osmolarity than binding. The data presented in Figure 4 illustrate the effect of increasing sucrose concentrations on D-lactate-induced, sodium-dependent TMG accumulation and NPG binding under the same conditions. Although increasing sucrose concentrations inhibit NPG binding to a significant extent, it is evident that TMG accumulation is affected much more severely. As such, these observations provide additional support for the contention that NPG is bound but not accumulated in the intravesicular space.

Effect of Variations in $\Delta\bar{\mu}_{H^+}$, $\Delta\Psi$, ΔpH, and ΔpNa on NPG Binding. Both *E. coli* (Ramos et al., 1976; Ramos & Kaback, 1977a,b) and *S. typhimurium* (Tokuda & Kaback, 1977) membrane vesicles generate a large ΔpH (interior alkaline) as a result of D-lactate or reduced phenazine methosulfate oxidation, and this component of $\Delta\bar{\mu}_{H^+}$ (and as a result, $\Delta\bar{\mu}_{H^+}$ itself) varies dramatically with external pH, while $\Delta\Psi$ remains essentially constant. D-Lactate-induced, sodium-dependent NPG binding does not vary markedly with external pH, but exhibits a relatively flat pH profile (data not shown), suggesting preferential dependence on $\Delta\Psi$.

ΔpH and $\Delta\Psi$ can be varied reciprocally in the presence of the ionophores valinomycin and nigericin, and these manipulations have provided a powerful means of investigating the coupling between individual transport systems and $\Delta\bar{\mu}_{H^+}$ or its components (Ramos et al., 1976; Ramos & Kaback,

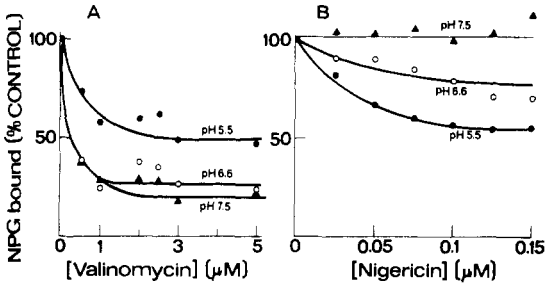

FIGURE 5: D-Lactate-induced, sodium-dependent NPG binding in the presence of valinomycin and nigericin. Induced G-30 vesicles were suspended in 0.1 M potassium phosphate at given pH values as described previously (Tokuda & Kaback, 1977). NPG binding was determined by flow dialysis at pH 5.5 (●), 6.6 (○), and 7.5 (▲) as described in Figure 6. After binding equilibrium was achieved in the presence of sodium D-lactate, valinomycin (A) or nigericin (B) was added to given final concentrations, and the amount of NPG binding was determined. In the absence of ionophores, the following quantities of NPG were bound (in nmol per mg of membrane protein): 0.41 at pH 5.5; 0.54 at pH 6.6; 0.37 at pH 7.5.

TABLE I: Effect of Valinomycin and Nigericin on ΔpH, ΔΨ, $\Delta\bar{\mu}_{H^+}$, and NPG Binding at pH 6.6.

Ionophore	ΔpH[a]	ΔΨ[b] (% control)	$\Delta\bar{\mu}_{H^+}$[c]	NPG binding[d]
Valinomycin, 3 μM	74	29	54	26
Nigericin, 0.1 μM	0	135	58	79

[a] ΔpH was determined from flow dialysis experiments carried out with [1,2-^{14}C]acetate as described previously (Tokuda & Kaback, 1977) and in Methods. The control value obtained in the absence of ionophores was about −65 mV. [b] ΔΨ was determined from flow dialysis experiments carried out with [^{3}H]TPMP$^+$ as described previously (Tokuda & Kaback, 1977) and in Methods. The control value obtained in the absence of ionophores was about −50 mV. [c] $\Delta\bar{\mu}_{H^+}$ was calculated from ΔpH and ΔΨ as described previously (Tokuda & Kaback, 1977). The control value obtained in the absence of ionophores was −115 mV. [d] NPG binding was measured as described in Figure 3, except that 20 mM sodium D-lactate was used rather than potassium D-lactate and sodium chloride, and 0.1 M potassium phosphate (pH 6.6) was pumped from the lower chamber. The control value was 0.5 nmol per mg of membrane protein.

