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## Neutral Amino Acid Transport by Membrane Vesicles of *Streptococcus cremoris* Is Subject to Regulation by Internal pH

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The pH dependence of transport of the neutral amino acids L-serine and L-alanine by membrane vesicles of *Streptococcus cremoris* have been studied in detail. The rates of four modes of facilitated diffusion (e.g., influx, efflux, exchange, and counterflow) of L-serine and L-alanine increase with increasing  $H^+$  concentration. Rates of artificially imposed electrical potential across the membrane ( $\Delta\Psi$ )-driven transport of L-serine and L-alanine show an optimum at pH 6 to 6.5. Under similar conditions,  $\Delta\Psi$ - and pH gradient across the membrane ( $\Delta pH$ )-driven transport of L-leucine is observed within the pH range studied (pH 5.5 to 7.5). The effect of ionophores on the uptake of L-alanine and L-serine has been studied in membrane vesicles of *S. cremoris* fused with proteoliposomes containing beef heart mitochondrial cytochrome *c* oxidase as a proton motive force ( $\Delta p$ )-generating system (Driessen et al., Proc. Natl. Acad. Sci. USA 82:7555–7559, 1985). An increase in the initial rates of L-serine and L-alanine uptake is observed with decreasing pH, which is not consistent with the pH dependency of  $\Delta p$ . Nigericin, an ionophore that induced a nearly complete interconversion of  $\Delta pH$  into  $\Delta\Psi$ , stimulated both the rate and the final level of L-alanine and L-serine uptake. Valinomycin, an ionophore that induced a collapse of  $\Delta\Psi$  with a noncompensating increase in  $\Delta pH$ , inhibited L-alanine and L-serine uptake above pH 6.0 more efficiently than it decreased  $\Delta p$ . Experiments which discriminate between the effects of the internal pH and the driving force ( $\Delta pH$ ) on solute transport indicate that at high internal pH the transport systems for L-alanine and L-serine are inactivated. A unique relation exists between the internal pH and the initial rate of uptake of L-serine and L-alanine with an apparent  $pK$  of 7.0. The rate of L-alanine and L-serine uptake decreases with increasing internal pH. The apparent complex relation between the  $\Delta p$  and transport of L-alanine and L-serine can be explained by a regulatory effect of the internal pH on the activity of the L-serine and L-alanine carriers.

Homofermentative, lactate-producing streptococci like *Streptococcus cremoris* and *S. lactis* are able to maintain their internal pH at a value of about 7.0 to 7.5 when the external pH is varied between 5.5 and 8.0 (B. Poolman, unpublished results). The internal pH in these bacteria is about 0.5 pH unit lower than in *Streptococcus faecalis* (10, 11). Although the phenomenon of pH homeostasis has been recognized for already a number of years in fermentative bacteria, the exact regulatory mechanisms involved are still obscure. An important regulatory role has been suggested for the  $F_1F_0$ -ATPase (9–11). In combination with electrogenic  $K^+$  uptake via a system called KtrI (8), which functions as either a  $K^+$ - $H^+$  symporter regulated by ATP or an ATP-driven potassium pump, regulated by the proton motive force ( $\Delta p$ ) (7), these bacteria would be able to regulate internal pH and adjust the magnitude of the  $\Delta p$ .

A physiological role of pH homeostasis has up to now only been implied for cytoplasmic enzymes. These proteins often show a pH optimum that is near the internal pH. Participation of internal pH in the regulation of transport systems has only been suggested for those systems which seem to be involved in pH regulation, such as the  $F_1F_0$ -ATPase of *S. faecalis* (9), the  $Na^+$ - $H^+$  antiporter (2), and the TrkA system (1, 3) in *Escherichia coli*.

Recently, we have shown that internal and external pH can act differently on solute transport in *S. cremoris* (A. J. M. Driessen, S. de Jong, and W. N. Konings, submitted for publication; A. J. M. Driessen, K. J. Hellingwerf, and W. N. Konings, submitted for publication) and *S. lactis* (15a). L-glutamate and L-glutamine transport is facilitated by a

system that requires ATP or another form of phosphate bond energy. A unique relation was found between the rate of L-glutamate transport and the internal pH (15a). The rate of L-glutamate uptake increased more than 30-fold when the internal pH was raised from 6.0 to 7.4. Since  $H^+$  does not seem to be catalytically involved in the translocation process, this pH effect was assigned as a regulatory effect of internal pH on the activity of the transport system. A similar role of internal pH has been found for  $P_i$  (B. Poolman, unpublished results) and dipeptide uptake (A. van Boven, unpublished results).

