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Quantitative analysis of proton movements associated with the uptake of weak carboxylic acids. The yeast *Candida utilis* as a model

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A quantitative analysis of the proton movements associated with the initial uptake rate of weak short-chain carboxylic acids was developed in order to estimate proton/carboxylate symports stoichiometries. The yeast *Candida utilis* was used as a biological model and the deduced equations were applied on the elucidation of the proton/carboxylate symports stoichiometries of lactate, succinate and citrate in a strain of that yeast species at different pH values. At pH 5.0, the proton/lactate and the proton/succinate symport stoichiometry was 1:1. In the cases of the proton/lactate and proton/citrate symports it appears that the stoichiometry ratio increased with increasing extracellular pH.

Introduction

Earlier studies on the transport of short-chain mono-, di- and tricarboxylic acids have provided evidence that in several yeast species the transport of anionic(s) form(s) of the acids across the plasma membrane was performed by distinct proton/carboxylate symports which involve a transmembrane proton motive force and a carrier mediated flow of proton down their electrochemical gradient (see Ref. 1 and references therein). However, in most cases the question of the stoichiometry remains to be elucidated. Various ways of estimating the co-substrate/substrate stoichiometry have been used. In general, the conceptual basis for the prediction of the stoichiometry lies in the equation derived from Mitchell relating the accumulation ratio of metabolite and the electrochemical gradient through the proton/substrate stoichiometry of the substrate. In this way for the proton/lactate stoichiometry of *Saccharomyces cerevisiae*, using a nonmetabolizable analog of lactate, it was possible to predict a stoichiometry of 1:1 [2]. However, in *Candida utilis* and in a number of other yeast species, nonmetabolizable analogs are not available for those purposes. The ratio between the initial rate of co-substrate uptake and the initial rate of substrate uptake is also a common procedure to estimate a co-transport mechanism stoichiometry but, in the case of a proton/carboxylate symport it is necessary to take in account the particular nature of the substrate. Actually, most of the carboxylic acids are weak or intermediate electrolytes which implies that in aqueous solutions they are incompletely dissociated. Thus, in opposite to the case of a neutral substrate, the ratio between the initial proton uptake and the initial uptake rate of labelled weak carboxylic acid can not be used to estimate the proton/substrate stoichiometry since the initial slopes of the experimental pH curves are the result of two components: the proton flux associated to the transport and the proton flux associated to the re-establishment of the extracellular acid-base equilibrium disturbed by the transport of the acid. Besides its considerable interest in what concerns the characterization of such a secondary active transport system, to date no information seems to have been published on a quantitative analysis of the experimental proton flux associated to the initial uptake of weak short-chain carboxylic acids.

In the present work we present a theoretical study which can be used to estimate the proton flux exclusively associated to the transport and thus the proton/carboxylate stoichiometry. The use of that theoretical study as a potential tool to distinguish in which form the acid is transported is also discussed.

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Materials and Methods

Microorganism and growth conditions

Candida utilis IGC 3092 used in previous work [1] was maintained on a medium containing glucose (2% w/v), peptone (1%, w/v), yeast extract (0.5%, w/v), agar (2% w/v). For growth, a mineral medium with vitamins and 0.5% (w/v) lactate, succinate or citrate [1], pH 4.8, was used, at 25°C, with mechanical shaking (120 rev/min).

