Oscillatory model of crassulacean acid metabolism with a dynamic hysteresis switch

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The mechanism of endogenous circadian photosynthesis oscillations of plants performing crassulacean acid metabolism (CAM) is investigated in terms of a nonlinear theoretical model. Unlike previous CAM models containing a discrete element, we use throughout continuous time differential equations which more adequately reflect the CAM dynamics. By incorporating results from both a complementary and a continuous membrane model, a detailed description of the molecular malate transport in and out of the vacuole through the tonoplast membrane is achieved. Our analysis shows that the membrane effectively acts as a hysteresis switch regulating the oscillations. It thus provides a molecular basis for the circadian clock. The model shows regular endogenous limit cycle oscillations that are stable for a wide range of temperatures, in a manner that complies well with experimental data. The circadian period length is explained simply in terms of the filling time of the vacuole. The results emphasize the central role of membrane dynamics for the generation of circadian oscillations and thus have general relevance for the explanation of biological clocks.

Keywords: biological clock; crassulacean acid metabolism; endogenous rhythm; hysteresis switch; limit cycle oscillation; lipid membrane order parameter

1. INTRODUCTION

Crassulacean acid metabolism (CAM) is a special mode of photosynthesis providing a mechanism for plants to concentrate CO₂ and economize water use (Kluge & Ting 1978; Osmond 1978; Lüttge 1987; Winter & Smith 1996). CAM plants take up external CO₂ during the night while the stomata are open, and water loss by transpiration is low due to reduced nocturnal leaf-to-air water vapour pressure difference. The CO₂ is fixed in the plant in the form of organic acids, normally malate, via the enzyme phosphoenolpyruvate carboxylase (PEPc) and stored overnight in the cell vacuole. During the daytime, malate is remobilized from the vacuole and decarboxylated again. The resulting CO₂ is refixed by ribulose-bis-phosphate carboxylase-oxygenase (RubisCO) for photosynthetic assimilation.

As a result, CAM plants show a diurnal rhythm of CO₂ uptake and acidification, which is entrained by the day–night cycle. What is particularly surprising is that this same rhythm persists for many periods when the day–night cycle is artificially removed and replaced by environmental conditions that are constant in time (Wilkins 1959, 1992; Nuernbergk 1961). For instance, the net CO₂ exchange of the CAM plant *Kalanchoë daigremontiana* can show regular oscillations over several weeks under conditions of continuous illumination (Lüttge & Beck 1992). It is this characteristic that makes the CAM cycle a free-running endogenous circadian rhythm, and this is the motivation for our model.

There are a number of other fascinating aspects of the natural CAM cycle that are challenging to model. In recent years, considerable experimental effort has been devoted to studying the response of CAM plants to varying environmental conditions and control parameters, such as temperature, irradiation and CO₂ supply from the surrounding atmosphere (Anderson & Wilkins 1989a,b; Grams *et al.* 1997). The endogenous oscillations of CAM plants are known to be stable for only a limited region in the space of these control parameters. For instance, if temperature or light intensity are increased above a certain level the endogenous rhythm in continuous light disappears (Buchanan-Bollig & Smith 1984). At a critical threshold level, very small changes of temperature or light intensity are sufficient to destroy rhythmicity (Lüttge & Beck 1992). With respect to temperature, the loss of regular oscillations occurs not only when a critical high level, but also when a critical low level, is passed. It has been shown that these transitions between rhythmicity and steady states are reversible (Grams *et al.* 1996). Thus, temperature, *T*, and light intensity, *L*, appear as important state-determining control parameters of the endogenous CAM rhythm.

Already as early as 1984 a first model of CAM was established in terms of a set of coupled nonlinear differential equations, describing the varying concentrations in the major metabolites of CAM during a day–night cycle (Nungesser *et al.* 1984). Recently, the model has been refined to allow simulations of endogenous oscillations under continuous conditions, as well as the breakdown of rhythmicity by a change of control parameters (Lüttge &

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Beck 1992; Blasius *et al.* 1997, 1998). There have also been considerable simplifications regarding the dynamically independent constituents. Compared to the original version (Nungesser *et al.* 1984) which employed six pools of metabolites, it has been shown by stepwise reduction in the number of pools that only three reactants are essential for a successful description of CAM in terms of a skeleton model; these are internal CO_2 , w ; malate in the cytoplasm, x ; and malate in the vacuole, y .

