Progress in Botany

Ulrich Lüttge
Wolfram Beyschlag
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Progress in Botany 74

Springer

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Volume 74

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ISSN 0340-4773
ISBN 978-3-642-30966-3 ISBN 978-3-642-30967-0 (eBook)
DOI 10.1007/978-3-642-30967-0
Springer Heidelberg New York Dordrecht London

Library of Congress Control Number: 2012950375

Printed on acid-free paper

Springer is part of Springer-Science+Business Media (www.springer.com)

Series information

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Part I
Review

Half a Century of Pursuing the Pervasive Proton

John A. Raven

Contents

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U. Lüttge et al. (eds.), *Progress in Botany*, Progress in Botany 74,
DOI 10.1007/978-3-642-30967-0_1,

Abstract Acid–base regulation is probably a universal attribute of life, and energy coupling via transmembrane H^+ gradients is very widespread. Much of my academic career has been related to these two processes and to their interactions. Highlights from my studies of acid–base regulation are the quantitative resolution of the challenges for acid–base regulation in land plant shoots when metabolism involving net H^+ production (e.g. primary assimilation of NH_4^+, NH_3 or N_2) occurs there, quantitation of the energy costs of acid–base regulation for different locations and mechanisms of acid–base regulation for the assimilation of a range on N sources and the interaction of CO_2 concentrating mechanisms in aquatic photosynthetic organisms with acid–base regulation. Research on the significance of transmembrane H^+ gradients has included a significant contribution to the early development of chemiosmotic hypothesis of polar transport of indoleacetic acid, the evolutionary significance of chemiosmotic coupling and the role of H^+ leakage relative to other processes which consumed energy at an essentially constant rate regardless of the rate of light energy supply in determining the minimum photon flux density at which photolithotrophic growth can occur. On a global scale, work on the effects of anthropogenic CO_2 production on ocean acid–base balance has helped to set limits on the significance of this 'ocean acidification' for marine algae. A final point covered in the chapter is an analysis of the continuing attempts to determine precisely what is being regulated, e.g. the pH of the intracellular compartment or the ionisation state of one or more of weak electrolytes in the compartment.

1 Introduction

The role of pH in ecology and physiology has been understood for well over half a century when I began my PhD in 1963, and by this time both animal and plant physiologists recognised that acid–base regulation at the cell and organisms level was an important aspect of homeostasis. The plant work was led by crop physiologists who recognised the role of organic acid synthesis in acid–base balance (often termed charge balance) in plants growing with NO_3^- as N source. A role for protons in bioenergetics had been hinted at by R. N. Robertson, but it was the insight of Peter Mitchell of chemiosmotic coupling of oxidation–reduction energy and hydration–dehydration (ATP–ADP) energy (Mitchell 1961, 1966, 1968) that put protons centre stage in bioenergetics, not only with endergonic and exergonic fluxes of H^+ across proteolipid bilayer membranes involved in coupling of oxidation–reduction and hydration–dehydration energy but also, as we now know, endergonic solute fluxes coupled to exergonic H^+ fluxes and exergonic H^+ fluxes coupled to cell motility in archaeal and bacterial flagella. These ideas found an especially receptive home among those interested in plants since the publication of the Hill and Bendall (1960) 'Z scheme' for two photochemical reactions in series in photosynthetic electron transport from H_2O to CO_2, and a chloroplast research community less steeped in hypothesised chemical rather than H^+ electrochemical

potential differences across membranes, as 'high energy' intermediates, and with experimental material (thylakoid membrane preparations) in which it could be shown that H^+ energy gradients across membranes were a, if not the only, component of the interchange of oxidation–reduction and hydration–dehydration energy.

The 1960s were, then, an exciting time to work on plant energetics and solute transport, and my earliest pH related work was on the use of exogenous HCO_3^- as well as CO_2 in photosynthetic inorganic C assimilation in cells of the giant alga *Hydrodictyon africanum* (Raven 1968), although my PhD under the supervision of the incomparable Professor Enid MacRobbie was mainly about transport of Cl^-, K^+ and Na^+. A referee for that paper asked how direct effects of pH could be distinguished from effects on changed fractions of the various forms of inorganic C as external pH changes: this is a question which still bothers us today (Raven et al. 2005a; Raven 2011). The HCO_3^- work led to the review (Raven 1970) which attempted to relate the physiology of C_3 and C_4 land plants and of aquatic algae and embryophytes to the kinetics of Rubisco and to what was known of the transport of CO_2, HCO_3^- and H^+/OH^-. This attempt was premature, not least because the isolation methods for the Rubisco (not known as a Form IB Rubisco) from C_3 terrestrial plants did not yield enzyme preparations which showed the relatively high CO_2 affinity which is accepted today (Tcherkez et al. 2006). This certainly did not help my arguments that organisms like *Chlorella*, when acclimated to low CO_2, might need to accumulate CO_2, since if the then known in vitro kinetics of C_3 land plants were taken as representative of the in vivo state than a means of concentrating CO_2 would also be needed in those plants too in order to explain the dependence on external CO_2 in vivo. At least there was recognition of the possible widespread occurrence of what are now termed CO_2 concentrating mechanisms (CCMs) among photosynthetic organisms, but also the relation to acid–base regulation (not just 'charge balance') during HCO_3^- with subsequent assimilation of CO_2 (Raven 1968, 1970). This work set the pattern of a rather large number of review, synthesis and conceptual and quantitative modelling papers relative to primary data papers that has characterised my published output.