1977a–c; Tokuda & Kaback, 1977). When this approach is applied to D-lactate-induced, sodium-dependent NPG binding, the results shown in Figure 5 are obtained. NPG binding responds to increasing concentrations of valinomycin (Figure 5A) and nigericin (Figure 5B) in a manner that is generally similar to the effect of these ionophores on $\Delta\bar{\mu}_{H^+}$ (Tokuda & Kaback, 1977). That is, NPG binding is progressively inhibited or relatively unaffected by increasing concentrations of either ionophore at external pH values of 5.5, 6.6, and 7.5. Moreover, it is clear that the inhibitory effect of valinomycin increases with external pH, while inhibition by nigericin disappears as external pH is increased. Thus, as external pH is increased, NPG binding becomes progressively more dependent upon ΔΨ (which remains constant) and less dependent upon ΔpH (which decreases drastically as external pH is increased over this range).

Although NPG binding appears to vary with $\Delta\bar{\mu}_{H^+}$ primarily, there is a bias toward ΔΨ even at pH values below pH 7.5. This point is illustrated best by the data presented in Table I where the effects of fixed concentrations of valinomycin and nigericin on NPG binding and $\Delta\bar{\mu}_{H^+}$, ΔΨ, and ΔpH at pH 6.6 are tabulated. Inhibition of NPG binding by valinomycin correlates reasonably well with the effect of the ionophore on ΔΨ. Nigericin does not stimulate binding as it does ΔΨ, but it is evident that this ionophore completely dissipates ΔpH and

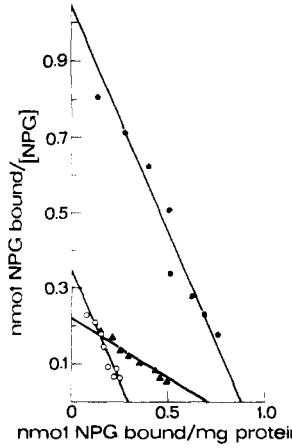

FIGURE 6: Effect of valinomycin and monensin on the kinetics of D-lactate-induced, sodium-dependent NPG binding. NPG binding by induced G-30 vesicles was measured at given concentrations of [6-^{3}H]NPG as described in Figures 3B and 6. Studies were carried out in the absence of ionophores (●) and in the presence of 0.45 μM valinomycin (▲) or 0.45 μM monensin (○). The data obtained were plotted according to Scatchard (1949). Although the vesicles used previously were relatively insensitive to 0.45 μM valinomycin (cf. Figure 5 in Tokuda & Kaback, 1977), this concentration of valinomycin reduced ΔΨ by about 70% in the preparations used in these experiments.

that it inhibits $\Delta\bar{\mu}_{H^+}$ more effectively than NPG binding.

The observations presented in Figure 6 are derived from experiments in which [6-^{3}H]NPG binding was determined at various concentrations in the presence of sodium D-lactate alone and with given concentrations of valinomycin or monensin. Data obtained with control samples incubated in the absence of ionophores are similar to those presented in Figure 2 (i.e., the K_D is about 0.8 μM and the number of binding sites at saturation is 0.85 nmol per mg of membrane protein). When ΔΨ is diminished by addition of valinomycin (Tokuda & Kaback, 1977), the K_D increases to approximately 3.2 μM, while the number of binding sites observed at saturation remains relatively high (i.e., about 0.7 nmol per mg of membrane protein). On the other hand, when the sodium gradient (ΔpNa) is dissipated by addition of monensin (Ashton & Steinrauf, 1970), the K_D remains unaltered, while the maximum number of binding sites is diminished to about 0.3 nmol per mg of membrane protein.

Since a significant number of high affinity binding sites is observed in the presence of monensin (i.e., under conditions where ΔpNa is abolished), it is possible to determine the effect of sodium itself on the kinetics of NPG binding in the absence of ΔpNa. Thus, when NPG binding is studied in the presence of monensin and two concentrations of sodium chloride (Figure 7), it is apparent that sodium decreases the K_D for NPG but has little or no effect on the number of binding sites at saturating NPG concentrations.

