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Transport of Branched-Chain Amino Acids in Membrane Vesicles of *Streptococcus cremoris*

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The kinetics, specificity, and mechanism of branched-chain amino acid transport in *Streptococcus cremoris* were studied in a membrane system of *S. cremoris* in which beef heart mitochondrial cytochrome *c* oxidase was incorporated as a proton motive force (Δp)-generating system. Influx of L-leucine, L-isoleucine, and L-valine can occur via a common transport system which is highly selective for the L-isomers of branched chain amino acids and analogs. The pH dependency of the kinetic constants of Δp-driven L-leucine transport and exchange (counterflow) was determined. The maximal rate of Δp-driven transport of L-leucine (V_{max}) increased with increasing internal pH, whereas the affinity constant increased with increasing external pH. The affinity constant for exchange (counterflow) varied in a similar fashion with pH, whereas V_{max} was pH independent. Further analysis of the pH dependency of various modes of facilitated diffusion, i.e., efflux, exchange, influx, and counterflow, suggests that H^+ and L-leucine binding and release to and from the carrier proceed by an ordered mechanism. A kinetic scheme of the translocation cycle of H^+–L-leucine cotransport is suggested.

Streptococci are fastidious bacteria. They are auxotrophic for most amino acids and therefore strongly dependent on the exogenous supply of these nutrients. Several aspects of amino acid transport have been studied in intact cells of *Streptococcus faecalis* (3, 9), *S. lactis* (19, 20, 23, 24), *S. cremoris* (19), *S. agalactiae* (15), *S. thermophilus* (1), *S. pneumonia* (25), and *S. pyrogenes* (22). The proton motive force (Δp) in these bacteria can be generated by the proton-translocating ATPase or by end-product efflux (17). The generation of Δp in several *Streptococcus* species has been studied under different conditions (12, 13). Δp functions as the driving force for the uptake of several solutes (13a). The mechanism of energy coupling to solute transport in streptococci appears to be more complex than was initially thought. Conclusions about the mode of energy coupling are usually drawn from the effects of uncouplers, ionophores, and inhibitors of the membrane-bound H^+-translocating ATPase and glycolytic enzymes. In intact cells, these inhibitors usually exert their effect on more than one metabolic process. For example, uncouplers and ionophores which dissipate ΔpH also cause changes in internal pH and can deplete the ATP pool to a significant extent (20). Furthermore, manipulation of the ΔpH can result in complex changes in the activity of the transport system which are not related to ΔpH as a driving force (24) but are due to a regulatory effect of the internal pH (8).

Recently, we described a method to study secondary transport of solutes in isolated membrane vesicles that lack a suitable primary proton pump (6, 7). This method uses a membrane fusion step to insert a reconstituted primary proton pump into the bacterial membrane. Membrane fusion can be accomplished by a freeze-thaw-sonication step (6–8; A. J. M. Driessen, K. J. Hellingwerf, and W. N. Konings, J. Biol. Chem., in press) which results in the formation of tightly sealed vesicles. In this way, beef heart mitochondrial cytochrome *c* oxidase has been incorporated into membrane vesicles from *S. cremoris*. Since the electron donor-reduced cytochrome *c* only binds to cytochrome *c* oxidase molecules with their binding sites located on the outer surface of the

membrane, Δp generation with a defined polarity (interior, negative and alkaline) is assured. With the electron-donor system consisting of ascorbate-N,N,N',N'-tetramethyl-*p*-phenylene diamine (TMPD)-cytochrome *c*, a Δp of considerable magnitude can be generated and is maintained for at least 60 min. With membrane vesicles, a reliable and accurate determination of the magnitude of the Δp is possible, and the composition of the Δp can be easily manipulated by the use of ionophores. This well-defined fused membrane system is more amenable to detailed experimental studies of the kinetics, the specificities, and the mechanism of solute transport in streptococci.

The mechanism of energy coupling to entry and exit of neutral and branched-chain amino acids has been studied with such fused membranes (Driessen et al., in press). It was concluded that these amino acids are translocated in symport with one proton-amino acid molecule. The apparent complex relationship between the Δp and transport of neutral amino acids (8; Driessen et al., in press) could be explained by a regulatory effect of the internal pH on the activity of the L-serine and L-alanine carrier (8). In this paper, we describe the specificity, kinetic properties, and pH dependence of branched-chain amino acid transport in *S. cremoris*. A scheme is presented of the sequential events in H^+-L-leucine cotransport based on the impact of the pH and Δp on facilitated diffusion.

MATERIALS AND METHODS

Materials. ^{14}C-labeled amino acids were obtained from Amersham Corp., Buckinghamshire, England, or from Du Pont N.E.M., Dreieich, Germany. The following uniformly ^{14}C-labeled compounds were used: L-leucine (12.4 TBq/mol), L-isoleucine (13.3 TBq/mol), and L-valine (10.7 TBq/mol). Octyl-β-D-glucopyranoside, crude asolectin, and horse heart cytochrome *c* were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of reagent grade and were acquired from commercial sources.

Growth of *S. cremoris* and isolation of membrane vesicles. *S. cremoris* Wg2 (Prt⁻) was grown anaerobically in MRS broth (4) at a controlled pH of 6.4 in a 5-liter fermenter.

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Membrane vesicles were prepared as previously described (16) and stored in liquid nitrogen in 50 mM potassium phosphate (pH 7.0) containing 10 mM $MgSO_4$ at a concentration of 10 to 15 mg of protein per ml.

Purification and reconstitution of cytochrome c oxidase. Cytochrome c oxidase was isolated from beef heart as previously described (30) and reconstituted into asolectin liposomes by detergent dialysis (5–7).

Fusion of liposomes with S. cremoris membrane vesicles. Fusion of S. cremoris membrane vesicles and cytochrome c oxidase proteoliposomes or oxidase-free liposomes was effected by freeze-thaw sonication as described (6, 7).

Transport assays. Amino acid transport upon imposition of a $\Delta\psi$ or ΔpH (6) or driven by a Δp generated by cytochrome c oxidase activity (6–8; Driessen et al., in press) was assayed as described previously. More than 98% of liposomal phospholipid, estimated from the fluorescence of residual N-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine incorporated into the asolectin liposomes (5 mol% phospholipid), was retained on the filters under these conditions.