After discussing some aspects of my published work that relate to the roles of protons in plant biology, I address the question of what is being controlled in acid–base regulation in algae and plants without, alas, coming to a clear conclusion.

2 Acid–Base Regulation as a Function of Nitrogen Source

The work on inorganic C assimilation in *Hydrodictyon africanum* (Raven 1968) followed work (also in Professor MacRobbie's laboratory) by Andrew Smith (Smith 1967) on *Nitella translucens* in which he focussed on the products of assimilation of ^{14}C inorganic C and their distribution between cytoplasm and vacuole. However, it was not until Andrew had moved to Adelaide and I had moved to Dundee that we collaborated on acid–base regulation and related matters. Raven and Smith (1973, 1974, 1976b) set the scene with respect to the inadequacy

of 'passive' buffering, i.e. uptake of excess H^+ or OH^- by pre-existing weak electrolytes in intracellular compartments with pK_a values close to those of the set point pH of the compartment, for anything but short-term response to perturbation on the acid–base balance of a compartment. Net metabolic generation of OH^- (assimilation of exogenous HCO_3^-, NO_3^- and SO_4^{2-} in primary metabolism) or H^+ (net assimilation of NH_4, NH_3 and N_2 in primary metabolism) at the rates seen during growth, and observed rates of passive entry of H^+ in some organisms, demands the involvement of enzymes and/or integral membrane transporters in, respectively, the biochemical and the biophysical pH stats. Raven and Smith (1973, 1974, 1976b) point out the spatial restrictions on the occurrence of these pH stats: the biophysical pH stats need a large external phase to which excess intracellular H^+ or OH^- can be transported, which restricts this mechanism to aquatic organisms and the below-ground parts of terrestrial organisms. For the biochemical pH stat, the net synthesis of organic acids from a neutral ultimate precursor (atmospheric CO_2) can generate H^+ as means of neutralising excess OH^- in cells in any environment. This is especially important in land plant shoots, with accumulation of the resulting salt of the organic anion with the cations accompanying the NO_3^- and SO_4^{2-} up the xylem to the shoot in the shoot cell vacuoles or their transport to the roots in phloem for 'further treatment'. Metabolic production of OH^- is only quantitatively important as a means of neutralising excess H^+ when there is the salt of an organic anion which can be metabolised to CO_2 and OH^-, neutralising the H^+ and 'replacing' it with the cation which accompanied the organic anion up the xylem to the shoot, having been taken up by the root in exchange (in terms of charge, if not mechanism) for the H^+ produced in net organic acid synthesis in the shoots. Excess H^+ generated in the shoot cannot be transported to the roots in the phloem. The cases for the absence of significant H^+ transport in the phloem, and for the absence of OH^- generation in plants other than in assimilation of HCO_3^-, NO_3^- or SO_4^{2-}, or the catabolism of organic anions, have been further developed in, respectively, Raven (1977) and Raven (1986, 1988). Raven et al. (1990a) considered the evidence on acid–base regulation in symbiotically N_2-fixing vascular plants, while Raven and Farquhar (1990) have considered organic acid production in acid–base regulation in the context of the impact on the C stable isotope natural abundance of the plants. Raven and Farquhar (1989) present data from a 'null point' method of estimating leaf apoplasm pH. The energy and water costs of the various acid–base regulation processes related to N assimilation have been quantitatively modelled by Raven (1985) and most recently updated in Andrews et al. (2009).

These various predictions rationalising the distribution of N assimilation processes in algae and vascular plants in relation to acid–base regulation related to the assimilation of various N sources were, for NO_3^-, relatively well-quantified by experiment by the 1970s. However, some experimentation remained to be done for NO_3^-, and more for N_2 and, especially, NH_4^+ and NH_3. Some of these tests of predictions have been carried out, with general support for the predictions, by a series of excellent post-doctoral fellows. Acid–base regulation in *Hydrodictyon africanum* was investigated by Ida De Michelis and Hemal Jayasuriya (De Michelis et al. 1979; Raven and De Michelis 1979, 1980). Other work investigated the roles

of long-distance transport in acid–base regulation in NO_3^- and NH_4^+ assimilation in *Ricinus communis*, and in NO_3^-, NH_4^+ and N_2 (in root nodules) assimilation in *Phaseolus vulgaris*, by Susan Allen (Allen and Raven 1984, 1987; Raven et al. 1984; Allen and Allen 1987; Allen and Smith 1987; Allen et al. 1988). The problems posed by N_2-fixing symbionts in nodules on stems in air, rather than on roots in soil, in *Sesbania rostrata* was examined by Richard Parsons (Parsons et al. 1993, 1995). Acid–base regulation related to assimilation of gaseous NH_3 through shoots of two C_3 and one C_4 grass was investigated by Bernd Wollenweber and by Zu-Hua Yin (Wollenweber and Raven 1993a, b; Yin et al. 1996; Yin and Raven 1997, 1998), and the graminoid (Juncaceae) *Luzula sylvatica* was investigated by PhD student Paul Hill (Hill et al. 2001, 2002). Subsequent work on gaseous NH_3 has been less focussed on acid–base regulation and has a more ecological and environmental bias with PhD students Jennifer Carfrae and Matt Jones (Carfrae et al. 2004, 2007; Sheppard et al. 2004; Jones et al. 2007a, b, 2008).