Given the observation that valinomycin increases the K_D for NPG binding without significant change in the number of binding sites, the ionophore would be expected to inhibit the initial rate of TMG transport in an apparently competitive fashion. This predicition is borne out by the data presented in Figure 8 where it is shown that valinomycin increases the apparent K_m for TMG transport without a discernible change in V_{max}.[6] Although not shown, monensin inhibits noncom-

[6] On the other hand, when the steady-state level of TMG accumulation is measured as a function of TMG concentration, valinomycin does not affect the TMG concentration at which half-maximal steady-state levels of accumulation are observed, and double-reciprocal plots exhibit a "noncompetitive" pattern. Thus, if NPG binding represented accumulation in the intravesicular pool, valinomycin would be expected to alter the number of binding sites rather than the K_D.

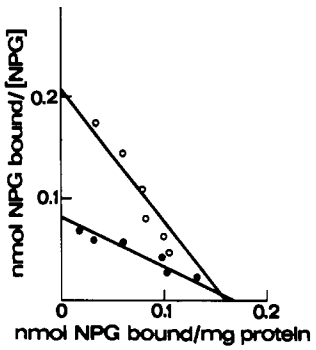

FIGURE 7: Effect of sodium on the kinetics of NPG binding in the presence of monensin. NPG binding at given concentrations of [6-^{3}H]NPG was measured in the presence of potassium D-lactate as described in Figure 3B, except that 0.5 μM monensin was present and the sodium chloride concentration was 20 mM (○) or 0.5 mM (●). The data are plotted as described by Scatchard (1949).

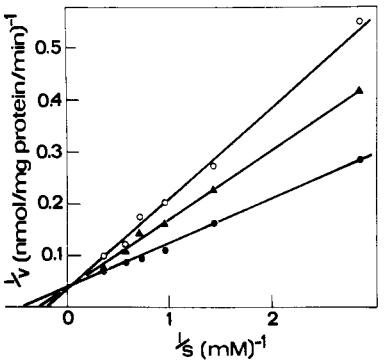

FIGURE 8: Effect of valinomycin on initial rates of TMG uptake by induced G-30 vesicles. Initial rates of [^{14}C]TMG uptake were measured as described in Figure 2, except that valinomycin was also added at the following final concentrations: (●) no valinomycin added; (▲) 0.2 μM; (○) 0.4 μM. The values obtained are presented as a double reciprocal plot.

petitively (i.e., V_{max} is depressed without a significant change in the apparent K_m).

NPG Binding in the Absence of D-*Lactate.* Addition of 20 mM sodium chloride to a concentrated suspension of induced G-30 vesicles which have been equilibrated with [6-^{3}H]NPG results in a sharp decrease in the concentration of ligand in the dialysate, followed by partial release of ligand and a new equilibrium (Figure 9A). Addition of excess unlabeled NPG at this point causes release of ligand, and the concentration of NPG in the dialysate quickly returns to the control level. The data presented in Figures 9B and 9C indicate that binding induced by addition of sodium chloride is not due merely to the presence of sodium per se, but to ΔpNa. Thus, CCCP inhibits the absolute amount of binding by about 35% without altering the shape of the curve, while monensin inhibits binding much more potently and abolishes the "overshoot". Since CCCP increases the proton permeability of the vesicles, it seems likely that its effect on NPG binding is related to collapse of $\Delta\Psi$ (interior negative) induced by a chloride diffusion potential (Grollman et al., 1977; Lever, 1978). Monensin, on the other hand, catalyzes proton/sodium exchange (Ashton & Steinrauf, 1970) and thereby collapses ΔpNa. The small amount of binding observed in the presence of monensin approximates the difference in the curves obtained without and with CCCP (i.e., curve A minus curve B) and presumably represents the contribution of the chloride diffusion potential in the presence of sodium. It is also important that NPG binding is not observed on addition of potassium or choline chloride (D) nor with vesicles prepared from uninduced cells (E).