Measurement of acid uptake rate

The methodology used was that previously described [1]. Typically the cells were harvested in the mid-exponential phase, centrifuged, washed twice with ice-cold distilled water and suspended in ice-cold distilled water to a final concentration of about 25 mg dry wt./ml. The uptake rates of labelled lactic, succinic or citric acids were estimated using 10 ml conical centrifuge tubes containing 10 μl amounts of the yeast suspension and 30 μl 0.1 M KH_2PO_4 buffer at the different experimental pH values. The buffer capacity, for each experimental pH, was tested and in these buffering conditions the extracellular reaction mixture pH was constant during the time period assays of 5 and 10 s. After 2 min of incubation at 25°C in a water bath, the reaction was started by the addition of 10 μl of an aqueous solution of D,L-[1-^{14}C]lactic acid (3000 dpm/nmol), [2,3-^{14}C]succinic acid (3000 dpm/nmol) or [1,5-^{14}C]citric acid (2100 dpm/nmol) at the desired concentration and stopped by dilution with 5 ml of cold water. Sampling times for all the uptake assays were 0, 5 and 10 s. The reaction mixtures were filtered immediately through Whatman GF/C membranes, washed on the filter with 10 ml ice-cold water, and counted in a scintillation fluid (Opti-Phase HiSafe II; LKB FSA Laboratory Supplies, Loughborough, UK). For non specific ^{14}C absorption, labelled carboxylic acid was added after cold water. Radioactivity was measured in a Packard Tri-Carb 2200 CA liquid scintillation spectrophotometer, with corrections for disintegrations per min.

Measurement of proton uptake rate

Proton uptake rates were calculated using a standard pH meter PHM 62 (Radiometer, Copenhagen) connected to a flatbed Perkin-Elmer 024 Recorder. The pH electrode was immersed in a water-jacketed chamber provided with magnetic stirring. To the chamber were added 4.5 ml of KH_2PO_4 10 mM and 0.5 ml yeast suspension. The pH was adjusted to the desired value and a base line was obtained. The desired amount of lactic, succinic or citric acid (adjusted to the experimental pH value) was added and the subsequent alkalization was followed in the recorder. The experimental initial uptake rate was calculated from the slope of the tangent to the initial part of the pH trace obtained with the pH recorder. Active ejection of protons, by energy dependent mechanisms, which could interfere with the proton influx induced by the acid uptake was assumed to be insignificant since all the cell suspensions used displayed slowly alkalization base lines. Furthermore, an inversion of the alkalization trace after the addition of the acid, consistent with active proton pumping [3] was never observed suggesting that this mechanism was absent or not measurable under the experimental conditions.

Different calibration procedures were tested and compared in order to be the more rigorous as possible in the estimation of the initial proton uptake. It was selected the calibration with NaOH in the cell suspensions for the different experimental pH. The buffering capacity of the weak carboxylic acids, was also estimated for the experimental concentration range in each assay and showed to be insignificant.

TABLE I

Equations for the variation of the different equilibrium concentrations of the acids expressed as a function of the radioactive flux of the transported form

$M_1 = K_1/[H^+]$; $M_3 = K_1K_2/[H^+]^2$; $M_4 = K_1K_2K_3/[H^+]^3$, where K_1, K_2, K_3 are the first, second and third dissociation constant of the acid, respectively.

	Monocarboxylic acid	Dicarboxylic acid	Tricarboxylic acid
Undissociated acid	$\frac{\Delta[HA]}{\Delta t} = \frac{1}{1+M_1} f$	$\frac{\Delta[H_2A]}{\Delta t} = \frac{1}{1+M_1+M_3} f$	$\frac{\Delta[H_3A]}{\Delta t} = \frac{1}{1+M_1+M_3+M_4} f$
Monoanionic form	$\frac{\Delta[A^-]}{\Delta t} = \frac{M_1}{1+M_1} f$	$\frac{\Delta[HA^-]}{\Delta t} = \frac{M_1}{1+M_1+M_3} f$	$\frac{\Delta[H_2A^-]}{\Delta t} = \frac{M_1}{(1+M_1+M_3+M_4)} f$
Dianionic form		$\frac{\Delta[A^{2-}]}{\Delta t} = \frac{M_3}{1+M_1+M_3} f$	$\frac{\Delta[HA^{2-}]}{\Delta t} = \frac{M_3}{(1+M_1+M_3+M_4)} f$
Trianionic form			$\frac{\Delta[A^{3-}]}{\Delta t} = \frac{M_4}{(1+M_1+M_3+M_4)} f$

The theoretical proton flux, defined as the proton flux exclusively associated to the transport, was estimated as described in Results and Discussion.

