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Thermodynamics and Energetics of the Tonoplast Membrane Operating as a Hysteresis Switch in an Oscillatory Model of Crassulacean Acid Metabolism

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Abstract. The observed endogenous circadian rhythm in plants performing Crassulacean acid metabolism is effected by malate transport at the tonoplast membrane. Experimental and theoretical work asks for a hysteresis switch, regulating this transport via the ordering state of the membrane. We apply a schematic molecular model to calculate the thermally averaged order parameter of the membrane lipid structure in its dependence on external parameters temperature and area per molecule. The model shows a first order structural phase transition in a biologically relevant temperature range. Osmotic consequences of malate accumulation can trigger a transition between the two phases by changing the surface area of the cell vacuole. Estimation of the energy needed to expand the vacuole under turgor pressure because of osmotic changes while acidifying shows that energy needed as latent heat for the calculated change between phases can easily be afforded by the cell. Thus, malate content and the coexisting two phases of lipid order, showing hysteretic behavior, can serve as a feedback system in an oscillatory model of Crassulacean acid metabolism, establishing the circadian clock needed for endogenous rhythmicity.

Key words: Crassulacean acid metabolism — Endogenous rhythm — Lipid membrane structure — Phase transition — Osmotic cell pressure

Introduction

Crassulacean acid metabolism (CAM) is a special mode of photosynthesis, where inorganic carbon is concentrated and water use economized (Osmond, 1978; Lüttge, 1987). CO_2 is fixed nocturnally and stored in the cell vacuoles in the form of organic acids, normally malic acid, and remobilized again during the light period for photosynthetical assimilation behind closed stomata. This cycle of CO_2-exchange and organic acid accumulation/remobilization also occurs under constant external conditions as a free-running endogenous rhythm (Wilkins, 1984; Lüttge & Beck, 1992). Hence, it is clearly necessary to postulate the existence of an endogenous oscillator. This can also be readily demonstrated by a computer model of CAM (Blasius, Beck & Lüttge, 1997) having four major pools of metabolites connected via metabolite flows as well as positive and negative feedback loops, where incorporation of a 'hysteresis switch', i.e., a mechanism which regulates the passive efflux of malate in dependence on the malate content in the vacuole, leads to a stable oscillatory behavior (Blasius, Beck & Lüttge, 1998). In our modeling we realized this beat oscillator by two discrete states of the tonoplast which could be switched to efflux 'on', or 'off', respectively, by increasing or decreasing the malate filling level of the vacuole. Though this model reproduces many empirical observations very well (Blasius et al., 1997, 1998), the effective beat oscillator has no physiological explanation so far.

It has been debated vividly, what the cellular, physiological or molecular hysteresis switch of CAM plants might be. The tonoplast membrane, which mediates energy-dependent vacuolar malate accumulation and passive malate efflux, and the enzyme phosphoenolpyruvate carboxylase (PEPC) serving nocturnal CO_2-fixation have both been considered most seriously as the possible candidates (Wilkins, 1984; Anderson & Wilkins, 1989; Lüttge & Beck, 1992; Grams et al., 1997). PEPC-activity regulated by phosphorylation/dephosphorylation in the

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day/night rhythm of CAM and the phosphorylating PEPC-kinase have in fact been demonstrated to show endogenous circadian oscillations (Nimmo et al., 1987; Carter et al., 1991; Kusumi et al., 1994; Carter et al., 1996).