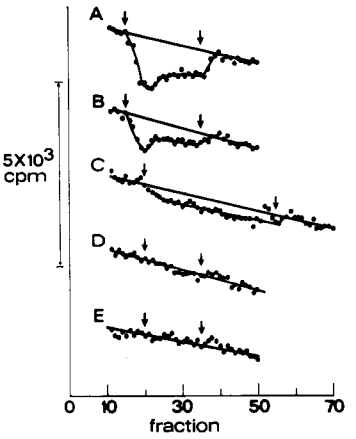

FIGURE 9: Sodium gradient-induced NPG binding. Induced (A–D) and uninduced (E) G-30 vesicles were suspended in 0.1 M potassium phosphate (pH 6.6) containing 0.01 M magnesium sulfate to a final concentration of 12 mg of protein per mL. Aliquots (0.8 mL) were placed in the upper chamber of a flow dialysis apparatus and 0.1 M potassium phosphate (pH 6.6) was pumped from the lower chamber. At the inception of the experiments (i.e., 0 on the abscissa), [6-^{3}H]NPG (2380 mCi/mmol) was added to the upper chamber to a final concentration of 3 μM. After equilibrium was achieved, sodium chloride (A, B, C, and E) or potassium (or choline) chloride (D) [first arrow] and then nonradioactive NPG (second arrow) were added to final concentrations of 20 mM and 0.94 mM, respectively. In the experiments shown in B and C, CCCP (B) and monensin (C) were added to the upper chamber to final concentrations of 13 μM and 5 μM, respectively, 5 min prior to the start of the experiment. Although curves A through E are displayed in decreasing order, the levels of radioactivity obtained initially in the dialysate (fraction 10) were approximately 6500 cpm in each case (i.e., curves A through E should be superimposed).

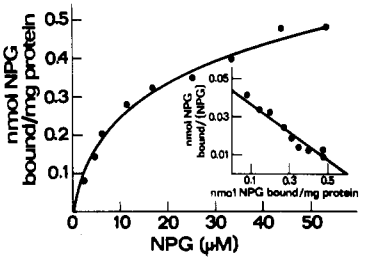

FIGURE 10: Effect of ΔpNa on the kinetics of NPG binding. For each experiment point shown, a separate flow dialysis experiment was carried out with induced G-30 vesicles in the presence of CCCP as described in Figure 9B, except that [6-^{3}H]NPG was added to the upper chamber at the concentrations given on the abscissa. Maximum binding of NPG was observed at around fraction 25 (cf. Figure 9B) in each case, and the quantity of NPG bound was calculated from this point. Inset: Data plotted according to Scatchard (1949).

ΔpNa-Induced binding of NPG in the presence of CCCP can be quantitated as described in Figure 10.The data presented in the body of the figure demonstrate that NPG binding under these conditions is a saturable function of NPG concentration. Furthermore, when the data are treated according to Scatchard (1949) [Figure 10, inset], the K_D is calculated to be about 14 μM and there are approximately 0.6 nmol of NPG bound per mg of membrane protein at saturation. Thus, imposition of ΔpNa induces the appearance of a large number of binding sites but their affinity is relatively low.

Discussion

NPG as a Nonpenetrating Ligand Which Binds to TMG Permease II. Although not as complete as the arguments that certain ligands bind to the *lac* carrier protein in *E. coli* membrane vesicles but are not translocated (for reviews, cf.

TABLE II: Summary of NPG Binding under Various Conditions.

Experimental conditions	ΔΨ	ΔpNa	Na	NPG binding K_D (μM)	NPG binding No. binding sites (nmol/mg of protein)
1. D-Lactate[a]	+	↓[b]	↓	3.2	0.2
2. D-Lactate + NaCl	+	+	+	0.7	0.8
3. (2) + monensin[c]	+	−	+	0.7	0.3
4. (2) + valinomycin[c]	↓[d]	+	+	3.2	0.7
5. NaCl + CCCP[e]	−	+	+	13.8	0.6

[a] Data obtained from Figure 2. [b] The sodium concentration under these conditions is 0.5 mM (cf. footnote 4 in the text). [c] Data obtained from Figure 6. [d] Under these conditions, ΔΨ is reduced by about 70% (cf. legend to Figure 6). [e] Data obtained from Figure 10.