Dramatic effects of the external pH have also been observed on the rate of several modes of facilitated diffusion of the amino acids L-leucine and L-alanine (Driessen et al., submitted) in membrane vesicles of *S. cremoris*. The rate of L-leucine efflux increased with increasing pH with a  $pK_a$  of 6.8 to 6.9, whereas the rate of exchange was found to be pH independent. A similar  $pK_a$  was determined for the pH dependence of L-alanine efflux, although the pH effect was opposite to the effect on L-leucine efflux. In addition, L-alanine exchange showed a pH dependence similar to that of efflux. An asymmetry in the interaction of pH was observed with the L-leucine carrier. An increase in the external  $H^+$  concentration resulted in an increase in the  $K_i$  (affinity constant) for  $\Delta p$ -driven transport of L-leucine, whereas an increase in the internal  $H^+$  concentration resulted in a decrease in  $V_{max}$ . We concluded that internal pH acts on the rate of deprotonation of the carrier on the inner surface of the membrane, whereas external pH acts on the binding of L-leucine to the carrier on the outer surface of the membrane. These pH effects were therefore defined as catalytic

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effects. However, it was not possible to define the pH effect on the L-alanine carrier as being catalytic or allosteric.

In this paper we have investigated the role of pH in secondary transport of L-alanine and L-serine in more detail. These two neutral amino acids are transported by different carriers (Driessen et al., submitted). L-alanine and L-serine transport are subjected to regulation by the internal pH. This regulatory pH effect is the reason for abnormalities observed in the coupling between the  $\Delta p$  and neutral amino acid uptake and exit in *S. cremoris*.

## MATERIALS AND METHODS

**Materials.**  $^{14}\text{C}$ -labeled amino acids were obtained from Amersham (Buckinghamshire, United Kingdom). The following uniformly  $^{14}\text{C}$ -labeled compounds were used: L-leucine (12.4 TBq/mol), L-alanine (6.6 TBq/mol), and L-serine (6.6 TBq/mol). Octyl- $\beta$ -D-glucopyranoside, crude asolectin, and horse heart cytochrome *c* were obtained from Sigma Chemical Co. (St. Louis, Mo.). Pyranine was obtained from Eastman Kodak Co. (Rochester, N.Y.). All other materials were reagent grade and obtained from commercial sources.

**Growth of *S. cremoris* and isolation of membrane vesicles.** *S. cremoris* Wg2 (Prt<sup>-</sup>) was grown as described previously (15). Membrane vesicles were obtained by osmotic lysis as described previously (6, 15).

**Purification and reconstitution of cytochrome *c* oxidase.** Cytochrome *c* oxidase was isolated from beef heart as described previously (19) and reconstituted into asolectin liposomes by dialysis as described previously (5, 6; Driessen et al., submitted).

**Fusion of liposomes with *S. cremoris* membrane vesicles.** *S. cremoris* membrane vesicles were fused with cytochrome *c* oxidase proteoliposomes or asolectin liposomes by freeze-thaw-sonication as described previously (5, 6). Cytochrome *c* oxidase proteoliposomes (10 mg of phospholipid) were mixed with *S. cremoris* membrane vesicles (1 mg of protein) in a final volume of 0.6 ml. The suspension was rapidly frozen and stored in liquid nitrogen until use. Fused membranes were slowly thawed at room temperature, sonicated for 8 s in a plastic tube with a MSE probe-type sonicator at full amplitude, and suspended in a 20-fold volume of a given buffer. Fused membranes were collected by centrifugation for 1 h at 55,000 rpm (maximally 280,000  $\times g$ ) in a Beckman type 75 Ti rotor at 5°C and suspended to a protein concentration of about 8 mg/ml unless indicated otherwise.

**Determination of the  $\Delta\psi$ .** The electrical potential across the membrane ( $\Delta\psi$ ) (interior negative) was determined from the distribution across the membrane of the lipophilic cation tetraphenylphosphonium by using a tetraphenylphosphonium-selective electrode (16) as described previously (4–6). Membranes (0.8 mg of protein) were suspended in 50 mM potassium phosphate of the indicated pH, supplemented with 5 mM  $\text{MgSO}_4$ , 200  $\mu\text{M}$  *N,N,N',N'*-tetramethyl *p*-phenylene diamine, 20  $\mu\text{M}$  cytochrome *c*, and 2  $\mu\text{M}$  tetraphenylphosphonium in a final volume of 1 ml. Energization was started by the addition of 10 mM ascorbate, adjusted to the desired pH with 10 N KOH. Nigericin and valinomycin were used at concentrations of 10 and 100 nM, respectively.  $\Delta\psi$  was calculated from the steady-state level of tetraphenylphosphonium uptake and corrected for concentration-dependent binding of tetraphenylphosphonium to membranes (4, 13).

The pH gradient across the membrane ( $\Delta\text{pH}$ ; interior alkaline) was determined from the fluorescence of pyranine

entrapped in the intravesicular space as described previously (4, 6). Pyranine (100  $\mu\text{M}$ ) was added to the fused membranes before the sonication step. Pyranine-loaded membranes were washed with a 20-fold volume of 50 mM potassium phosphate buffer of the desired pH and collected by centrifugation for 1 h at 55,000 rpm (maximally 280,000  $\times g$ ) in a Beckman type 75 Ti rotor at 5°C. Membranes with entrapped pyranine (0.4 mg of protein) were suspended into 2 ml of 50 mM potassium phosphate buffer of the desired pH, containing 5 mM  $\text{MgSO}_4$ , 200  $\mu\text{M}$  *N,N,N',N'*-tetramethyl-*p*-phenylene diamine and 20  $\mu\text{M}$  cytochrome *c*. Ascorbate (10 mM), adjusted to the desired pH with 10 N KOH, was added, and the internal pH was estimated from the fluorescence of pyranine (excitation, 450 nm; emission, 508 nm) as described previously (4, 5). Nigericin and valinomycin were used at final concentrations of 10 and 100 nM, respectively. A conversion factor *Z* of 59 at 25°C was used to express  $\Delta\text{pH}$  in millivolts.