For kinetic analysis of amino acid uptake, experiments were performed in tubes (7) and rates of Δp-driven uptake of branched-chain amino acids were determined from the amount of label accumulated during the first 15 s, using a concentration range between 1.5 and 100 μM. For imposed Δp-driven uptake and counterflow experiments, the transport rates were determined from the amount of label accumulated within 5 and 10 s, respectively. Results were analyzed by Eady-Hofstee plots. Inhibitor constants (K_i) were determined by the method of Dixon. Apparent inhibitor constants (K_i') were estimated from the inhibition of L-leucine uptake (J_0) at fixed 3 μM L-leucine ([Leu]) and 3 μM to 5 mM inhibitor ([I]) concentration by using the following relation (21):

$$K_i' = \frac{J_I}{(J_0 - J_I)} \cdot \frac{(K_t \cdot [I])}{(K_t + [Leu])}$$

where leucine uptake was measured in the absence (J_0) and presence (J_I) of inhibitor and K_t is the affinity constant of L-leucine uptake in the absence of inhibitor. Inhibitors were used at concentrations which inhibited leucine transport by approximately 50%.

L-Leucine efflux, exchange, and counterflow experiments were performed as described previously for L-alanine and L-serine (8), except that S. cremoris membrane vesicles were loaded with 1 mM L-[^{14}C]leucine, unless indicated otherwise. All facilitated diffusion experiments performed in the absence of Δp were carried out in the presence of the uncoupler 5-chloro-3-tert-butyl-2'-chloro-4'-nitrosalicyl-anilide (5 μM). For artificially imposed $\Delta\psi$, valinomycin was added to a suspension of membrane vesicles to a final concentration of 2 nmol/mg of protein. The membrane vesicles were washed and suspended in 50 mM potassium phosphate or 50 mM sodium phosphate of the desired pH supplemented with 25 mM methylamine and 1 mM L-[^{14}C]leucine. Samples (2 μl) of potassium-loaded vesicles (25 mg of protein per ml) were either diluted into 400 μl of potassium phosphate of similar pH (no gradient) or 50 mM sodium phosphate of similar pH ($\Delta\psi$; interior negative) supplemented with 5 mM $MgSO_4$. Sodium-loaded membrane vesicles were diluted into sodium-containing buffer (no gradient), or potassium-containing buffer ($\Delta\psi$; interior positive) as described for potassium-loaded vesicles. During exchange, 1 mM L-leucine was present in the external solution.

For the imposition of Δp, membrane vesicles were washed and suspended in 20 mM potassium phosphate of the desired pH, supplemented with 100 mM potassium acetate, in the presence of valinomycin. For exchange and efflux, 1 mM L-[^{14}C]leucine was present during equilibration. Subsequently, membrane vesicles were diluted into 20 mM sodium phosphate of the desired pH containing 100 mM sodium piperazine-N,N'-bis(2-ethanesulfonate) (PIPES) (Δp; interior negative and alkaline), 20 mM sodium phosphate containing 100 mM sodium acetate ($\Delta\psi$; interior negative), 20 mM potassium phosphate containing 100 mM potassium PIPES (ΔpH; interior alkaline), or 20 mM potassium phosphate containing 100 mM potassium acetate (no gradient), in all cases supplemented with 5 mM $MgSO_4$.

For calculations, a value of 8 μl/mg of protein was used as the internal volume of the fused membranes (6, 7). Efflux and exchange experiments were analyzed by subtracting the amount of radioactivity retained on the filter after a 60-min incubation from the samples assayed at different times. The logarithm of the intravesicular solute concentration was plotted as a function of time.

Binding of L-leucine. L-Leucine binding was measured by flow dialysis (29). L-[^{14}C]leucine (5 to 15 μl; 0.15 mM) was added to 0.4 ml of a suspension of S. cremoris membrane vesicles (20 mg of protein per ml) fused with asolectin liposomes in 50 mM potassium phosphate (pH 6.0). After 20 min, bound L-leucine was determined by the addition of 4 μl 50 mM L-leucine (final concentration, 0.5 mM). Flow dialysis was performed as described (11).

Measurement of $\Delta\psi$ and ΔpH. $\Delta\psi$ was determined from the distribution of tetraphenylphosphonium (Ph$_4$P$^+$) assessed with a Ph$_4$P$^+$-selective electrode (5, 7). ΔpH was determined from the fluorescence of pyranine entrapped in membrane vesicles as described previously (5, 7).

Protein determination. Protein was assayed by the method of Lowry et al. (14) with bovine serum albumin as a standard.

RESULTS

Kinetic analysis of branched-chain amino acid transport. The branched-chain amino acids L-leucine, L-isoleucine, and L-valine were accumulated 10- to 20-fold at pH 6.0 by S. cremoris membrane vesicles fused with cytochrome c oxidase proteoliposomes when these were supplied with the electron donor system ascorbate-TMPD-cytochrome c (not shown). A steady-state level of accumulation was reached in 4 to 6 min. Eady-Hofstee plots of the initial rates of L-leucine uptake measured as a function of increasing L-leucine concentrations are shown in Fig. 1. When the L-leucine concentration was varied from 1.5 to 250 μM, biphasic kinetics of L-leucine transport were observed. At pH 7.0, the high-affinity transport system exhibited a K_t of 6.5 μM and a V_{max} of 1.9 nmol of L-leucine per min per mg of protein (Fig. 1), whereas the low-affinity component exhibited an apparent K_t of at least 300 μM and a V_{max} of at least 6 nmol of L-leucine per min per mg of protein. L-Valine and L-isoleucine uptake also exhibited biphasic kinetics (data not shown). Both amino acids inhibited L-leucine transport competitively with K_i values identical to the K_t values found for uptake of these amino acids by the high-affinity system (Table 1).