Schuldiner et al., 1976b; Schuldiner & Kaback, 1977), the evidence presented here suggests that NPG behaves similarly with respect to the α-galactoside porter in *S. typhimurium* G-30 vesicles. Clearly, the ligand competitively inhibits TMG transport with a K_i that is comparable to its binding constant. Moreover, TMG competitively inhibits NPG binding with a K_i that is similar to the K_m of the transport system for TMG. The contention that NPG is not translocated is somewhat more tenuous, however, and is based on the following evidence: (i) The ligand is not accumulated to a significant extent by either whole cells or membrane vesicles when transport is assayed by standard filtration techniques, and there is no stimulation by sodium or lithium ion; (ii) NPG is hydrolyzed very slowly by intact cells;[2] (iii) although lithium stimulates TMG accumulation significantly better than sodium, both cations stimulate NPG binding to essentially the same extent;[5] (iv) steady-state levels of TMG accumulation are inhibited more drastically than NPG binding by increasing osmolarity; and (v) valinomycin increases the K_D for NPG binding and the K_m for TMG transport, but has no effect on the TMG concentration which yields half-maximal steady-state levels of accumulation.[6] Unfortunately, neither NPG binding nor TMG accumulation is inhibited by *N*-ethylmaleimide or *p*-chloromercuribenzenesulfonate, both of which react directly with the *lac* carrier protein in *E. coli* and displace bound ligand (Reeves et al., 1973; Schuldiner et al., 1975b; Rudnick et al., 1976). Thus, it has not been possible to demonstrate directly that chemical inactivation of the α-galactoside porter causes release of bound ligand.

Previous studies on the β-galactoside transport system indicate that there are 1 or 2 nmol of *lac* carrier protein (i.e., M protein) per mg of membrane protein (Jones & Kennedy, 1969; Reeves et al., 1973; Schuldiner et al., 1975a,c, 1976a, 1977; Rudnick et al., 1976). On the other hand, the studies reported here demonstrate that there are about 0.8 nmol of TMG permease II per mg of membrane protein (Figures 3 and 8). It is important to note, however, that most of the studies on the *lac* carrier protein were carried out with membrane vesicles prepared from the ML strain of *E. coli*. These vesicles contain little contaminating outer membrane protein (Kaback, 1971), while vesicles prepared from *S. typhimurium* by the methods used here may contain as much as 50% outer membrane (Osborn et al., 1972). Thus, it seems probable that both porters are present to approximately the same extent in their respective membranes.

NPG Binding. Studies on the *lac* carrier protein (cf. Schuldiner et al., 1976b; Schuldiner & Kaback, 1977) and other transport systems (Ramos & Kaback, 1977b,c; Tokuda & Kaback, 1977) in isolated membrane vesicles indicate that carrier molecules play a dynamic role in coupling the transport of solutes to the electrochemical ion gradients which provide the driving force for accumulation. Experiments presented here (pertinent results of which are summarized in Table II) provide a strong indication that a similar statement is justified with regard to the α-galactoside porter in *S. typhimurium* vesicles. In order to generate a maximum number of high affinity binding sites for NPG, it is apparent that $\Delta\bar{\mu}_{H^+}$, sodium, and ΔpNa are necessary. Moreover, it seems highly likely that these components of the total driving force function in different capacities. Monensin, an ionophore which collapses ΔpNa and stimulates or has no effect on ΔΨ,[7] diminishes the available number of binding sites without altering their affinity in the presence of D-lactate. Sodium, when added in the presence of D-lactate and monensin (i.e., when ΔΨ is present but ΔpNa cannot be established), increases the affinity of the available binding sites. On the other hand, imposition of ΔpNa ($Na_{out} > Na_{in}$) in the absence of ΔΨ gives rise to a large number of binding sites which have relatively low affinity for NPG. In summary, therefore, it appears that sodium per se increases the affinity of the porter for ligand but only if ΔΨ (interior negative) is present and ΔpNa increases the number of binding sites independent of ΔΨ. These conclusions are consistent with the effect of valinomycin which diminishes (but does not abolish) ΔΨ and presumably has little or no effect on ΔpNa (Tokuda & Kaback, 1977). In the presence of this ionophore, the vesicles exhibit little change in the number of binding sites, but the K_D for NPG is increased about fourfold. Stated otherwise, diminution in ΔΨ appears to block sodium from increasing the affinity of the carrier for NPG, although ΔpNa which is unaffected maintains a relatively large number of NPG binding sites.

