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Role of Scalar Protons in Metabolic Energy Generation in Lactic Acid Bacteria

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Lactic acid bacteria are able to generate a protonmotive force across the cytoplasmic membrane by various metabolic conversions without involvement of substrate level phosphorylation or proton pump activity. Weak acids like malate and citrate are taken up in an electrogenic process in which net negative charge is translocated into the cell thereby generating a membrane potential. The uptake is either an exchange process with a metabolic end-product (precursor/product exchange) or a uniporter mechanism. Subsequent metabolism of the internalized substrate drives uptake and results in the generation of a pH gradient due to the consumption of scalar protons. The generation of the membrane potential and the pH gradient involve separate steps in the pathway. Here it is shown that they are nevertheless coupled. Analysis of the pH gradient that is formed during malolactic fermentation and citrate fermentation shows that a pH gradient, inside alkaline, is formed only when the uptake system forms a membrane potential, inside negative. These secondary metabolic energy generating systems form a pmf that consists of both a membrane potential and a pH gradient, just like primary proton pumps do. It is concluded that the generation of a pH gradient, inside alkaline, upon the addition of a weak acid to cells is diagnostic for an electrogenic uptake mechanism translocating negative charge with the weak acid.

KEY WORDS: Protonmotive force; scalar protons; metabolic energy generation; malolactic fermentation; citrate fermentation; lactic acid bacteria.

INTRODUCTION

The electrochemical gradient of protons across the cytoplasmic membrane (protonmotive force, pmf) forms together with the ATP pool in the cytoplasm the major metabolic energy store of the bacterial cell. Both are intermediate between catabolism and anabolism and drive important processes like the uptake of nutrients. The pmf and the ATP pool communicate through the action of F_0F_1-ATPase that couples the movement of protons across the membrane to the synthesis/hydrolysis of ATP. The pmf is composed of a chemical (pH gradient, ΔpH) and an electrical (membrane potential, $\Delta\psi$) part:

$$\frac{\Delta\tilde{\mu}_{H^+}}{F} = \frac{\Delta\tilde{\mu}_{H^+}}{F} - \frac{2.3RT}{F}\Delta pH + \Delta\psi \qquad (1)$$

in which R, T, and F are the gas constant, the temperature, and the Faraday constant, respectively. The electrochemical gradient is generated by pumping protons out of the cell. Proton translocation is driven by redox energy, light, ATP hydrolysis, or some other exergonic chemical reaction. The consequence of the movement of the protons is the generation of a pH gradient (inside alkaline relative to outside) because the chemical proton concentration at either side of the membrane is affected and an electrical gradient (inside negative relative to outside) because charge is translocated. In pmf generation by these primary proton pumps, the generation of the pH gradient and the membrane potential are directly coupled events (primary pmf generation). Fermentative bacteria like lactic acid bacteria (LAB) have deviced secondary pmf generating systems that

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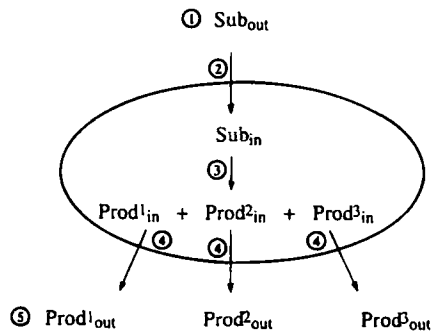
do not involve direct transport of protons across the membrane (for a general review on energetics in LAB, see Poolman, 1993). The two components of the pmf are generated in different steps. The membrane potential is generated during electrogenic transport of substrates and/or products into and out of the cell, whereas the pH gradient is generated as a consequence of the conversion of substrate into products by the cell, i.e., by scalar protons. Typical for these systems is that in addition to the gradient formation, the overall proton concentration of the system changes because the products formed are less acidic than the substrates used. During malolactic fermentation (Poolman *et al.*, 1991), malate is taken up by the cell and subsequently decarboxylated by malolactic enzyme to yield lactate and CO_2. The malate transporter couples the uptake of negatively charged malate ($Hmal^-$) to the excretion of protonated lactate (Hlac) thereby generating a membrane potential, alkaline inside (precursor/product exchange). Proton consumption in the decarboxylation reaction results in the formation of a pH gradient, alkaline inside. Similar mechanisms involving decarboxylation reactions have been shown for oxalate/formate exchange (Anatharam *et al.*, 1989) and histidine/histamine exchange (Molenaar *et al.*, 1993) and suggested for a number of other biogenic amines (Poolman, 1993). More recently, uniporters were described that serve the same function as these exchangers: these systems generate a membrane potential, inside negative, by translocation of negatively charged species. For instance, in the wine bacterium *Leuconostoc oenos* the uptake of monoanionic malate ($Hmal^-$) is not coupled to the excretion of lactic acid but proceeds as uniport (Salema *et al.*, 1994). In the same organism, the uptake of citrate is catalyzed by a uniporter translocating monoanionic citrate (H_2cit^-) and metabolism of citrate resulted in the formation of a pH gradient, inside alkaline (Ramos *et al.*, 1994). The latter example shows that the generation of pH gradients is not restricted to simple, one-step metabolic pathways like decarboxylation reactions and suggests that secondary metabolic energy generation may also occur in other more complex pathways.