The work described earlier has been followed up, with criticism and extension, by a number of workers (Sanders and Slayman 1982; Sakarno 1998; Kronzucker et al. 2001; Britto and Kronzucker 2002, 2005). Sanders and Slayman (1982) showed that oxidative metabolism was more important than the plasmalemma H^+ efflux ATPase in removing H^+ from inside cells, but in the long term there must be export of excess H^+ from the cells, though not necessarily via the H^+ ATPase (Raven 1986). Britto and Kronzucker (2005) make important points about the activity and regulatory properties of phosphoenolpyruvate carboxylase which do not readily fit the requirements of a biochemical pH stat, generating H^+ and neutralising OH^- generated in NO_3^- assimilation, and the clear anaplerotic involvement of this enzyme in nitrogen assimilation in producing the suite of carbon skeletons needed for amino acid and pyrimidine synthesis. However, it is clear that there is a role for additional organic acid synthesis from (ultimately) neutral substrates specific to NO_3^- as opposed to NH_4^+ assimilation in vascular embryophytes, especially when assimilation of NO_3^- occurs in shoots (Raven and Smith 1976b; Raven and Farquhar 1990).

An important point for the work on nitrogen assimilation but also relevant to the remaining sections is that work from the author's laboratory does not explicitly use the Strong Ion Difference procedures of Stewart (1978, 1981) but comes to the same conclusions.

3 Acid–Base Regulation in Algae and Aquatic Plants with an Emphasis on Carbon Source

The other main line of research on acid–base regulation concerns inorganic carbon acquisition. In the decade after Raven (1970) there were great advances in our understanding of the kinetics of Rubisco, including the finding that the enzyme has an oxygenase as well as a carboxylase activity, and culminating in a mechanistic

model of gas exchange in C_3 land plants (Farquhar et al. 1980), and the discovery of CO_2 concentrating mechanisms in a cyanobacterium (Kaplan et al. 1980) and a green microalga (Badger et al. 1980). Work with post-doctoral fellow Sheila Glidewell (Raven and Glidewell 1978) suggested that the C_4-like physiology but C_3 biochemistry of the freshwater colonial giant-celled alga *Hydrodictyon africanum* was due to intracellular accumulation of inorganic carbon in the cells based on active influx of HCO_3^-, but we did not attempt to measure the intracellular inorganic carbon concentration. Subsequent work involving the author has dealt with freshwater macrophytes and with marine macrophytes collected from the field and cultured for various periods in the laboratory and cultures of microalgae.

The work on freshwater macrophytes with post-doctoral fellows John Beardall, Howard Griffiths and Jeffrey MacFarlane showed that the red algae examined relied on diffusive entry of CO_2 (Raven and Beardall 1981a; Raven et al. 1982, 1994, 2000b, 2005a); MacFarlane and Raven 1985, 1989, 1990. For the red alga *Lemanea mammilosa* a detailed analysis of boundary layer effects under natural flow conditions of intracellular inorganic C transport and Rubisco kinetics was undertaken, with a satisfactory fit of the data to a mechanistic model (MacFarlane and Raven 1985, 1989; Raven et al. 2005a). The freshwater green alga *Cladophora glomerata* and the flowering plant *Ranunculus penicillatus* ssp. *pseudofluitans* (Raven et al. 1982, 1994) have CCMs and can use HCO_3^- as an inorganic carbon source and so share in the complications for acid–base regulation attendant on expressing a CCM (Raven 1999). PhD student Jonathan Newman investigated the mechanism of HCO_3^- use in *Ranunculus penicillatus* ssp. *pseudofluitans*: this seems to involve the conversion of HCO_3^- to CO_2 in invaginations in the radial walls of leaf epidermal cells (Prins and Elzenga 1989; Rascio et al. 1999). These transfer cell-like invaginations have carbonic anhydrase activity in the apoplasm, and presumably are acidified by a plasmalemma H^+ efflux pump (Raven 1976), with uptake of CO_2 (Newman and Raven 1993, 1999). This mechanism would be a variant on that found in ecorticate freshwater and brackish water characean algae and submerged flowering plants of the elodeid life form (Arens 1939; Walker et al. 1980; Price and Badger 1985; Price et al. 1985; Maberly and Madsen 2002; Ray et al. 2003).

The freshwater flowering plant *Crassula helmsii* exhibits Crassulacean Acid Metabolism (CAM) (Newman and Raven 1995), with its regulated variation of pH in the cell vacuole, a 'P' compartment in the sense of Mitchell (1966, 1968), i.e. a compartment into which H^+ is pumped and containing no functional nucleic acids and low diversity and concentration of proteins, which can tolerate larger excursions of pH than can the 'N' phases (Mitchell 1966, 1968) of cytosol, chloroplast stroma and mitochondrial matrix. More recent work on inorganic carbon assimilation by *Crassula helmsii* is that of Klavsen and Maberly (2009, 2010). Other work on submerged freshwater vascular plants involving post-doctoral fellows Howard Griffiths and Katherine Richardson confirmed that both *Isoetes lacustris* (a lycophyte at the pteridophyte grade of organisation) and *Lobelia dortmanna* (a flowering plant) took up much, or most, of their CO_2 through their roots and that *I. lacustris* uses CAM (Richardson et al. 1984). It was shown in work

with Professor Jon Keeley and Barry Osmond that the amphibious *Stylites* (= *Isoetes*) *andicola* took up most of its CO_2 through the root system and used CAM when submerged and when emerged, and did not produce functional stomata when growing on land (Keeley et al. 1984), while submerged seedlings of the flowering plant *Eriocaulon decangulare* took up much of their CO_2 through the roots and did not exhibit CAM (Raven et al. 1988). For the freshwater aquatic flowering plants this work is put into a broader context by Maberly and Madsen (2002).