A similar picture emerged when the kinetics of facilitated diffusion of L-leucine were studied in the absence of Δp at pH 7.0 (Fig. 1, inset). In these experiments, the external L-leucine concentration was varied over a broader range (3 μM to 4 mM). Initial rates of L-leucine influx were determined from time points taken during the first 20 s, yielding a
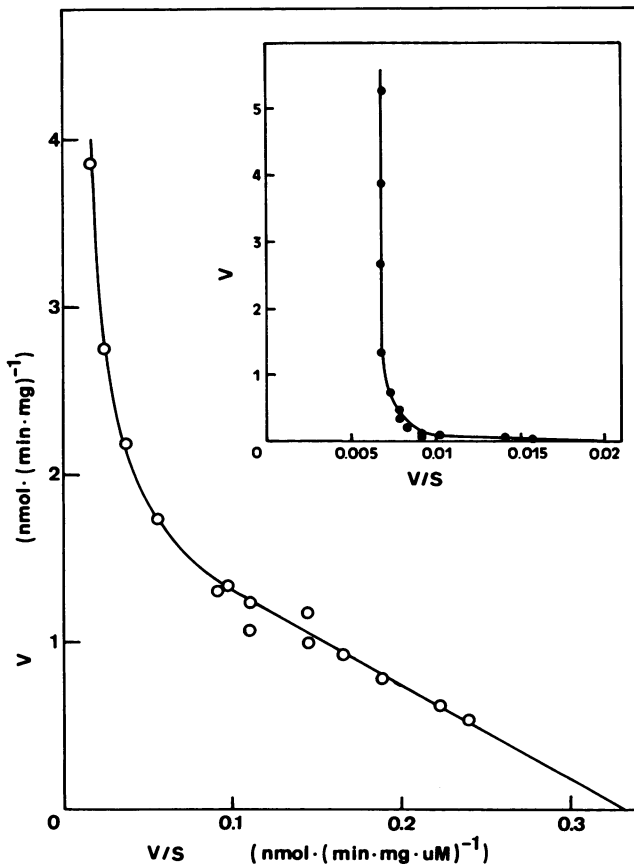
FIG. 1. Kinetic analysis of Δp-driven transport of L-leucine by membrane vesicles of *S. cremoris* fused with proteoliposomes containing beef heart cytochrome *c* oxidase at pH 7.0. Inset, Kinetics of L-leucine influx in the absence of Δp at pH 7.0.

K_t value of 8 to 10 μM, which is nearly identical to the K_t value found for Δp-driven transport. However, V_{max} was significantly lower (0.2 nmol of L-leucine per min per mg of protein). At high external L-leucine concentrations (>400 μM), kinetics are clearly nonsaturable; this finding suggests that at high L-leucine concentration, influx occurs via passive diffusion.

Inhibition of transport by analogs. The specificity and structural requirements of the branched-chain amino acid carrier were further analyzed by studying the effect of a number of L-leucine analogs on the initial rate of L-leucine uptake at pH 6.0. The apparent K_i' (see Materials and Methods) was used as a relative index of the specificity of the carrier, assuming competitive inhibition. Strong inhibi-

tion of L-leucine transport was observed with L-isomer amino acid analogs containing either a branched side chain or an aliphatic side chain with at least three methyl groups (Table 2). The inhibitory capacity of norvaline and norleucine was lost when a oxygen (*O*-methylthreonine) or sulfur atom (methionine) was inserted in the side chain. Modification of the amino group resulted in a complete loss of inhibitory effect of the analogs. Esters of L-leucine showed moderate inhibition but were not transported, as indicated by an absence of counterflow transport (data not shown). Elimination of the carbonyl group (e.g., leucinol) dramatically increased the K_i'. From this study, the following structural requirements for substrate recognition can be inferred. (i) The carrier must function stereospecifically; e.g., L-isomers are preferred. (ii) An aliphatic side chain of at least three methyl groups is essential, whereas branching of the side chain is preferred to unbranched side chains. (iii) A free amino group is necessary. (iv) A hydrogen atom is necessary. (v) A carbonyl group bound to C-α is essential.

Number of branched-chain amino acid-binding sites. The number of branched-chain amino acid-binding sites in membrane vesicles of *S. cremoris* was estimated from the amount of specific L-leucine binding as determined by flow dialysis (29). Membrane vesicles fused with asolectin liposomes were incubated with L-[^{14}C]leucine, and the amount of bound ligand was determined after addition of surplus nonlabeled L-leucine. To correct for nonspecific binding to the vesicles, binding was also determined for a similar quantity of soybean lipid liposomes, thereby obviating possible nonspecific binding to *S. cremoris* membranes, which contributes approximately 8% of total phospholipid in the reconstituted system. Experiments were performed with an L-leucine concentration of between 0.74 and 4.5 μM. The results indicate that cytoplasmic membranes of *S. cremoris* contain approximately 42 pmol of L-leucine-binding sites per mg of protein, with a dissociation constant for L-leucine of 1.5 μM at pH 6.0. The Hill coefficient was 0.99, indicating that only one class of binding sites was present.

TABLE 1. Kinetic constants for transport of branched-chain amino acids by membrane vesicles of *S. cremoris* fused with cytochrome *c* oxidase proteoliposomes[a]

Transported substrate	K_t (μM)	V_{max} (nmol/min per mg of protein)	K_i (μM)	
			Ile	Val
L-Leucine	6.5	2.0	9	15
L-Isoleucine	8	2.1	ND	ND
L-Valine	12	0.9	ND	ND

[a] Kinetic and inhibitor constants were determined from the initial rate of ^{14}C-labeled amino acid uptake at pH 7.0 as described in Materials and Methods. ND, Not determined.