The formation of the membrane potential in the secondary pmf generating systems is immediately evident from the transport mechanism by which the substrate enters the cell. In this paper we analyze how the pH gradient is formed. It will be demonstrated that even though pH gradient and membrane potential are generated in separate steps, the formation of the pH gradient is dependent on the transport mechanisms by which the substrate and products are transported in and out of the cell, respectively. The examples of malate and citrate utilization will be used to analyze how the transport systems determine the contribution of the scalar protons in the generation of metabolic energy.

RESULTS

Overall Alkalinization in the Steady State

The metabolic pathways that are considered here convert a weak acid substrate, originally outside the cell, into products that all leave the cell. Apart from the secondary metabolic energy conservation discussed above, there are no energy-conserving steps in the pathway like substrate level ATP synthesis, chemiosmotically coupled decarboxylation reactions (Lauβermair *et al.*, 1989), or generation of redox equivalents. The cell functions as a catalyst by taking up the substrate, converting it into products which are subsequently excreted (Scheme 1).



Scheme 1. The cell as a catalyst. General scheme indicating the steps involved in the conversion of a substrate into products by the cell.

The overall effect on the pH is independent of the metabolic pathway via which the cell converts substrate into products. Instead, proton consumption is solely determined by the chemical properties of substrate and products. In the kinetic steady state, the conversion of substrate into products will be accompanied by a constant proton consumption in the medium. The overall reaction describing malate fermentation (malolactic fermentation) in lactic acid bacteria is

$$H_2mal \rightsquigarrow Hlac + CO_2 \qquad (2)$$

in which H_2mal and Hlac are fully protonated malic acid and lactic acid, respectively.

Similarly, citrate fermentation in resting cells of *L. oenos* is described by

$$2\ H_3cit \rightsquigarrow 2\ Hac + acetoin + 4\ CO_2 \quad (3)$$

in which H_3cit and Hac are fully protonated citric acid and acetic acid, respectively (Ramos *et al.*, 1995). Under conditions of constant partial carbon dioxide pressure, pCO_2, the production of CO_2 in reactions 2 and 3 will have no effect on the pH of the medium. Both the malate and citrate conversions are pH neutral at very low pH. At high pH, malate and citrate fermentation result in the consumption of 1 and 2 protons per molecule of substrate, respectively.

$$mal^{2-} + H^+ \rightsquigarrow lac^- + CO_2 \quad (4)$$

$$2\ cit^{3-} + 4\ H^+ \rightsquigarrow 2\ ac^- + acetoin + 4\ CO_2 \quad (5)$$

At intermediate, more physiological pH values the alkalinization is determined by differences in pK between substrate and products, i.e., the substrates malate and citrate are more dissociated than the products lactate and acetate, respectively. The proton consumption per molecule of substrate as a function of medium pH is demonstrated for malolactic fermentation (Fig. 1, ●) and citrate metabolism (Fig. 2, ●).