The values of the dissociation constants utilized in the calculations were the following: lactic acid $K_1 = 1.38 \cdot 10^{-4}$; succinic acid $K_1 = 6.17 \cdot 10^{-5}$ and $K_2 = 2.29 \cdot 10^{-6}$; citric acid $K_1 = 7.41 \cdot 10^{-4}$, $K_2 = 1.74 \cdot 10^{-5}$ and $K_3 = 3.98 \cdot 10^{-7}$.

Results and Discussion

Theoretical proton flux associated to the initial uptake rate of the acid

Most of the carboxylic acids are weak or intermediate electrolytes. As a consequence, in aqueous solutions they are incompletely dissociated, the equilibrium concentrations being a function of the pH and of the total acid concentration, $[T_{eq}]$. In accordance to the analysis presented in the Appendix, the equilibrium concentrations of the different carboxylic acids can be expressed as a function of the total acid concentration. On the other hand, when transport of a weak carboxylic acid, through the plasma membrane takes place with a radioactive flux f, during a time period Δt the total acid concentration in the extracellular medium after transport will be $[T_{eq}] - f\Delta t$. Then and following the analysis presented in the Appendix we can express the variation, per unit time, of the equilibrium concentrations of each acid form after transport as a function of the flux of the transported form. Table I summarizes the equations for the amounts of the equilibrium forms of the acids, that will have disappeared per unit time, expressed as a function of the flux of the transported form.

Now let us consider that proton signals were observed when a weak carboxylic acid was added to suspensions, in weak buffer, of cells that are able to transport the acid by a proton/symport mechanism. The observed experimental proton flux, in opposition to the case of a neutral substrate, does not represent the proton co-transport flux (see Appendix). On the other hand, assuming a given proton/negative charge stoichiometry (n), the theoretical proton flux f_t^H can be estimated either from the experimental radioactive flux (f) or from the experimental proton flux (f_{app}^H).

The equations for the several anion transport hypothesis either of mono-, di- and tricarboxylic acids, were deduced (see Appendix). These equations could be used to estimate the respective theoretical proton fluxes and thus the proton/negative charge stoichiometry. Table II exemplifies the most representative transport situations.

As it was previously reported [4], the observation of proton movements, when a weak carboxylic acid was added to cell suspensions, would also be consistent with the facilitated diffusion of the undissociated acid. However, the observed proton movements would not be related to proton influx but to the re-establishment of the acid-base equilibrium disturbed by the transport of the acid into the cell. Although, in this case, the theoretical proton flux, f_t^H is zero by definition, it is also possible, using the methodology described above to deduce the equations for the theoretical apparent proton flux, f_{app}^H, estimated from the experimental ra-

TABLE II

Equations for the theoretical proton fluxes associated to the initial uptake rate of the different weak acids, for the several transport hypothesis

The equations were deduced in Appendix. M_1, M_3 and M_4 are as defined in Table I.

I. Monocarboxylic acids	II. Dicarboxylic acids	III. Tricarboxylic acids
Proton/symport of the monoanionic form		
$f_{t1}^H = nf_{A^-}$	$f_{t1}^H = nf_{HA^-}$	$f_{t1}^H = nf_{H_2A^-}$
$f_{t2}^H = \frac{n(1+M_1)}{n(1+M_1)-1}$	$f_{t2}^H = \frac{n(1+M_1+M_3)}{(n-1)+nM_1+(n+1)M_3} f_{app}^H$	$f_{t2}^H = \frac{n(1+M_1+M_3+M_4)}{(n-1)+nM_1+(n+1)M_3+(n+21)M_4} f_{app}^H$
Proton/symport of the dianionic form		
	$f_{t1}^H = nf_{A^{2-}}$	$f_{t1}^H = nf_{H_2A^{2-}}$
	$f_{t2}^H = \frac{n(1+M_1+M_3)}{(n-2)+(n-1)M_1+nM_3} f_{app}^H$	$f_{t2}^H = \frac{n(1+M_1+M_3+M_4)}{(n-2)+(n-1)M_1+nM_3+(n+1)M_4} f_{app}^H$
Proton/symport of the trianionic form		
		$f_{t1}^H = nf_{A^{3-}}$
		$f_{t2}^H = \frac{n(1+M_1+M_3+M_4)}{(n-3)+(n-2)M_1+(n-1)M_3+nM_4} f_{app}^H$
Facilitated diffusion of the acid		
$f_{app'}^H = \frac{M_1}{1+M_1} f_{HA}$	$f_{app'}^H = \frac{M_1+2M_3}{1+M_1+M_3} f_{H_2A}$	$f_{app'}^H = \frac{M_1+2M_3+3M_4}{1+M_1+M_3+M_4} f_{H_3A}$