For several reasons, however, we favor the tonoplast as the key oscillator of the endogenous CAM-rhythm (Lüttge, 1997). Both in vivo (Carter et al., 1995) and in the model (Blasius, 1997) the endogenous rhythm functions without the PEPC-kinase rhythm, although the latter somewhat stabilizes the oscillatory behavior (Blasius, 1997). Endogenous PEPC-kinase oscillation may be an epiphenomenon (Grams et al., 1997). Conversely, the model does need the tonoplast hysteresis switch (Blasius, 1997). Furthermore, regular endogenous oscillations of CAM in *Kalanchoë daigremontiana* under the influence of the external control parameter temperature are driven into arrhythmic behavior. This occurs both at a critical low and at a critical high temperature (Grams et al., 1997). When the endogenous rhythm is reinitiated again by increasing temperature from a temperature too low for regular oscillations, it sets in with decreased CO_2-uptake and malate mobilization. Conversely, when the reinitiated endogenous rhythm is due to lowering of the temperature from a level too high for regular oscillations, it begins with an increase in CO_2-uptake and malate accumulation. This can be easily explained by temperature effects on the physical state of the tonoplast membrane affecting passive malic acid efflux from the vacuole. At low temperature efflux is low and the rhythm stops with the vacuole remaining full of malate, at high temperature the vacuole is emptied when the rhythm goes into arrhythmicity. The phenomenon is observed both in vivo and in the performance of the CAM model (Grams et al., 1997). Finally, the critical temperature thresholds for the change between rhythmicity and arrhythmic behavior in continuous light are dependent on the growth temperatures of the plants (Grams, Kluge & Lüttge, 1995). Measurements with isolated tonoplast membranes have shown that this is due to homeoviscous adaptation; measured at the same temperature the tonoplast membranes from plants grown at higher temperatures have a higher state of order than those kept under lower temperatures during growth (Schomburg & Kluge, 1994). Thus, tonoplast functions must play a decisive role in driving endogenous circadian CAM oscillations.

In the present work we used a theoretical approach to find out if it is thermodynamically and energetically feasible that the lipid order of the tonoplast functions as a hysteresis switch. The highly simplified and schematic membrane model of Jähnig (1977, 1979) could be parameterized so that it gave two coexisting phases, one in which the order of lipid molecules in the membrane is high, and one in which it is low. The set of parameters is in a temperature range relevant for CAM plants, and where transitions between the two states are thermodynamically easily feasible in view of known biophysical behavior of CAM-plant vacuoles and their tonoplast membrane during acidification and deacidification in vivo (Lüttge, Kluge & Ball, 1975; Steudle, Smith & Lüttge, 1980; Lüttge, 1986).

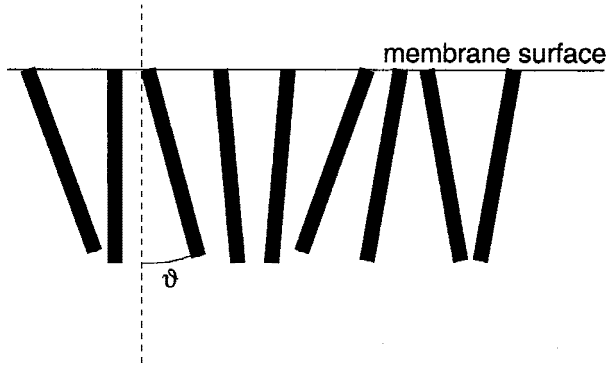

Fig. 1. Schematic representation of a monolayer of the lipid molecules as rigid rods reflecting the excluded volume of the hydrocarbon chains in the lateral plane of the membrane. ϑ is the angle of deviation from the membrane normal.

Materials and Methods

The schematic membrane model developed by Jähnig (1977, 1979) concerns a pure lipid matrix. The headgroups of the lipid molecules are neglected, and only a monolayer is taken into account, which, nevertheless, is proven generally to give reasonable results also in application to the basic thermodynamics of bilayers (Nagle, 1976), because only a constant surface pressure has to be added to transfer all physical properties from a monolayer. The model thus consists of single hydrocarbon chains with equal length, arranged in one plane and interacting with each other by van-der-Waals forces and mutual steric hindrance (see Fig. 1).The phase character of the system remains qualitatively the same regarding short chains without elastic energy or infinitely long chains with elasticity (*cf.* Figs. 5 and 6 in Jähnig, 1979). Changing the effective temperature scale only transfers one case into the other (*see below*). Thus, in the calculations presented here, the hydrocarbon chains are regarded as rigid, because with this assumption the thermodynamic averages can be evaluated almost completely in analytic form. The chain-chain interaction is substituted by a self-consistent potential acting on a specific chain, i.e., a mean field approximation is employed. According to Jähnig (1977, 1979), the energy of one chain in the molecular field is