In the model, malate transport regulation between cytoplasm and vacuole is the key process for establishing the observed endogenous rhythm in CAM, and it inevitably requires a switching of the passive malate efflux which shows a strong hysteretic behaviour. In the earlier models of CAM this was realized by the rather crude approximation in terms of a discrete function. For the purpose of structural analysis, and for the calculation of stability boundaries in the CAM cycle, this proved most suitable (Blasius *et al.* 1998). However, discretely separated hysteresis states are far from the realistic intrinsic behaviour of a living system. Moreover, it gives little insight into the biophysical mechanism of malate transport regulation and into the nature of the endogenous clock.

Recent experimental progress has shown that conformation changes in the order structure of lipid membranes may play an important role in their *in vivo* behaviour in CAM plants (Kliemchen *et al.* 1993). In a complementary theoretical study based on a schematic membrane model (Jähnig 1979), we have shown that under natural thermodynamic and energetic conditions for the leaves of CAM plants their tonoplast membrane can effectively function as a continuous hysteretic switch (Neff *et al.* 1998). In this work we proceed one step further and demonstrate that the results from the schematic membrane model can also be coupled to the CAM model in a quantitatively correct way. Thus, the main objective of this paper is to introduce an extended model of CAM which enlarges our successful earlier models with a detailed description of molecular malate transport. This provides a concrete biophysical example for the mechanism of the underlying biological clock. The model of the CAM cycle which we derive consists of continuous variables only, and allows us to learn many new details about CAM dynamics which were missing in the earlier discrete models. Our analysis shows that regular endogenous oscillations of the limit cycle type occur for a wide range of temperature and other control parameter values, created by super- and subcritical Hopf bifurcations. The circadian period length of the oscillations emerges in a natural way within the framework of the membrane model.

2. CONSTRUCTION OF A MINIMAL MODEL

The CAM model, shown schematically in figure 1, describes the carbon flow of the CAM cycle and its regulation at the cellular level. This is achieved by introducing concentrations in the pools of the major reactants of CAM: w , internal CO_2 concentration; x , malate concentration in the cytoplasm; y , malate concentration in the vacuole (see figure 1); and the order of the tonoplast membrane, z , as time-dependent variables of the cyclic process. As explained in detail in Blasius *et al.* (1997), the

Figure 1. Block diagram of the CAM model showing as dynamic variables (encircled) the three reactant pools (internal CO_2 concentration, w ; malate concentration in the cytoplasm, x ; and in the vacuole, y) and the order of the tonoplast, z , within a CAM cell. They are connected by the flows, u_1, u_2, u_3 (solid arrows). Regulatory feedback loops are indicated as dashed lines. The model depends on the three external control parameters: temperature, T ; light intensity, L ; and external CO_2 concentration, C_{ext} (dotted arrows).

reactant pools are connected by flows, u_1, u_2, u_3 , describing the gain and loss terms of the metabolites. This, in turn, leads to time changes of the pool concentrations given by the net flow into a specific pool

$$\begin{aligned}\epsilon \dot{w} &= -u_2 + u_3, \\ \epsilon \dot{x} &= -u_1 + u_2, \\ \dot{y} &= +u_1.\end{aligned}\quad (1)$$

Here, the smallness parameter ϵ takes care of the volume ratio of cytoplasm to vacuole which is typically about 1/100 in CAM plants. (Steudle *et al.* 1980; Blasius *et al.* 1998). The introduction of this small parameter is necessary since x, y, z are expressed as concentrations rather than as the absolute amount of reactants. The following form for the flows is proposed (Blasius *et al.* 1998):

$$\begin{aligned}u_1 &= c_1 x - c_2 P(z) y, \\ u_2 &= c_3 w/x - c_4 x, \\ u_3 &= c_5 \frac{(C_{\text{ext}}(t) - w)}{\exp(\alpha w)} - c_6 L(t) w + c_7 \left(\frac{L_K}{L(t) + L_K} \right) \left(\frac{w_\Gamma}{w + w_\Gamma} \right).\end{aligned}\quad (2)$$

In these equations, u_1 is given by the difference of malate influx and efflux into and out of the vacuole. $P(z)$ describes the tonoplast permeability for malate efflux out of the vacuole as explained below (equation 6).

The flow u_2 denotes the difference of malate production from CO_2 fixation by PEPc minus malate depletion by decarboxylation. The well-known feedback inhibition of PEPc by malate is described in the form $1/x$. This regulation leads to rhythmic changes in the activity of PEPc, and it is essential for CAM in order to prevent refixation of CO_2 , which is released during the day, by PEPc rather than via RubisCO in the Calvin cycle.