dioactive flux (Table II). However, the transport hypothesis of the facilitated diffusion of the undissociated acid, was eliminated based on the experimental evidences previously reported [1].

Stoichiometries of the proton / carboxylate symports of C. utilis by the use of the deduced theoretical proton flux equations

In order to estimate proton/carboxylate symports stoichiometries by the use of equations presented in Table II, we choose the yeast strain *C. utilis* as a biological model. The yeast was grown in a medium with mono-, di- or tricarboxylic acids as a sole carbon and energy source. Under these growth conditions, mid-exponential harvested cells of this yeast strain are capable to transport the anionic(s) form(s) of weak carboxylic acids through different proton/carboxylate symports, with their highest activity [1] and [5,6]. Although these mediated transport systems have been characterized from a kinetic and energetic point of view, the proton/carboxylates stoichiometries were not elucidated at that time. We repeat the measurement of the activities of the reported proton/carboxylates symports of that yeast strain either by the use labelled acids or following the proton movements associated to the acid uptake, in cell suspensions from the same original culture. In this way it was possible to minimize experimental variations associated with the use of yeast cells coming from different cultures.

The problem of distinguishing the proton through the symport from the basal proton flow outside it [7], was taken in account. In our experimental conditions the observed proton flux did not differs significantly from the proton flux corrected for the basal proton flux. Typically and as expected, in accordance with the published data, transient external alkalization indicative of proton uptake was observed when either lactic acid, succinic acid as well as citric acid was added to a suspension of cells, in weak buffer (from pH 5.0 to 6.0), that had been grown in mono-, di-, or tricarboxylic acids (Fig. 1). At these buffer pH values the relative undissociated acid concentration was near zero being possible to measure the activity of the transport system for the anionic form of the acid without contamination of the possible mediated transport for the undissociated form. At pH values lower than 5.0 and at higher acid concentrations, such as those corresponding to the postulated transport of the undissociated acid by facilitated diffusion [1], it was not possible to estimate proton uptake rates due to the buffering capacity of the acid itself and/or to the high extracellular proton concentration. Thus, the estimation, by iterative procedures, of the proton flux associated exclusively to the proton/carboxylate symport activity was not feasible and, as a consequence it was not possible to estimate the proton/carboxylate stoichiometry in that pH range.