$$E(\cos\vartheta) = -N\,\Gamma\cos\vartheta - N\Lambda\,\langle S\rangle\,\frac{1}{2}(3\cos^2\vartheta - 1), \qquad (1)$$

where ϑ is the polar angle of the chain defined with respect to the inward surface normal (*cf.* Fig. 1). N defines the chain length, expressed as the number of carbon atoms. The first term in Eq. 1 reflects the steric hindrance exerted by the surrounding lipid chains, with parameter Γ giving its strength. The second term is the mean field of the van-der-Waals forces of all surrounding chains with a constant strength parameter Λ (*cf.* Table) and the dynamical parameters $\langle S\rangle$ describing the mean orientational order of all chains (for detailed explanation of

Table. Parameters occurring in the membrane model

Symbol	Calculatory Relation	Value	Unit	Meaning
T	External		K	Temperature
f	External		m^2	Mean area per molecule
ρ	External	$6.25 \cdot 10^{-30}$	m^{-3}	Chains per volume
k	Constant	$1.381 \cdot 10^{-23}$	$J\,K^{-1}$	Boltzmann constant
Λ	Constant	2,800	$J\,mol^{-1}$	Strength of van-der-Waals forces
M	Constant	10,500	$J\,mol^{-1}$	Elasticity constant for bending
N_{eff}	Constant	4	None	Effective chain length
Γ	Calculated		$J\,mol^{-1}$	Strength of steric hindrance
$\langle\vartheta\rangle$	Calculated		None	Mean angle to membrane normal
$\langle x\rangle$	Calculated	$\langle\cos\vartheta\rangle$	None	Mean unitary dipole moment of the chains
$\langle S\rangle$	Calculated		None	Order parameter (Eq. 2)

The external parameters are not used directly in the calculation, but they are needed to obtain realistic values from modeling. The calculated quantities depend on the effective parameters t (Eq. 5) and f' (Eq. 6). (Values of M and ρ from Jähnig, 1977, p. 59 and p. 104; value of Λ from Marcelja, 1974)

the energy terms *see* Jähnig 1977, 1979 and the references given therein). The cornered brackets of $\langle S\rangle$ denote the thermodynamic average. Thus, the microscopical energy of one chain is not only dependent on its own orientation but also on the orientation of the ensemble of chains as a whole. Calculating the thermodynamic averages using $E(\cos\vartheta)$ (*see* Eqs. 3 and 4), leads to a self consistency problem (*see* Eqs. 11) because of this dependency on the thermodynamically averaged $\langle S\rangle$. Equation 1 implies, that the chains at low temperature, and by that at low excitation, tend to straighten rectangular to the membrane surface.

The orientations of all chains are summed up in $\langle S\rangle$, called the order parameter of the membrane lipids. It is derived from the quadrupole tensor of rodlike molecules. In the case of uniaxial symmetry, as we assume it here, the tensor has only one free parameter, which determines $\langle S\rangle$ in the form

$$\langle S\rangle = \frac{1}{2}\left(3\langle\cos^2\vartheta\rangle - 1\right) \tag{2}$$

This order parameter $\langle S\rangle$ characterizes by the second order term $\langle\cos^2\vartheta\rangle$ the orientational order of the system. If all chains orientate themselves parallel to the membrane normal, the maximum value $\langle S\rangle = 1$ is reached. On the other hand, a completely random orientation with $\vartheta < \pi/2$ for each lipid chain yields $\langle S\rangle = 0$. $\langle S\rangle$ can be measured directly by electron paramagnetic resonance (EPR), nuclear magnetic resonance (NMR), or by fluorescence anisotropy (FA), using probe molecules.

