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Editors

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Review

Curriculum Vitae

1936	Manfred Kluge was born in Zschopau (Saxonia; Germany)
1957	Graduation from the Gymnasium
1957–1962	Undergraduate studies at the University of Frankfurt and the Darmstadt University of Technology in Biology, Chemistry and Geography. Examination for Teachers Profession (Gymnasium) in 1962
1962–1964	Graduate student in Botany with Professor Hubert Ziegler at the Darmstadt University of Technology
1964	Doctor's degree (Faculty for Chemistry, Biology and Geosciences of the Darmstadt University of Technology), Scientific Assistant at the Institute of Botany in Darmstadt
1966	Married to Helga Müller, also graduate student of Hubert Ziegler
1969	Habilitation (Botany) at the Darmstadt University of Technology, Lecturer at the Institute of Botany; birth of son Christoph
1970	Birth of son Andreas, Postdoctoral Research Fellow at the Australian National University, Canberra
1971–1973	Associate Professor at the Institute of Botany of the Technical University Munich
1974	Full Professor of Botany, Faculty Biology of Darmstadt University of Technology
2002	Retirement

1985 Guest Professor at the Botany Department of the National University Singapore. 1989, 1991 and 1993 Guest Docent of the German Academic Exchange Service (DAAD) at the Botany Department of the University Antananarivo (Madagascar). Repeatedly elected Director of the Institute of Botany and the Botanical Gardens of the Darmstadt University of Technology and Dean of the Faculty of Biology of this University. Chairman of the DFG Schwerpunktsprogramm "Biochemical fundaments of ecological adaptation in plants"(1974–1979), and the DFG Graduate School 340 "Communication in biological systems: from the molecule to the organism in its environment"; Vice Chairman of the DFG Center of Excellence 199 "Molecular ecophysiology of plants: acquisition of resources, membrane transport, regulation of resource consumption". Tudor of the Studienstiftung des Deutschen Volkes at the Darmstadt University of Technology (1976–2003).

Ecophysiology: Migrations Between Different Levels of Scaling

Manfred Kluge

Contents

Abstract Causal explanation of the mechanisms behind ecological adaptation in plants requires research on different levels of scaling, ranging from molecules to plants in their natural environment. In the following article this is illustrated by a description of the author's scientific life-work mainly with respect to the crassulacean acid metabolism (CAM) and research on *Geosiphon*, an endosymbiotic consortium of a fungus and a cyanobacterium.

Introduction

The title chapters of *Progress in Botany* provide a forum where in the past celebrities of the plant sciences explored important fields of their scientific life-work. Thus, the invitation by the Editors of *Progress in Botany* to write the title chapter of

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volume 69 honors me beyond measure. Simultaneously it confronts me with a dilemma because, with the title chapter of the preceding volume, Barry Osmond provided an excellent survey of the world of crassulacean acid metabolism (CAM), a topic which concerns also the major part of my scientific work. Therefore I cannot avoid that CAM plays an important role also in my treatise, but I want to consider this topic from a different point of view and to include also aspects other than CAM.

In his paper Barry explored CAM in all its diversity of recent and future aspects and the implications of CAM research for other disciplines of the plant sciences, and he expressed his "indebtedness to very many companions in CAM research". The latter holds very much true also for me and even concerns largely the same circle of personalities to whom I am indebted and whom I have to thank. Thus, in order to avoid repetition and hoping that it will not be misunderstood as immodesty, I will try to illustrate my personal adventure of nearly four decades of research on CAM. In particular I would like to show the logic which pushed me during my involvement in CAM to exciting migrations through the various levels of scaling, ranging from the plants in their environment down to the world of molecules.

From my beginning I considered CAM primarily as a matter of ecophysiology, irrespective of its implication also for other disciplines of plant sciences (compare Osmond 2007). Ulrich Lüttge (1997) clearly characterized the hierarchy of approaches required aiming to extend ecophysiology beyond the descriptive level. One has:

- "To start by depicting habitats and plants physiognomically,
- To deduce problems from such observations in the field as are suited for physiological, biochemical and biophysical and perhaps even molecular experimentation in the laboratory, and
- To return from the laboratory to the field with increasing sophisticated technologies for measurements and analyses applicable to field conditions."

My migrations included all these steps and thus are largely symptomatic for the path one has to follow when aiming to really understand even single facets of the breathtaking diversity of mechanisms evolved in plants to cope with the demands of their environments.

1 First Steps Towards Biology

My interest in biology emerged from two important experiences of my youth. When I was 12 years old, as a Christmas gift I got a little microscope. It opened to me a new exciting world of sometimes bizarre beauty; and to put anything under the microscope which looked somehow interesting became besides playing the piano a stern hobby.

The next, and this time a more serious impetus in the direction of biology, came from my school days. I graduated from a gymnasium practicing so-called "reformed senior classes" where I enjoyed excellent instruction in biology which not only comprised the prescribed topics of the gymnasial biology curriculum for the final two years, i.e. genetics and phylogeny, but also introduced us, based on the work of

L. v. Bertalanffy, to theoretical biology. Thus, I began to understand organisms as a steady state – maintaining dynamic open systems. Important for the direction of my later work was the introduction to classic ecology. It provided me first insights into the structure of ecosystems, food webs and matter cycling, and I began to suspect that, because of its complexity, ecology is a particularly demanding discipline within the biosciences. For me this seemed to be more attractive than deterrent.

For the development of the capability of scientific thinking and working it was very important that, in the "reformed senior class" system for the matriculation examination, a thesis based on own independent work had to be written. Within the frame of this thesis I conducted a limnological study on a pond where I investigated during the cycle of a whole year the changes in the biocenosis parallel to the alterations in water temperature, oxygen content, pH values and other environmental parameters. This study was a veritable introduction into research work. It provided me with a considerable knowledge of species, strengthened skills in handling microscopic and analytical techniques, developed the ability for independent scientific working and gave me the first feeling of satisfaction to find something that nobody had known before. Altogether, in particular the reformed senior classes of the gymnasium laid a sound fundament for my later studies, thus I have all reason to look gratefully back to my school days.