These results do not lead to an elucidation of sodium/TMG symport in molecular terms, but they do suggest certain important conclusions regarding the translocation mechanism. From this and previous work (Tokuda & Kaback, 1977), it seems apparent that $\Delta\bar{\mu}_{H^+}$ has both direct and indirect effects on the α-galactoside porter. Through the mediation of the proton/sodium antiporter, $\Delta\bar{\mu}_{H^+}$ is responsible for the generation of ΔpNa, the immediate thermodynamic driving force for sodium/TMG symport. In addition, ΔpNa in itself can increase the number of NPG binding sites on the external surface of the membrane. However, $\Delta\bar{\mu}_{H^+}$ (preferentially ΔΨ) also has a direct effect on the porter which alters its interaction with sodium so as to allow the cation to effect an increase in binding affinity for NPG. Since sodium-dependent NPG binding is induced by an artificially imposed ΔΨ (interior negative), it is tempting to suggest that this porter, like the *lac* carrier protein (Schuldiner et al., 1975b; Rudnick et al., 1975a,b, 1976), may be negatively charged, and that the electric field across the membrane induces a conformational change which exposes or increases the affinity of a sodium binding site. Although the observation that ΔpNa increases the number of NPG binding sites in the absence of $\Delta\bar{\mu}_{H^+}$ would seemingly favor the latter interpretation, it is premature to speculate since we have not measured sodium binding per se. In any event, the results taken as a whole provide an indication that sodium binding occurs prior to binding and translocation of solute.

Despite certain obvious conceptual similarities between sodium/TMG symport and proton/lactose symport, obser-

[7] Monensin has effects on ΔΨ, ΔpH, and $\Delta\bar{\mu}_{H^+}$ which are essentially the same as those reported for nigericin (cf. Figure 5 in Tokuda & Kaback, 1977).

vations presented here indicate that some of the analogies may break down when the mechanisms are scrutinized in more detail. Transport of β-galactosides (Kepes, 1960; Barnes & Kaback, 1970) and binding of various nonpenetrating ligands by the *lac* carrier protein (Schuldiner et al., 1975a–c, 1976a; Rudnick et al., 1976) are blocked by sulfhydryl reagents, and it has been demonstrated directly (Fox & Kennedy, 1965) that the *lac* carrier protein contains a sulfhydryl group which is essential for activity. In contrast, sodium-dependent TMG transport and NPG binding are completely impervious to inhibition by *N*-ethylmaleimide, *p*-chloromercuribenzenesulfonate, and other sulfhydryl reagents. With the β-galactoside transport system, only a small number of binding sites are detected in the absence of energization, and generation of $\Delta\bar{\mu}_{H^+}$ causes an increase in the number of binding sites without a change in K_D (Rudnick et al., 1976). Furthermore, variations in $\Delta\Psi$ and addition of inhibitors such as dinitrophenol, cyanide (Schuldiner et al., 1975a), and nigericin (Robertson, D. E., Tokuda, H., & Kaback, H. R., in preparation) decrease the number of binding sites without a change in K_D. Alternatively, dissipation of $\Delta\Psi$ in this system effects a decrease in affinity for NPG with little change in the number of binding sites and collapse of ΔpNa decreases the number of binding sites without a change in K_D. Finally, acidification of the external medium causes no increase in binding of 2′-(*N*-dansyl)aminoethyl 1-thio-β-D-galactopyranoside by the *lac* carrier protein (Schuldiner et al., 1975a), while ΔpNa increases the number of NPG binding sites in this system.

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References

Ashton, R., & Steinrauf, L. K. (1970) *J. Mol. Biol. 49,* 547.

Barnes, E. M., Jr., & Kaback, H. R. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66,* 1190.