The probability $p(x)$ of a chain to have a unitary dipole moment $x = \cos\vartheta$ is given by the Boltzmann distribution:

$$p(x) = \frac{\exp\left[-\dfrac{E(x)}{kT}\right]}{\displaystyle\int_{-1}^{1}\exp\left[-\frac{E(x)}{kT}\right]dx} \tag{3}$$

The thermodynamic average of any quantity $A(x)$ in thermodynamic equilibrium is defined by the integral

$$\langle A\rangle = \int_{-1}^{1} A(x)p(x)\,dx \tag{4}$$

The macroscopic observables for such an ensemble are the temperature T, and the mean area per molecule f. They are not introduced explicitly in the computation. Rather, the temperature T is merged with the parameters N and Λ in the dimensionless parameter t, called reduced temperature:

$$t := \frac{kT}{N\Lambda} \tag{5}$$

The other macroscopic quantity, the mean area per molecule, f, is strongly related to the average $\langle x\rangle$ of the unitary dipole moment of the chains due to the fact, that a tilted chain consumes more membrane surface area than a rectangular one. Simple geometric reasoning (Jähnig, 1979; Neff, 1997) gives:

$$f' := N\rho \cdot f = (b \cdot \langle x\rangle)^{-1}, \tag{6}$$

where ρ is the number of chains per volume and b is the effective length of one C—C bond. For the calculations, the left hand side of Eq. 6 is combined into the second free varying parameter f'.

The scale of the reduced temperature t depends on the assumption about the chain flexibility. As pointed out earlier in this section, the calculations performed by Jähnig (1979) show that the thermodynamic properties for two limiting cases, the short rigid chain and the (infinitely) long elastic chain, are very much the same (Figs. 5 and 6 of this article, *loc.cit.*). They can be transformed into each other by only changing the reduced temperature scale. This can be seen immediately from the underlying physical mechanisms. For the rigid chain the thermodynamic excitations of a given chain in its mean field surroundings are the bending modes, described by the bending angle θ (Eq. 1), and a mean field strength parameter Λ. For the long elastic chain this mode is replaced by the relative bending modes of the individual parts of the chain, once more characterized by a bending angle, but additionally also by the elastic bending constant M. Thus, the reduced temperature for rigid chains, Eq. 5, is replaced for elastic chains by (Jähnig, 1979)

$$t_{el} := \frac{kT}{\sqrt{M\Lambda}}, \tag{7}$$

where the denominator now contains the geometric mean of the two force constants, M and Λ. Eqs. 5 and 7 allow one to scale the two cases into one another by only shifting the relative temperature appropriately.

Real tonoplast membranes contain lipid chains with between 14 and 26 carbon atoms (Haschke et al., 1990) which is certainly closer to the second of the two limiting cases. To apply our results to the tonoplast membrane we therefore have to relate the scales to each other by defining an effective chain length, N_{eff}, for the short rigid chain which brings the phase tansition to the same value of the reduced temperature as for the long elastic chain (where it is uniquely determined by the realistic force parameters M and Λ, *cf.* Table). This results in

$$t \approx 0.5\, t_{el}, \tag{8}$$

or

$$N_{eff} \approx 2\sqrt{\frac{M}{\Lambda}} \tag{9}$$

The advantage of the drastically simplified membrane model with rigid chains is that nearly all steps of the calculation of thermodynamic averages can be done analytically; only one Dawson-Integral has to be solved numerically. Besides that, a numerical procedure is also needed to fit the two self-consistency conditions: The first one results from the fact that the thermodynamic average $\langle x\rangle$ has to fulfill Eq. 6, where the parameter f' is chosen as starting value in the calculation. Using Eq. 4 to express $\langle x\rangle$ leads to the consistency requirement

$$\langle x\rangle = \frac{\int_{-1}^{1} x \cdot \exp\left[-\frac{E(x)}{kT}\right] dx}{\int_{-1}^{1} \exp\left[-\frac{E(x)}{kT}\right] dx} \stackrel{!}{=} \frac{1}{b \cdot f'} \tag{10}$$

This equation figures the dependence of the steric hindrance on the area available for the molecules expressed by f', and therefore the unkown steric hindrance parameter Γ (*cf.* Eq. 1) is adjusted to fulfill Eq. 10. The second self-consistency follows since the order parameter $\langle S\rangle$ influences the strength of the molecular field, as expressed in Eq. 1, while it is simultaneously dependent on this interaction, as $\langle S\rangle$ is a thermodynamic average. Formally this is done by inserting Eq. 4 into 2 and therefore the following equation has to be solved:

$$\langle S\rangle \stackrel{!}{=} \frac{1}{2}\left(3 \cdot \frac{\int_{-1}^{1} x^2 \cdot \exp\left[-\frac{E(x)}{kT}\right] dx}{\int_{-1}^{1} \exp\left[-\frac{E(x)}{kT}\right] dx} - 1\right) \tag{11}$$