**Transport assays.** Amino acid transport driven by a  $\Delta p$  generated by cytochrome *c* oxidase activity was assayed as described previously (5, 6). Membranes (1 mg of protein) were suspended into 2 ml of 50 mM potassium phosphate of given pH, containing 5 mM  $\text{MgSO}_4$ , 200  $\mu\text{M}$  *N,N,N',N'*-tetramethyl-*p*-phenylene diamine, and 20  $\mu\text{M}$  cytochrome *c*.  $^{14}\text{C}$ -labeled amino acids were added 30 s after the addition of 10 mM ascorbate (adjusted to the desired pH with 10 N KOH) to the following concentrations: 3.1  $\mu\text{M}$  L- $^{14}\text{C}$ leucine, 5.9  $\mu\text{M}$  L- $^{14}\text{C}$ alanine, and 5.9  $\mu\text{M}$  L- $^{14}\text{C}$ serine. At intervals, samples of 100  $\mu\text{l}$  were taken, 2 ml of ice-cold 0.1 M LiCl was added, and the samples were filtered immediately (6).

Kinetic analysis of amino acid uptake was performed in tubes. Uptake rates were determined from the uptake values obtained at time points up to the first 60 s by using a concentration range between 1.5  $\mu\text{M}$  and 1 mM. Results were analyzed by Eady-Hofstee plots.

For  $\Delta\psi$ -driven amino acid accumulation, *S. cremoris* membrane vesicles fused with asolectin liposomes were suspended into 20 mM potassium phosphate of the desired pH, which was supplemented with 100 mM potassium acetate. Valinomycin was added to a final concentration of 2 nmol/mg of protein. Fused membranes were collected by centrifugation as described above and suspended to a final protein concentration of 20 mg/ml. A sample (4  $\mu\text{l}$ ) was then diluted 50-fold into 20 mM sodium phosphate of the desired pH, which was supplemented with 100 mM sodium acetate, 5 mM  $\text{MgSO}_4$ , and 20  $\mu\text{M}$   $^{14}\text{C}$ -labeled amino acid at 25°C. For  $\Delta\text{pH}$ -driven amino acid accumulation, samples (4  $\mu\text{l}$ ) of potassium-loaded membrane vesicles were diluted 50-fold into 20 mM potassium phosphate of the desired pH, which was supplemented with 100 mM potassium PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)], 5 mM  $\text{MgSO}_4$ , and 10  $\mu\text{M}$   $^{14}\text{C}$ -labeled amino acid. At intervals, 2 ml of ice-cold 0.1 M LiCl was added, and the sample was filtered immediately (6). Radioactivity was determined by liquid scintillation spectrometry.

Amino acid efflux, exchange, and counterflow were performed as follows. *S. cremoris* membrane vesicles (10 mg of protein) were washed twice in a 20-fold volume of 50 mM potassium phosphate of the desired pH. Concentrated membrane suspensions (25 mg of protein per ml) were equilibrated with 1 mM  $^{14}\text{C}$ -labeled amino acids (for counterflow 5 mM nonlabeled amino acid was used). After 3 to 5 h of incubation at 25°C, 4- $\mu\text{l}$  samples were rapidly diluted into 400  $\mu\text{l}$  of 50 mM potassium phosphate of the pH indicated and containing 5 mM  $\text{MgSO}_4$ . Nonlabeled amino acid (1 mM)