After the matriculation examination in spring 1957 it was clear to me to become teacher at a gymnasium, less because this was already tradition in my family, rather because I myself felt, strengthened by the example of my father, that teaching can be a passion and source of deep satisfaction. Briefly I was not sure whether I should choose music or biology as the main topic of my studies for the teacher's profession, but finally I decided myself in favor of science and kept music as a hobby. Thus, for the summer semester 1957 I enrolled in the Faculty of Natural Sciences of the University at Frankfurt.

2 Academic Studies and Entrance into Professional Activity

As topics for the academic studies for teacher's profession beside biology I chose chemistry and geography. In Frankfurt my studies were focused on zoology, but in 1959 I changed to the Darmstadt University of Technology where for the following three semesters I was engaged mainly in botany.

In Darmstadt the biology departments were quite small, and in particular the Institute of Botany was excelled by a close contact between the at this time sole professor, the scientific staff and the students, creating a nearly family-type and stimulating atmosphere. When I came to Darmstadt, Otto Stocker, one of the great pioneers and celebrities of experimental plant ecology, was just retired and Hubert Ziegler had become his successor. Although being still very young, H. Ziegler had already a great reputation as researcher on phloem transport, but his wide spectrum of interest included also physiological aspects of plant ecology, so that in Darmstadt the long tradition in this field was not only continued rather but, due to the introduction

of modern biochemical methodology, extended to the quest for the biochemical fundaments of ecological adaptation in plants.

H. Ziegler was an excellent, electrifying teacher, and due to him my main interest soon shifted to plant physiology and biochemistry, although on purpose I tried to avoid too early and too narrow a specialization and to retain curiosity towards the diversity of the biological world. Also in this respect H. Ziegler was a great mentor and exemplary for me.

In 1961 I won a scholarship of the Studienstiftung des Deutschen Volkes, and I began to work in H. Ziegler's group on the chemical composition of phloem exudates of trees. After I passed the teacher's examination in 1962, H. Ziegler accepted me as a candidate for a doctorate. He was a great mentor and really a friend to his pupils. Within the frame of my doctoral thesis I continued my investigation on phloem transport. In summer 1964 I finished my studies by taking the Doctor's degree. Moreover, that year became particularly important for me, because a new PhD student, Helga Müller, joined the group of Hubert Ziegler to conduct comparative studies on the carbohydrate composition of sieve tube saps. She not only appreciated my help in the time-consuming gathering of phloem exudates from the trees of the Botanical Garden but also found a partner who was happy to accompany her excellent playing of the flute on the harpsichord. It became a partnership for the whole life. Thank you, Helga.

3 Research on CAM

3.1 Initial Physiological Studies at the Level of the Entire Plant

After I took the Doctor's degree H. Ziegler encouraged me to enter an academic career and offered me the position of Scientific Assistant at his institute which I happily accepted. Looking for my own field of research, accidentally I read a paper by Nishida (1963) which trapped me so much that it determined the topic of my main future scientific work: the crassulacean acid metabolism (CAM). In 1965, when I began my investigations on CAM, at the level of science the phenomenon had already been known for 150 years, but it was still considered more or less as a physiological curiosity exhibited by certain specialists among plants. In the mentioned publication Nishida showed by viscous flow porometry that CAM plants perform a pattern of stomatal movement inverse to that of non-CAM plants, i.e. CAM plants open the stomata at night and close them during the day. This pattern coincides with typical CAM CO_2 exchange, namely net CO_2 uptake during the night (Phase I, denotation after Osmond 1978), a short period of enhanced net CO_2 uptake after the onset of the light period (Phase II), depression of net CO_2 uptake during several hours of the day (Phase III), and recommencement of net CO_2 uptake towards the end of the day (Phase IV). However, the question remained open whether the unusual stomatal behavior is the primary event determining the unique course of CAM CO_2 exchange, or whether this CO_2 exchange is otherwise controlled.

The attempt to answer this question was the beginning of my love story with CAM lasting now more than 40 years.

My start into CAM research was facilitated by several lucky circumstances. First, in 1965, because of its reputation as a physiological curiosity (Osmond 1978), CAM kept world-wide only few researchers busy. Thus, speaking in terms of ecology, for a young researcher CAM provided still a kind of niche without too much competitive pressure. Triggered by the discovery of C4-photosynthesis in 1966, this situation later changed considerably. Second, I had access to an efficient system of modern infrared gas analyzers already established by O. L. Lange when he worked from 1961 until 1963 in Darmstadt. This system allowed one to measure continuously net CO_2 exchange and transpiration by plants under controlled conditions. Finally, in the mid-1960s young scientists still at the beginning of their work like me had a good chance to get research funds from the Deutsche Forschungsgemeinschaft just by presenting a convincing research program, without the pressure that prior results, best published in the journals with highest impact factors, had to be shown.

Furnished with the mentioned equipment and a DFG grant of 5000 DM for three years, I started a study aiming to provide a causal explanation of the CAM gas exchange phenomena.

I began my investigations with a simple, now I would say brutal, experiment in one of the classic CAM plants, *Kalanchoë daigremontiana*, where fortunately it is possible to strip off the abaxial epidermis of the planar leaves remaining attached to entire plant, without substantial damage to the leaf mesophyll. Net CO_2 exchange and transpiration (the latter as an index for the stomatal behavior) were measured under constant water vapor pressure deficit of the ambient air before and after the leaf epidermis had been stripped off. The result was very clear (Fig. 1). As to be expected from the results by Nishida (1963), in the intact leaves both during day and night there was a perfect coincidence between net CO_2 exchange and the stomatal movements indicated by transpiration, whereas in the leaves without epidermis the typical CAM pattern of CO_2 exchange was preserved whilst expectedly transpiration stayed constant. My interpretation was “that not the stomatal behavior is the primary event determining the CO_2 exchange in CAM plants rather than that the CO_2 exchange of the mesophyll cells, which itself is the direct consequence of CAM, determines whether the stomata close or open at a given time” (Kluge and Ting 1978). However, the strict synchronization of CO_2 exchange and stomatal movement in the intact leaves implied that there must be a coupling between the mesophyll-inherent CO_2 exchange and the movements of the stomatal guard cells. I found a plausible explanation on the basis of the pioneering publication by K. Raschke (1966) showing in *Zea mays* that the stomata are links in a regulatory circuit which stabilizes the CO_2 concentration in the subepidermal intercellular spaces ($[CO_2]_{int}$). A consequence of such a circuit is that an increase in the internal CO_2 concentration leads to a closure of the stomata, and vice versa. On the basis of Raschke’s regulatory circuit model I proposed that also in CAM plants $[CO_2]_{int}$ is the signal factor. For me it was just logic that CAM plants open their stomata at night because, due to dark CO_2 fixation, $[CO_2]_{int}$ in the mesophyll should be low, and they close them during the day because malic acid decarboxylation would lead