Finally, u_3 is the sum of CO_2 uptake from outside minus the CO_2 consumption by photosynthesis plus the CO_2 production by respiration. Regulation of CO_2 uptake by stomatal opening and closing is due to a stomatal resistance, ρ_s , related to internal CO_2 concentration, w , (Cockburn *et al.* 1979). As explained in more detail in Blasius *et al.* (1998), simulations with low values of external CO_2 concentration require a stomatal resistance which grows faster than linearly with w , as is also observed experimentally. In the model we use an exponential growth $\rho_s \sim \exp(\alpha w)$. The simulation of a CO_2 source given by respiration is provided by the last term of the third of equations (2). Respiration is assumed to be inhibited by light and internal CO_2 , with inhibition constants L_K and w_r , respectively. As a consequence, the respiration term has no influence under normal light intensities and in normal air, but it is necessary for simulations in CO_2 -free air and darkness (Blasius *et al.* 1998). Besides these regulation elements between pools, indicated as dashed lines in figure 1, flows depend linearly on substrate concentrations, with material constants, $c_1, \dots, c_7$, which represent flow resistances, diffusion coefficients, etc. For quantitative comparisons with experimental results, these parameters should be adjusted to fit measured properties of the plant in question.

(a) Regulation of malate transport

In our model, regulation of the transport of malate between cytoplasm and vacuole is the key process for establishing the observed endogenous rhythm in CAM, and, as has been pointed out earlier, requires a hysteretic switching of the passive malate efflux. In the earlier models of CAM (Lüttge & Beck 1992; Blasius *et al.* 1997, 1998) this hysteretic behaviour was introduced in the simple form of a discrete beat oscillator, i.e. as a function, $f(y)$, which switched the passive malate efflux to 'on', ($f(y) = f_1$), or to 'off', ($f(y) = f_0$), depending on the malate content in the vacuole. For reasons outlined in §1, we model here the mechanism of passive malate efflux in more detail by employing a continuous hysteresis switch, based on results described in Neff *et al.* (1998). To this end we introduce the order parameter of the tonoplast membrane lipids, z , as an additional dynamical variable, defined by

$$z = \frac{1}{2} (3 \langle \cos^2 \theta \rangle - 1). \quad (3)$$

Here θ denotes the polar angle of a lipid chain against the membrane surface normal. Neff *et al.* (1998) used a schematic molecular membrane model (Jähnig 1979) to obtain the thermal average of z in its dependence on temperature, T , and mean area per lipid molecule, ϕ .

The combination of these results with the CAM process is based on the following assumptions: the osmotic consequences of malate accumulated in the cell vacuole lead to an increase of the surface area of the vacuole which, via a change in the mean area per lipid ϕ , can then trigger a first-order structural phase transition from high to low order in the conformation of the membrane; the reverse process, switching from low to high order, is characterized by strong hysteresis (Neff *et al.* 1998). On the other hand, the hysteretic phase behaviour

Figure 2. Nullclines for the variables malate content in the vacuole, y (dashed line) (equation 13) and for the tonoplast order, z (solid line) (equation 14). The nullclines are calculated for different relative temperatures T , ranging from $T = 0.2238$ to $T = 0.2254$. (Set of constants and control parameters as in figure 3).

of the membrane has a strong influence on the passive malate transport out of the vacuole, inasmuch as higher membrane order reduces the fluidity of the tonoplast barrier, and irrespective of whether transport occurs by diffusion or via a carrier.

To integrate this behaviour of the tonoplast into our model, the following functional dependencies have to be specified.

- (i) The increase of the mean area per lipid molecule, ϕ , by raising malate concentration in the vacuole, y . This dependence can be reasonably assumed to be linear (Blasius 1997)

$$\phi(y) = \phi_0 + \lambda y. \quad (4)$$

ϕ_0 describes the minimal area per lipid for the tonoplast of the empty vacuole.

- (ii) The dependence of the order parameter of the tonoplast membrane, z , on the mean area per lipid molecule, ϕ ,

$$\phi = \phi(z, T). \quad (5)$$

This is given by the numerical results in Neff *et al.* (1998) in dependence on reduced temperature, T , which was introduced there as a dimensionless parameter proportional to Boltzmann's constant times real temperature, and containing two lipid chain parameters (see figure 2).