For marine macrophytes the occurrence of diffusive CO_2 entry and of CCMs has been investigated in green, red and brown seaweeds and in an intertidal cyanolichen. The work of PhD student Andrew Johnston on the intertidal brown fucoid alga *Ascophyllum nodosum* showed that it had physiological characteristics of an alga with a CCM, apparently based on active transport at the plasmalemma of an inorganic C species (the alga can use HCO_3^-) although there is evidence of significant short-term incorporation of inorganic ^{14}C into dicarboxylic organic acids under some situations; the alga showed very substantial photosynthetic uptake of CO_2 from the atmosphere at natural CO_2 concentrations when emersed but still hydrated, and also exhibited very low-amplitude CAM, in part involving PhD student Misni Surif (Johnston et al. 1986a, b, c, 1987, Surif and Raven 1989a, b, 1990; Johnston 1991).

The occurrence of CAM was investigated in a range of brown algae and other submerged and intertidal macrophytes, and the low amplitude CAM was only found in a limited range of the Fucales (Raven et al. 1985, 1988, 1990a, b, 1995a, b, 1996; Johnston and Raven 1986c; Surif and Raven 1989b; Raven and Johnston 1991). However, in his excellent and comprehensive review of aquatic CAM, Keeley (1998) points out that the fate of the compounds labelled in the dark from inorganic ^{14}C in *Ascophyllum nodosum* is not consistent with typical CAM, so the role, if any, of the diel changes in titratable acidity and in malate in the fucoid brown algae in the carbon balance of the organisms is not clear. Metabolomic and genomic studies give no evidence of CAM-like metabolism (or C_4-like metabolism) in the (non-fucoid) brown alga *Ectocarpus siliculosus* (Cock et al. 2010; Gravot et al. 2010).

CCMs were found on the basis of physiological evidence, and correlated data on the natural abundance of stable isotopes, in all marine brown algae, in almost all of the green algae, and most of the red algae examined, as well as in the only marine lichen examined, *Lichina pygmaea* (Johnston and Raven 1986a, b, 1987; Raven and Samuelsson 1988; Surif and Raven 1989a, 1990; Raven et al. 1989, 1990a, b, 1994, 1995a, b, 2002, 2005a; Johnston et al. 1992; Maberly et al. 1992, 2009; Raven and Osmond 1992; Kübler and Raven 1994, 1995, 1996; Kübler et al. 1999; Sherlock and Raven 2001; Kevekordes et al. 2006; Hepburn et al. 2011; Marconi et al. 2011). The inspiration for the Johnston et al. (1992) and Maberly et al. (1992, 2009) work came from the 'pH drift' studies of Professor Stephen Maberly (1990). While a few of the organisms studied had been previously investigated, in most cases the data were first reported on inorganic carbon assimilation for the algae. The assignment of algae to 'CO_2 diffusion' or 'CCM' categories on the basis of pH drift and C stable isotope natural abundance measurements is generally robust, although there are some caveats (Raven et al. 2005a; Kevekordes et al. 2006; Midelboe and Hansen 2007a, b; Hepburn et al. 2011; Marconi et al. 2011; Moulin et al. 2011). The occurrence of CO_2 diffusion

macroalgae generally correlates with lower irradiances as predicted by Johnston et al. (1992), Maberly et al. (1992) and Raven et al. (2000a, 2002a, b), although this correlation is by no means perfect (Johnston et al. 1992; Maberly et al. 1992; Raven et al. 2000a, b; Kevekordes et al. 2006. Hepburn et al. 2011; Marconi et al. 2011; Moulin et al. 2011).

The work on cultured microalgae has a number of proton-related components. Post-doctoral fellows Richard Geider and Bruce Osborne showed that cultures of the diatom *Phaeodactylum tricornutum* at low (Geider et al. 1985) or very low (Geider et al. 1986) irradiances showed high yields of growth on the basis of absorbed photons. This is of interest in view of the 'photons and protons' predictions of Raven and Beardall (1981b, 1982) on the effects of H^+ permeability of thylakoid and other membranes becoming a multiplicative factor with other energy costs which are essentially independent of irradiance. These other energy costs are back-reactions in photosystem II, slippage in the ATP synthetase, leakage of CO_2 from the CCM if this machinery is present and expressed at low irradiances, and protein turnover and, with H^+ leakage, should restrict growth at low irradiances (Raven et al. 2000a, b; Quigg et al. 2006). The discussion earlier suggests that CCMs might be less common in algae adapted to low irradiances, and expression of CCMs might be decreased. Work by post-doctoral fellow Janet Kübler showed that CCM expression is decreased, at least when judged by the decreased affinity of cells for inorganic C, when they are cultured at low irradiances (Kübler and Raven 1994, 1995, 1996; see also Young and Beardall 2005). It would be helpful to follow up these studies with measurements of the intracellular:extracellular ratio of CO_2 as a measure of CCM function, as originally performed by Badger et al. (1980) and Kaplan et al. (1980) and some of their early followers (e.g. Beardall 1981; Beardall and Raven 1981; Beardall et al. 1982) and recently by Spijkerman (2011).