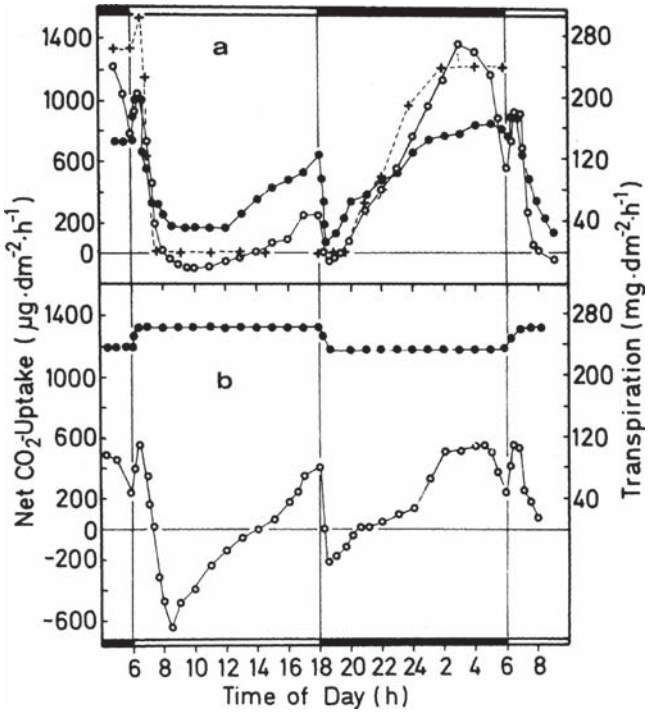

Fig. 1 Net CO_2 exchange and transpiration in *Kalanchoe daigremontiana*. **a** Coincidence of CO_2 exchange and transpiration in an intact plant. ○—○ net CO_2 exchange, ●—● transpiration, +—+ porometric values (relative units) obtained in the same species under the same conditions by Nishida (1963). **b** CO_2 exchange and transpiration in the same plant as **a**, however with the lower epidermis of all leaves having been stripped off (from Kluge and Fischer 1967)

to high $[CO_2]_{int}$. In the early years of my struggling with CAM I made a great effort to verify this hypothesis by following two strategies, namely (a) trying to measure $[CO_2]_{int}$ directly and to correlate it with the actual status of the stomatal aperture, and (b) trying to influence the duration of stomatal closure during the day (Phase III) by experimentally manipulating the source for a high internal CO_2 concentration, i.e. the malic acid pool built up during the previous night.

Already in our first CAM experiment (Fig. 1b) we observed a massive CO_2 outburst occurring upon removal of the epidermal diffusion barrier during Phase III. This outburst was drastically lower or even absent with intact leaves. Thus, for us this outburst was indicative of massive CO_2 enrichment built up behind the closed stomata. Actually, this observation was the first hint in favor of the now widely accepted view that CAM is also a CO_2 enrichment mechanism (see e.g. Osmond 2007). Later it could be demonstrated by direct measurements in air samples (Cockburn et al. 1979) taken along the day/night cycle from the succulent *Kalanchoë* leaves that, in the night, when the stomata were shown to be open,

$[CO_2]_{int}$ dropped close to zero, while during Phase III, when the stomata were closed, the leaf internal CO_2 was enriched by a factor of ten with respect to the ambient air (Kluge et al. 1981a).

The next step was to corroborate systematically the hypothesis that, optimal water status provided, the duration of stomatal closure in CAM plants during Phase III is mainly determined by the production of CO_2 from the previously synthesized and stored malic acid. I could directly prove this assumption by ^{14}C-pulse-chase experiments (Kluge 1968a).Subsequently a large series of gas exchange measurements followed which were paralleled by estimation of biochemical parameters in the plants under variation of the environmental conditions. These experiments not only uncovered the extraordinary phenotypic flexibility of CAM performance, but also showed that all experimental treatments which prevent filling of the malic acid storage pool during the night and all treatments which accelerate the consumption of the previously stored malic acid during the day shorten the duration of Phase III. After this we were quite sure that Phase III of CAM is a function of malate consumption (Kluge 1968a).

It was an interesting side-issue of the above-mentioned studies to find that application of CO_2-free air during the night inhibits not only the nocturnal synthesis of malate, but also the depletion of starch (Kluge 1969a). This showed that the breakdown of starch to generate phosphoenol pyruvate (PEP) as a CO_2 acceptor of the PEP carboxylase (PEPCase) reaction must be coupled rather directly to the availability of the substrate CO_2. Looking back I regret that neither we nor other researchers followed this finding further immediately, because presumably it could have guided us quite early into the investigation of carbohydrate metabolism in CAM which currently collects more attention (Osmond 2007).

We also observed that, at the beginning of the light period, a lag-phase in the onset of malic acid consumption occurred which coincided with Phase II. It was for me the first hint that, under the experimental condition applied, at the onset of light malate stored in the vacuole is not immediately disposable for decarboxylation. The duration of the lag phase turned out to depend on the actual vacuolar malate concentration and temperature. Thus it was reasonable to conclude that these two factors might be important for the determination of the malate release from the vacuole. Some years later I came back to these findings (Kluge et al. 1991d), and the investigation of temperature effects on the tonoplast became an important aspect in my research on CAM.