- (iii) Dependence of the tonoplast permeability, P , for malate efflux out of the vacuole on the tonoplast membrane order, z . In simplest approximation, the transport resistance, $\rho = 1/P$, is proportional to the tonoplast membrane order, z , so that

$$P = P(z) = \frac{P_0}{z}. \quad (6)$$

- (iv) The dynamics of the tonoplast order, z , can not be derived from the thermal averages, as obtained by Neff *et al.* (1998). Rather it is given by the membrane's relaxation behaviour. For this we assume a changing rate proportional to the distance of malate concentration in the vacuole, y , from its thermodynamic equilibrium value, $g(z, T)$: $\tau \dot{z} = g(z, T) - y$, with $g(z, T)$ given by

$$g(z, T) = \frac{\phi(z, T) - \phi_0}{\lambda}. \quad (7)$$

Here we have introduced the relaxation time constant, τ , which is unknown *a priori* and has to be adjusted later.

Putting everything together, the time evolution of our model is governed by the following system of four coupled nonlinear differential equations of first order in time:

$$\begin{aligned} \varepsilon \dot{w} &= -u_2 + u_3, \\ \varepsilon \dot{x} &= -u_1 + u_2, \\ \dot{y} &= +u_1, \\ \tau \dot{z} &= g(z, T) - y. \end{aligned} \quad (8)$$

We now introduce dimensionless variables and parameters in the following way:

$$\begin{aligned} x' &= \frac{c_4}{c_3} x, & y' &= \frac{c_2 P_0}{c_3} y, & w' &= \frac{c_4}{c_3} w, & z' &= z, & t' &= c_2 P_0 t, \\ \tau' &= \frac{c_2 P_0^2}{c_3} \tau, & \epsilon' &= \frac{c_2 P_0}{c_4} \epsilon, & T' &= T, & L' &= \frac{c_6}{c_4} L, & C'_{\text{ext}} &= \frac{c_4}{c_3} C_{\text{ext}}, \\ \alpha' &= \frac{c_3}{c_4} \alpha, & L'_K &= \frac{c_6}{c_4} L_K, & w'_\Gamma &= \frac{c_4}{c_3} w_\Gamma, & \lambda' &= \frac{c_3}{c_2 P_0} \lambda, & \phi'_0 &= \phi_0. \end{aligned} \quad (9)$$

Here the dimensionless smallness parameter ϵ is also rescaled to take into account the large difference (several orders of magnitude) between the malate concentrations in the vacuole and in the cytoplasm. As a consequence, $\epsilon' \ll 1$, and the dynamics of the reactant pools in the cytoplasm, w, x , is permanently held in a pseudo steady state (Blasius *et al.* 1998).

In the following we rewrite variables and constants omitting from now on the primes. In dimensionless variables the flows are then given by

$$\begin{aligned} u_1 &= cx - y/z, \\ u_2 &= w/x - x, \\ u_3 &= c_J \frac{(C_{\text{ext}}(t) - w)}{\exp(\alpha w)} - L(t)w + c_R \left(\frac{L_K}{L(t) + L_K} \right) \left(\frac{w_\Gamma}{w + w_\Gamma} \right), \end{aligned} \quad (10)$$

where the abbreviations

$$c = \frac{c_1}{c_4}, \quad c_J = \frac{c_5}{c_4}, \quad c_R = \frac{c_7}{c_3}, \quad (11)$$

have been used.

Equations (8) and (10) completely define our CAM model, which contains four dynamic variables w, x, y, z , and depends on three control parameters, T, L, C_{ext} ; two short time-scales, ϵ, τ ; six constants, $c, c_J, c_R, L_K, w_\Gamma, \alpha$; and on a function describing the equilibrium state of the membrane order $g(z, T)$.

3. FIXED POINT ANALYSIS

In order to study the regions of rhythmic oscillations and of steady states, the fixed points of equation (8) are calculated (Blasius *et al.* 1998). The fixed points of the reactant pools $\bar{w}, \bar{x}, \bar{y}$ can all be obtained by setting each flow to zero individually,

$$u_i = 0, \quad (i = 1, \dots, 3), \quad (12)$$

whereas the equilibrium of the membrane order is defined by the condition $\dot{z} = 0$. Using equations (8) and (10) this leads to the following results.