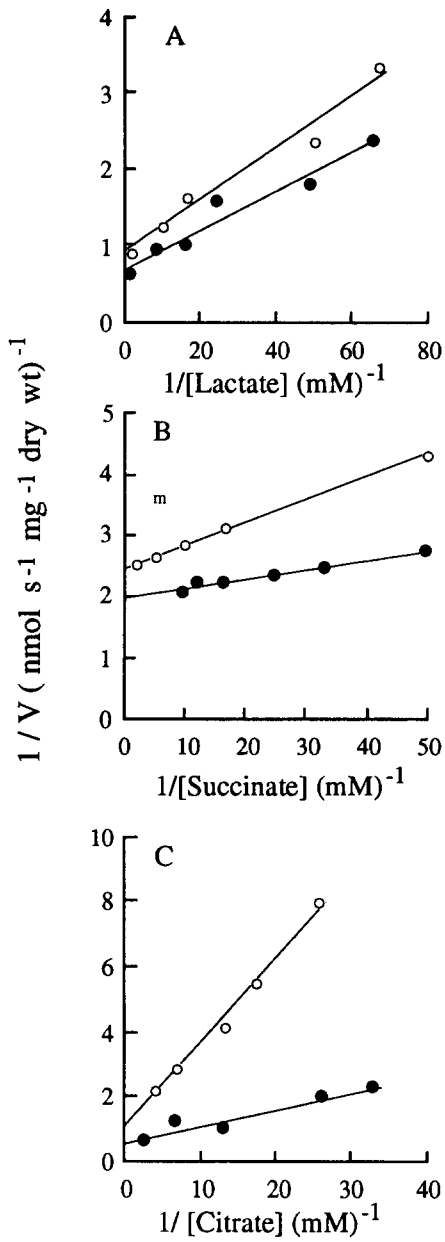

Fig. 1. Lineweaver-Burk plots of initial rates of uptake of labelled acids and protons by lactic acid grown cells of *C. utilis* 3092 as a function of a acid concentrations. (A) ○, Labelled lactic acid; ●, protons, pH 5.5. (B) ○, Labelled succinic acid; ●, protons, pH 5.0. (C) ○, Labelled citric acid; ●, protons, pH 5.5.

For the different acids studied, the Lineweaver-Burk plots of the initial acid uptake calculated from the slopes of the initial part of the experimental pH curves, as well as, of the initial rates of labelled acids, were linear. Fig. 1 shows results representative of these experiments which agree with those published in this field. The initial uptake rates of labelled acid and of protons estimated in this way, correspond to what we considered in the previous section as the radioactive fluxes (f) and the apparent proton fluxes f^{H}_{app}, respectively. The methodology used to estimate the apparent

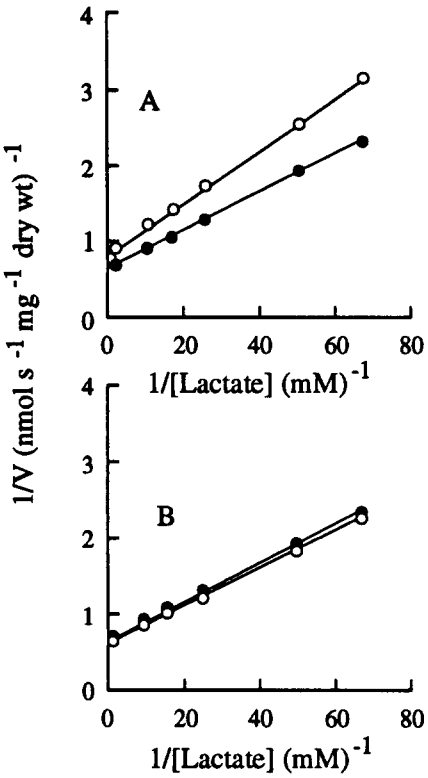

Fig. 2. Lineweaver-Burk plots of the theoretical proton fluxes of lactate at pH 5.5 as a function of lactate concentration by lactic acid grown cells of *C. utilis* 3092. (A) For an initial proton/carboxylate stoichiometry of 1:1. (B) For the most probable proton/carboxylate stoichiometry of 1.4:1. ○, f_{t1}^{H}; ●, f_{t2}^{H}.

proton fluxes through the variation of the extracellular pH could be considered not consistent with equations, such as those presented in Table II, which were deduced assuming a constant pH value. However, in our experimental conditions the proton fluxes were calculated from the initial proton movements corresponding to a variation of pH never above 0.002 pH units under the experimental time of 10 s.

