Progress in Botany 68

68 PROGRESS IN BOTANY

Review
Genetics
Physiology
Ecology

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Editorial

FORTSCHRITTE DER BOTANIK was founded in 1932 by Fritz von Wettstein. The aim of this series, which today is published annually, is clearly stated in the preface to the first volume: "The large number of journals, books and monographs currently being published, prohibits individuals from maintaining an overview of the progress in all the specialised areas of botany, let alone from keeping abreast of the results from related areas. The necessary interconnections between different specialised areas can only thrive, or be revealed, if one is able to maintain an overview of the whole field of botany."

Since its first appearance more than 70 years ago, FORTSCHRITTE DER BOTANIK or PROGRESS IN BOTANY, as it was later called, has made an effort to meet the high standards it set for itself. As a result, the series stands out from other Annual Reviews in the English-speaking world, in which articles pertaining to a particular theme just tend to be listed and briefly summarised.

Following the untimely death of von Wettstein in 1945, the series was brought back to life by Erwin Bünning (Tübingen) and Ernst Gäumann (Zürich) in 1948. After Ernst Gäumann's death in 1963, Heinz Ellenberg took his place in 1964 (volume 26). As the field of botany was being transformed more and more into an experimental science, it seemed appropriate for this

to be reflected in the series. It was with this in mind that Erwin Bünning, and the publishers Dr. Heinz Götze and Dr. Konrad Springer invited me to take charge of the area of Genetics as an editor. In order to be able to follow the whole spectrum of botanical research, Dr. Heinz Götze and Dr. Konrad Springer followed my suggestion of expanding the editorial board to include 5 members. Thus, from volume 27 onwards, Peter Sitte (Freiburg) was responsible for Anatomy and Morphology, Erwin Bünning (Tübingen) for Physiology, Karl Esser (Bochum) for Genetics, Hermann Merxmüller (Munich) for Systematics, and Heinz Ellenberg (Göttingen) for Ecology.

The idea of structuring the series into 5 sections not only revitalised the series but also proved successful over the following decades. There was of course turnover of the members of the editorial board. It was not until 1998 (volume 60) that the editorial board was reduced to 4 members. Over the last few years, we have broadened the scope of PROGRESS IN BOTANY by giving retired colleagues the opportunity to publish overview articles about their life's work.

In order to communicate the botanical nature of the series to the public, the chlorophyll molecule was used as a logo on the cover from volume 28 onwards. To keep pace with the increasing use of English in the field of natural sciences, from 1974 (volume 36) more and more articles were published in English. This trend was further reflected by adopting the English title "Progress in Botany". From volume 47 (1985), all articles were published in English. As a consequence, the German subtitle "Fortschritte der Botanik" was completely removed. From volume 59 (1997), the cover illustration was "modernised".

After 42 years of service to Fortschritte der Botanik/Progress in Botany and having reached almost 83 years of age, I feel the time has come for me to retire and to make way for a younger editor.

I would like to take this opportunity to thank the countless authors who supported me over the last decades with their contributions to the series. Moreover, I would like to thank my many colleagues who served with me on the editorial board for their cooperation. I would also like to acknowledge Dr. Czeschlik and Mrs. Gramm who, over the last few years, were involved with the series on behalf of the publisher.

Bochum, autumn 2006 Karl Esser

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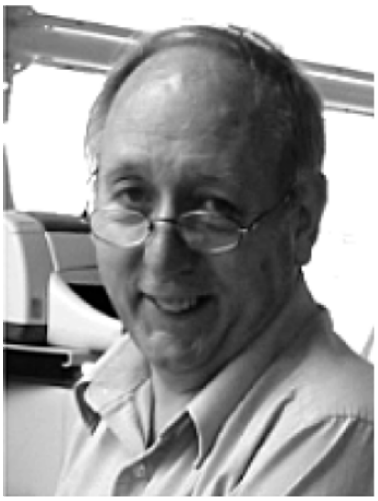

Curriculum Vitae

Charles Barry Osmond was born 20 September 1939 in Cooranbong, a small town in the foothills of the central coastal range New South Wales (NSW), Australia, to Joyce Daphne (nee Krauss) and Edward (Edmund) Charles Osmond, carpenter.