One should bear in mind that $E(x)$ contains $\langle S\rangle$. The combination of the Eqs. 10 and 11 is equivalent to the mathematical problem of finding the roots of a function with two variables, here Γ and $\langle S\rangle$. A numeric computer algorithm was invented to determine these variables employing all relevant parameters summarized in the Table.

Results and Discussion

Jähnig's model in the form adapted here contains two free varying parameters: the reduced temperature t (Eq. 5) and the reduced surface area per molecule f' (Eq. 6). The calculation starts by choosing values for both. Then calculating the thermodynamic averages by solving the self-consistency conditions, Eqs. 10 and 11, results in values for the order parameter $\langle S\rangle$ of the membrane for each pair of t and f' (Fig. 2). There, a coexistence region shows up, i.e., a region in the parameter space where $\langle S\rangle$ has three different solutions. Only the two outer states (the upper and lower values for $\langle S\rangle$) are relevant, because the middle one is unstable:

$$\frac{d\langle S\rangle}{df'} > 0, \text{ or } \frac{d\langle S\rangle}{dt} > 0 \Rightarrow \textit{unstable} \tag{12}$$

The remaining two stable states establish two phases, distinguished by the order parameter $\langle S\rangle$: a high and a low ordered phase between which the system can switch. These phases can be considered as the two states of the hysteresis switch needed to explain the endogenous CAM rhythm (*see* Introduction). The jumb from one phase to the other is a first order phase transition with latent heat. At the critical point the coexistence region vanishes, and a phase transition of second order takes place.

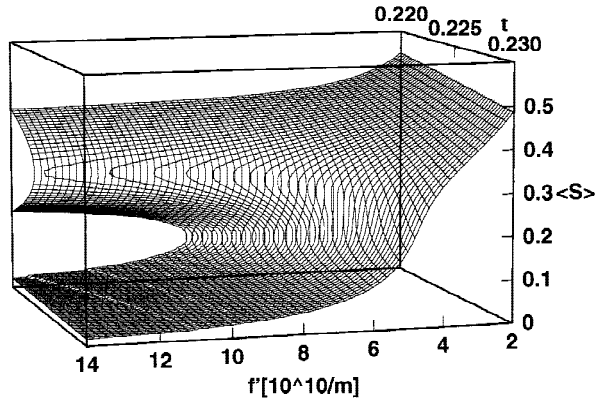

Fig. 2. The order parameter $\langle S\rangle$ in its dependence on the calculation parameters f' and t, showing a region in the parameter space with three coexisting different solutions for $\langle S\rangle$ at a given combination of f' and t.

Taking cuts across the parametric space at constant temperature results in the isotherms as shown in Fig. 3. Here we discuss only the case of constant temperatures, since the endogenous rhythm of CAM is normally defined under constant environmental conditions. Starting on a Z-like curve, i.e., in the coexistence region (e.g., $t = 0.224$ in Fig. 3), with a low value of f', and thus a high one of $\langle S\rangle$ indicating a high orientational order of the lipid chains, increasing f' causes at first only a slight decrease in $\langle S\rangle$, and thus only a slight weakening of the rectangular orientation of the chains until the system reaches the turning point. Further increase of f' forces the system to jump down on the lower branch of the curve into a disordered state having a much lower value of $\langle S\rangle$. In this state the chains are more excited, and in consequence the system has a higher internal energy U. This consumption of energy ΔU is overcompensated by the disordering process incrementing the entropy S_E by ΔS_E resulting in a net decrease of the Helmholtz free energy $\Delta F = \Delta U - T\Delta S_E$ at the transition according to the fundamental thermodynamic law, that the energy potential F has to be minimized. Decreasing f' drives the system back towards the lower turning point and in result to a reversal transition to a high ordered state at a much lower f' than at the first jump establishing the hysteresis.