The condition $u_3 = 0$ defines the fixed point of the internal CO_2 concentration, $\bar{w}(L, C_{\text{ext}})$, which has to be evaluated graphically or numerically, and it depends on the control parameters L and C_{ext} (Blasius *et al.* 1998). Since $u_2 = 0$ yields $\bar{x} = \sqrt{\bar{w}}$, the fixed points of the reactants in the cytoplasm are directly dependent on each other, and by this the steady-state value of the malate concentration in the cytoplasm, $\bar{x}$, is also fixed. Setting the malate transport through the tonoplast to zero, $u_1 = 0$, yields $c\bar{x} = \bar{y}/\bar{z}$. Thus, the equilibrium malate content in the vacuole, $\bar{y}$, is directly proportional to the steady state of the tonoplast membrane, $\bar{z}$,

$$\bar{y} = c\bar{x}\bar{z} = c\sqrt{\bar{w}(L, C_{\text{ext}})} \cdot \bar{z}. \quad (13)$$

The equilibrium value for the tonoplast order, $\dot{z} = 0$, is of course defined by the result obtained with the schematic membrane model (equations 5, 7)

$$\bar{y} = g(\bar{z}, T). \quad (14)$$

Equations (13) and (14) define two null clines. The steady states of the system lie on the intersection of the null clines in the y, z -phase plane. As a first consequence of the dynamic nature of the hysteresis switch employed here, we obtain two null clines governing the fixed points of the system instead of only one null cline in the case of the discrete hysteresis switch (Blasius *et al.* 1998). The null clines are plotted in figure 2 for different temperatures, T . The y -null cline, equation (13), is just a straight line through the origin. It describes the membrane-dependent malate transport through the tonoplast in such a way that malate in the vacuole, y , tends towards an equilibrium value proportional to the membrane order, z . The temperature-dependent z -null cline, equation (14), describes the equilibrium value of the membrane order as a function of accumulated malate. Its cubic form corresponds to the hysteretic behaviour of the phase diagram in the membrane model of Neff *et al.* (1998). For very high or very low temperatures, the intersections of the null clines lie on the branches with negative slope; conversely, for medium temperatures the cuts lie on the unstable branches with positive slope, allowing for oscillating solutions. In a small temperature regime (here from $T = 0.224179$ to $T = 0.224188$) there even exist three intersections of the null clines.

Figure 3. Simulation of time courses of sustained endogenous rhythms in continuous light. Simulated values (relative units) of: (a) CO_2 uptake, J_{CO_2} ; (b) malate content in the vacuole, y (solid line), and malate content in cytoplasm, x (dashed line); and (c) tonoplast order, z . (Control parameters: $T = 0.2246$, $C_{\text{ext}} = 1$, $L(t) = 1$; constants: $c = 5.5$, $c_j = 1$, $c_R = 1$, $\epsilon = 0.001$, $\tau = 0.35$, $\alpha = 1.5$, $w_T = 0.1$, $L_K = 0.5$, $\phi_0 = 2$, $\lambda = 7$.) Note: the use of relative units obscures the fact that the physiological ratio malate in cytoplasm to malate in vacuole is always much smaller than one; cf. the remark at the end of the first paragraph in § 2.

4. ENDOGENOUS RHYTHM AS A LIMIT CYCLE OSCILLATOR

Numerical integration of equations (8) under the condition of continuous light reveals a regime of sustained, periodic oscillations in a large domain of parameter values and temperatures. The temporal variations of CO_2 uptake and of the metabolic pools are shown in figure 3. The time course of the order parameter, z , shows the typical form of a relaxation oscillation (Murray 1993), with distinct plateaus of high and low values, intersected by fast transition phases. This behaviour is a direct consequence of the small time-scale, τ , introduced in equation (8), in comparison with the relative large influx and efflux times of the vacuole (see below).

A typical cycle starts with the empty vacuole and a high value of tonoplast order, z . Because of the high ordering of the tonoplast, the permeability for malate efflux is low (equation 6) and the vacuole is filled by active influx of malate from the cytoplasm, leading to a relatively small malate concentration in the cytoplasm and a high value of CO_2 uptake. With the filling of the vacuole, the mean area of the membrane surface increases. Once the value of malate concentration in the vacuole and, due to its osmotic consequences, also of the membrane surface area has risen above a critical value, a first-order phase transition in the tonoplast membrane is

Figure 4. Simulation results of (relative units) CO_2 uptake, J_{CO_2} (solid line), and malate concentration in the vacuole, y (dashed line), in dark/light cycles. Times of simulated darkness, $L(t) = 0$, are indicated as black bars. The trajectories are obtained as in figure 3, for the same set of constants and control parameters.

induced (Neff *et al.* 1998). This is seen in figure 3 in the fast decrease of the order parameter z . Since after reaching a small value of z the tonoplast permeability, P , is high, fast efflux sets in. As a result the vacuole is soon emptied again to a low level. In this phase of CAM, at times of highest efflux rates, the malate concentration in the cytoplasm is high and CO_2 uptake is depressed. Later, when the vacuole is emptied to a certain level, further decrease of vacuolar malate is only very slow which is shown in a broad minimum plateau in figure 3. Once the malate concentration has fallen down to a critical value, a phase transition in the membrane is once again initiated, the order parameter increases very rapidly, malate permeability decreases and the cycle starts anew.