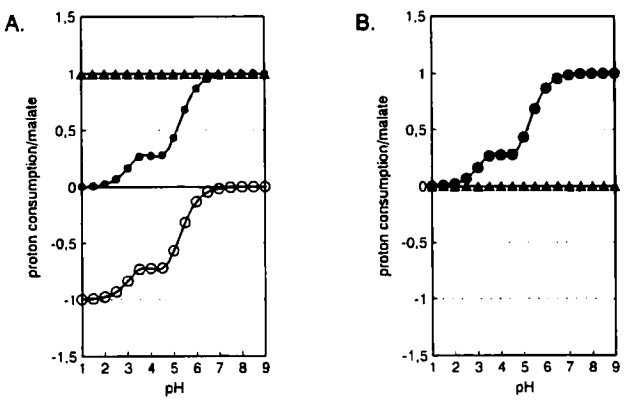


Fig. 1. Proton consumption during malolactic fermentation. Proton consumption during malate fermentation as a result of the overall process (●), the redistribution of malate and lactate over the different protonated species in the external medium (○), and the conversion of malate into lactate and carbon dioxide inside the cell (▲). Net proton consumption in the overall process was calculated at each pH from the fractions of the involved weak acids in the different protonated species and the overall reaction stoichiometry (reaction 2). Net proton consumption per molecule of malate equals $2 \times mal^{2-} + Hmal^- - lac^-$. The two p$K$'s for malate are 3.4 and 5.2. The pK for lactate is 3.86. (A) Malate fermentation according to Scheme 2A. Proton consumption due to redistribution in the external medium is given by $mal^{2-} - H_2mal - lac^-$. (B) Malate fermentation according to Scheme 2B. Proton consumption in the external medium follows from the same expression as for net proton consumption.

Alkalinization of the medium during malate and citrate fermentation by lactic acid bacteria is well documented in the literature (Poolman *et al.*, 1991; Molenaar *et al.*, 1993; Ramos *et al.*, 1994).

Effect on the Transmembrane pH Gradient

The formation of a pH gradient across the membrane during the metabolism of a weak acid is a property of the mechanism by which the cell (the catalysts) catalyzes the overall reaction. The mechanisms by which substrate and products are transported into and out of the cell, respectively, determine where the actual proton consumption takes place and, consequently, the direction of the proton flux over the cytoplasmic membrane in the steady state. Alkalinization of the cytoplasm relative to the medium will result in a flux of protons from out to in either by passive diffusion ("leak") or catalyzed by transporters. Similarly, alkalinization of the medium relative to the cytoplasm results in a proton flux from in to out. In either case the medium pH rises, but the pH gradient across the membrane is of opposite sign. Therefore, the energetic consequences of proton consumption is determined by the properties of the transport systems that are responsible for the uptake and excretion of substrate and products. Factors that determine the effects on the internal and external pH can be dissected in a number of steps (Scheme 1).

Step 1. Redistribution of the substrate. The substrate in the medium is distributed over the different protonated species depending on pH. Transport of one of the species into the cell disturbs the equilibrium and, consequently, results in redistribution of the substrate over the different states of protonation. The transported species functions as a sink for the other protonation states. Higher and lower protonated states result in proton release and consumption, respectively. The net effect of the redistribution on the pH depends on the initial distribution which in turn is determined by the pH.

Step 2. Uptake of the substrate. The number of protons that enter the cell with the substrate is determined by the uptake mechanism. The substrate may enter the cell by passive diffusion of the fully protonated, uncharged form. The high energy barrier of the cytoplasmic membrane to charged species will prevent the diffusion of the dissociated species at significant rates. Alternatively, the substrate may be taken up by a carrier protein in the membrane. The transported