TABLE 2. K_i' values for inhibition of L-leucine transport by branched-chain amino acid analogs

Inhibitor(s)	$K_i'^a$ (μM) of stereoisomer:	
	L-	D-
γ-Methylleucine	2.8	52
Leucine	3.2	65
Isoleucine	5.4	70
2-(*t*-Butyl)glycine	6.4	87
Allo-isoleucine	7.6	115
Valine	10.7	300
Norvaline	14	ND
Norleucine	21	ND
Methionine	113	ND
c-Allylglycine	130	ND
O-Methylthreonine	138	ND
Leucine-*t*-butylester, -methylester, -benzylester, -ethylester	150–170	ND
Leucineamide, leucinol	400–500	ND
α-Ketoisocaproate, leucic acid, α-methylleucine, α-aminoisobutyric acid, threonine, serine, alanine, lysine, glycine, *N*-methylleucine, *N*-acetylleucine, cycloleucine	>1,000	ND

[a] K_i' values were determined from the initial rate of L-leucine uptake at pH 6.0 in the presence and absence of inhibitor as described in Materials and Methods. ND, Not determined. Coefficient of variation, ±20%.
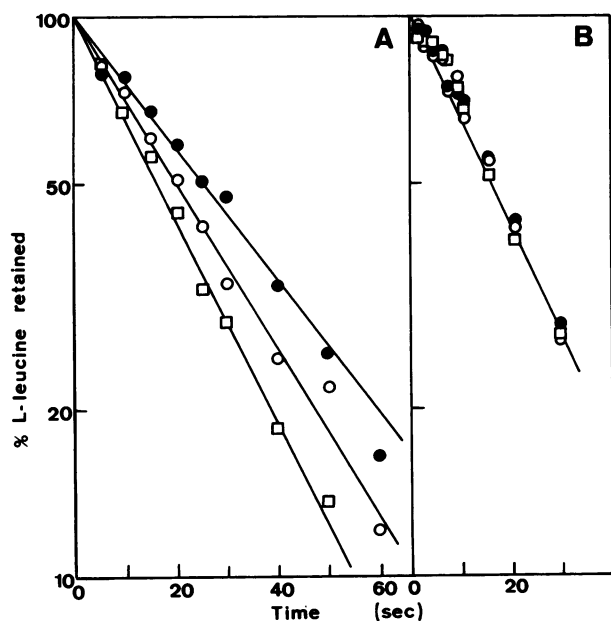
FIG. 2. Effect of pH on L-leucine efflux (A) and exchange (B) by membrane vesicles of *S. cremoris*. Experiments were performed in 50 mM potassium phosphate, pH 6.0 (●), 6.7 (○), or 7.5 (□), as described in Materials and Methods.

pH dependency of facilitated diffusion in the absence of Δp. The effect of pH on L-leucine efflux, influx, exchange, and counterflow was examined under conditions in which Δp was zero (short-circuited by 5 M of the uncoupler 5 chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide). Membrane vesicles of *S. cremoris* were equilibrated with 1 mM L-[¹⁴C]leucine, a concentration considerably greater than the K_t for influx or exchange (see below). After 200-fold dilution of the loaded vesicles into a medium free of L-leucine, efflux of L-leucine occurred with pseudo-first-order-rate kinetics (Fig. 2A). The rate of L-leucine efflux increased with increasing pH (Fig. 2A) with an apparent pK of about 6.8 (not shown). The rate of L-leucine influx showed a pH dependency (Fig. 3, inset) qualitatively similar to the rate of efflux (Fig. 2A).

Efflux can be described as consisting of at least four steps in the transport cycle: (i) binding of the solute and cotransported ion (H⁺) to the carrier on the inner surface of the membrane; (ii) translocation of the solute-H⁺ complex across the membrane; (iii) release of substrate and H⁺ from the carrier on the outer surface of the membrane; and (iv) reorientation of the free carrier to the inner surface of the membrane. To test whether the rate of efflux is limited by the return of the free carrier, efflux of L-[¹⁴C]leucine was studied under conditions identical to those described above, except that an equimolar concentration of nonlabeled L-leucine was present in the external medium (i.e., under exchange conditions). At all pH values tested, the exchange rates were faster than the efflux rates (Fig. 2B), indicating that the rate-limiting step during efflux is the return of the free carrier to the inner surface of the membrane. At high pH (>7.0), efflux was almost as fast as exchange. In contrast with efflux, L-leucine exchange exhibited virtually no pH dependence, suggesting that the carrier remains protonated during exchange.

Another method to study exchange is counterflow. Since a more precise resolution of the initial rate of exchange is

possible in counterflow experiments, this method was used to study the pH dependency of the kinetic constants of exchange. Membrane vesicles were equilibrated with 50 μM L-leucine and subsequently diluted 50-fold into a solution containing various concentrations of L-[¹⁴C]leucine (1 to 20 μM). By this means, an outwardly directed L-leucine concentration gradient was imposed, and rapid exchange and efflux occurred in which the initial phase caused a transient accumulation of labeled L-leucine (Fig. 3). The K_t for exchange (counterflow) increased with increasing pH (Fig. 4), whereas the V_{max} remained essentially constant. At high pH, the kinetic analysis of counterflow was relatively inaccurate; therefore, counterflow was assayed under conditions in which the external L-leucine concentration was saturating (100 μM). The initial rate of uptake was essentially independent from pH, albeit the rate of decay after reaching the maximal uptake level displayed a pH dependency similar to that observed for L-leucine efflux (Fig. 2A).

Effect of Δp on L-leucine influx, efflux, and exchange. The effect of the magnitude and composition of Δp on the kinetic constants of L-leucine transport at pH 6.0 was investigated. A ΔpH was imposed by the use of an acetate diffusion gradient. The V_{max} increased with increasing ΔpH, whereas K_t remained largely unchanged (Table 3). Similar results were obtained when the driving force was supplied by a Δψ, imposed by the use of a valinomycin-mediated potassium diffusion gradient (Table 3). The effect of Δψ on L-leucine efflux and exchange was examined with a saturating L-leucine concentration. Imposition of an outwardly directed
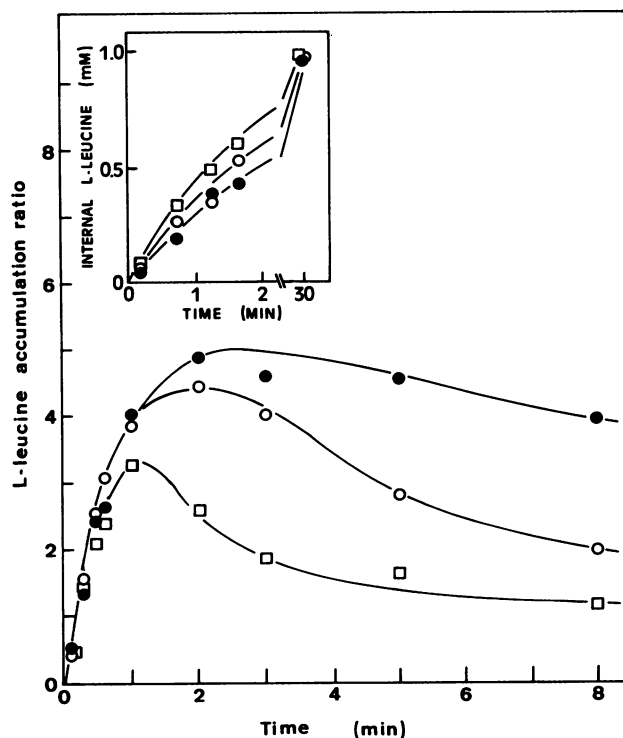