1946–55	Schooling at Morisset Central and Wyong High Schools, NSW
1956–62	BSc (Hons) and MSc, Department of Botany, University of New England, Armidale NSW, with plant ecophysiologist, Noel C.W. Beadle.
1963–65	PhD, Department of Botany, University of Adelaide, South Australia, with plant physiologist-biochemist Sir Rutherford Ness (Bob) Robertson.
1965–66	Postdoctoral research associate with plant physiologist George G Laties, UC Los Angeles USA.
1966–67	CSIRO overseas postdoctoral scholarship with plant biochemist Tom ap Rees, Botany School, University of Cambridge, UK.
1967–87	Successively, non-tenured Research Fellow, tenured Fellow, Senior Fellow and Professor (1978) Department of Environmental Biology, Research School of Biological Sciences (RSBS), Australian National University (ANU), Canberra Australia.
1980–86	Director, Biosciences Center, Desert Research Institute, University of Nevada-Reno NV USA (joint appointment with ANU)
1987–91	Arts and Sciences Distinguished Professor, Botany Department, Duke University, Durham NC, USA.

1991–2001	Director, RSBS (until 1998) then Professor, Photobioenergetics Group, RSBS ANU.
2001–03	President and Executive Director, Biosphere 2 Center, Columbia University, Oracle AZ, USA.
2003–	Visiting Fellow, School of Biochemistry and Molecular Biology, ANU, and Research Professor (adjunct) Nicholas School of the Environment, Duke University
2005	Visiting scientist, Institut Phytosphäre (ICG III) Forschungszentrum Jülich, Germany.

Elected Fellow, Australian Academy of Science (1978), Royal Society of London (1984) and Mitglied, Deutsche Akademie der Naturforscher, Leopoldina (2001). Edgeworth David (1972) and Clarke (1997) Medals, Royal Society of NSW. Fulbright Senior Scholar UC Santa Cruz and Carnegie Institution Fellow, Department of Plant Biology Stanford; Richard Merton Gastprofessor, Institut für Botanik, Technische Universität München (1974). Overseas Fellow, Churchill College, Cambridge (1980); Inaugural Distinguished Alumnus, University of New England (1994); Forschungspreis, Alexander von Humboldt Stiftung (1996).

Review

Crassulacean Acid Metabolism: Now and Then

Charles Barry Osmond

Previous title chapters in Progress in Botany, from giants of European botanical research in the latter half of the twentieth century, have explored significant areas of plant science. I am honoured, and more than a little over-awed, by the Editor's invitation to contribute in this context (and alarmed to discover that I am only, but precisely, a decade younger than the previous contributor!!). Although this chapter may not present the long view of the discipline offered by others, any perspective on crassulacean acid metabolism (CAM), a pathway of photosynthetic carbon metabolism that occurs in about 5% of vascular plants (Winter and Smith 1996; Lüttge 2004), reveals much of wider significance in plant physiology and biochemistry. When Clanton Black and I prepared a brief historical overview of CAM (Black and Osmond 2003), we emphasized the close relationship between these succulent plants and humans through the romantic paintings of Carl Spitzweg. We noted that the taste–test diagnostic of CAM may have been known to the Romans, and that accounts of morning acidity in leaves of succulents that disappeared by evening were published by Grew in the seventeenth century and Heyne in the early nineteenth century. The early literature on CAM into the 1960s was highlighted, but space constraints relegated most of the influential studies of the last 40 years to little more than a few citations from more than 40 mentors, former students and colleagues.

This chapter provides an opportunity to make amends by recording my indebtedness to very many companions in CAM research. It is a personal view of an active and exciting area of plant biology since about 1970. Indeed, Lüttge (2004) cited a selection of more than 20 reviews, edited volumes and books on CAM (notably Kluge and Ting 1978; Winter and Smith 1996) over the last 2–3 decades. My reminiscences will be largely confined to areas in which my companions and I have published, but the temptation to range more broadly sometimes will be difficult to resist. Throughout, I will link to important current developments, and emphasize some broader implications that have emerged. As will become evident, my peripatetic research on photosynthesis in succulent plants with CAM continues to depend on stimulus from many colleagues in plant science, particularly those in Germany.

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1.1 A pathway to CAM via oxalate and malate in *Atriplex*

There was little in my family or educational background to suggest any particular scholastic ability or affinity with plant biology. Alfred Kurtz, a distant relative of my mother, and a well known second generation viticulturist in the Mudgee region of central western NSW, was the only strong family connection to things botanical. His vineyard is generally credited as the source (in the 1950s) of robust Chardonnay root stocks that supported the worldwide expansion of this variety in the last half of the twentieth century (Halliday 1985). However, since both sides of my family were teetotal for two generations, I did not think to explore viticulture or enology. Rather, my botanical career emerged accidentally. It was made more likely by a spectacular collapse in mathematical ability between high school and university that terminated my aspiration to qualify as a teacher of math and science. After 3 undistinguished years, I began again, and in 1960 did well enough in botany and in natural products organic chemistry to commence graduate research in botany at the University of New England, Armidale NSW, then the only university in Australia outside a State capital city.