From the beginning I guessed that the pattern of the CAM gas exchange must be meaningful for the carbon and water balance of the concerned plants growing in the field (Kluge and Fischer 1967) so that CAM can reasonably be interpreted in terms of ecophysiological adaptation to environments where water is in short supply. I had the dream to get once the opportunity to verify this interpretation by studying the behavior of CAM plants in their natural habitats. A couple of years later this dream became reality. However, first we wanted to prove the ecophysiological relevance of CAM at least in the laboratory, and thus we conducted the (to my knowledge) first stress experiment on a CAM plant (*Kalanchoë daigremontiana*; Kluge and Fischer 1967). It turned out that just a few days cessation of watering

were sufficient to completely suppress opening of the stomata during the day (i.e. Phases II, IV), whereas nocturnal opening of the stomata with net CO_2 uptake continued, although on a somewhat lower level. All stress effects on CAM gas exchange disappeared within one hour after re-watering the plant. These findings suggested that CAM allows the plants to retain a positive carbon balance even if carbon acquisition during the day is prevented by water deficiency stress.

Because of compassion with our experimental plants we were not cruel enough to extent withholding of water for longer than five days. This was a pity because otherwise we would have discovered that, upon long-lasting water deficiency stress, CAM plants finally close their stomata also during the night but continue CAM behind the closed stomata by nocturnal recycling of respiratory CO_2 ("CAM idling"). Szarek and Ting (1975), who discovered CAM idling, observed that in the field it is ecophysiologically highly relevant for CAM plants which have to overcome long-lasting drought seasons.

Our stress experiments showed us also that the $[CO_2]_{int}$ regulatory circuit controlling the aperture of stomata operates undisturbed only if the plants do not suffer water deficiency stress. The conclusion was that drought factors which were still unknown to us at that time override the CO_2 regulatory mechanism. Today it is textbook knowledge that a drop in the plant water potential, with abscisic acid as molecular messenger, leads to hydroactive closure of the stomata dominating the other regulation circuits.

3.2 Investigations at the Metabolic Level: the Problem of CAM Regulation

Orderly functioning of CAM requires that during the day the malic acid derived free CO_2 is directed quantitatively to the Calvin cycle for final assimilation. From the beginning it was a striking question for me how it is prevented that during Phase III the malate derived intermediary CO_2 is trapped by PEPCase and thus futilely recycled into the malate pool instead of entering correctly the photosynthetic pathway. By means of $^{14}CO_2$ tracer experiments I could show that in the light during Phase III the PEPCase-mediated primary CO_2 fixation is switched off. In contrast, during the night even under illumination CO_2 is nearly exclusively fixed by PEPCase thus practically not available to the Calvin cycle (Kluge 1969b; Fig. 2). These in vivo changes in the activity of the primary CO_2 fixation turned out to be correlated with the diurnal malic acid rhythm of CAM. In other words: the PEPCase pathway is in an active state when the malate level in the CAM performing cells is low, and vice versa (Kluge 1971). This correlation was strictly retained when the course and the amplitude of the malic acid fluctuations were experimentally manipulated.

Looking for an explanation of the diel changes in the activity of the primary CO_2 fixation, the lucky circumstance came to my assistance that, at the same time when we discovered the in vivo down-regulation of PEPCase during Phase III, Queiroz (1966) found that in vitro this enzyme can be inhibited by L-malate. This led me to

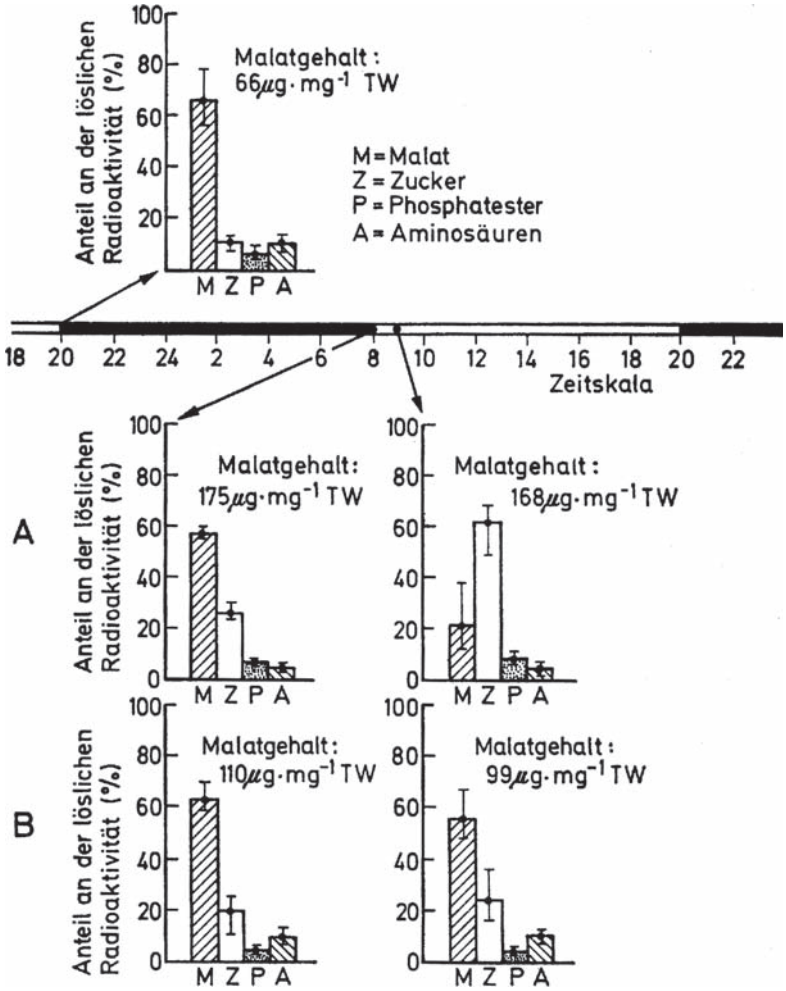

Fig. 2 *Kalanchoe tubiflora*: evidence for the inhibition in vivo of primary CO_2 fixation by PEPCase during Phase III of CAM. Indicative of this inhibition were changes in the labeling patterns in the soluble fraction after 15min $^{14}CO_2$ fixation in the light by phyllodia detached from the plant and labeled at the time points indicated by the *arrows*. The values indicate arithmetic means of three independent extractions. *Graph at top of diagram*: labelling pattern obtained at the beginning of the night. **A** Phyllodia with uninhibited nocturnal malic acid accumulation. In contrast to the labeling pattern obtained during the night (*closed horizontal bar*), one hour after the beginning of the light period (*open horizontal bar*) most of the tracer was trapped by sugars instead of malate, indicating that PEPCase was switched off. **B** Phyllodia with inhibited nocturnal malic acid accumulation. The labeling pattern in the light remained unchanged and indicative of PEPcase being still active. *TW* Dry weight, *M* malate, *Z* sugars (mainly sucrose), *P* phosphate esters, *A* amino acids (from Kluge 1971)