A clearly proton-related aspect of inorganic carbon acquisition by microalgae relates to a carbonic anhydrase (Cah3) expressed in the thylakoid lumen of *Chlamydomonas reinhardtii* and especially, in cells grown in low CO_2, in the part of the thylakoids which penetrate the pyrenoid (Karlsson et al. 1998; Moroney and Ynalvez 2007). Following the results and suggestions of Pronina and colleagues (Pronina and Semenenko 1988, 1990; Pronina and Borodin 1993), Raven (1997a, b) proposed a quantitative model of how HCO_3^- could move from the medium to the thylakoid lumen, where, catalysed by Cah3 and using the high H^+ concentration generated by the light-driven H^+ pumps located in the thylakoid membrane, a CO_2 concentration well in excess of that in the medium could be achieved, i.e. a CCM. The CO_2 could then diffuse through the thylakoid membrane to Rubisco in the pyrenoid. The proposed mechanism is essentially an internalisation of that proposed by Walker et al. (1980) with the addition of carbonic anhydrase (CA), as is now the case for variants of the Walker et al. (1980) model for characean cells and elodeid leaves (Price and Badger 1985; Price et al. 1985; Maberly and Madsen 2002; Ray et al. 2003). This mechanism is now part of the best-accepted model of the CCM of *Chlamydomonas reinhardtii* (Moroney and Ynalvez 2007; Markelova et al. 2009), although not all of the components have yet been identified. Furthermore, Cah3 has at least one more H^+-related role in photosynthesis, i.e. the enhancement of the rate of O_2 evolution by H^+ removal (Shutova et al. 2008). Finally, it must be

remembered that this kind of CCM is, as far as is known, only found in the well-investigated *Chlamydomonas reinhardtii* (Giordano et al. 2005; Roberts et al. 2007a, b. Raven 2010, 2011).

A final aspect of microalgal CCMs in relation to acid–base regulation is the interaction of carbon and nitrogen acquisition. Beardall et al. (1982) found that growth of *Chlorella emersonii* under nitrogen (supplied as NO_3^-) limitation with a high CO_2 concentration led to the expression of a CCM, just as did decreasing the CO_2 concentration at high (or low) nitrogen availability. This effect of nitrogen supply was related by Beardall et al. (1982) to the high nitrogen use efficiency (biomass increase rate per unit nitrogen in the cells) predicted for algae expressing a CCM, although this argument only strictly applies to growth in air levels of CO_2 (at lower CO_2). Regardless of the evolutionary explanation of this phenomenon, it clearly influences acid–base regulation to the extent that there is HCO_3^- influx as part of the CCM, adding OH^- efflux related to HCO_3^- entry followed by CO_2 assimilation to the OH^- efflux related to NO_3^- (and SO_4^{2-}) assimilation. A different situation occurs in a strain of *Chlamydomonas reinhardti* lacking the capacity to grow on NO_3^- and so grown with NH_4^+ (Giordano et al. 2003; see Raven 2001), where CCM expression (again judged from inorganic carbon affinity) is mainly regulated by nitrogen supply, with the lowest expression of the CCM under nitrogen limitation, paralleling decreased expression of a mitochondrial carbonic anhydrase. Here the decreased H^+ generation rate in the NH_4^+-limited cells is paralleled by a decreased rate of OH^- generation from HCO_3^- entry and CO_2 assimilation with decreased CCM expression. Also not related to a change in inorganic carbon supply, Giordano et al. (2007) examined metabolic responses of *Dunaliella parva* to a gradual change from NO_3^- to NH_4^+ as nitrogen source with a change from a net intracellular OH^- production to a net intracellular H^+ production from nitrogen assimilation against a (presumed) constant intracellular net OH^- production related to HCO_3^- entry in the CCM (Raven 2009, 2011).

4 An Acid–Base Dimension to Environmental Change

Increasing anthropogenic CO_2 production from fossil fuel burning and land use change has increased CO_2 input to the atmosphere over the last 250 years, and at least a quarter of this CO_2 has dissolved in the surface ocean. Through interaction with the existing inorganic carbon system in the ocean has produced a pH decrease of about 0.1 unit since 1750, with another 0.4 unit decrease predicted by 2100 (Raven et al. 2005b; Doney et al. 2009). My attention was focussed on this ‘ocean acidification’ in 2004 when I was asked to chair the Royal Society of London panel which produced the 2005 report (Raven et al. 2005b), although I had published on the effects of increasing CO_2 and temperature on microalgae (Raven 1991a, c; Raven et al. 1993) and macroalgae (Raven and Johnston 1991) as well as more general accounts (Beardall et al. 1998a, b). The predictions from the effects of increased CO_2 and temperature on extant genotypes show a variety of responses, with generally negative responses on growth of calcified algae and no effect, or a

stimulation, of growth for non-calcified algae (Raven et al. 2005b; Doney et al. 2009; Crawfurd et al. 2011; Gattuso and Hansson 2011; Raven et al. 2011, 2012).

A number of points need to be made. One is that 'ocean acidification' means a decrease in pH relative to the interglacial value before 1750; it is not predicted that surface ocean pH will fall below pH 7.0 even with continued fossil fuel burning (Raven et al. 2005b; Diaz-Pulido et al. 2007; Falkowski and Raven 2007; Doney et al. 2009; Gattuso and Hansson 2011). Another point is that the decrease in pH is not necessarily, or even probably, the major influence on photosynthetic aquatic organisms of the effects of increased atmospheric CO_2 on the CO_2–H_2CO_3–$HCO_3^-$$CO_3^=$–$H^+$–$OH^-$ system. For calcified organisms the decrease in $CO_3^=$ is very important, while for non-calcified organisms the increase in the concentration of the other inorganic carbon species is important. A third point is that very little of the currently available data used in modelling involves genetic adaptation (Collins 2011; Collins and Bell 2004, 2006; Collins and Gardner 2009; Collins et al. 2006a, b; Huertas et al. 2011). Such experiments are difficult to perform, but more are in progress. A fourth point is that few of the experiments involve changes to both inorganic carbon and temperature in factorial experiments (Hurd et al. 2009; Finkel et al. 2010; an exception is the work of Fu et al. 2007; Feng et al. 2008 and Fu et al. 2008). A related, and very important, point is that warming will mean a shoaling of the thermocline, with decreases in the nutrient flux from the deep ocean to the upper mixed layer and increases in the mean flux of ultraviolet and photosynthetically active radiation in the upper mixed layer (a point of relevance to phytoplankton but not phytobenthos) (Raven et al. 2011, 2012), requiring even more complex multifactorial growth experiments to provide data for modellers. Many models of future ocean primary productivity emphasise warming and the shoaling of the thermocline, with little or no account taken of the effects of ocean acidification (Steinacher et al. 2010).