I was influenced by the ecological focus of the Armidale Botany Department especially its interest in halophytes of the genus *Atriplex*, the "saltbushes" from semi-arid ecosystems in southern Australia. Physiological plant anatomy featured in the curriculum, and *Atriplex* leaves were fascinating for their large crystals of calcium oxalate, their huge epidermal bladders that proved to be salt secreting systems, and their "Kranz" arrangement of mesophyll and bundle sheath tissues that proved to be the foundation of C_4 pathway of photosynthetic metabolism. One could not have been presented with a more fascinating complex of leaf physiological anatomy, and all three features were to provide this starting graduate student with significant opportunities for original research. My first encounter with a CAM plant in the wild (a huge specimen of "tree pear" introduced O*puntia tomentosa* Salm-Dyck) took place about 1960 during an excursion to the arid shrublands of south west Queensland as a field assistant to Professor Noel Beadle, a pioneering Australian plant ecophysiologist. Sadly, it was a decade or more before I rediscovered the impact of CAM on the Australian landscape.

Presented with equipment for ether extraction of organic acids, I found oxalate to be the balancing anion for the inorganic cation excess in *Atriplex* leaves. Subsequently, as a PhD student in the laboratory of Professor "Bob" Robertson in the University of Adelaide, it was possible to explore the synthesis of oxalate following $^{14}CO_2$ fixation in the light and dark, using ion-exchange and paper chromatography. Malic acid, exclusively labelled in

the 4-C carboxyl was the most abundant early labelled product in the dark (the terms malic acid and malate will be used interchangeably throughout this chapter). Unexpectedly, 4-C labelled malic and aspartic acids, were also the most abundant initial products of $^{14}CO_2$ fixation in the light in *Atriplex* leaves. As related elsewhere (Osmond 1997), I had stumbled across the "β-carboxylation" pathway of primary CO_2 fixation, subsequently associated with "Kranz" anatomy, in this large genus of C_4 plants.

These early adventures in intermediary metabolism were stimulated by P.N. ("Danny") Avadhani, who was visiting Adelaide from the University of Singapore. Danny considered himself an "ideas man" and he occupied the chalkboard in the Departmental tearoom for days on end with a frequently amended forerunner of the metabolic wall charts that Boehringer-Mannheim later supplied to decorate laboratories throughout the world. Our interpretation then of the pathway of oxalate synthesis in *Atriplex* leaves (Osmond and Avadhani 1968) was based on analogies with the isocitrate cycle and was probably incorrect. Danny had taken his PhD in the University of Newcastle upon Tyne, where Thomas and Beevers (1949) had introduced the term Crassulacean acid metabolism. In the same laboratory, David Walker (1956) had demonstrated that phosphoenolpyruvate carboxylase (PEPCase), the legendary "wouldn't work man!" reaction (Wood and Werkmann 1938), was involved in the pathway to malic acid in CAM. It is a particular pleasure now to observe that another generation of researchers has been "taking coal to Newcastle" in the form of highly original physiological biochemical and unequivocal molecular evaluations of CAM (Griffiths et al. 1989; Borland et al. 1999; Borland and Dodd 2002).

I had been introduced to plant physiology through the 1956 edition of Thomas' textbook but its description of CAM in terms of respiratory quotients probably explains why I did not readily connect to the photosynthetic implications of this pathway. Even now, with O_2- and CO_2-specific electrodes (Osmond et al. 1996) and mass spectrometers (Maxwell et al. 1998), the stoichiometries of net CO_2 and O_2 exchanges in CAM are difficult to interpret. Ranson and Thomas (1960) provided the authoritative source on CAM in English, but it had been reported that malic acid accumulating in CAM in the dark was labelled in both 1-C and 4-C carboxyl positions, in the ratio 1:2 (Bradbeer et al. 1958). At the time, the "Newcastle overall hypothesis" seemed rather perplexing and only remotely connected to my observations in *Atriplex*. Nevertheless, Danny led me through the simple diel routine of acid extraction in boiling water and titration to phenolphthalein end-points that even now draws me through nights of interrupted sleep. There is no escape from this fundamental reference for the temporal expression of CAM in different conditions, a simple reference that could

bring greater rigor to contemporary studies of regulatory cascades of gene expression.