assume that feed-back inhibition of PEPCase by the nocturnally synthesized and stored malate might be important for suppressing CO_2 fixation by PEPCase during Phase III. However, it was also evident to me that an enzymatic feed-back inhibition alone cannot provide the complete explanation. Logically, there must be a factor which during the night keeps the malate level low in the cytoplasm, otherwise the PEPCase located there would be inhibited. In contrast, during Phase III the cytoplasmatic malate level has to be high to bring about feed-back inhibition of the enzyme. I proposed that diel changes in the source-sink behavior of the storage compartment for malate, notably the vacuole, could be the factor which determines the fluctuation of the cytoplasmatic malate level important for the feed-back control of PEPCase (Kluge 1969b). Without having already clear ideas about the mechanism behind this, I imagined that in CAM the vacuole acts with respect to malate in analogy to a siphon which is a highly attractive sink, thus keeping the cytoplasm free of malate, as long it is not filled, but which overflows thus enriching the cytoplasm with malate when it reaches a critical state of filling. In this model the sink situation corresponds to the CAM processes during Phase I and the overflow to the processes during Phase III (Kluge 1969a).

Although the model of co-operation between the vacuole and feed-back inhibition of PEPcase in CAM regulation appeared to be altogether conclusive, I was aware of the many questions remaining open and that the effort to answer them required to learn more: (a) on the structural, kinetic and regulatory properties of PEPCase, and (b) on the mechanisms controlling the reversible transport of malic acid across the tonoplast. Consequently mainly of these two complexes of questions determined my research on CAM during the following years.

3.3 Biographic Insert: Visiting Research Fellow at the ANU in Canberra, Associate Professor in Munich, Return to Darmstadt

The outlines in Sections 1.3.1 and 1.3.2 describe my struggle with CAM until summer 1969 when I finished my qualification for an academic career by Habilitation at the Darmstadt University of Technology. Thereafter I became a Lecturer in the Institute of Botany of this university, and I held this position until spring 1971. During this time I won a stipend from the Deutsche Forschungsgemeinschaft which allowed me to spend six months as Visiting Research Fellow in the group of Ralf Slatyer and Barry Osmond (Research School of Biological Sciences, ANU, Canberra). Barry introduced me to enzymology, and our work was mainly dedicated to the PEPCase of CAM plants. We estimated kinetic and regulatory properties of the semi-purified enzyme and found first indications that PEPCase is allosterically regulated, mainly by malate as negative effector (Kluge and Osmond 1972), We also demonstrated that pyruvate-P_i-dikinase, previously supposed to be a marker enzyme of C4-plants, is active also in CAM plants and permits in the light their incorporation of malate-derived pyruvate into carbohydrates (Kluge and Osmond 1971).

One of the highlights of my stay in Australia was the international meeting "Photosynthesis and Photorespiration" held in November 1970 in Canberra. Since this meeting the view has emerged and is now generally accepted that CAM represents an ecophysiologically relevant mode of photosynthetic carbon assimilation equivalent to C3- and C4-photosynthesis. Thus, by no means could CAM be any longer considered as a physiological curiosity featured by some specialists among higher plants, and in the following decades this new understanding initiated a boom in international CAM research. In this context it is worth mentioning that later we could demonstrate that even classic C3 plants such as spinach can be driven experimentally to a photosynthetic pathway analogous to CAM, with CO_2 fixed in darkness into malate entering the Calvin cycle during light (Böcher and Kluge 1978).

I owe to my stay in Australia also my first impressions of tropical rain forests, coral reefs and deserts, really unforgettable key experiences for me as an ecologically interested biologist.

After my stay in Canberra I went to the Technical University Munich and became Associate Professor at the Institute of Botany. The Director of this institute was Hubert Ziegler who, briefly before I departed to Australia, had moved from Darmstadt to Munich. There I stayed until 1974, and my work during this time strengthened my conviction that transport across the tonoplast is a key factor in the metabolic regulation of CAM. To get deeper insights into this matter I looked to contact experts in cellular transport. In this situation I was offered by the Darmstadt University of Technology the position of a Full Professor of Botany, freshly established parallel to the traditional chair held by Ulrich Lüttge as a follower of H. Ziegler. Ulrich, a leading transport physiologist, was already successfully active in CAM research. So, the call to Darmstadt opened to me the ideal possibility for co-operation and to learn more on vacuolar transport. I was the more pleased since I already knew Ulrich from our common time in Darmstadt as pupils of H. Ziegler and I was sure that we were scientifically and personally on the same wavelength.

Thus, my family and I decided to follow the call to Darmstadt, a decision which we never repented. Compared with other universities, in Darmstadt the Faculty of Biology was quite small, but it provided me such a beneficial environment for my work that in 1978 I refused to follow a call to the University of Essen. In particular the friendship and effective co-operation with Ulrich in research, teaching and administration belong to the most valuable experiences of my scientific career. This co-operation was the fundament that over the years could be founded first a DFG Researcher Group, later the DFG Center of Excellence 199 and finally the DFG Graduate School 340 at the Faculty of Biology in Darmstadt.

Towards the end of my stay in Munich the international boom in CAM research had increased the knowledge on CAM to such an extent that a comprehensive consideration of the many new results was highly desired. This was for me the impetus to publish the first monograph on CAM (Kluge and Ting 1978) which was then followed by the book by Winter and Smith (1996).

3.4 Investigations at the Level of Enzymes: the PEP Case

The central importance of PEPCase not only for CAM and C4-photosynthesis but also for many other metabolic processes in plants has triggered internationally a vast number of investigations on this enzyme, covering the whole range from its function and regulation to its molecular structure and genomic expression. Here I want to stress briefly our own involvement in the PEPCase story.