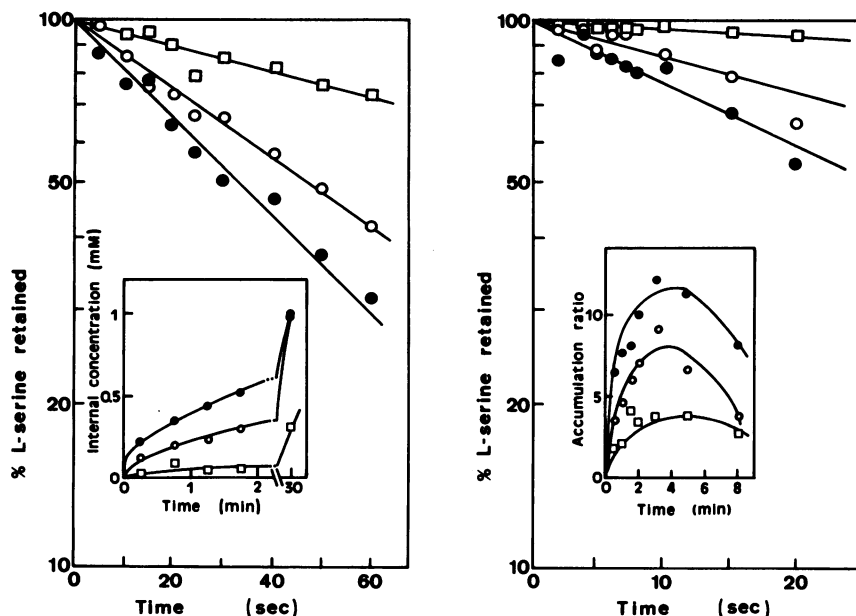


FIG. 1. Effect of pH on L-serine efflux (A), L-serine influx (inset A), and exchange (B) by membrane vesicles of *S. cremoris* and effect of pH on the time course of L-serine counterflow (inset in B). Experiments were performed in 50 mM potassium phosphate at pH 6.0 (●), pH 6.7 (○), or pH 7.5 (□) as described in Materials and Methods.

was present during exchange conditions. 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. For counterflow samples of 4  $\mu$ l were diluted into 200  $\mu$ l of potassium phosphate of the desired pH, containing 5 mM  $\text{MgSO}_4$  and 3  $\mu$ M  $^{14}\text{C}$ -labeled amino acid (103  $\mu$ M, final concentration). Influx measurements were performed by adding 1 mM  $^{14}\text{C}$ -labeled amino acid to a concentrated suspension of *S. cremoris* membrane vesicles (8 mg of protein per ml) which were fused with asolectin liposomes to increase the internal volume. All facilitated diffusion experiments were performed in the presence of 5  $\mu$ M 5-chloro-2'-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide.

An internal volume of 8  $\mu$ l/mg of protein was used for fused membranes (6) and a value of 4.3  $\mu$ l/mg of protein was used for *S. cremoris* membrane vesicles (15).

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

## RESULTS

**pH dependence of facilitated diffusion of L-alanine and L-serine in the absence of a  $\Delta p$ .** The pH dependence of transport of L-serine and L-alanine was studied under conditions such that  $\Delta p$  was clamped to zero by the addition of the uncoupler 5-chloro-2'-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide. Membrane vesicles of *S. cremoris* were loaded with 1 mM L-[ $^{14}\text{C}$ ]serine, and L-serine efflux was measured after diluting the vesicles 200-fold into an L-serine-free solution. Efflux occurred with pseudo-first-order rate kinetics (Fig. 1A). The rate of L-serine efflux dramatically decreased with increasing pH (Fig. 1A). At pH 7.5, L-serine efflux became extremely slow. A similar pH dependence was observed for L-serine influx (inset, Fig. 1A).

Exchange rates were measured by diluting the L-[ $^{14}\text{C}$ ]serine-loaded membrane vesicles into a solution con-

taining an equimolar concentration of unlabeled L-serine. Exchange rates (Fig. 1B) were faster than the efflux rates at similar pHs. However, the pH dependence was similar. In addition, rates of counterflow were studied as a function of pH. Membrane vesicles were loaded with 5 mM nonlabeled L-serine and diluted 50-fold into a solution containing 0.1 mM L-[ $^{14}\text{C}$ ]serine, a concentration exceeding the  $k_t$  for  $\Delta p$ -driven transport (see Table 2). In the initial phase, rapid exchange (inset, Fig. 1B) occurred, which shows a pH dependence similar to that of the exchange reaction (Fig. 1B). A similar pH dependence of four modes of facilitated diffusion was observed for L-alanine (data not shown). These results demonstrate that transport of neutral amino acids in the absence of a  $\Delta p$  is pH dependent.

**Effect of pH on artificially imposed  $\Delta\Psi$ - and  $\Delta\text{pH}$ -driven uptake of L-alanine and L-serine.** Imposition of an outwardly directed, valinomycin-mediated potassium diffusion gradient across membranes of *S. cremoris* membrane vesicles fused with asolectin liposomes leads to a transient generation of  $\Delta\Psi$ , interior negative (6). The rate of artificially imposed  $\Delta\Psi$ -driven uptake of L-alanine (Fig. 2B) and L-serine (Fig. 2C) showed an optimum at pHs around 6.0 to 6.5. At high pH, uptake rates became extremely slow, but still a significant rate of  $\Delta\Psi$ -driven L-leucine uptake was found (Fig. 2A).

The effect of an imposed  $\Delta\text{pH}$  at different external pHs has been tested by the use of an acetate diffusion gradient (12). When membrane vesicles are equilibrated with relatively high concentrations of acetate and diluted into a medium containing a less permeant anion such as gluconate or PIPES, acetate will diffuse across the membrane in its protonated form. This results in an alkalization of the intravesicular space. L-Alanine (Fig. 2B) and L-serine (Fig. 2C) uptake driven by an artificially imposed  $\Delta\text{pH}$  was only observed at low external pH values. L-Leucine uptake driven by an artificially imposed  $\Delta\text{pH}$  was observed at all pHs tested (Fig. 2A).