These comments are NOT an attempt to underplay the importance of ocean acidification for the future of marine photosynthetic organisms or of inland water phototrophs. However, it is essential that there is a multifactorial approach to both experimentation and modelling, bringing in all relevant components of environmental change and the significance of genetic adaptation (Raven et al. 2011, 2012).

5 Acid–Base Regulation, Chemiosmotic Coupling, and the Last Universal Common Ancestor

Raven and Smith (1976a, 1981, 1982) and Smith and Raven (1978) suggested sequences of evolutionary events which could have occurred in acid–base homeostasis, bioenergetics and membrane transport, based on the then-popular 'chemoorganotrophy (= heterotrophy) first' hypothesis for the energetic basis of the earliest organisms on Earth. The scenario of Raven and Smith (1976a, 1981, 1982) and Smith and Raven (1978) has the Last Universal Common Ancestor (LUCA) of all extant organisms on Earth as a fermenting anaerobic organotroph with active, ATP-powered H^+ efflux related originally to acid–base regulation,

necessitated in part by acidic fermentation products. LUCA is now thought of by many scientists, including the author, as a chemolithotroph (Lane et al. 2010). However, intracellular acid–base homeostasis, in addition to redox homeostasis, would still have been an important factor in LUCA (Allen 2010).

6 Protons and Plant Growth Substances

Many natural plant growth substances are weak electrolytes with pK_a values close to the pH of intracellular and extracellular compartments (Raven and Rubery 1982). Since many of them also have relatively high lipid:water partition coefficients, the unionised form of, for example, auxin (indoleacetic acid), abscisic acid (ABA) and gibberellins would have significant lipid solution permeability through membranes and tend to be accumulated in alkaline compartments ('alkaline trap') as the anion. These effects must, to at least some extent, influence the distributions brought about by any other transport processes which exist for the growth substances.

In the case of auxin, the other transporters are those associated with the polar transport of auxin: this is now known (Goldsmith 1977; Estelle 1998) to involve the 'chemiosmotic' mechanism (Rubery and Sheldrake 1973, 1974; Raven 1975). Some commentators (e.g. Estelle 1998; Abel and Theologis 2010) are kind enough to give the author equal credit with Rubery and Sheldrake, despite the obvious lack of synchrony of publication. The proton efflux pump, the neutral auxin influx at the upstream end of the cell and the auxin anion channels at the downstream end of the cell are the essential components of the mechanism; none of these were well characterised when the mechanism was proposed.

For short-term regulatory ABA responses of stomata, Cowan et al. (1982) suggested that the increased stromal pH upon illumination acted as an 'alkaline trap' for the ABA anion. With a fixed (in the short term) ABA pool in the leaf, the trapping of ABA would decrease ABA in the rest of the leaf and, since ABA inhibits stomatal opening and promotes stomatal closing, stomata would open in the light. The reverse of this process could occur in the dark. Whilst this may not be a significant mechanism of stomatal control in diel (or sunfleck-shade) cycles, pH is a significant regulator in the role of ABA as a drought signal (Williamson and Davies 1997, 2002). Regardless of the role of pH, stomatal responses do provide very good regulation of water loss in transpiration per carbon gain in photosynthesis (Cowan 1977, 1986; Cowan and Farquhar 1977; Vico et al. 2011).

7 Circulating Currents Carried By H^+ and Their Role in Algal and Plant Biology

Currents circulate through and outside growing, polar, eukaryotic cells and organs, generally with positive charge influx near the extending tip and positive charge efflux in more mature regions (Raven 1991b). In most cases, and universally in

terrestrial rhizophytic plants, most of the current is carried by protons. The positive charge efflux in the more mature regions involves active H^+ efflux, while positive charge influx in apical regions involves a H^+ channel; buffered H^+ moves apically through the cytosol including, in multicellular structures, in plasmodesmata and basally in the aqueous medium.

What do these circulating H^+ do? In characeans, the acidic and basic zones on the internode, with H^+ efflux in the acid zones and H^+ influx in the alkaline zones, would produce circulating H^+ currents were it not for the intervention of the external inorganic carbon system. In the acid zones, H^+ plus HCO_3^- produce CO_2, while in alkaline zones removal of H^+ from HCO_3^- produces $CO_3^=$. The $CO_3^=$ in the alkaline zone then precipitates as $CaCO_3$, using half a Ca^{2+} which balances the HCO_3^- consumed in the acid zone; this half $Ca^{2=}$ carries a positive change as it diffuses into the alkaline zone, where it is used in $CaCO_3$ precipitation with the half Ca^{2+} which balances the now-deprotonated $HCO_3^=$ (Raven 1991b). A similar process occurs in polarised elodeid leaves.