The broad minimum of malate content in the vacuole, shown in figure 3, is also observed experimentally (Klug & Ting 1978) but it cannot be simulated, even in principle, with a model containing a discrete hysteresis switch. A discrete hysteresis switch requires the start of the next filling phase immediately after emptying of the vacuole (Blasius *et al.* 1997). This deficit of a discrete two-state switch is reflected in the simulation results of the previous CAM models; in particular the simulation of day–night cycles shows a qualitatively incorrect structure of phase I of CAM, i.e. nocturnal CO_2 uptake (Blasius *et al.* 1997). Figure 4 shows that this deficiency is largely removed by our version of a continuous hysteresis switch, as introduced in the present work. The simulation results show the typical gas exchange pattern of CAM plants with peaks of CO_2 uptake at the beginning and the end of the light phase. Furthermore, the simulation results show a pronounced maximum of CO_2 uptake during nocturnal phase I of CAM in good agreement with experimental findings (Klug & Ting 1978). Consequently, the new model of CAM presented here leads to much improved simulations in normal day–night cycles compared with the former models using a discrete hysteresis switch (Blasius *et al.* 1997).

Further insight into the difference between a discrete and a continuous hysteresis switch is possible by visualization of the numerical results in a phase-space portrait. Figure 5 shows the projection of the trajectory in the four variable system into the z, y -phase plane, together with the two null clines, equations (13, 14). The trajectory is plotted for different values of the tonoplast response time, τ , starting in all cases at the same initial conditions. For a

Figure 5. Influence of the tonoplast response time, τ , on the stability of the endogenous oscillations. Plotted are (relative units) the null clines for the variables malate content in the vacuole, y (dashed line) (equation 13), for the tonoplast order, z (dotted line) (equation 14), and the simulated trajectories (solid line) in phase space for different values of τ . (a) $\tau = 3$; (b) $\tau = 0.35$; and (c) $\tau = 0.03$. The trajectories are obtained as in figure 3, for the same set of constants and control parameters.

rather large value of the time constant, $\tau = 3$, representing a very slow response of the tonoplast order to changes in the malate content in the vacuole, the system moves towards a stable fixed point (figure 5a). The location of the steady state in phase space is determined by the intersection of the two null clines. If the time constant of the tonoplast is reduced, corresponding to faster tonoplast dynamics, the transition towards the equilibrium becomes slower (not shown). Finally, when τ is decreased below a critical value, the steady state loses its stability, and the stable spiral changes into an unstable one, surrounded by a stable limit cycle (figure 5b, $\tau = 0.35$). In this case, for all initial conditions the trajectory evolves towards the same, unique closed curve in phase space. With very small values of τ , $\tau \ll 1$, the dynamics of the system are characterized by two widely separated time-scales: a long circadian time-scale, given by the time needed for emptying and filling the vacuole; and a short time-scale characterizing the changing rate of the tonoplast order z . As a consequence of the fast membrane relaxation, the trajectory nearly always resides in thermodynamic equilibrium, i.e. in close vicinity to the z -null cline. After reaching the upper or lower threshold level, the membrane undergoes a first-order phase transition, seen in a fast jump of the trajectory towards the new temporary equilibrium value

Figure 6. Simulation results in continuous light in phase space. Plotted are (relative units) the malate concentration in the vacuole, y , versus the tonoplast order, z , for different relative temperatures, T : $T = 0.2239$ (dashed and dotted line), $T = 0.2244$ (dashed line), $T = 0.2249$ (dotted line), and $T = 0.2254$ (solid line). Other control parameters and set of constants as in figure 3. The fixed points reached by the system for both high and low temperature are indicated by black dots.

(figure 5c, $\tau = 0.03$). These periodic solutions of alternating fast and slow motion characterize our system as a relaxation oscillator.