Colowick, S. P., & Womack, F. C. (1969) *J. Biol. Chem. 244,* 774.

Crane, R. K. (1978) in *Reviews on Physiology, Biochemistry, and Pharmacology,* Springer-Verlag, Heidelberg (in press).

Fox, C. F., & Kennedy, E. P. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54,* 891.

Grollman, E. F., Lee, G., Ambesi-Impiombato, F. S., Meldolesi, M. F., Aloj, S. M., Coon, H. G., Kaback, H. R., & Kohn, L. D. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 2352.

Harold, F. M. (1972) *Bacteriol. Rev. 36,* 172.

Hengstenberg, W., Egan, J. B., & Morse, M. L. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58,* 274.

Hopfer, U., & Sigrist-Nelson, K. (1974) *Nature (London) 252,* 422.

Jones, T. H. D., & Kennedy, E. P. (1969) *J. Biol. Chem. 244,* 5981.

Kaback, H. R. (1971) *Methods Enzymol. 22,* 99.

Kaback, H. R. (1976) *J. Cell. Physiol. 89,* 575.

Kennedy, E. P., & Scarborough, G. A. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58,* 225.

Kennedy, E. P., Rumley, M. K., & Armstrong, J. B. (1974) *J. Biol. Chem. 249,* 33.

Kepes, A. (1960) *Biochim. Biophys. Acta 40,* 70.

Lever, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 2614.

Lever, J. (1978) *J. Biol. Chem.* (in press).

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193,* 265.

Mitchell, P. (1973) *J. Bioenerg. 4,* 63.

Osborn, M. J., Gander, J. E., Parisi, E., & Carson, J. (1972) *J. Biol. Chem. 247,* 3962.

Prestidge, L. S., & Pardee, A. B. (1965) *Biochim. Biophys. Acta 100,* 591.

Ramos, S., & Kaback, H. R. (1977a) *Biochemistry 16,* 848.

Ramos, S., & Kaback, H. R. (1977b) *Biochemistry 16,* 854.

Ramos, S., & Kaback, H. R. (1977c) *Biochemistry 16,* 4271.

Ramos, S., Schuldiner, S., & Kaback, H. R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 1892.

Reeves, J. P., Shechter, E., Weil, R., & Kaback, H. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70,* 2722.

Rudnick, G., Weil, R., & Kaback, H. R. (1975a) *J. Biol. Chem. 250,* 1371.

Rudnick, G., Weil, R., & Kaback, H. R. (1975b) *J. Biol. Chem. 250,* 6847.

Rudnick, G., Schuldiner, S., & Kaback, H. R. (1976) *Biochemistry 15,* 5126.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51,* 660.

Schuldiner, S., & Kaback, H. R. (1975) *Biochemistry 14,* 5451.

Schuldiner, S., & Kaback, H. R. (1977) *Biochim. Biophys. Acta 472,* 399.

Schuldiner, S., Kerwar, G. K., Weil, R., & Kaback, H. R. (1975a) *J. Biol. Chem. 250,* 1361.

Schuldiner, S., Kung, H., Weil, R., & Kaback, H. R. (1975b) *J. Biol. Chem. 250,* 3679.

Schuldiner, S., Spencer, R. D., Weber, G., Weil, R., & Kaback, H. R. (1975c) *J. Biol. Chem. 250,* 8893.

Schuldiner, S., Weil, R., & Kaback, H. R. (1976a) *Proc. Natl. Acad. Sci. U.S.A. 73,* 109.

Schuldiner, S., Rudnick, G., Weil, R., & Kaback, H. R. (1976b) *Trends Biochem. Sci. 1,* 41.

Schuldiner, S., Weil, R., Robertson, D. E., & Kaback, H. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 1851.

Schultz, S. G., & Curran, P. F. (1970) *Physiol. Rev. 50,* 637.

Small, H., Stevens, T., & Bauman, W. C. (1975) *Anal. Chem. 47,* 1801.

Stock, J., & Roseman, S. (1971) *Biochem. Biophys. Res. Commun. 44,* 132.

Tokuda, H., & Kaback, H. R. (1977) *Biochemistry 16,* 2130.