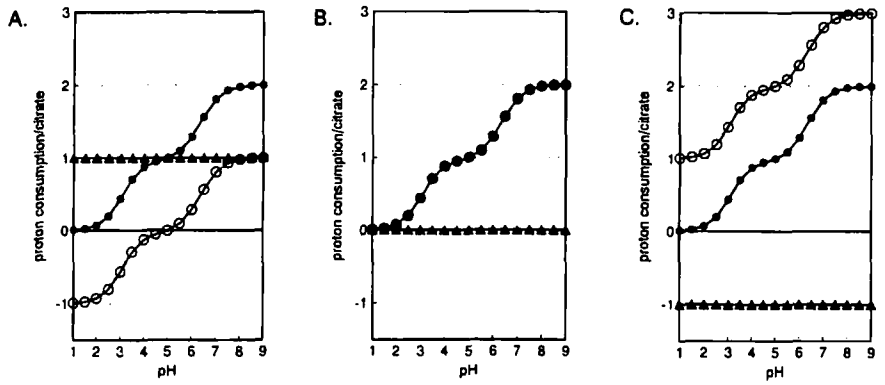


Fig. 2. Proton consumption during citrate fermentation. Proton consumption during citrate fermentation as a result of the overall process (●), the redistribution of citrate and acetate over the different protonated species in the external medium (○), and the conversion of citrate into acetate, acetoin, and carbon dioxide inside the cell (▲). Net proton consumption per molecule of citrate was calculated as described in the legend to Fig. 1 (see reaction 3) and equals $3 \times cit^{3-} + 2 \times Hcit^{2-} + H_2cit^- - ac^-$. The three p$K$'s for citrate are 3.1, 4.8, and 6.4. The pK for acetate equals 4.76. (A) Citrate fermentation according to Scheme 3A. Proton consumption due to redistribution in the external medium is given by $2 \times cit^{3-} + Hcit^{2-} - H_3cit - ac$. (B) Citrate fermentation according to Scheme 3B. Proton consumption in the external medium follows from the same expression as for net proton consumption. (C) Citrate fermentation according to Scheme 3C. The expression for external proton consumption is $4 \times cit^{3-} + 3 \times Hcit^{2-} + 2 \times H_2cit^- + H_3cit - ac^-$.

particle is determined by the substrate specificity of the carrier and is one of the protonation states of the substrate. The carrier may be a uniporter catalyzing facilitated diffusion of the transported particle, a symporter catalyzing the coupled uptake of substrate and proton, or an exchanger catalyzing the coupled uptake of the substrate and the excretion of one of the products. For the present discussion about the effect on internal and external pH, only the net proton stoichiometry of the transport mechanism is important. A uniporter transporting diprotonated citrate (H_2cit^-) is equivalent to a symporter transporting monoprotonated citrate ($Hcit^{2-}$) with a proton (H^+), and the combination of the uptake of monoprotonated malate ($Hmal^-$) by a uniporter and passive diffusion of lactic acid (Hlac) out of the cell is equivalent to the precursor/product exchanger that couples the uptake of $Hmal^-$ to the excretion of Hlac.

Step 3. Conversion. Upon entering the cell, the substrate will be converted into the products in a given metabolic pathway. A deficit or excess of protons to run the overall reaction in the cytoplasm follows from the conversion of the net protonated state of the entering substrate into the net protonated states in which the products will leave the cell.

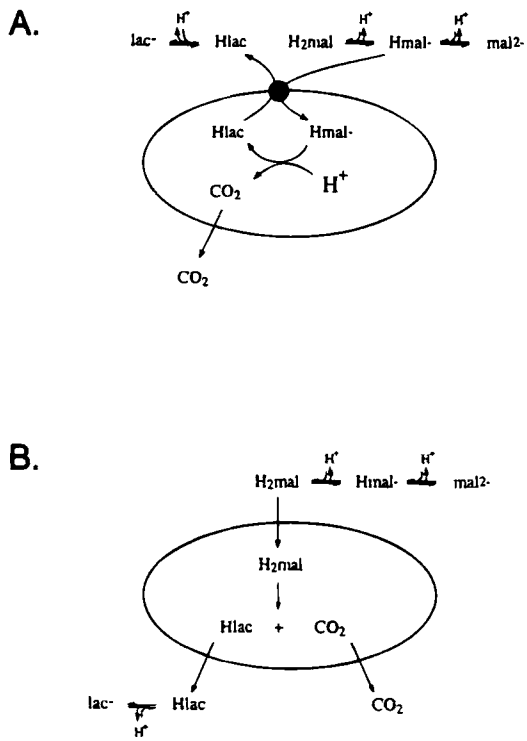
Step 4. Excretion of the products. Like the substrate, the products may pass the cell membrane either passively or facilitated. The cytoplasmic membrane of bacteria is usually quite permeable to typical end-products like lactic acid, acetic acid, and carbon dioxide as long as the molecules are uncharged.

Step 5. Redistribution of the products. The products enter the external medium in the protonation state in which they are excreted. The molecules will immediately redistribute over the different states of protonation which involves the release or binding of protons from the medium. If a weak acid is excreted in the protonated state, this will result in proton release into the medium. The extent of proton release depends on the pK of the weak acid and the medium pH.

In conclusion, the external pH is affected by redistribution of substrate and products over the different protonation states following uptake and excretion of the transported species and by the energy coupling mechanism of the transport processes, if any. The effect on the internal pH is determined by the state of protonation of the substrate and products that have entered and will leave the cell which, in turn, is determined by the mechanisms of uptake and excretion. The consequences of different transport mechanisms on the pH gradient generation will be demonstrated for malate and citrate utilization as described in overall equations (2) and (3).