Calculation of the proton/carboxylate stoichiometry was done as follows. Firstly, a given proton/carboxylate stoichiometry was arbitrarily selected. Then, for each acid concentration, the theoretical proton fluxes, as defined in the preceding section, were calculated for the different anionic transport hypothesis. In this way the respective initial radiolabelled acid uptake rate and the initial proton uptake rate were introduced in the equations presented in Table II. As an example we represent in Fig. 2 the Lineweaver-Burk plots of the theoretical proton fluxes, f_{t1}^{H} and f_{t2}^{H} of lactate, pH 5.5, obtained for an initial proton/carboxylate stoichiometry arbitrarily choosed of 1:1 as well as the Lineweaver-Burk plots of those theoretical fluxes obtained for the proton/carboxylate stoichiometry of 1.4:1. In this last situation both theoretical plots appeared to be near coincident, which was confirmed by the application of a χ^2 test for the similarity of the two regression lines obtained from the above plots. We concluded that at pH 5.5, the most probable transport H^+/lactate stoichiometry was 1.4:1.

Table III summarizes the estimated proton/carboxylate stoichiometries, at different extracellular pH, using the methodology described above. In accordance to these results, at pH 5.0, the proton/lactate and the proton/succinate stoichiometry was 1:1 with a neutral net charge. In the case of the proton/lactate and proton/citrate it appears that the stoichiometry ratio increased with increasing extracellular pH. The variations of the proton/carboxylates stoichiometries with the external pH could be explained if we take in account that in yeasts at high pH values, $\Delta\Psi$ is the main component of the proton electrochemical gradient while at low pH the value of $\Delta\Psi$ is near zero [8]. At pH 6.0 a proton/carboxylate stoichiometry of $3H^+$/substrate was obtained for the transport of citrate in the monoanionic form. A similar proton/carboxylate stoichiometry was obtained for the transport of citrate in *Klebsiella pneumoniae* vesicles [9]. Although we consider that more evidences are necessary to support the variation of the proton/carboxylate stoichiometry with the external pH, the results obtained with whole cells can be considered as a first approximation.

When the results presented in Table III were analyzed as whole we can also conclude that the developed quantitative analysis of the proton movements associated to the initial uptake rates of the acid did not allow, for a given experimental pH, to distinguish in which form the acid was transported since several hypothesis were equally probable. Thus, in the case of succinate both mono- and dianionic forms transport hypothesis were probable and a similar behavior occurs if we consider the transport possibility of the different

TABLE III

Estimated proton/carboxylate stoichiometry in the yeast C. utilis for different extracellular pH

Substrate	pH	Anion transport hypothesis	Estimated stoichiometry (H^+/ negative charge)	Net charge
Lactate	5.0	A^-	1:1	0
	5.5	A^-	1.4:1	+0.4
Succinate	5.0	HA^-	1:1	0
		A^{2-}	1:1	0
Citrate	5.5	H_2A^-	2.5:1	+1.5
		HA^{2-}	3.5:2	+1.5
		A^{3-}	4.5:3	+1.5
	6.0	H_2A^-	3:1	+2
		HA^{2-}	4:2	+2
		A^{3-}	5:3	+2

anionic forms of citrate. However the deduced equations revealed to be adequate for estimating proton/carboxylates stoichiometries. At present we are trying to test the methodology developed, with results obtained with membrane vesicles.

Appendix

Equilibrium concentrations of the different carboxylic acid forms before transport

Most of the carboxylic acids are weak or intermediate electrolytes. As a consequence, in aqueous solutions they are incompletely dissociated, the equilibrium concentrations being a function of the pH and of the total acid concentration. For the different weak carboxylic acids, at equilibrium, we have:

Case I. Monocarboxylic acids

$$\frac{[A^-]}{[HA]} = \frac{K_1}{[H^+]} = M_1 \tag{1}$$

where K_1 is the dissociation constant of the acid and [HA], $[A^-]$ are the equilibrium concentrations of the undissociated and monoanionic forms of the acid, respectively.