FIG. 3. Effect of pH on counterflow of L-leucine at saturating concentrations by membrane vesicles of *S. cremoris*. Experiments were performed in 50 mM potassium phosphate, pH 6.0 (●), 6.7 (○), or 7.5 (□), as described in Materials and Methods. Membrane vesicles were loaded with 5 mM L-leucine. Inset, Effect of pH on L-leucine influx into membrane vesicles of *S. cremoris* fused with asolectin liposomes.
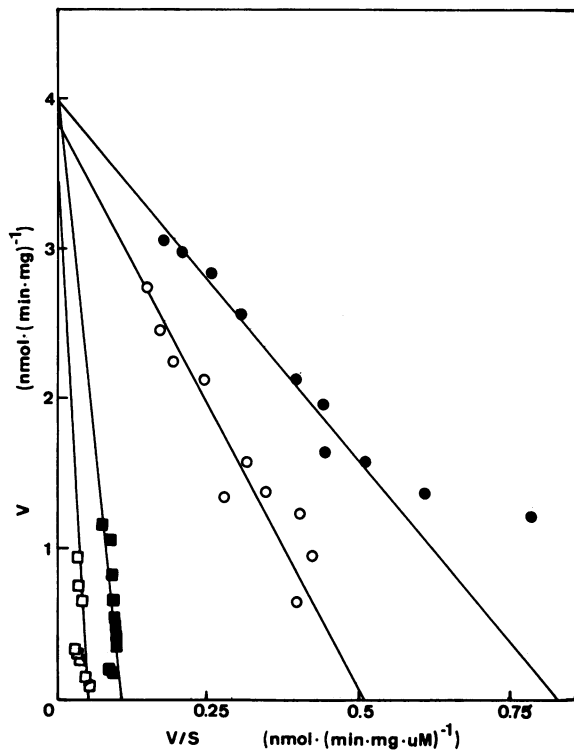
FIG. 4. Effect of pH on the kinetic constants of L-leucine counterflow. Membrane vesicles fused with asolectin liposomes were loaded with 50 μM L-leucine and subsequently diluted 50-fold into 50 mM potassium phosphate, pH 5.5 (●), 6.3 (○), 7.0 (■), or 8.0 (□), supplemented with L-[^{14}C]leucine in a concentration range of 1.5 to 20 μM.

potassium diffusion gradient in the presence of valinomycin (Δψ; interior negative) at pH 6.7 retarded L-leucine efflux by a factor of 4 (Fig. 5A). On the other hand, imposition of an inwardly directed potassium diffusion gradient (Δψ; interior positive) enhanced the rate of L-leucine efflux almost to the rate of exchange. Δψ had no effect on L-leucine exchange (Fig. 5B). The effects of Δψ and ΔpH on L-leucine efflux were additive. Imposition of an outwardly directed acetate diffusion gradient (ΔpH; interior alkaline) (Fig. 5C) retarded efflux only by a factor of 2. Simultaneous imposition of Δψ and ΔpH (interior negative and alkaline) resulted in a more than eightfold inhibition of the rate of L-leucine release.

pH dependency of the kinetic parameters of Δp-driven transport. The pH dependency of the kinetic parameters of L-leucine transport was studied in more detail. K_t values were determined over an extended pH range for L-leucine uptake driven by an imposed Δψ (Fig. 6, open circles) or ΔpH (Fig. 6, closed circles). A sigmoidal increase of the affinity with increasing H$^+$ concentration was observed, which was independent of the nature of the driving force. An apparent pK of about 6.9 was calculated from these data. These K_t values fit well with the K_t values determined for Δp-driven transport of L-leucine when the driving force was furnished by cytochrome c oxidase (Tables 1 and 4).

The pH dependency of L-leucine efflux (Fig. 2) suggested that deprotonation of the carrier on the outer surface of the membrane is one of the rate-limiting steps of efflux. Under conditions of Δp-driven transport, deprotonation occurs on the inner surface of the membrane. Thus, one would anticipate that the rate of transport is affected by the internal pH.

The effect of the internal pH on L-leucine uptake was studied under conditions in which the magnitude of Δp was kept constant, whereas the internal pH was varied by the addition of the ionophore nigericin. Kinetic experiments were performed at pH 6.0 (Δp equaled −105 to −115 mV) in the absence (internal pH 6.6) and presence (internal pH 6.0) of nigericin, with a L-leucine concentration range of 1.5 to 25 μM. The V_{max} of L-leucine uptake decreased markedly when the internal pH was lowered because of the action of nigericin (Table 4). A similar experiment was performed at pH 7.0. Since ΔpH is small at this pH, very little effect was observed on V_{max} with the addition of nigericin (Table 4). It is remarkable that the V_{max} was approximately twofold higher at pH 7.0 compared with pH 6.0 in the absence of nigericin, despite a lower magnitude of Δp.

DISCUSSION

In this study, the properties of the branched-chain amino acid carrier of *S. cremoris* were analyzed with respect to the substrate specificity and the pH dependency of various modes of carrier-mediated transport.