**$\Delta p$  and the effect of ionophores.** The relation between the  $\Delta p$  and uptake of neutral amino acids was studied in more

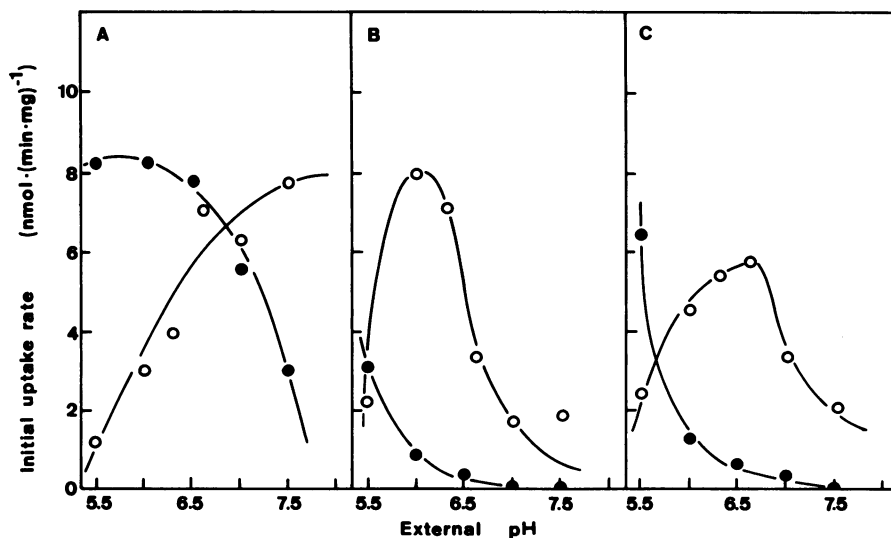


FIG. 2. Effect of the external pH on the initial rate of  $\Delta\Psi$ -driven (O) and  $\Delta pH$ -driven (●) amino acid uptake by membrane vesicles of *S. cremoris* fused with asolectin liposomes. (A) L-Leucine uptake; (B) L-alanine uptake; (C) L-serine uptake. Uptake was assayed as described in Materials and Methods. A final amino acid concentration of 20  $\mu M$  was used.

detail with *S. cremoris* membrane vesicles that had been fused with cytochrome *c* oxidase proteoliposomes (5, 6). The use of the ionophores nigericin and valinomycin in this system allows a more detailed investigation of the mode of energy coupling to neutral amino acid transport. To explain the effects of selective dissipation of either  $\Delta\Psi$  or  $\Delta pH$  on amino acid translocation, the magnitude and composition of  $\Delta p$  generated as a result of oxidase-mediated oxidation of the electron donor system ascorbate-*N,N,N',N'*-tetramethyl-*p*-phenylene diamine-cytochrome *c* was determined at pHs between 5.5 and 8.0.

In the absence of ionophores,  $\Delta p$  mainly consists of a  $\Delta\Psi$  (Fig. 3A). The contribution of  $\Delta pH$  to  $\Delta p$  increased with decreasing external pH. In the presence of potassium ions, the addition of the ionophore valinomycin resulted in a

collapse of  $\Delta\Psi$  with an increase in  $\Delta pH$  at pH 6.5 (Table 1). Interconversion was far from complete, and therefore the overall effect of valinomycin was a decrease in  $\Delta p$ . Similar observations were made at other pHs (data not shown). When the valinomycin concentration was further increased (>2.5 pmol/mg of phospholipid), also a collapse of  $\Delta pH$  was observed (data not shown). As previously reported (6), a nearly complete conversion of  $\Delta pH$  into  $\Delta\Psi$  was observed upon the addition of the ionophore nigericin (Table 1). Nigericin catalyzes an electroneutral exchange of  $K^+$  for  $H^+$ . However, high nigericin concentrations (>2.0 pmol/mg of phospholipid) resulted in an uncoupling of  $\Delta p$ . Thus the use of these ionophores permits a selective dissipation of either one of the components of  $\Delta p$  at low concentrations. Titration is necessary.

**Effects of ionophores and pH on the steady-state level of accumulation of L-serine and L-alanine.** Steady-state accumulation levels of L-serine and L-alanine were determined at various pHs. Only low levels of uptake were observed above pH 6.5 (Fig. 3B). If L-alanine and L-serine are translocated in symport with  $H^+$ , apparent  $H^+$ -amino acid stoichiometry ( $n_{app}$ ) for these neutral amino acids can be determined from the steady-state level of accumulation and the  $\Delta p$  according to the following equation:  $n_{app} = \Delta\bar{\mu}_{solute}/(F \cdot \Delta p)$ , in which  $\Delta\bar{\mu}_{solute}$  is the chemical gradient of solute across the membrane. The  $n_{app}$  for L-alanine (Fig. 4B), and L-serine (Fig. 4C) rapidly drops to values of 0.6 or lower above pH 6.5.