The concentrations of the two different forms at equilibrium as a fraction of the total acid concentration ($[T_{eq}]$), are the following:

$$[HA] = \frac{[HA]}{[HA]+[A^-]}[T_{eq}] \tag{2}$$

$$[A^-] = \frac{[A^-]}{[HA]+[A^-]}[T_{eq}] \tag{3}$$

and by substituting Eqn. 1 in Eqns. 2 and 3 we have:

$$[HA] = \frac{1}{1+M_1}[T_{eq}] \tag{4}$$

and

$$[A^-] = \frac{M_1}{1+M_1}[T_{eq}] \tag{5}$$

Case II. Dicarboxylic acids

$$\frac{[HA^-]}{[H_2A]} = \frac{K_1}{[H^+]} = M_1 \tag{6}$$

$$\frac{[A^{2-}]}{[H_2A]} = \frac{K_1K_2}{[H^+]^2} = M_3 \tag{7}$$

where K_1 and K_2 are the first and second dissociation constant of the acid, respectively; $[H_2A]$, $[HA^-]$ and $[A^{2-}]$ are the equilibrium concentrations of the undissociated, monoanionic and dianionic forms of the acid, respectively.

In a similar way to that described above for the monocarboxylic acids and using Eqns. 6 and 7, the concentration of the different forms at equilibrium, expressed as a fraction of $[T_{eq}]$, are as follows:

$$[H_2A] = \frac{1}{1+M_1+M_3}[T_{eq}] \tag{8}$$

$$[HA^-] = \frac{M_1}{1+M_1+M_3}[T_{eq}] \tag{9}$$

$$[A^{2-}] = \frac{M_3}{1+M_1+M_3}[T_{eq}] \tag{10}$$

Case III. Tricarboxylic acids

$$\frac{[H_2A^-]}{[H_3A]} = \frac{K_1}{[H^+]} = M_1 \tag{11}$$

$$\frac{[HA^{2-}]}{[H_3A]} = \frac{K_1K_2}{[H^+]^2} = M_3 \tag{12}$$

$$\frac{[A^{3-}]}{[H_3A]} = \frac{K_1K_2K_3}{[H^+]^3} = M_4 \tag{13}$$

where K_1, K_2 and K_3 are the first, second and third dissociation constant of the acid, respectively; $[H_3A]$, $[H_2A^-]$, $[HA^{2-}]$ and $[A^{3-}]$ are the equilibrium concentrations of the undissociated, monoanionic, dianionic and trianionic forms of the acid, respectively.

Then, using Eqns. 11, 12 and 13, the concentration of the different forms at equilibrium, expressed as a fraction of the total acid concentration $[T_{eq}]$, are as follows:

$$[H_3A] = \frac{1}{1+M_1+M_3+M_4}[T_{eq}] \tag{14}$$

$$[H_2A^-] = \frac{M_1}{1+M_1+M_3+M_4}[T_{eq}] \tag{15}$$

$$[HA^{2-}] = \frac{M_3}{1+M_1+M_3+M_4}[T_{eq}] \tag{16}$$

$$[A^{3-}] = \frac{M_4}{1+M_1+M_3+M_4}[T_{eq}] \tag{17}$$

In all the cases the equations deduced for the equilibrium forms concentration of the acid are valid for constant pH, since M_1, M_3 and M_4 are a function of pH.

Equilibrium concentrations of the different carboxylic acid forms after transport

Let us suppose that transport of a weak carboxylic acid, through the plasma membrane takes place with a

radioactive flux f, during a time period Δt. Then, the total acid concentration in the extracellular medium after transport will be $[T_{eq}] - f\Delta t$. Following the analysis presented in the preceding section we can express the variation, per unit time, of the equilibrium concentrations of each acid form after transport as a function of the flux of the transported form. As an example, suppose we focus our attention on the transport of a tricarboxylic acid. The amounts of the four forms of the acid that will have disappeared per unit time can easily be obtained as follows. For the undissociated acid will be:

$$\frac{\Delta[H_3A]}{\Delta t} = \left(\frac{1}{1 + M_1 + M_3 + M_4}[T_{eq}] - \frac{1}{1 + M_1 + M_3 + M_4}([T_{eq}] - f\Delta t) \right) \frac{1}{\Delta t} \quad (18)$$