Malolactic Fermentation

Scheme 2A shows the mechanism by which *Lactococcus lactis* catalyzes malolactic fermentation (reaction 2) (Poolman *et al.*, 1991).



Scheme 2. Malolactic fermentation. (A) Malate fermentation as described for *L. lactis*. The malate carrier catalyzes electrogenic exchange of $Hmal^-$ and Hlac. (B) Malate fermentation in the absence of a transporter. Fully protonated malate diffuses through the membrane into the cell.

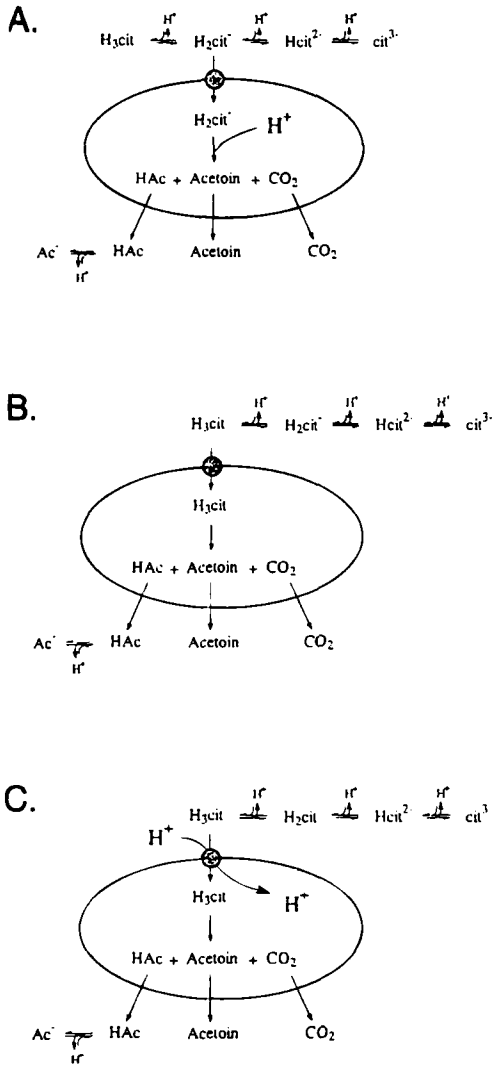
Monoanionic malate is transported into the cell by the carrier. The fraction of malate in the H_2mal state will release a proton and the fraction in the mal^{2-} state will take up a proton before being transported. The product lactate leaves the cell in the protonated state and will in part dissociate in the external medium. At low pH the redistribution results in the release of one proton per molecule of malate, and at high pH the redistribution is pH neutral. Redistribution of substrate and product results initially in an acidification of the external medium up to a medium pH of about 7 (Fig. 1A, ○). According to overall reaction 2, 1 proton is needed inside the cell to convert $Hmal^-$ into the protonated products (Fig. 1A, ▲). The overall result is the generation of a pH gradient of physiological polarity ($-Z\Delta pH < 0$). Note that at low pH the combined scalar reactions that take place outside and inside the cell mimic vectorial proton transport of a proton pump, i.e., the proton that dissapears inside, reappears outside with no net proton consumption (Fig. 1A, ●).

It has been suggested that at the low medium pH found in wine, malolactic fermentation by the wine bacterium *L. oenos* would proceed via the passive uptake of uncharged malate (H_2mal) (Loubiere *et al.*, 1993). The energetic consequences of this pathway (Scheme 2B) are analyzed in Fig. 1B. Obviously, the electroneutral diffusion of the substrate into the cell does not result in a membrane potential. Inside the cell, fully protonated malate is converted into the protonated products without the need for a proton and, therefore, the internal pH is not affected (▲). Redistribution of substrate and products results in a net proton consumption (○) that follows the net proton consumption in the overall reaction (●). Consequently, a pH gradient is generated, inside acid ($-Z\Delta pH > 0$), which is inverted relative to the physiological pH gradient and consequently, will reduce the pmf across the membrane. Malolactic fermentation involving passive uptake of malic acid into the cell requires input of metabolic energy from other sources that restore the proton gradient across the membrane.

Citrate Fermentation

Scheme 3 shows three possible mechanisms for the uptake of citrate in combination with the metabolism according to overall reaction 3.