As already outlined, aiming to understand the role of PEPCase in the regulation of the complex CAM carbon flow, it is necessary to study the kinetic and regulatory properties of the enzyme. I began such studies during my sabbatical in Canberra. There we worked with plant extracts freed from low molecular substances by Sepadex filtration aiming to measure pH optimum curves of enzyme activity, K_m values and K_i values. Usually professional enzymologists refuse to take such kinetic data into account if they are not derived from measurements on the highly purified enzyme protein. Of course, such data are available also from PEPCase of CAM plants. However, it turned out that sometimes it can be useful not to obey the experts too much. Namely, it was discovered independently and nearly simultaneously by three laboratories (including ours), that PEPcase extracted from the plants at different times during the diurnal CAM cycle showed different K_i values for malate, provided the enzyme extracts were quickly desalted by passing through molecular sieves and measuring strictly within a few minutes after the plant material had been collected (v. Willert et al. 1979; Kluge et al. 1980; Winter 1980). Upon purification of the protein the differences vanished. In close co-operation with Jeanne Brulfert and Orlando Queiroz (CNRS, Gif-sur-Yvette, France) we then found that not only the sensitivity of PEPCase towards malate varies along the diurnal CAM cycle but also its sensitivity towards the activator glucose-6-phospahte (G-6-P) and the K_m for the substrate PEP. Thus, in the early 1980s it became clear that, during the diurnal CAM cycle, PEPCase switches reversibly from a physiologically active night form to a physiologically inactive day form. In vivo measurements of gas exchange, $^{14}CO_2$ labeling patterns and malic acid fluctuations parallel to the enzyme extraction supported the view that the changing properties of PEPCase are relevant for up- and down-regulation of the enzyme in vivo and with it for the regulation of the carbon flow in CAM (Kluge et al. 1981b).

It is worth mentioning that our work on the PEPCase of CAM plants founded a long-lasting co-operation and friendship with French colleagues around Orlando Queiroz in Gif-sur-Yvette, in particular with Jeanne Brulfert to whom I owe important stimulation and input for my work. Jeanne and I organized in 1983 a first international workshop on PEPCase which assembled world-wide most of the researches involved in this enzyme. During this workshop the idea was first discussed that the diurnal changes in the regulatory properties of CAM PEPCase might be due to post-translational modification of the enzyme protein. Indeed, Nimmo et al. (1986) then discovered that in the PEPCase of *Bryophyllum fedtschenkoi* the night-form is phosphorylated, not however the day-form. Soon after we found the same in other *Kalanchoë* species (Brulfert et al. 1986) and could demonstrate that the protein is

predominantly labeled with $^{32}PO_4$ in serine residues. The discovery of reversible PEPCase phosphorylation was also per se interesting because in the mid-1980s only few plant enzymes were known to be regulated by this way, and it was the only known example of a plant enzyme where protein phosphorylation leads to up- instead of down-regulation. Now it is generally accepted that not only in CAM is the reversible phosphorylation of PEPCase part of a complex regulatory network which includes also the involved PEPCase specific protein kinases and phosphatases (Nimmo 2000).

We have also conducted studies on PEPCase on the molecular level. Already by means of serological techniques we could show that, in the leaves of the facultaltive CAM-plant *Kalanchoë blossfeldiana* upon photoperiodic induction of CAM, a new isoform of PEPCase appeared additionally to that already present in the C3-state of the plant (Brulfert et al. 1982). This finding was then specified on the level of PEPCase gene sequences (Gehrig et al. 1995). We also compared in the CAM performing orchid *Vanilla planifolia* the amino acid sequences of the PEPCase isoforms (deduced from the c-DNA) present in the different organs of the plant (Gehrig et al. 1998a, b). We found that in all organs a constitutive PEPCase isoform (a "house-keeping" form) is expressed functioning most likely in context with anaplerotic reactions, whereas in the CAM-performing organs (leaves and stem) besides the housekeeping isoform an additional one occurs specifically responsible for the start reaction of CAM. Finally, a comparison of PEPCase nucleotide and aminoacid sequences in 50 plant species comprising the whole range from Bryophyta to Spermatophyta (Gehrig et al. 1998a, b, 2001a, b) convinced us that PEPCase sequences are valuable markers for the reconstitution of phylogenetic relations between organisms and metabolic pathways. In this context it was interesting to learn that in the spermatophytes the PEPCase isoforms supposed to be functionally related to CAM are widely dispersed over the different levels of taxa which support the view (Cushman and Bohnert 1999) that CAM is of polyphyletic origin.

3.5 Investigations at the Level of Cellular Compartmentation: the Role of the Vacuole and of Tonoplast Properties in CAM

As outlined in Section 1.3.2 already the results of my early studies on CAM forced me to the conclusion that diel oscillation in the PEPcase activity is the consequence of the rhythmic changes of the source/sink behavior of the vacuole for malate. This assumption pushed me into a long-lasting friendly controversy with Orlando Queiroz. Orlando was convinced that the diurnal CAM carbon flow is principally regulated by an endogenous circadian rhythm of the PEPC activity (Queiroz 1979), following a master oscillator intrinsic to the enzyme system. Finally he believed to have evidence "that an enzyme molecule behaves by itself as an oscillator shifting between different conformations" (Queiroz and Queiroz-Claret 1992).

This controversy forced me to look for more theoretical and experimental arguments in favor of my conviction that in CAM the vacuole is the basic oscillator.

Stimulated by discussions with the great cyberneticist W. Oppelt and his colleague H. Tolle, both professors of the Department of Regulation Techniques of the Technical University Darmstadt, and in co-operation with them we developed a dynamic computer model of the metabolic and regulatory processes in CAM (Nungesser et al. 1984). The model was based on a minimalistic metabolic scheme of CAM and allowed a quite realistic simulation of its typical phenomena such as diurnal acid fluctuation and gas exchange patterns. Later Freder Beck (Department of Physics, Darmstadt University of Technology) in co-operation with Ulrich Lüttge and his team developed a more sophisticated and thus more efficient theoretical model, which also identified the vacuole as master switch governing the circadian rhythmicity of CAM (Lüttge 2000).