In the limit of vanishing tonoplast response times, the trajectory resides nearly always in two distinct regions in phase space characterized by a low ($z \approx 0.1$) or a higher value ($z \approx 0.35$) of membrane order (figure 5c). This behaviour and also the form of the limit cycle resembles very much the situation in the case of a discrete hysteresis switch (Blasius *et al.* 1997), where only two values for the tonoplast state are allowed. In this sense the discrete hysteresis switch of the previous CAM models is contained as the limit $\tau \rightarrow 0$ in the continuous model presented here.

5. INFLUENCE OF TEMPERATURE

The influence of temperature as an external control parameter is investigated in figure 6. Simulation results in continuous light are plotted in the (y, z) phase plane for different effective temperatures. For low temperatures the trajectory evolves towards stable fixed points characterized by a high malate content of the vacuole. Conversely, at the upper end of the temperature range the rhythm stops with an 'empty' vacuole. Limit cycle oscillations are found in the medium temperature range only. This is obvious from figure 2, since limit cycle oscillations are only possible if the two null clines intersect in the positive slope region of the cubic null cline (Murray 1993).

The influence of temperature can best be illustrated in the bifurcation diagram (figure 7) showing the stable and unstable fixed points and the maximum and minimum values of stable limit cycles for the malate content in the vacuole, y , in its dependence on temperature, T . The diagram starts at low temperatures with a branch of stable fixed points characterized by high malate content in the vacuole. With increasing temperature the steady state malate level decreases, until at the critical temperature $T_1 = 0.224072$, the system undergoes a

Figure 7. Bifurcation diagram: values obtained for the malate concentration in the vacuole, y , in stable (solid line) and unstable (dotted line) fixed points, and maximal and minimal values in the stable limit cycle (dashed line), in their dependence on temperature T . $T_1, \dots, T_5$: bifurcation points as discussed in text. The open circles mark the (only slightly pronounced) back bending part of the unstable fixed points where three points lie on top of each other. (Set of constants and control parameters as in figure 3, $\tau = 0.1$.)

supercritical Hopf bifurcation, and the stable fixed point changes into an unstable one surrounded by a small-amplitude limit cycle. The minimum level of the malate oscillations changes abruptly from $y_{\min} = 1.07$ to $y_{\min} = 0.61$ (relative units) in the small temperature range from $T = 0.2240850$ to $T = 0.2240855$. At $T_2 = 0.224181$, two additional unstable fixed points, an unstable spiral and a saddle point, are born at $y = 0.86$ in a saddle-node bifurcation. If temperature is increased further to $T_3 = 0.224188$, the saddle point collides with the upper unstable fixed point at $y = 0.93$ and both fixed points disappear in a second saddle-node bifurcation. The occurrence of the two new unstable fixed points corresponds to a regime with three intersections of the null clines in figure 2. With increasing values of temperature at $T_4 = 0.225325$, the unstable fixed point changes stability and becomes a stable spiral. At this temperature the stable fixed point and the stable limit cycle coexist and are separated by an unstable limit cycle, which is born in a subcritical Hopf bifurcation. Finally at $T_5 = 0.225386$, the stable limit cycle suddenly disappears, when the unstable and the stable limit cycles collide.

Figure 7 shows that, starting from a medium temperature in the regime of regular oscillations, exposure to very high or very low temperatures drives the oscillator to fixed points characterized by low or high values of malate in the vacuole. The transition regime between steady states and high-amplitude oscillations is very small. This means that near the bifurcation points a small temperature change suffices to destroy or initiate rhythmicity. This same behaviour has also been found in experiments with the CAM plants *Kalanchoë daigremontiana* (Lüttge & Beck 1992; Grams *et al.* 1997) and *Kalanchoë fedtschenkoi* (Anderson & Wilkins 1989b). Besides the difference in malate levels, experimentally the two states can also be distinguished by a 180° difference in phase when the rhythm is reinitiated after moving temperature back to the rhythmic regime. From the model this is obvious since at very high or very low temperatures the rhythm is stopped at opposite points in phase space (figure 6).