The effects of the ionophores valinomycin and nigericin on L-alanine and L-serine uptake was investigated between pH 5.5 and 8.0. L-Serine uptake at pH 6.5 was stimulated by the addition of nigericin (Table 1), which is unexpected for a

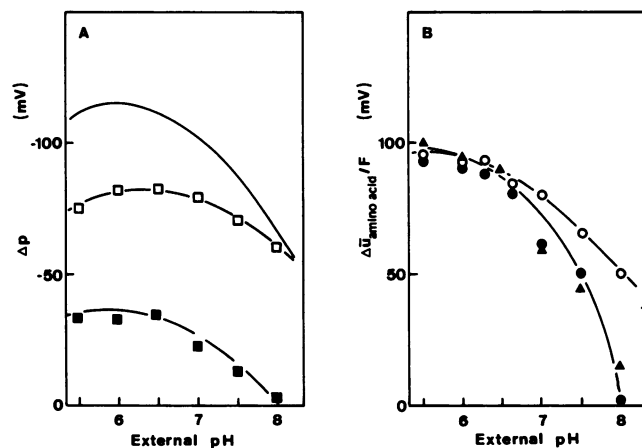


FIG. 3. Relation between the  $\Delta p$  and uptake of L-serine, L-alanine, and L-leucine in membrane vesicles of *S. cremoris* fused with proteoliposomes containing cytochrome *c* oxidase. (A) Effect of external pH on magnitude and composition of  $\Delta p$  generated by cytochrome *c* oxidase (—),  $\Delta\Psi$  (□), and  $\Delta pH$  (■). (B) Effect of external pH on the steady-state accumulation of L-leucine (○), L-alanine (●), and L-serine (▲). Uptakes and  $\Delta p$  measurements were performed as described in Materials and Methods.

TABLE 1. Relation between the  $\Delta p$  and L-serine uptake<sup>a</sup>

Addition	$\Delta\Psi$	Z $\Delta pH$ (mV)	$\Delta p$ (mV)	$\Delta\bar{\mu}_{solute}/F$	$n_{app}$
None	-82	34	-116	91	0.79
10 nM nigericin	-104	0	-104	103	0.99
100 nM valinomycin	0	49	-49	25	0.52

<sup>a</sup> Uptake and  $\Delta p$  measurements were performed at pH 6.5 with membrane vesicles of *S. cremoris* fused with proteoliposomes containing cytochrome *c* oxidase as described in Materials and Methods.

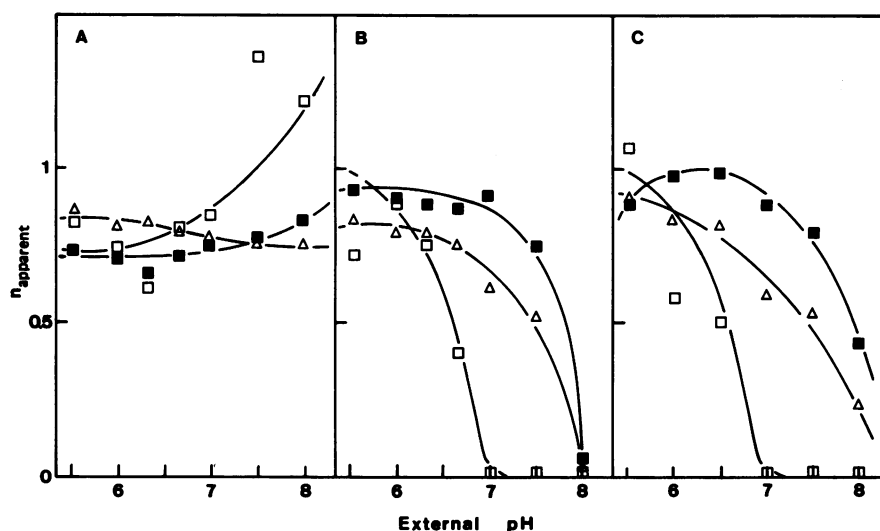


FIG. 4. Effect of pH on the  $n_{app}$  in the absence of ionophores ( $\Delta$ ) and in the presence of valinomycin ( $\square$ ) or nigericin ( $\blacksquare$ ). (A) L-leucine stoichiometry; (B) L-alanine stoichiometry; (C) L-serine stoichiometry. Valinomycin and nigericin were used at concentrations of 100 nM and 10 nM, respectively.

neutral amino acid translocated in symport with  $H^+$ . A stimulatory effect of nigericin was also observed on L-alanine uptake within the pH range pH 5.5 to 8.0 (data not shown). The magnitude of  $\Delta p$  was hardly affected by the addition of nigericin; thus the  $n_{app}$  for L-alanine (Fig. 4B) and L-serine (Table 1 and Fig. 4C) was increased. In the presence of nigericin, final accumulation levels of L-alanine and L-serine decreased strongly with increasing pH. However,  $n_{app}$  decreased steeply at higher pHs. In the presence of valinomycin, significant accumulations of L-alanine and L-serine were only observed below pH 7.0, which is consistent with the pH dependence of artificially imposed  $\Delta p$ -driven amino acid uptake. Consequently  $n_{app}$  already equals zero above pH 6.5 (Fig. 4B and C).