Leucine, isoleucine, and valine are transported by a common stereospecific transport system. The kinetics of Δp-driven leucine transport are biphasic, suggesting the existence of two transport systems. Biphasic kinetics are even more pronounced when facilitated diffusion of leucine is examined. However, the low-affinity component displayed nonsaturability, with an apparent first-order-rate constant of 0.85 min^{-1}. This value is in reasonable agreement with the diffusion constant calculated from steady-state accumulation studies (e.g., 0.9 to 1 min^{-1}) (Driessen et al., in press). Δp-driven leucine uptake results in a steady state in which the rate of carrier-mediated influx is balanced by an outwardly directed passive flux of leucine across the membrane. Consequently, a kinetic steady state is reached which is lower than the solute accumulation ratio predicted from thermodynamic equilibrium (8; Driessen et al., in press). It is therefore concluded that there is only one transport system for branched-chain amino acids in *S. cremoris*. Similar

TABLE 3. Effect of the magnitude of Δψ and ΔpH on the kinetic constants of L-leucine uptake[a]

Imposed Δp (mV)	K_t (μM)	V_{max} (nmol/min per mg of protein)
Δψ[b]		
−80	3.2	0.8
−100	2.8	1.1
−120	2.9	1.6
−140	3.2	2.0
−ZΔpH[c]		
−80	3.1	0.7
−100	2.7	1.5
−120	2.4	2.6
−140	2.9	2.9

[a] Kinetic experiments were performed with *S. cremoris* membrane vesicles, loaded with 20 mM potassium phosphate (pH 6.0), supplemented with 100 mM potassium acetate in the presence of 2 nmol of valinomycin per mg of protein.

[b] Δψ was imposed by diluting membrane vesicles into 20 mM sodium phosphate (pH 6.0), supplemented with 100 mM sodium acetate. The magnitude of Δψ was varied by varying the external potassium concentration.

[c] −ZΔpH was imposed by dilution of membranes into 20 mM potassium phosphate (pH 6.0), supplemented with 100 mM potassium PIPES. The magnitude of −ZΔpH was varied by varying the external acetate concentration. L-[^{14}C]leucine concentration was varied between 1.5 and 15 μM. Imposed Δp was calculated with the Nernst equation.
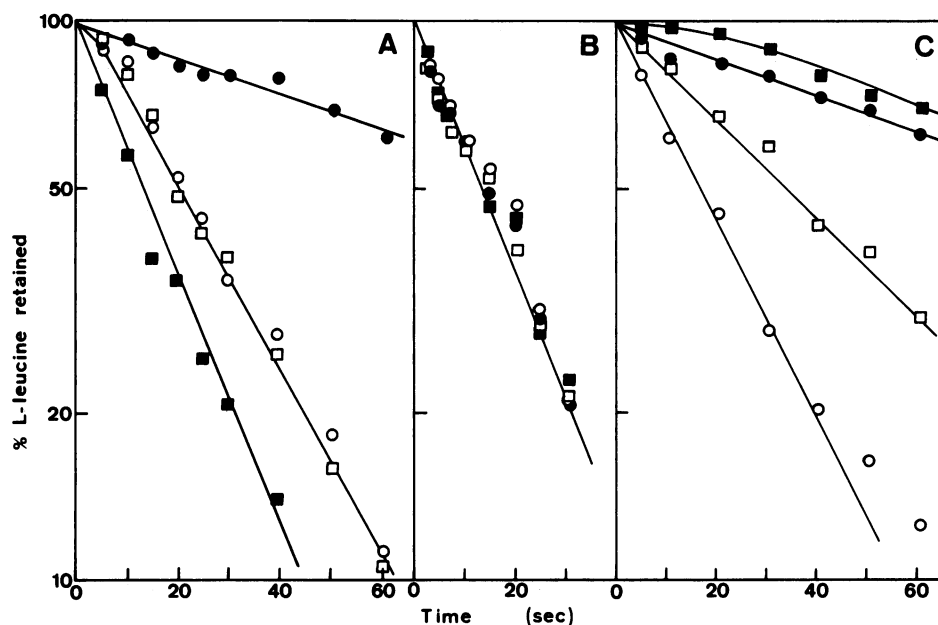
FIG. 5. Effect of Δp on L-leucine efflux (A and C) and exchange (B) at pH 6.7 by membrane vesicles of *S. cremoris*. Efflux (A) and exchange (B) in the presence of a $\Delta\psi$, interior negative (●); $\Delta\psi$, interior positive (■); or in the absence of a potassium (○) or sodium (□) diffusion gradient. Efflux (C) in the presence of a Δp, interior negative and alkaline (■); ΔpH, interior alkaline (□); $\Delta\psi$, interior negative (●); or no gradient (○). Experiments were performed as described in Materials and Methods.

phenomena have been observed for L-proline uptake by intact cells of *Escherichia coli* (2).

Leucine is transported in symport with H^+ with a mechanistic stoichiometry of 1 (8; Driessen et al., in press). Carrier-mediated leucine efflux down a concentration gradient should thus occur in symport with H^+. Under conditions in which the leucine concentration on the inside of the membrane was saturating, the rate of leucine efflux increased with increasing pH, with a pK_a of about 6.8 to 6.9. Translocation appears to be limited by deprotonation of the carrier on the outer surface of the membrane. When saturating amounts of leucine are present both on the inside and on the outside (e.g., exchange and counterflow), rates of transport are essentially pH independent and faster than rates of efflux. This observation is consistent with the notion that the carrier recycles in the protonated form during exchange and the initial events of counterflow. Consequently, it can be concluded that efflux occurs by an ordered mechanism in which leucine is released first from the carrier, followed by release of the symported H^+ on the outer surface of the membrane (Fig. 7). During efflux, reorientation of the free carrier is necessary to bind another H^+ and leucine molecule on the inside of the membrane. The rate of efflux is inhibited by $\Delta\psi$, inside negative. In contrast, exchange is unaffected by $\Delta\psi$. This observation is consistent with the conclusion that the reaction catalyzed by the free carrier involves net movement of negative charge (Fig. 7).

In analogy with efflux, leucine influx down a concentration gradient appears to be limited by the rate of deprotonation of the carrier on the inner surface of the membrane. The rate of influx increases when the internal pH is raised (Table 4).
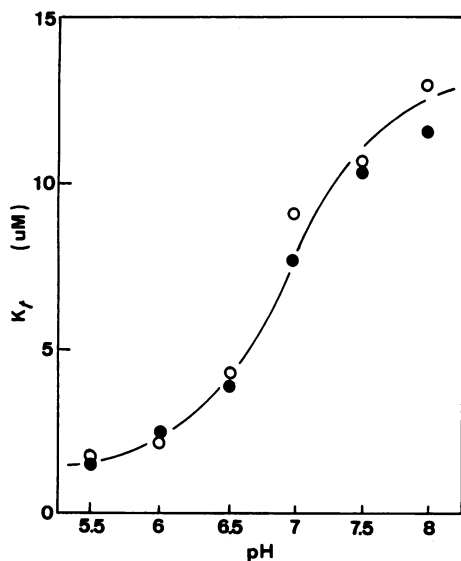

FIG. 6. Effect of pH on the K_t of L-leucine transport by membrane vesicles of *S. cremoris* fused with asolectin liposomes. Transport was assayed in the presence of an imposed ΔpH (●) or $\Delta\psi$ (○).