6. DISCUSSION

The main objective of this paper is to present an improved model of the CAM cycle which extends our successful earlier models by a detailed description of molecular malate transport, and by this provides a concrete biophysical example for the mechanism of the underlying biological clock. The endogenous CAM oscillations are described in terms of malate transport through the tonoplast membrane regulated by the order state of the membrane itself, which depends on the malate concentration in the vacuole. The circadian time stems from the interplay between the periods needed to fill and empty the vacuole, and it is influenced by the reservoir surrounding the membrane. This phase transition leads to the inclusion of a dynamic hysteresis switch. It allows for the first time to describe the CAM cycle as a fully autonomous dynamic system. We could show that the discrete hysteresis switch of the earlier CAM models is still contained as the $\tau \rightarrow 0$ limit in the present dynamic model. On the other hand, even in this limit there is still an important qualitative difference between the two cases. For instance, the model with the discrete hysteresis switch does not contain the unstable steady state. This has direct dynamical consequences, for example in comparison with phase resetting experiments (Anderson & Wilkins 1989c). The explanation for the suppression of oscillations by short high or low temperature pulses relies on the fact that, given the appropriate phase point, the perturbation can drive the system on to the unstable steady state. Such a behaviour can in principle not be modelled with a discrete hysteresis switch. It could, however, be readily tested in experiments.

The model presented in this paper describes the circadian rhythm of CAM as a limit cycle oscillator. Limit cycle oscillators are particularly stable since the amplitude and period of the oscillations are uniquely defined for a given set of control parameters independent of initial conditions. This behaviour is far more appropriate for a description of a circadian system than, for instance, a conservative model with a neutrally stable fixed point as, for example, the Lotka–Volterra system, which results in structurally unstable oscillations.

Regarding the simplicity of the skeleton model, the simulated time course of CO_2 exchange and its dependence on external control parameters shows good agreement with experimental findings. Both in experiment and in simulations, increase or decrease of temperature above or below certain critical threshold levels destroys rhythmicity and drives the system to a well-defined steady state characterized by low or high malate content, respectively. In the high temperature regime, the CAM model is an excitable system since a small perturbation from the stable fixed point undergoes a large excursion in phase space before returning to the fixed point (figure 6). This highly non-linear response to perturbations is well known also from other excitable systems, as for example the FitzHugh–Nagumo model (FitzHugh 1961; Nagumo *et al.* 1962). To our knowledge, it is discussed here for the first time in the context of a higher plant system.

The model of the circadian rhythm presented here emphasizes the importance of feedback mechanisms as an essential ingredient for the function of biological clocks. It

should also play an important role in genetic explanations of circadian clocks or biological rhythms of shorter timescales, such as protein synthesis and regulation on the mRNA level (Goldbeter 1995). With respect to the regulation of the endogenous CAM rhythm at the transcriptional (mRNA) and translational level, much excellent work has been presented by Nimmo and co-workers regarding PEPc-modulations (for a review, see Hartwell *et al.* 1996). We have also considered this in previous modelling and summarized our reasons why we assume that it is an epiphenomenon—albeit important—and favour the dominant beat oscillator at the tonoplast level (Lüttge 1998). In our CAM model, the biological clock crucially involves the participation of a membrane, and in fact shows many similarities to other membrane models discussed in the literature (Njus *et al.* 1974). Generally, membrane models of biological clocks have striking attractive features. For instance the large circadian time-scale emerges naturally as a consequence of the filling time of the membrane environment, as described above. Furthermore, temperature compensation of period length can be easily explained by a futile recycling process (Blasius *et al.* 1997). In some cases the exact details of a membrane clock remain relatively vague. In our present work, however, we demonstrate the possibility of such a clock using a specific concrete example. In the CAM cycle the central role of the tonoplast membrane is motivated from CAM physiology, resulting in a simplified but realistic membrane model, and our simulation results which provide excellent agreement with observed experimental findings.

Biological endogenous rhythm research has long been determined by the quest for the endogenous clock, the oscillator as a single concrete entity. So far it has never been found. The discovery of rhythm genes, e.g. in *Drosophila* and *Neurospora* (Dunlap 1993), is often presented in a way which implies that these would be in fact the oscillators. However, considering the complexity of the regulation of gene expression, it is immediately obvious that system networks with positive and negative feedbacks and hence nonlinear dynamics exist at this level just like those at the membrane and metabolic levels studied in this report. In this respect, dynamics of structures in time as analysed here have principal similarities to dynamics of structures in space studied by developmental biology in other plant systems (Stone & Ezrati 1996; Theißen & Saedler 1998). Thus, the interplay of empirical and theoretical analyses together with modelling of a nonlinear oscillating system, as we have practised in previous work, as well as the development of a dynamic beat oscillator resulting from the analysis of nonlinear systems, as presented here, may prove to be of general pertinence to biological rhythm research.

This work was supported by the Sonderforschungsbereich 199 (Teilprojekt B5) of the Deutsche Forschungsgemeinschaft, Bonn, Germany.

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