It is assumed that the end-products leave the cell in their uncharged state. In Scheme 3A citrate is taken up by an electrogenic uniporter that transports H_2cit^-. Redistribution of citrate and acetate over the different species results in net proton release in the medium below pH 5 and proton consumption above pH 5 (Fig. 2A, ○). In the cell 1 proton per molecule of citrate is needed to convert the transported species into the products (▲). The net result is generation of a pH gradient, alkaline inside ($-Z\Delta pH < 0$) which together with the membrane potential generated in the uptake step serves the cell as metabolic energy. The same metabolic energy generation is obtained when, instead of uniport of H_2cit^-, symport of $Hcit^{2-}$ plus H^+ or exchange of $Hcit^{2-}$ plus Ac^- (Hugenholtz *et al.*, 1993) is considered. Electroneutral uptake of citrate (Scheme 3B) results in pH neutral conversion inside the cell (Fig. 2B, ▲) and, as a result of the redistribution of substrate and products (○), the alkalinization of the overall reaction (●) takes place completely outside



Scheme 3. Citrate fermentation in *L. oenos*. The schemes differ in the way citrate is transported into the cell. (A) Uniport of H_2cit^-. (B) Electroneutral citrate uptake. (C) Pmf-driven citrate uptake. The uptake mechanism has consequences for the pH of the internal medium.

the cell. As was observed for electroneutral uptake of malate, the result is an inverted pH gradient ($-Z\Delta pH > 0$). This is even more prominent when citrate is transported into the cell via a pmf driven transporter, e.g., symport of $Hcit^{2-}$ plus $3H^+$, which is the mechanism described for citrate uptake in Gram(−) bacteria (Scheme 3C) (van der Rest *et al.*, 1991; Ishiguro and Sato, 1985). The surplus of protons that drive the uptake of citrate result in acidification of the cytoplasm (Fig. 2C, ▲) and, together with the redistribution of substrate and products, in a strong alkalinization of the medium (○). The net effect is dissipation of the pmf both at the level of charge and proton translocation in the transport step and the formation of an inverted pH gradient by the scalar protons in the metabolism. Citrate addition to resting cells of *L. oenos* results in the formation of a pH gradient of physiological polarity (alkaline inside). The above analysis shows that this is only consistent with electrogenic uptake of citrate during which negative charge is translocated into the cell (Scheme 3A). In agreement with this, studies of citrate transport in isolated membrane vesicles of this organism have shown that the citrate carrier of *L. oenos* catalyzes uniport of H_2cit^- (Ramos *et al.*, 1994).

DISCUSSION

Primary transport systems generate a pmf by pumping protons across the cytoplasmic membrane, which results in a membrane potential and a pH gradient. The pump couples in a single step an exergonic metabolic reaction to the translocation of a proton. Secondary pmf generation proceeds via a number of steps involving more than one enzyme, and the membrane potential and pH gradient are formed in separate steps. The membrane potential is formed during the uptake of the substrate, and the pH gradient is formed as a result of the consumption of a scalar proton in the metabolism of the substrate. However, the analysis of malate and citrate fermentation given here shows that also in these systems the generation of membrane potential and pH gradient are coupled events. Only in those cases where the uptake of the weak acid results in a membrane potential of physiological polarity, negative inside, is a pH gradient formed that is alkaline inside and, therefore, adds to the pmf formed by the transporter. When the uptake of the substrate is not electrogenic, metabolism results in an inverted pH gradient that will have a dissipative effect on the pmf. Such an uptake will only be possible when other pmf generating systems are active in the cell. The coupling between the formation of the membrane potential and the pH gradient can be understood by inspection of the overall reactions 2 and 3, which show a pH neutral conversion of the fully protonated weak acid into the products. An internal proton is only needed for the metabolism to proceed when a deprotonated state of the substrate enters the cell. The negatively charged, deprotonated state results in the membrane potential, negative inside, upon entering the cell.

Turning around the argument, the formation of a pH gradient, outside acid, upon addition of a weak

acid to cells is diagnostic for an uptake mechanism that involves the translocation of net negative charge. The generation of a pH gradient in resting cells of *L. oenos* upon addition of citrate is consistent with the transport of monoanionic citrate that followed from studies of the transport mechanism in membrane vesicles (Ramos *et al.*, 1994).

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