Evidently a slow learner, I should have recognized the research potential of CAM during a postdoctoral year in George Laties' laboratory at UC Los Angeles exploring ion transport and malate compartmentation in beet discs (Osmond and Laties 1967). Ulrich Lüttge occupied the other side of the lab bench, and although I doubt we spoke of it then, our lifelong friendship later came to be entwined with CAM. A second post-doc year with Tom ap Rees in Cambridge introduced me to enzymology and the use of specifically labelled substrates for evaluation of metabolic pathways. I then had the good fortune to join Ralph Slatyer's Department of Environmental Biology in the new Research School of Biological Sciences (RSBS) at the Australian National University (ANU) in Canberra, and returned to Australia in 1967. The "research only" appointments in the Max Planck-like Research Schools embedded in a university environment, with limited opportunities for tenure but access to front-line equipment of the day, provided privileged starts for many research careers in Australia at the time. I had been hired to work on starch to malate metabolism in stomatal guard cells, but any links to CAM research that may have occurred to me at the time were soon put to one side by the wave of interest in C_4 photosynthesis. The Research School was just across the street from CSIRO Plant Industry, the nation's strongest concentration of plant physiologists and biochemists, notably Jan Anderson, Keith Boardman and Hal Hatch.

Ralph's prestige, and the popularity of environmental science at the time, may have conspired to grant us the opportunity to organize the first workshop sponsored by the US–Australia bilateral programme in science and technology. With Ralph's deft handling, the programme was expanded to include some leading scientists from the UK, Germany and Japan. The ensuing workshop on photosynthesis and photorespiration was most timely and evidently of lasting impact (Sage and Monson 1999). It was a very exciting time in photosynthetic metabolism, and the meeting afforded excellent early career opportunities to build enduring networks. My latent interest in CAM was stimulated by this meeting when a plant anatomist (Laetsch 1970) provocatively declared the C_4 pathway to be "CAM mit Krantz". A better understanding of CAM in relation to C_3 and C_4 pathways of metabolism was obviously needed, especially after the surprising observation of Klaus Winter that the ice-plant *Mesembryanthemum crystallinum* L. could be converted from C_3 to CAM patterns of CO_2 fixation by salt stress (Winter and von Willert 1972). Hal Hatch was providing inspirational research leadership in C_4 metabolism in CSIRO, and the time seemed ripe to explore CAM as a photosynthetic process.

1.2 Sorting the phases of CAM

We now know that C_4 and CAM pathways of photosynthetic carbon metabolism are both based on largely analogous preliminary CO_2 concentrating mechanisms (CCMs) in which primary carboxylation leads to 4C acids (and amino acids) that serve as intermediate, internal stores of carbon. These substrates are subsequently decarboxylated to generate internal CO_2 concentrations of 1000–25,000 ppm (Cockburn et al. 1979) that largely mitigate the oxygenase activity of Rubisco (Leegood et al. 1997). From an evolutionary perspective, these CCMs recreate the atmospheric CO_2 concentrations of the Cretaceous, a time of grand expansion of terrestrial plants under conditions of CO_2 saturation in which O_2 fixation by Rubisco oxygenase and subsequent C recycling in photorespiration would not have carried the same penalty, in energetic terms, as it does for C_3 photosynthesis today. Simply put, CCMs of C_4 plants are based on small (about 1–10 mM), spatially separated cytoplasmic pools of 4C acids that turn over rapidly ($t_{1/2}$ about 1–10 s). These CCMs can be distinguished from those of CAM plants which are based on larger (100–500 mM) pools of 4C (and 6C) acids in the vacuoles, that turn over much more slowly ($t_{1/2}$ about 5000–50,000 s) with complex, temporally separated, patterns of acid synthesis and degradation.