TABLE 4. Effect of pH on the kinetic constants of L-leucine uptake[a]

Nigericin	pH		$\Delta\psi$[b] (mV)	Δp (mV)	K_t (μM)	V_{max}[c]
	External	Internal[d]				
−	6.0	6.6	−79	−115	2.5	0.8
+	6.0	6.0	−109	−109	2.6	0.4
−	7.0	7.1	−82	−88	7.1	1.7
+	7.0	7.0	−92	−92	7.9	1.6

[a] Kinetic experiments were performed in the absence (−) and presence (+) of 10 nM nigericin.
[b] $\Delta\psi$ was estimated from the distribution of Ph_4P^+ as measured with a Ph_4P^+-selective electrode.
[c] V_{max} is expressed as nanomoles per minute per milligram of protein.
[d] Internal pH was estimated from the fluorescence of membrane vesicle-entrapped pyranine as described in Materials and Methods.
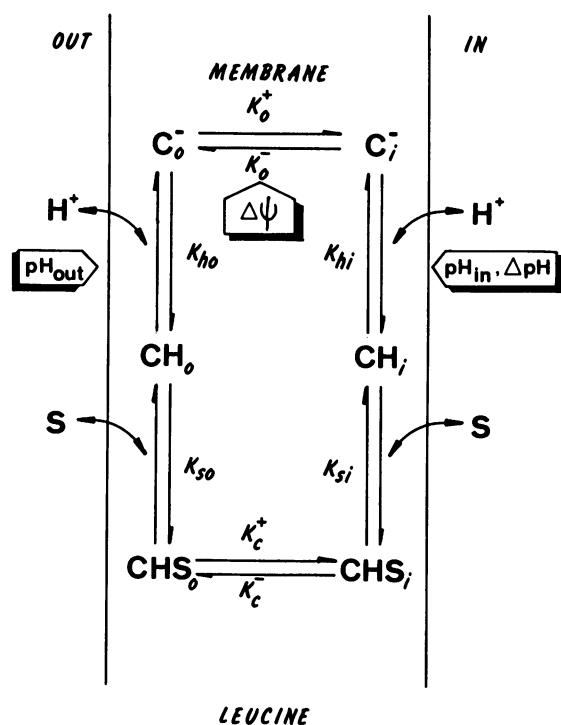
LEUCINE

FIG. 7. Model for the transport cycle by a carrier (C) catalyzing H^+-L-leucine cotransport. L-Leucine is indicated by S (solute), and protons are indicated by H^+. During Δp-driven leucine transport, the carrier is first protonated on the outer surface, followed by leucine binding. Reorientation of the carrier-H^+-leucine complex takes place, and leucine is released on the inner surface, followed by deprotonation of the carrier. Reorientation of the free carrier is accelerated by $\Delta\psi$. Deprotonation and protonation of the carrier are affected by the internal and external pH, respectively.

Deprotonation becomes faster at higher internal pH, and ΔpH will cause a shift in the equilibrium between the protonated and unprotonated forms of the carrier (Fig. 7), favoring the unprotonated form at high internal pH. Imposition of $\Delta\psi$, inside negative, accelerates influx, which is consistent with the previously discussed effect of $\Delta\psi$ on efflux, and most likely affects the return of the free carrier.

Analysis of our data according to the general model for H^+ (ion)-solute cotransporters described by Wright (28) supports the proposed order of H^+ and L-leucine binding to and release from the carrier.

Internal pH effects on leucine transport are also notable in intact cells. The rate of leucine transport by K^+-depleted glycolyzing cells of *S. cremoris* is stimulated by K^+ (unpublished results). Addition of K^+ results in a depolarization of $\Delta\psi$ and a compensatory increase in ΔpH (19). Despite the fact that Δp remains constant, the rate of leucine transport becomes higher as a result of a increase in internal pH, which is consistent with the model depicted in Fig. 7.

The pH dependence of leucine transport shows many analogies with lactose transport in *E. coli* (10, 26, 27). However, the pK_a of the H^+-binding group of the lactose carrier is much higher, i.e., 8.3. In analogy with leucine transport, the rate of lactose efflux increases with increasing pH, whereas the rate of exchange is pH independent. Deprotonation of the carrier and a reaction involving the return of the free carrier act as rate-limiting steps. It was concluded that H^+ and lactose binding to and release from the carrier are ordered processes (10, 26). However, recent

studies indicate that the pK_a of the H^+-binding group is also visible during exchange transport (18, 27). Therefore, random binding of the substrates to the carrier on both sides of the membrane seems more likely. The low dissociation constant for the symported H^+ forces the carrier in a state in which H^+ binding before substrate binding is favored.

In conclusion, L-leucine, L-isoleucine, and L-valine transport in *S. cremoris* is catalyzed by a single transport system, highly specific for L-isomers of branched-chain amino acids and analogs. H^+ and amino acid binding to and release from the carrier occur by an ordered mechanism in which proton release and reorientation of the free carrier are rate limiting.