Already our first computer model, and even more so the version developed by Freder Beck, clearly showed that proper simulation of the diurnal CAM behavior can be achieved by only inserting the changing flux behavior of the vacuole as an input variable into the model. Other approaches, where rhythmic changes of enzyme activities instead of the vacuole were supposed to represent the oscillator, failed nearly completely.

At the experimental level we could directly demonstrate by ^{14}C-tracer studies the changing source-sink behavior of the vacuole for malate (Fig. 3). Then the question arose for determining the factors controlling this changing behavior. The mechanisms behind the loading of the vacuole were studied by Ulrich Lüttge and his group (see e.g. Lüttge 2000, 2004; Osmond 2007). I was more interested in the mechanisms controlling the efflux of malate. It had been shown that this efflux is thermodynamically passive and it was assumed that at the end of the night, with vacuolar pH being low, malic acid efflux proceeds presumably by diffusion of the undissociated molecule through the lipid phase of the membrane (Lüttge and Smith 1984). Looking for the factors which might influence this diffusion I paid attention mainly to cell water relations and temperature.

1. *Water relations*. Still during my time in Munich we could show that the efflux of malate from the vacuole might be under the control of osmotic gradients (Kluge and Heininger 1973). In co-operation with Ulrich later in Darmstadt these investigations were continued and confirmed the view that osmotic gradients between the vacuole and its environment, thus finally the turgor, are important factors determining the acidification and deacidification of the vacuole (Lüttge et al. 1975).
2. *Temperature*. For a long time it was known that the gas exchange and carbon flow of CAM are phenotypically affected by temperature. As the literature shows, this phenomenon can be explained partially at the level of the enzymes involved in CAM. In this context we studied the temperature response of PEPCase in relation to its allosteric effectors (Buchanan-Bollig and Kluge 1984). However, there were also observations suggesting that the vacuole is the target of temperature effects (Kluge and Ting 1978). For instance, one of my PhD students, Detlev Ritz, found by ^{13}C mass-spectrometric analysis that a high temperature during the night prevents the storage of malate, while its synthesis is still possible (Ritz et al. 1987). Then we could show that temperature, via the

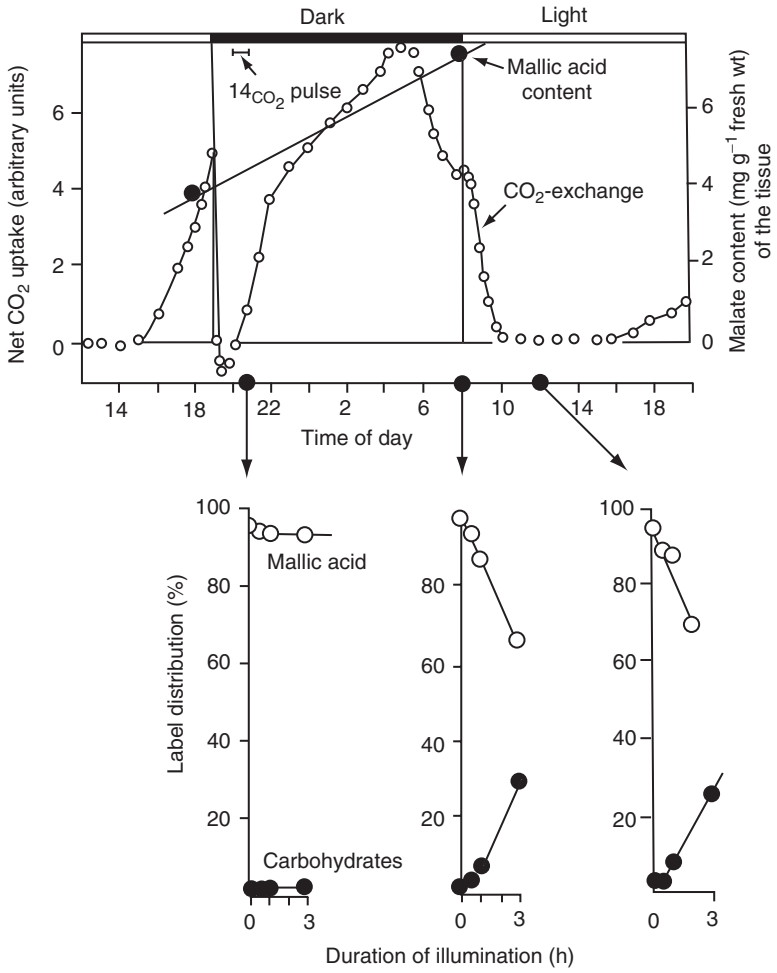

Fig. 3 ^{14}C pulse-chase experiments to show changes in the storage behavior of the vacuole during the night and the first hours of the following day. About 50 phyllodia of *Kalanchoe tubiflora* were detached from a single plant and enclosed in a glass cuvette to follow the CAM gas exchange and the nocturnal malic acid accumulation (*upper panel*). At the beginning of the night 30 phyllodia were exposed for 1 h in darkness to $^{14}CO_2$ as indicated in the upper panel ("$^{14}CO_2$ pulse"). After the pulse the labeled phyllodia were transferred back to normal air and remained in darkness. At the time points indicated by the *arrows* subsamples were taken from the labeled phyllodia and exposed to light for 3 h. During this time the phyllodia were continuously analyzed with respect to changes in the label distribution. In the *lower panel* it can be seen that with empty vacuoles at the beginning of the night even under illumination the labeled malate is retained in the vacuole, thus its label is not transferred to other metabolites. In contrast, at the end of the night, when the vacuole is filled up, upon illumination malate-derived label appears quickly in carbohydrates as photosynthetic end products. The upper panel shows the net CO_2 exchange during the CAM cycle and the nocturnal malic acid accumulation by the detached phyllodia. *Closed bar at top of the graph*: duration of the night (from Kluge 1977)

malic acid efflux from the vacuole, affects the carboxlation pathways of CAM (Friemert et al. 1988), and clear-cut evidence came also from studies on temperature effects on the onset of malic acid release from the vacuole at the beginning of the light period (Kluge et al. 1991d). This release showed at 10 °C a lag-phase of more than two hours, while at temperatures higher than 30 °C no lag-phase occurred, suggesting that at high temperatures malic acid easily leaks out from the vacuole and thus becomes instantaneously available for decarboxylation. The Arrhenius plot of the temperature effects on the duration of the lag-phase showed a break in the slope at about 15 °C. We interpreted this physiological effect in terms of phase separation of the tonoplast lipids beginning in the range of 15 °C. At temperatures higher than this critical value the tonoplast becomes increasingly fluid, thus leaky for malic acid. At temperatures lower than the phase separation range the opposite holds true (Kluge et al. 1991d).