Aside from inorganic carbon acquisition and calcification, circulating currents have been suggested, by setting up external electrical potential gradients (negative at the tip, positive in the more mature regions) to attract or repel microorganism which are galvanotactic (free-swimming cells, such as diazotrophic rhizobia) or galvanotropic (hyphae of mycorrhizal fungi such as glomeromycotes). So far, these suggestions remain without adequate testing, despite the best efforts of PhD student Andrew Miller (Miller et al. 1986, 1991). A further possibility is that it is power which is being transmitted, i.e. chemiosmotic energy flow with a long distance between the generator of the H^+ transmembrane energy gradient and the consumer of the H^+ gradient. An example is motility in filaments of motile (gliding) oscillatorean cyanobacteria. Calculation (Raven 1983) showed that earlier suggestions of long-distance (mm) transport of proticity along trichomes were exaggerated.

8 Intracellular Acid–Base Regulation: What Is Being Regulated?

8.1 Methodology

The methods for measuring the pH of various intracellular components are outlined in Table 1, showing that there are a range of methods available which are divisible into two categories. One is the use of pH-selective microelectrodes, which have high temporal and spatial resolution, can cover the whole pH range, and can be used best in larger cells. The other three methods involve the use of the degree of ionisation of weak electrolytes. The ionisation state, as a function of pH, of endogenous inorganic phosphates and phosphate esters are detectable with ^{31}P NMR. Exogenously supplied organic weak electrolytes can be detected by a

Table 1 Methods of measuring intracellular pH and their applicability

Method	pH range	Spatial resolution	Temporal resolution	Range of cell sizes	References
pH		microelectrode	0–14	Very good (vacuole, cytosol, plastid) in larger cells	Excellent
Better spatial resolution in larger cells	Davis (1974) and Spanswick and Miller (1977)				
^{14}C-labelled weak electrolyte distribution	1(−2) units above and below pK_a	Distinguish vacuole and cytoplasm in giant cells	Relatively poor	If cells are vacuolate, only fully applicable to giant cells	Walker and Smith (1975)
Weak electrolyte fluorescent imaging	1(−2) units above and below pK_a	Distinguish vacuole, cytosol, plastid in all but the smallest cells	Better then labelled weak electrolytes because applicable to smaller cell	All but the smallest cells	Dixon et al. (1989)
Weak electrolyte NMR (^{31}P)	1(−2) units above and below pK_a of endogenous inorganic and organic phosphates	None except through appeal to data from other sources	Depends on time needed to acquire sufficient data	All cell sizes	Roberts et al. (1980)

labelling with a radioactive tracer or by fluorescence imaging. The weak electrolytes can only be used effectively within a certain pH range close to their pK_a. As for terminology, a convention for the use of the weak organic acid DMO (2',2'-dimethyloxazalidine-2.4-di-one) in studies on giant algal cells is to distinguish the pH of the vacuole (determined by isolation of an aliquot) from that of the cytoplasm (Walker and Smith 1975). The cytoplasm here comprises the remainder of the protoplast, i.e. cytosol, nucleus, plastids, mitochondria and the endomembrane system other than the central vacuole, and its pH is determined by calculating the DMO content of the vacuole (minus that of the aliquot used to determine vacuolar pH) and subtracting this from the DMO content of the rest of the

cell, and using this DMO content and the cytoplasmic volume, with the external DMO concentration and external pH, to calculate the cytoplasmic pH. Note that this use of cytoplasm differs from the original use by cytologists who divided the protoplasm (everything contained by the cell wall of a walled cell) into the nucleus and the cytoplasm, i.e. the protoplasm minus the nucleus. The microelectrode technique measures the pH of the cytosol and the vacuole (Spanswick and Miller 1977) and, in some cases, that of the plastid stroma (Davis 1974). The same three compartments are also imaged and thus have their pH estimated using the fluorescent weak electrolyte technique (Dixon et al. 1989). The ^{31}P NMR technique shows differences in the pH of compartments containing the various phosphate compounds whose chemical shifts are measured, but the nature of the compartments has to be decided on the basis of other evidence, usually the range of phosphate compounds indicating a particular pH and value of the pH estimated. For a photosynthetic cell the typical pH values for the major compartments are plastid stroma > cytosol > vacuole, with inorganic and organic phosphates in the first two and only inorganic phosphates in the vacuole.

8.2 *External Influences on Cytoplasmic pH as a Means of Determining the Variable Being Regulated: General Considerations*

The techniques outlined earlier and in Table 1 give the pH of compartments (or, in the case of the 'cytoplasm', a combination of compartments) achieved by the various biochemical and biophysical mechanisms of acid–base regulation discussed by Smith and Raven (1979). The 'cytoplasmic pH' or, where separately measured, the plastid and the cytosol pH, is, for most of the plants, algae and cyanobacteria examined, a genotype- and environment-specific pH of between 7.0 and 8.0. For photosynthetic cells the cytoplasmic pH is higher in the light than the dark, and much of the increase in the light is related to the increased pH in the chloroplast stroma (Smith and Raven 1979; Raven and Smith 1980b). The vacuolar pH is almost invariably less than pH 6. Regulatory systems have, by definition, a value of the variable that they are controlling which is the one to which the variable is returned after a perturbation. This is known as the 'set point', and in general terms the set points for acid–base regulation are implicitly considered to be the pH value of the compartments which are measured under steady-state conditions of external pH and solute concentrations, light and temperature. Here we examine the extent to which this is the case, and the implications of regulating pH for pH-sensitive processes as a function of temperature and ionic strength. The importance of this procedure is that what is important for natural selection is not necessarily what we are accustomed to measure. This has, of course, been recognised for decades for marine invertebrates, based on the temperature effect on the pH of extracellular fluids and, to a lesser extent, the intracellular fluids. Here the effect of temperature on the pH of the body fluids suggests that the 'set point'

relates to keeping constant the ionisation state of histidine, the 'alpha stat' hypothesis (Wilson 1977; Hazel et al. 1978; Egginton et al. 1999; cf. Johnson et al. 1983).