With a lot of help from colleagues, I set out to impose some order on the carbon metabolism of these temporally separated processes (so-called phases I–IV) and to place the curiosity of CAM into the context of other pathways of photosynthetic metabolism. The 1970 workshop stimulated two CAM enthusiasts, Manfred Kluge and Irwin Ting to spent sabbatical periods in RSBS where they successfully demonstrated the presence of pyruvate Pi dikinase and the distinctive kinetic properties of PEPCase in extracts of these plants (see below). Bruce Sutton, my first PhD student, undertook a reassessment of the labelling patterns of malic acid in CAM plants exposed to $^{14}CO_2$ in the light by comparing the previously employed *Lactobacillus* culture degradation method and degradation with purified malic enzyme to remove the 4-C carboxyl of specifically labelled malic acid preparations (Sutton and Osmond 1972). These experiments strongly suggested that fumarase activity in *Lactobacillus arabinosus* (synonym for *L. plantarum* WCFS1) led to randomization of label from 4-C to 1-C in ^{14}C-malic acid prior to or during decarboxylation, especially when old cultures were used to degrade large amounts of malic acid. Indeed, using the purified enzyme, we found that malic acid from dark $^{14}CO_2$ fixation in CAM plants was initially and predominantly 4-C labelled, consistent with primary CO_2 fixation of unlabelled PEP by PEPCase. On the other hand, malate labelling in the light was closer to the 1-C to 4-C ratio of 1:2 observed by Bradbeer et al. (1958),

consistent with PEP formation from two molecules of PGA, one of which had been previously labelled as a result of prior $^{14}CO_2$ fixation by Rubisco (Osmond and Allaway 1974).

Generous sabbatical provisions in ANU (1 year in 4 for tenured staff; a legacy of the postwar sense of isolation down-under) enabled me to work in UC Santa Cruz and the Technische Universität, München in 1973–1974. Harry Beevers evidently had a soft spot for CAM from his days in Newcastle upon Tyne and was a most generous host in Santa Cruz. Although germinating castor beans were an ideal system for investigation of Rubisco in proplastids, like most others in the lab, I welcomed opportunities to escape the nauseous extraction process. It proved possible to commute over the coast range for nocturnal gas exchange experiments with CAM plants in Olle Björkman's lab at Carnegie Plant Biology, Palo Alto. By the time I joined Professor Hubert Ziegler in München, it was clear that CO_2 fixation in the dark in CAM plants was insensitive to O_2, whereas CO_2 fixation in the light was inhibited by O_2 (Björkman and Osmond 1974), further confirming that C_4- and C_3-like carboxylation systems were functioning in a temporally separated fashion.

I went to München because Professor Ziegler had excellent access to natural abundance ratio mass spectrometers. We and others had earlier speculated that the variable natural abundance $\delta^{13}C$ values of CAM plants might reflect the variable contributions of C_4- and C_3-like carboxylations in the dark and light (Bender et al. 1973; Osmond et al. 1973). With time to think and colleagues to challenge, it now seems natural that notions of the "phases of CAM" should have matured in München, to emerge then in Naturwissenschftliche Rundschau (Osmond and Ziegler 1975; Fig. 1), some time before their most commonly cited source (Osmond 1978). Much more comprehensive studies have subsequently refined the above simple interpretation of $\delta^{13}C$ values in different taxa in different environments (Winter and Holtum 2002; Holtum et al. 2005a).

Unequivocal and independent confirmation of the labeling patterns in Fig. 1 followed later from gas chromatograph-mass spectrometry (GCMS) analysis of ^{13}C-malate extracted from CAM plants after exposure to $^{13}CO_2$. Only singly labelled malic acid molecules were detected in the dark (Cockburn and MacAuley 1975), with doubly and multiply labelled molecules appearing during $^{13}CO_2$ fixation in the light (Ritz et al. 1986; Osmond et al. 1988). Griffiths et al. (1990) provided the ultimate proof of the shifting carboxylation activities in the phases of CAM in-vivo with elegant on-line natural abundance isotope discrimination studies, and these also sealed the interpretations of shifting $\delta^{13}C$ values discussed below. Subsequent GCMS and nuclear magnetic resonance (NMR) studies facilitated assessment of the extent of fumarase randomization in CAM itself, and suggested that the