**Effects of ionophores and pH on the steady-state level of accumulation of L-leucine.** Accumulation levels of L-leucine were determined under the same conditions. In the absence of ionophores, the  $n_{app}$  for L-leucine uptake was rather constant at a value of about 0.8 (Fig. 4A). In the presence of nigericin,  $n_{app}$  was found to be somewhat lower compared with that when no ionophores were present. This inhibition was less pronounced at higher pHs. In the presence of valinomycin, an increase in  $n_{app}$  was observed with increasing pH (Fig. 4A). The  $n_{app}$  increased from 0.7 to 0.8 at pH 5.5 to about 1.3 to 1.4 at pH 8. It should be emphasized that the determination of  $n_{app}$  at high pH is inaccurate, since only a small  $\Delta p$  is present under those conditions ( $<0.3$  pH units). These results indicate that L-leucine is most likely transported in symport with one  $H^+$ .

**Effect of the internal pH on L-serine and L-alanine uptake.** The stimulatory effect of nigericin on L-alanine and L-serine uptake is in apparent conflict with the finding that  $\Delta\Psi$  and  $\Delta p$  (Fig. 2B and C) are both independently able to drive uptake of these two amino acids. The involvement of the internal pH in the uptake of L-alanine and L-serine was analyzed. The initial rates of L-alanine and L-serine uptake by membrane vesicles containing cytochrome *c* oxidase were measured as a function of the internal pH (Fig. 5). Internal pH was varied by the addition of nigericin ( $pH_{in} = pH_{out}$ ) or by varying the external pH ( $pH_{in} = \Delta p + pH_{out}$ ).

Under those conditions only the composition of  $\Delta p$  is altered, whereas the magnitude of  $\Delta p$  remains unchanged (6) (data not shown). Both in the presence and in the absence of nigericin, rates of L-alanine (inset of Fig. 5) and L-serine (data not shown) followed the external pH. However, these lines coincide when uptake rates of these amino acids are plotted as a function of internal pH (Fig. 5). A  $pK_a$  of about 7.0 can be estimated for the pH-sensitive group involved in this process. It should be emphasized that  $\Delta p$  varied ( $-110$  mV at pH 5.5 to  $-65$  mV and pH 8.0) with external pH, which could disturb an exact determination of the  $pK$ .

The kinetic parameters of L-alanine and L-serine transport were determined at pH 6.0 and pH 7.0 in the presence of nigericin (Table 2). No significant difference was observed between the  $K_s$  at both pHs. On the other hand, both transport systems showed a lower  $V_{max}$  at pH 7.0.

## DISCUSSION

In this report we have demonstrated that L-alanine and L-serine transport in *S. cremoris* is subjected to regulation by the internal pH.

Recently, we proposed a kinetic model for L-leucine transport, assuming that the observed pH dependence of L-leucine translocation is a result of an effect of pH on the catalytic  $H^+$  (Driessen et al., submitted). A similar approach was followed for L-serine and L-alanine transport. However, the pH dependence of efflux and exchange did not allow a distinction between an unordered model and an ordered model in which substrate binding is followed by  $H^+$  binding. In this paper we have shown that there is a unique relation between the internal pH and the rate of L-alanine and L-serine uptake. For both transport systems an apparent  $pK$  of about 7.0 was found. The apparent  $pK$  for L-alanine efflux equaled 7.0 (Driessen et al., submitted). Both the rate of uptake and the rate of efflux decreased with increasing (internal) pH. One would expect that the rate of deprotonation of the carrier on the inner surface of the membrane becomes faster with increasing pH. However, the internal pH dependence of L-alanine and L-serine transport

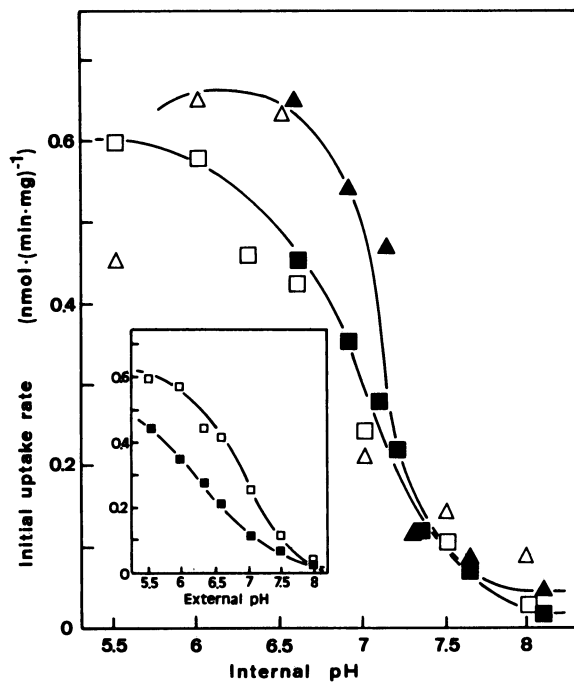


FIG. 5. Effect of the internal pH on the initial rate of uptake of L-serine ( $\Delta$ ,  $\blacktriangle$ ) and L-alanine ( $\square$ ,  $\blacksquare$ ) by membrane vesicles of *S. cremoris* fused with cytochrome *c* oxidase proteoliposomes. Uptake experiments were performed as described in the legend to Fig. 3 and Materials and Methods. In the absence of ionophores, the internal pH was calculated from the transmembrane  $\Delta\text{pH}$ , measured with pyranine and the external pH ( $\blacktriangle$ ,  $\blacksquare$ ). In the presence of nigericin (10 nM), internal pH was equal to the external pH ( $\Delta$ ,  $\square$ ). Inset, effect of external pH on the initial rate of L-alanine uptake in the absence ( $\blacksquare$ ) or presence of 10 nM nigericin ( $\square$ ).

is completely in the opposite direction. It is therefore more likely that the pH effect observed is an allosterical, rather than a catalytic, effect as suggested for L-leucine translocation. Possible pH effects on the catalytic  $\text{H}^+$  which is cotransported with L-alanine and L-serine might be obscured by the extreme internal pH dependence of these transport systems.