or in a simplified form

$$\frac{\Delta[H_3A]}{\Delta t} = \frac{1}{1 + M_1 + M_3 + M_4} f \quad (19)$$

In a similar way for the mono-, di- and trianionic forms, we have, respectively:

$$\frac{\Delta[H_2A^-]}{\Delta t} = \frac{M_1}{(1 + M_1 + M_3 + M_4)} f \quad (20)$$

$$\frac{\Delta[HA^{2-}]}{\Delta t} = \frac{M_3}{(1 + M_1 + M_3 + M_4)} f \quad (21)$$

$$\frac{\Delta[A^{3-}]}{\Delta t} = \frac{M_4}{(1 + M_1 + M_3 + M_4)} f \quad (22)$$

Similarly, we can consider the transport of a dicarboxylic acid or of a monocarboxylic acid. Table I summarizes the equations for the amounts of the equilibrium forms of the acids, that will have disappeared per unit time, expressed as a function of the flux of the transported form.

Theoretical proton flux associated to the initial uptake rate of the acid

Let us consider that proton signals were observed when a weak carboxylic acid was added to suspensions, in weak buffer, of cells that are able to transport the acid by a proton/symport mechanism. The observed experimental proton flux, in opposition to the case of a neutral substrate, does not represent the proton co-transport flux. Actually the observed experimental proton flux, here defined as the apparent proton flux, f_{app}^H, is the result of two components:

(1) The theoretical proton flux associated exclusively to the transport, f_t^H.

(2) The proton flux expressed by $\Delta[H^+]/\Delta t$ associated to the re-establishment of the extracellular acid-base equilibrium disturbed by the transport of the acid during the period time Δt.

Returning to our example, suppose that the weak acid transported is a tricarboxylic acid. Further assume that trianion is the acid form which entered the cell. Then the apparent proton flux can be written as:

$$f_{app}^H = f_t^H - \frac{\Delta[H^+]}{\Delta t} \text{ (associated to the dissociation of } H_3A \text{ for regeneration of } A^{3-}) - \frac{\Delta[H^+]}{\Delta t} \text{ (associated to the dissociation of } H_2A^- \text{ for regeneration of } A^{3-}) - \frac{\Delta[H^+]}{\Delta t} \text{ (associated to the dissociation of } HA^{2-} \text{ for regeneration of } A^{3-}) \quad (23)$$

Substituting Eqns. 19, 20 and 21 in Eqn. 23 we may write:

$$f_{app}^H = f_t^H - 3\frac{1}{1 + M_1 + M_3 + M_4} f_{A^{3-}} - 2\frac{M_1}{1 + M_1 + M_3 + M_4} f_{A^{3-}} - \frac{M_3}{1 + M_1 + M_3 + M_4} f_{A^{3-}} \quad (24)$$

In addition it is possible assuming a given proton/negative charge stoichiometry (n) to estimate the theoretical proton flux f_t^H either from the experimental radioactive flux (f) or from the experimental proton flux f_{app}^H.

Following the example of the transport of the trianionic form of a tricarboxylic acid, let us assume that the anionic form entered the cell by an electroneutral proton/tricarboxylate symport. Then, we can write:

$$f_{t1}^H = 3f_{A^{3-}} \quad (25)$$

$$f_{t2}^H = \frac{3(1 + M_1 + M_3 + M_4)}{M_1 + 2M_3 + 3M_4} f_{app}^H \quad (26)$$

Where f_{t1}^H and f_{t2}^H are the theoretical proton fluxes estimated from the experimental radioactive flux and the apparent proton flux, respectively. Using this methodology the equations for the several anion transport hypothesis either of mono-, di- or tricarboxylic acids, were deduced. These equations could be used to estimate the respective theoretical proton fluxes and thus the proton/negative charge stoichiometry. Table II exemplifies the most representative transport situations.

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