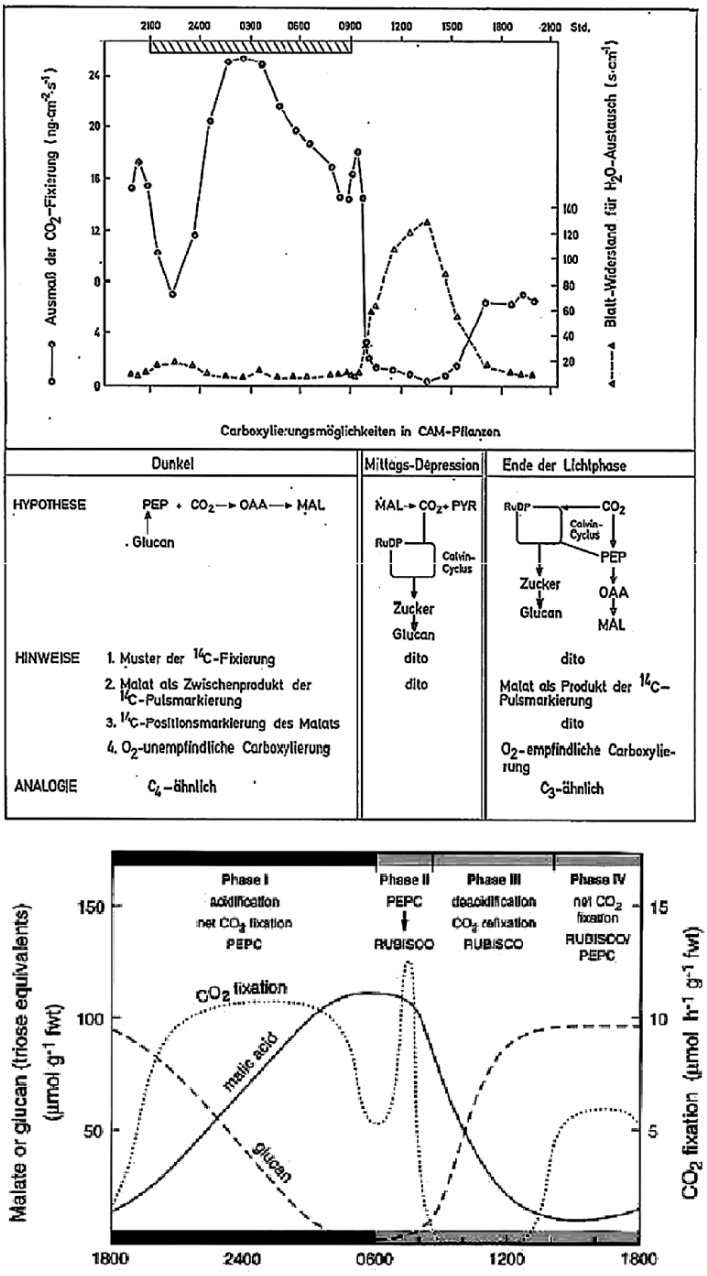

Fig. 1. Origins of the phases of CAM concept. An early summary of evidence (above) for C_4-like and C_3-like carboxylation events separated by deacidification of malic acid (Osmond and Ziegler 1975) corresponding to phases I, IV and III respectively, and (below) from a textbook chapter (Leegood et al. 1997). Phase II, the transition in carboxylation events early in the day, emerged from studies of plants in growth chambers exposed to sudden transitions in light. Perhaps one should also observe phase V, another period of transition in carboxylation events at the end of the day, especially in CAM plants exposed to slowly declining light under natural conditions. Diagrams reproduced by permission of the publishers; Wissenschaftliche Verlagsgesellschaft mbH and Pearson Education Ltd, respectively

equilibration of vacuolar, cytoplasmic and mitochondrial pools of malate changed during long-term exposures to $^{13}CO_2$ in the dark (Osmond et al. 1988). Much still remains to be done to convincingly evaluate these relationships. Although specification of the phases of CAM provided a helpful framework for a better understanding of CAM, it is important to recognize now that CAM can be much more plastic, indeed more fantastic than one then could have imagined (Dodd et al. 2003; Lüttge 2004).

1.3 Biochemistry and diffusion as determinants of the $\delta^{13}C$ value in CAM plants; improved understanding of water use efficiency in C_3 plants

Perhaps the most significant application arising from these studies of labelling patterns in CAM emerged from Marion O'Leary's interest in PEPCase in vivo. It is fair to say that interpretations of $\delta^{13}C$ values in C_3 and C_4 plants prior to 1980 were empirical, and lacked a rigorous mechanistic insight. When we were able to move the $\delta^{13}C$ value of *Kalanchoë daigremontiana* Hamet et Perrier de la Bâthie from about −16‰ to −29‰ by simply changing day-night temperature regimes and water stress exposures of plants in the same cross-gradient growth room of the Madison Biotron (Osmond et al. 1976), Marion became curious. An expert in heavy-isotope effects on enzyme kinetics, he immediately saw the merit of detailed evaluation of component processes (CO_2 diffusion, hydration to HCO_3^- and enzyme catalysis by PEPCase and malate dehydrogenase) contributing to the $\delta^{13}C$ value of CAM malate. From Marion's perspective of enzyme kinetic analyses, the carboxylation process in CAM in the dark was reporting in-vivo, as close as it gets a coupled PEPCase assay in-vitro. Many studies had shown that little else was labelled during dark $^{14}CO_2$ fixation, that there was little further metabolism of the product in the dark, and the isotopic composition of all C atoms in the product could be examined. Furthermore, because most CAM plants show substantial stomatal limitation to CO_2 diffusion, even when stomata are wide open in the dark, isotopic signatures due to diffusion were also readily detected.