The next step was to verify this hypothesis by measuring the fluidity of the tonoplast as a function of temperature directly. Thus, we had to shift our investigation to the level of the isolated vacuolar membrane. For this approach again co-operation with the group of Ulrich Lüttge was important because there we learned how to isolate, to purify and to characterize the tonoplasts from CAM plant cells. We learned in the laboratory of H.-J. Galla (Department of Organic Chemistry, Darmstadt University of Technology) the biophysical techniques required to measure the thermotropic phase behavior of membrane lipids (electron power magnetic resonance spectroscopy, measurement of fluorescence depolarization). The results of our studies were fully consistent with the predictions derived from physiological experiments (Kluge et al. 1991d). Namely, we found that in *K. daigremontiana*, grown at "standard conditions" (25 °C day, 17 °C night), below 10 °C the tonoplast is in a rigid state. With increasing temperature up to 52 °C tested so far, the membrane becomes steadily more fluid, with a break in the curve in the range of 14–18 °C, suggesting spontaneous phase separation (Fig. 4, upper panel; Kluge et al. 1991b). Moreover, we discovered that the fluidity of the tonoplast can adapt to the growth temperature (Kliemchen et al. 1989), a phenomenon called "homeoviscous adaptation" (HVA) and at that time not known to occur in plant biomembranes, except the thylacoids of *Nerium oleander*. Concerning the tonoplast of *K. daigremontiana*, the HVA to high temperatures means that in the acclimated plants much higher temperature than in the controls is required to reach a given degree of membrane fluidity, thus to elicit the phase transition in the membrane lipids. It was a great satisfaction for us to see that, indeed, in the plants acclimated to the higher temperature, thus having a more rigid tonoplast, the efflux from the vacuole at a given temperature was significantly slower with respect to the not-acclimated controls (Fig. 4). HVA of the tonoplast occurs also upon acclimation to low temperatures, but as to be expected in the other direction (Kliemchen et al. 1989). Finally, we could make clear that HVA of the tonoplast is due to changes in the membrane protein and phospholipid composition (Schomburg and Kluge 1994; Behzadipour et al. 1998).

Our data on the temperature response of tonoplast fluidity provided the experimental input needed by Ulrich Lüttge, Freder Beck and their groups for the development of

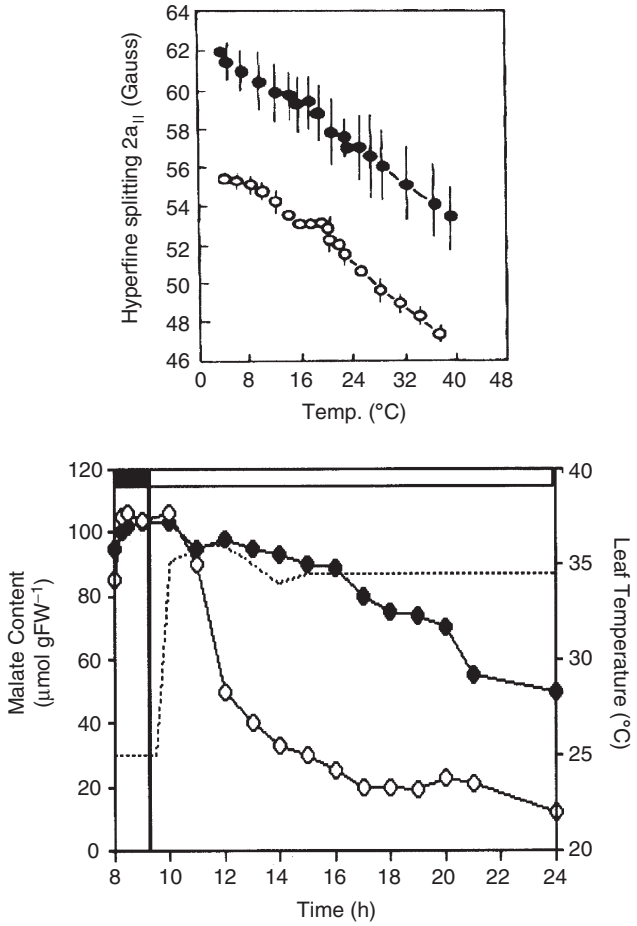

Fig. 4 Thermotropic changes of membrane fluidity and homeoviscous adaptation in the tonoplast in *Kalanchoe daigremontiana* upon acclimatization of the plants to higher growth temperature. *Upper panel* (from Kliemchen et al. 1993) shows the tonoplast fluidity, indicated by the electron power magnetic resonance (EPR) parameter "hyperfine splitting" ($2a_{II}$) in spin-labeled tonoplast preparations, in relation to the incubation temperature during EPR spectroscopy. The lower the $2a_{II}$ value, the more fluid is the tonoplast. *Open symbols*: tonoplasts from plants grown under standard conditions (25 °C day, 17 °C night), mean values of two independent repetitions of the experiment. *Closed symbols*: tonoplasts from plants grown for four weeks at "high temperature, HT" conditions (34 °C day, 25 °C night). Values represent arithmetic means with standard deviation of seven independent experiments. *Lower panel* (redrawn from Kliemchen et al. 1993): Course of malic acid depletion during the light period of CAM in *K. daigremontiana* under the HT conditions mentioned above. *Closed symbols*: plants grown already for four weeks under HT. *Open symbols*: plants grown under standard conditions and exposed without acclimation to HT. The figure shows that, in the plants having a more rigid tonoplast due to HT acclimation, depletion of the vacuolar malate in light is retarded compared with the plants grown at standard temperature thus having more fluid tonoplasts. *Dotted line*: leaf temperature. *Black bar on top of the graph*: Last hour of the dark period; *open bar*: light period