8.3 Influence of External Factors: pH of the Medium

For all of the cells examined, changes in external pH within the range normally encountered by the cell cause, to a greater or lesser extent, a change in cytoplasmic pH in the same direction as the change in external pH, and usually with a change of not more than 0.1 pH units per unit external pH change (Smith and Raven 1979; Kurkdian and Guern 1989; Egginton et al. 1999; Rengel 2002) in the bulk medium, noting that the pH in the diffusion boundary layer is higher (in the light in photosynthetic cells) or lower (during respiration) than in the bulk medium (e.g. Smith and Walker 1980; Kühn and Raven 2008; Hurd et al. 2011; Flynn et al. 2012). The extent to which these changes reflect variation in the set point (Cram 1976; Walker 1976; Raven and Smith 1978; Raven and Geider 1988, 2003) for cytoplasmic pH with varying external pH, the best compromise that the organism can make between achieving a constant set point pH and the costs of that regulation in, for example, metabolic energy input, or the use of the variation in intracellular pH as part of the signalling loops which control the pH-regulation apparatus, is not clear. At all events, the observed variation means that such important intracellular ionisation states as $[H^+]$:$[OH^-]$, [Histidine]:[Histidine$^+$], $[CO_2]$:$[HCO_3^-]$ and $[H_2PO_4^-]$-$[HPO_4^{2-}]$.

8.4 Influence of External Factors: N Source

It has frequently been observed that intracellular pH, including cytoplasmic pH, is slightly higher when NO_3^- is the N source (assimilation producing excess OH^-) than when NH_4^+ is the N source (assimilation producing excess OH^-) (e.g. De Michelis et al. 1979; Raven and De Michelis 1979, 1980). As with the variation in intracellular pH with extracellular pH, there are three possibilities as to why this variation in intracellular pH occurs as a function of N source.

8.5 Influence of External Factors: Temperature

An important environmental factor which has been much less investigated in cyanobacteria, algae and plants than light–dark changes or variations in external pH is temperature. Temperature effects on cytoplasmic pH have been specifically addressed by Raven and Smith (1978) for giant internodal cells of the charophycean alga *Chara corallina*, using the distribution of the weak organic acid DMO. Raven and Smith (1978) found a decrease in cytoplasmic pH of 0.05 units for a

temperature increase from 5 to 15° and of 0.15 units between 15 and 25°, i.e. cytoplasmic pH control was more precise over the lower temperature range. In any case the variation with temperature was less than the 0.17 pH units decrease per 10°C temperature increase typical of the extracellular fluids of marine invertebrates and, with more variability, intracellular pH of these organisms (Raven and Smith 1978). The temperature effects on the body fluid and intracellular pH of marine invertebrates keep constant the ionisation state of water ($[H^+]/[OH^-]$) and of nitrogenous bases with pK_a values close to the body fluid or cytoplasmic pH, predominantly histidine, in the 'alpha-stat' hypothesis (see Raven and Smith 1978, and Wilson 1977; Hazel et al. 1978; Egginton et al. 1999). The variation in cytoplasmic pH with temperature in *Chara corallina* is much closer to what is required to keep the ionisation state of weak acids and their conjugate bases such as CO_2:HCO_3^- and $H_2PO_4^-$:HPO_4^{2-} as well as certain organic acids and phosphate esters cannot all be held constant as the environment changes. In any case, no single temperature dependence of the cytoplasmic pH set point can achieve constancy in each of $[H^+]$:$[OH^-]$, [Histidine]:[Histidine$^+$], $[CO_2]$:$[HCO_3^-]$ and $[H_2PO_4^-]$-$[HPO_4^{2-}]$.

Adduci et al. (1982) have also examined the effects of temperature on cytoplasmic and vacuolar pH, in this case for *Zea mays* roots using ^{31}P nuclear magnetic resonance. The cytoplasmic pH decreases by 0.5 units with a temperature increase from 4 to 28 °C, i.e. a decrease of 0.21 units per 10 °C temperature increase. This is higher than the values found for *Chara corallina* and is even higher than the value typical of marine invertebrates. However, in these experiments it is possible that the values at the higher temperature are compromised by restricted O_2 supply as a result of decreased O_2 solubility to the roots in the nuclear magnetic resonance tube, combined with the higher potential metabolic rate at the higher temperature, resulting in the possibility of hypoxia and production of lactic or malic acid as fermentation products (Felle 2005; Greenway et al. 2011). The reversibility of the temperature change effects (Adduci et al. 1982) does not rule out this possible amplification of the temperature effect, since post-anoxia metabolism of organic acids could occur. The work on *Chara corallina* would not be influenced in this way, since photosynthetic cells were investigated in the light (Raven and Smith 1978).

8.6 *Influence of External Factors: Temperature in an Ecological Context*

Regardless of the magnitude of the effect, the restricted data available show a decrease in cytoplasmic pH with increasing temperature in photosynthetic organisms, qualitatively the same as in marine invertebrates. This could have significant implications for phytoplankton cells in this time of increased atmospheric CO_2, resulting in increased CO_2 in the surface ocean water resulting in a