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LITERATURE CITED

1. **Akpemado, K. M., and P. A. Bracquart.** 1983. Uptake of branched-chain amino acids by *Streptococcus thermophilus*. Appl. Environ. Microbiol. **45:**136–140.
2. **Anderson, R. R., R. Menzel, and J. M. Wood.** 1980. Biochemistry and regulation of a second L-proline transport system in *Salmonella typhimurium*. J. Bacteriol. **141:**1071–1076.
3. **Asghar, S. S., E. Levin, and F. M. Harold.** 1973. Accumulation of neutral amino acids by *Streptococcus faecalis*. J. Biol. Chem. **248:**5225–5233.
4. **DeMan, J. C., M. Rogosa, and M. Scharpe.** 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. **23:**130–135.
5. **de Vrij, W., A. J. M. Driessen, K. J. Hellingwerf, and W. N. Konings.** 1986. Measurements of the proton motive force generated by cytochrome c oxidase from *Bacillus subtilis* in proteoliposomes and membrane vesicles. Eur. J. Biochem. **156:**431–440.
6. **Driessen, A. J. M., W. de Vrij, and W. N. Konings.** 1985. Incorporation of beef heart cytochrome c oxidase as a proton-motive force-generating mechanism in bacterial membrane vesicles. Proc. Natl. Acad. Sci. USA **82:**7555–7559.
7. **Driessen, A. J. M., W. de Vrij, and W. N. Konings.** 1986. Functional incorporation of beef heart cytochrome c oxidase into membranes of *Streptococcus cremoris*. Eur. J. Biochem. **154:**617–624.
8. **Driessen, A. J. M., J. Kodde, S. de Jong, and W. N. Konings.** 1987. Neutral amino acid transport by membrane vesicles of *Streptococcus cremoris* is subject to regulation by internal pH. J. Bacteriol. **169:**2748–2754.
9. **Friede, J. D., D. P. Gilboe, K. C. Triebwasser, and L. M. Henderson.** 1972. Transport of lysine and hydroxylysine in *Streptococcus faecalis*. J. Bacteriol. **109:**179–185.
10. **Garcia, M. L., P. Viitane, D. L. Foster, and H. R. Kaback.** 1983. Mechanism of lactose translocation in proteoliposomes reconstituted with lac carrier purified from *Escherichia coli*. 1. Effect of pH and imposed membrane potential on efflux, exchange, and counterflow. Biochemistry **22:**2524–2530.
11. **Hellingwerf, K. J., and W. N. Konings.** 1980. Kinetic and steady state investigation of solute accumulation in bacterial membranes by continuously monitoring the radioactivity in the effluent of flow-dialysis experiments. Eur. J. Biochem. **106:**431–437.
12. **Kobayashi, H.** 1985. A proton-translocating ATPase regulates pH of the bacterial cytoplasm. J. Biol. Chem. **260:**72–76.
13. **Kobayashi, H., N. Murakami, and T. Unemoto.** 1982. Regulation of the cytoplasmic pH in *Streptococcus faecalis*. J. Biol. Chem. **257:**13246–13252.
13a.**Konings, W. N., W. de Vrij, A. J. M. Driessen, and B. Poolman.** 1987. Primary and secondary transport in gram-positive bacteria, p. 270–294. *In* J. Reizer and A. Peterkovsky (ed.), Sugar transport and metabolism in gram-positive bacteria. Ellis Horwood Ltd., Chichester, England.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.**

1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.

15. **Moran, J. W.** 1980. Branched-chain amino acid transport in *Streptococcus agalactiae*. Appl. Environ. Microbiol. **40**:25-31.

16. **Otto, R., R. C. Lageveen, H. Veldkamp, and W. N. Konings.** 1982. Lactate efflux-induced electrical potential in membrane vesicles of *Streptococcus cremoris*. J. Bacteriol. **149**:733-738.

17. **Otto, R., A. S. M. Sonnenberg, H. Veldkamp, and W. N. Konings.** 1980. Generation of an electrochemical proton gradient in *Streptococcus cremoris* by lactate efflux. Proc. Natl. Acad. Sci. USA **77**:5502-5506.

18. **Page, M. G. P.** 1987. The role of protons in the mechanism of galactoside transport via the lactose permease of *Escherichia coli*. Biochim. Biophys. Acta **897**:112-126.

19. **Poolman, B., K. J. Hellingwerf, and W. N. Konings.** 1987. Regulation of the glutamate-glutamine transport system by intracellular pH in *Streptococcus lactis*. J. Bacteriol. **169**: 2272-2276.

20. **Poolman, B., E. J. Smid, and W. N. Konings.** 1987. Kinetic properties of a phosphate-bond-driven glutamate-glutamine transport system in *Streptococcus lactis* and *Streptococcus cremoris*. J. Bacteriol. **169**:2755-2761.

21. **Preston, R. L., J. F. Schaeffer, and P. F. Curran.** 1974. Structure-affinity relationships of substrates for the neutral amino acid transport system in rabbit ileum. J. Gen. Physiol. **64**: 443-467.

22. **Reizer, J., and C. Panos.** 1982. Transport of α-aminoisobutyric

acid by *Streptococcus pyogenes* and its derived L-form. J. Bacteriol. **149**:211-220.

23. **Rice, G. H., F. H. C. Stewart, A. J. Hillier, and G. R. Jago.** 1978. The uptake of amino acids and peptides by *Streptococcus lactis*. J. Dairy Res. **45**:93-107.

24. **Thompson, J.** 1976. Characteristics and energy requirements of an α-aminoisobutyric acid transport system in *Streptococcus lactis*. J. Bacteriol. **127**:719-730.

25. **Trombe, M.-C., G. Lanéelle, and A. M. Sicard.** 1984. Characterization of a *Streptococcus pneumoniae* mutant with altered electric trans-membrane potential. J. Bacteriol. **158**:1109-1114.

26. **Viitanen, P., M. L. Garcia, D. L. Foster, G. J. Kaczorowski, and H. R. Kaback.** 1983. Mechanism of lactose translocation on proteoliposomes reconstituted with lac carrier purified from *Escherichia coli*. 2. Deuterium solvent isotope effects. Biochemistry **22**:2531-2536.

27. **Wright, K. J.** 1986. The kinetic mechanism of galactoside/H^+ cotransport in *Escherichia coli*. Biochim. Biophys. Acta **855**:391-416.

28. **Wright, K. J.** 1986. Experimental analysis of ion/solute cotransport by substrate binding and facilitated diffusion. Biochim. Biophys. Acta **854**:219-230.

29. **Wright, K. J., I. Reide, and P. Overath.** 1981. Lactose carrier protein of *Escherichia coli*: interaction with galactosides and protons. Biochemistry **20**:6404-6415.

30. **Yu, C. A., L. Yu, and T. E. King.** 1975. Studies on cytochrome c oxidase. J. Biol. Chem. **250**:1383-1392.