To find the temperature range, which the coexistence region with its hysteretic phase behavior belongs to, one has to adopt a value for the effective chain length N_{eff}. Taking $\Lambda = 2{,}800\ J\ mol^{-1}$ and $M = 10{,}500\ J\ mol^{-1}$

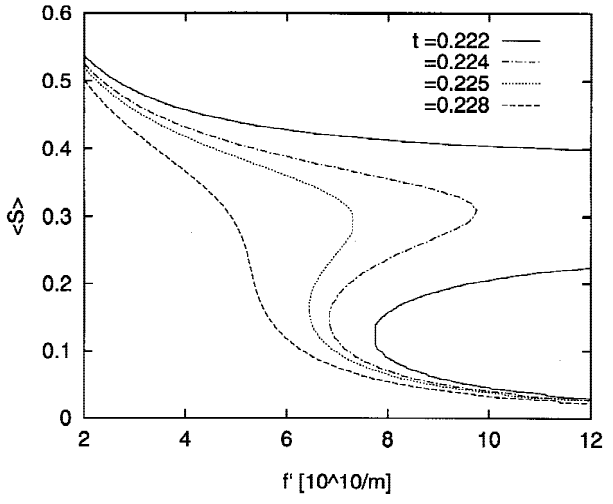

Fig. 3. Cuts across the parameter space (as shown in Fig. 2) showing the order parameter $\langle S \rangle$ in the coexistence region in its dependence on f' for different constant t. According to the values given in the Table, $t = 0.222$ and 0.2228 corresponds to temperatures $T = 299\ K$ and 307 K, respectively.

(*cf.* Table), one obtains $N_{eff} \approx 4$ (Eq. 9). The resultant temperatures lie in the interval between 299 and 307 K for the isotherms shown in Fig. 3, and this is well in the range of temperatures relevant for CAM plants. Thus, the Jähnig's model in this simple form becomes quite sufficient for our discussions of how the tonoplast could function as a hysteresis switch in endogenous CAM oscillations.

It is known (Lüttge et al., 1975; Steudle et al., 1980), that the night-time acidification and the day-time deacidification in CAM-plants lead to oscillating osmotic pressure and, in consequence, to oscillating turgor. Thus, not only the malate content of the vacuole, but also the vacuole volume changes periodically during the CAM rhythm. The nocturnal volume expansion, ΔV, of single cells of CAM plants has been measured, i.e., 4.6% for *Kalanchoë daigremontiana* and 2.3 to 10.7% for *Senecio medley-woodii* (Lüttge, 1986). The vacuole expansion can be regarded as being equal to the cell expansion, because the vacuole takes up more than 98% of the whole cell volume. Assuming that the vacuole has a spherical shape and stays spherical when expanding and shrinking, the volume expansion can be translated into a surface expansion. Correspondingly, the volume expansions of 2.3, 4.6 and 10.7% result in surface area expansions of 1.5, 3.0 and 7.0%, respectively. This is just in the range of permitted maximal surface expansion before bilayer vesicles burst (Evans & Needham, 1986). With constant chain density ρ the relative change of the whole surface of the vacuole is equal to the relative change of the mean are per molecule f. Thus, it is very probable, that the change of the malate content in the vacuole during the CAM cycle affects the area available per membrane lipid molecule. The first order phase transition calculated in the model will then establish a hysteresis, driven by the area change. In turn, the hysteretic phase behavior may influence the malate transport across the tonoplast, irrespective of whether it occurs by diffusion or via a carrier.