The observed internal pH dependence of the L-alanine and L-serine transport system can explain some peculiar observation concerning the relation between  $\Delta\text{p}$  and translocation of these amino acids. Translocation of neutral amino acids in symport with protons is expected to be coupled to  $\Delta\text{p}$ . Thus,  $\Delta\text{pH}$  and  $\Delta\psi$  should independently be able to drive amino

acid accumulation. As previously shown (5, 6; Driessen et al., submitted), L-leucine transport is coupled to  $\Delta\text{p}$ . The effects of nigericin and valinomycin on L-leucine uptake are consistent with their effects on the magnitude of  $\Delta\text{p}$ . The relation between the  $\Delta\text{p}$  and transport of L-alanine and L-serine appears to be much more complex. Only at low pH did  $\Delta\text{pH}$  appear to be capable to function as a driving force for L-alanine and L-serine uptake (Fig. 2A and B). From the pH dependence of the  $n_{\text{app}}$ , one tends to conclude that  $\Delta\psi$  is more efficient as a driving force than  $\Delta\text{pH}$ . A steep decrease in coupling efficiency is observed with increasing pH (Fig. 4B and C). On the other hand the  $n_{\text{app}}$  for L-leucine in the absence of ionophores remains constant at a value of 0.75 to 0.8. In the steady state, the rate of solute uptake is balanced by an outwardly directed solute flux. Recently, we have demonstrated that in *S. cremoris* membrane vesicles a significant fraction of solute exit occurs via an external leak (Driessen et al., submitted). Therefore, steady-state accumulation levels are proportional with the initial uptake rate and thus with the activity of the transport system. We have shown that pH exerts its effect on  $V_{\text{max}}$  of L-alanine and L-serine uptake, whereas  $K_t$  remains unaffected (Table 2). Although the external leak for L-alanine and L-serine is much smaller than for L-leucine (Driessen et al., submitted), the initial rates of L-alanine and L-serine uptake at high pHs were too low to reach thermodynamic equilibrium. Under those conditions a kinetic steady state is achieved. Since  $n_{\text{app}}$  approaches 1 at low pH values, it is most likely that the mechanistic  $\text{H}^+$ -L-alanine and  $\text{H}^+$ -L-serine stoichiometry equals 1.

The extreme internal pH dependency of the L-alanine transport system can also explain some observations on efflux of the L-alanine analog  $\alpha$ -aminoisobutyric acid from intact cells of *S. lactis* (17). The addition of a fermentable substrate enhanced both the rate of  $\alpha$ -aminoisobutyric acid uptake and efflux. It is difficult to envisage how metabolic energy, i.e.,  $\Delta\text{p}$ , can be coupled to solute translocation in two directions by the same carrier. Starved cells of *S. lactis* maintain a large phosphoenolpyruvate pool for several hours in the absence of an endogenous energy source (18). Due to a low pyruvate kinase activity, low concentrations of ATP are maintained in the cell for a prolonged period of time, which is used to generate a  $\Delta\text{pH}$  of about  $-120$  mV at pH 6.0, corresponding to an internal pH of 8.0 (B. Poolman, personal communications). The addition of a fermentable substrate like glucose or arginine results in a collapse of the  $\Delta\text{pH}$  with a concomitant decrease in internal pH to about 7.5 and a compensatory increase in  $\Delta\psi$ . This drop in internal pH might reactivate the L-alanine transport system, thereby stimulating  $\alpha$ -aminoisobutyric acid efflux until a new steady state is attained.

A large number of solute carriers in streptococci appear to be regulated by internal pH. Internal pH effects have now been observed on the activity of the L-glutamate acid-L-glutamine carrier (15a), the  $\text{P}_i$  carrier (B. Poolman, unpublished results), the dipeptide carrier (A. van Boven, unpublished results), and the branched-chain amino acid carrier (Driessen et al., submitted). The regulatory role of pH on neutral amino acid transport adds to the notion that internal pH participates in the regulation of solute transport.

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TABLE 2. Effect of pH on the kinetic constants of L-alanine and L-serine uptake<sup>a</sup>

Transported substrate	External pH	$V_{\text{max}}^b$	$K_t$ ( $\mu\text{M}$ )
L-Alanine	6.0	4.5	69
	7.0	2.4	68
L-Serine	6.0	6.4	51
	7.0	4.7	54

<sup>a</sup> Kinetic experiments were performed in the presence of 10 mM nigericin.

<sup>b</sup>  $V_{\text{max}}$  is expressed as nanomoles per minute per milligram of protein.

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