Arriving in Canberra one Christmas eve, having been rained-out of a camping and walking tour of New Zealand, Marion went to work on the natural abundance ^{13}C of carbons in malate accumulated in *K. daigremontiana* and *B. tubiflorum* Harvey. His analysis of dark CO_2 fixation in CAM showed us how to sum the biophysical and biochemical components of in-vivo isotope fractionation (O'Leary and Osmond 1980). These insights soon led to a new understanding of carbon isotope fractionation in C_3 and C_4 plants with

much more important consequences. It was recognized that integrated average stomatal conductance could be inferred from changes in $\delta^{13}C$ values in C_3 plants and correlated with water use efficiency (Farquhar et al. 1982). As a result, $\delta^{13}C$ values have been used in breeding programmes to select more water use efficient cultivars of wheat (Condon et al. 1990) and other crops, adding much to the value of marginal agriculture in Australia and elsewhere. At the time we were also engaged in ecophysiological studies on "prickly pear" [*Opuntia stricta* (Haw.) Haw.] that, by the 1930s, had denied access to otherwise productive land over large areas central-eastern Australia (see below). In retrospect, it is a delightful irony that, half a century later, insights from CAM should help advance cereal agriculture in the very same regions.

These insights into the importance of diffusion later led us to predict that the $\delta^{13}C$ value of malate should be somewhat less negative towards the centre of thick CAM tissues (Robinson et al. 1993). Indeed, slow diffusion of CO_2 in thick leaves of CAM plants, with low stomatal frequencies and intercellular airspaces often below 5% (Smith and Heur 1981), is manifest in photosynthetic metabolism in other interesting ways. Maxwell et al. (1997) estimated that intercellular CO_2 concentrations at the sites of Rubisco carboxylation were only 108 μbar in *Kalanchoë daigremontiana* with open stomata in air (380 μbar CO_2) during C_3 carbon assimilation in the afternoon. It should be no surprise then, that we had earlier noted clear labelling of intermediates of photorespiration during $^{14}CO_2$ feedings in phase IV (Osmond and Allaway 1974).

We now have other evidence that the high internal resistance to CO_2 diffusion also seems to manifest itself during decarboxylation in phase III. Chlorophyll fluorescence images of the efficiency of PSII are extremely heterogeneous in phase III (Rascher et al. 2001; Siebke and Osmond, unpublished), and are characterized by randomly arising and fading patches (or fronts) of higher efficiency. The heterogeneity persists during endogenous rhythms in continuous light. These suggest spatial and temporal differences in CO_2 concentration arise behind closed stomata as deacidification in some areas proceeds faster than in others. Remembering that CO_2 diffusion in wet cell walls is likely to be 3–5 orders of magnitude slower than in intercellular air spaces, the interpretation seems reasonable. Although Duarte et al. (2005) have recently demonstrated that lateral diffusion of CO_2 occurs in leaves of *K. daigremontiana* over periods of hours, the patterns observed during deacidification change with time constants of minutes. It seems likely that the smooth curves of deacidification are a product of local heterogeneity in the implementation of the regulatory cascade that control PEPCase sensitivity to malate and other elements of this part of the CAM cycle.