A necessary requirement for this possibility of the tonoplast membrane realizing a dynamic hysteresis switch, is the energy involved. The jump of the order parameter $\langle S \rangle$, when the membrane lipids are going from one phase to the other, is correlated with a jump in the Helmholtz free energy F of the system. In the membrane model this jump with the parameter values adopted here is around $5 \cdot 10^{-11}\ J$. An estimation of the expansion work, ΔW, which is needed to expand the whole cell against the turgor P, as it is experimentally observed to occur in CAM plants (*see above*), is about a factor of hundred times bigger than the theoretically evaluated energy above, as it is given by the following considerations: An amount of work $dW = P\ dV$ is needed to expand the CAM cell against the turgor, P, while malate accumulates in the vacuole and water is taken up osmotically. Assuming that the volumetric cell-wall elastic modulus ε is constant, the turgor P changes as:

$$P(V) = P_0 + \varepsilon\ ln\left(\frac{V}{V_0}\right), \tag{13}$$

where the index *0* indicates quantities at the start of the expansion. This results in a total work ΔW of the whole expansion ΔV:

$$\Delta W = \int_{V_0}^{V_0+\Delta V} P(V)dV$$

$$= V_0\left[(P_0 - \varepsilon)\frac{\Delta V}{V_0} + \varepsilon\left(1 + \frac{\Delta V}{V_0}\right) \cdot ln\left(1 + \frac{\Delta V}{V_0}\right)\right] \tag{14}$$

For values $\Delta V/V_0 = 4.6\%$ in *Kalanchoë daigremontiana* with $V_0 = 3.9 \cdot 10^{-13}\ m^3$ (Lüttge, 1986), and $P_0 = 1.82\ bar$, $\varepsilon = 42.4\ bar$ (Steudle et al., 1980) the expansion work is

$$\Delta W \approx 5 \cdot 10^{-9}\ J \tag{15}$$

Thus, the energy actually involved in the observed cell expansion and shrinking during acidification and deacidification, respectively, is much larger then the energy needed for the jump of the order parameter $\langle S \rangle$ between the two stable phases in the coexistence region of Jähnig's membrane model. This shows that the dynamic switch proposed here is indeed energetically possible.

Conclusion

Experimental as well as theoretical model studies of the endogenous CAM rhythm strongly support the role of the tonoplast as oscillatory generator. This implies that malate efflux out of the vacuole is regulated, depending on the malate content in the vacuole, between high and low states of membrane permeability. Since the latter depends on the ordering structure of the lipid chains in the membrane (Friemert et al., 1988; Kliemchen et al., 1993), two states of high and low order have to exist under the same external conditions to establish a hysteresis switch, or beat oscillator.

We have employed a molecular model of lipid membrane order, developed by Jähnig (1977, 1979), to investigate its dependence on the two external parameters, temperature and mean area per lipid molecule. Despite its highly schematic nature the model can be so manipulated that it serves our purpose to show a coexistence region of two stable states in parameter space, connected by a first order phase transition. With appropriate physiological parameters for the van-der-Waals interactions of the lipid molecules and the chain elasticity the coexistence region lies in a reasonable temperature range relevant for CAM plants. The switching between the two states occurs by the elastic stretch of the membrane layer resulting from osmotic and turgor pressure changes occurring during malate accumulation/remobilization in the vacuole. The energy needed for the order change in the membrane model is only about 1/100 of that actually afforded by the cells during pressure changes, thus they can easily initiate the order transitions in the tonoplast. This is not trivial, because the first energy is estimated by theory of molecular dynamics of the membrane lipids disregarding any osmosis and the latter is calculated from experimentally obtained physiological data of a whole cell.

Our analysis shows that the operation of the tonoplast membrane as a hysteresis switch in the circadian rhythm of CAM is a physically realistic possibility, and incorporation of the calculations shown here into the existing CAM computer model (Blasius et al., 1997) produces simulations of the metabolic cycle in total accordance to experimental data (Blasius, 1997, and paper in preparation). This beat oscillator indeed may be an important element of the endogenous oscillations.

Coming experimental work has to prove the existence of the proposed hysteresis switch by checking if a phase transition in the lipid order is responsible for the turnover from malate influx to malate efflux at the tonoplast. Therefore, the thermodynamic behavior of the tonoplast membrane has to be examined further and with special concern for the transmembrane flux. Furthermore it is decisive, if the osmotic expansion and shrinking of the vacuole drives indeed the flux regulation. This question depends on the pure mechanical properties, like shape and embedding of the tonoplast, and on physiological ones, like homeoviscious adaption.

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