1.4 Regulation of CAM PEPCase in the dark and light; its role in the diurnal rhythms of CAM and in C_4 plants

The desensitization of CAM PEPCase to end product inhibition by malate in the dark and its sensitization to the same process in the light is one of the most elegant, thoroughly and creatively documented, reversible regulatory cascades of a core physiological function in plant metabolism (Nimmo 2000). Early studies by Manfred Kluge (Kluge and Osmond 1971a,b) and Irwin Ting established the distinctive K_m, high V_{max} form of PEPCase in CAM (Ting and Osmond, 1973a) in which G-6-P desensitized the enzyme to the inhibitor malic acid (Ting and Osmond, 1973b). Thanks to Manfred and others, feedback inhibition of PEPCase by malic acid was soon implicated in the regulation of dark CO_2 fixation which tended to decline as malic acid accumulated in the vacuole towards the end of the dark period (Kluge et al. 1980). However, it was the experiments of Klaus Winter during his postdoc in Canberra (Winter 1981, 1982), and those of Jones et al. (1981) in Wilkins' laboratory in Glasgow, that stimulated the search for the PEPCase regulatory cascade. We now know that in CAM high affinity CO_2 fixation in the dark continues in the face of high malic acid contents because PEPCase is phosphorylated and desensitized to inhibition by the accumulating malic acid. In the light, PEPCase is de-phosphorylated, becomes more malic acid sensitive, and is largely prevented from competing with Rubisco in a futile carboxylation cycle during deacidification (Nimmo et al. 1986, 1987).

Damped diurnal rhythms of CO_2 evolution in CO_2-free air in continuous dark, of CO_2 exchange in air in continuous light, and their temperature responses, have been distinctive and enduring features of CAM research (Wilkins 1959; Nuernbergk 1961). The early acceptance of an overriding controller was best summed up by Queiroz (1974). He noted then that *"all the available data on CAM rhythms suggest that even if malate feedback inhibition operates under certain conditions in vivo, this effect should be superimposed on a basic oscillator (of unknown nature) which underlies the coherent operation of several enzymes of the pathway"* and that "control by feedback could be more efficient if applied to an already oscillating system". As is evident above, these thoughtful assertions provoked a determined and remarkably successful assault on PEPCase regulation from the likes of Klaus Winter and the biochemists in Glasgow. However, before these studies changed the way we think about the "CAM clock", a moment of levity intruded into this otherwise serious discussion of matters circadian. The first transmission EM pictures of the photosynthetic organelles in CAM plants were published from Eldon Newcomb's laboratory in 1975. These outstanding pictures also seemed to show a "CAM clock" in elegant physical

reality. The stained, ultra-thin sections revealed an "anomalous microcylinder" that presented a perfectly circular multi-point array in transverse section; quite clearly the 24-point cog of the "CAM clock" in *K. daigremontiana* (Kapil et al. 1975; see insert in their Fig. 13). In fact, the plant material had been maintained by vegetative propagation in the Madison greenhouses for decades, and presumably accumulated a virus (possibly a potyvirus; R. Milne personal communication) the structural proteins of which might have produced the "rifled" cylindrical structure responsible for the multi-pointed (18–24) cogwheel in transverse section.

In reality, explanations of the endogenous rhythms of CAM require nothing less than the careful interpolation and interpretation of the whole of CAM physiology and biochemistry. In those CAM plants that display damped diurnal rhythms of CO_2 exchange (only a handful are known in detail), we must embrace not only the coherent regulation of CO_2 uptake by PEPCase, deacidification and CO_2 release by malic enzyme, but also the refixation of CO_2 by PEPCase and Rubisco (Griffiths et al. 2002; Wyka and Lüttge 2003), as well as compartmentation dominated by malic acid fluxes into and out of the vacuole (Hafke et al. 2003), its relationships to metabolite fluxes among smaller organelles (Kore-eda et al. 2005) and of course, the even more complex coherent regulation of carbohydrate metabolism.

I confess to having long favoured the system view; that if left alone, the intricate network of physiology and biochemistry of CAM will oscillate of its own accord in continuous light and dark, so long as C-resources allow. The rather rapid dampening of the rhythm in CO_2-free air in the dark is almost certainly limited by carbohydrate reserves and respiration, and the numerous oscillations in continuous light in a variety of CAM plants obviously reflect the interactions of 2 carboxylases and differing decarboxylation options. The system view has taken strength from elegant temperature shift analyses augmented by on-line stable isotope discrimination and modelling (Grams et al. 1997), from biochemical and molecular evidence that malate overrides the circadian regulation of the PEPCase kinase (Carter et al. 1991; Borland et al. 1999), and from images of the entrainment of areas of low and high PSII efficiency during oscillations in continuous light (Rascher et al. 2001). Indeed, interpretation of these images in terms of the *"the biological clock as an assembly of coupled individual oscillators"* simply refers to independent nodes of the CAM system isolated by slow diffusion of internally generated CO_2 in a tissue with little intercellular air space connectivity.

The remarkable long-term commitment of Bohnert and Cushman to the molecular genetics of CAM in *M. crystallinum* (Cushman and Bohnert 1999) is now facilitating great progress on the circadian regulation of gene expression behind key components of the regulatory cascade (Hartwell et al. 2002;