Land plant biochemistry

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Biochemical studies have complemented ultrastructural and, subsequently, molecular genetic evidence consistent with the Charophyceae being the closest extant algal relatives of the embryophytes. Among the genes used in such molecular phylogenetic studies is that (*rbcL*) for the large subunit of ribulose biphosphate carboxylase-oxygenase (RUBISCO). The RUBISCO of the embryophytes is derived, via the Chlorophyta, from that of the cyanobacteria. This clade of the molecular phylogeny of RUBISCO shows a range of kinetic characteristics, especially of CO₂ affinities and of CO₂/O₂ selectivities. The range of these kinetic values within the bryophytes is no greater than in the rest of the embryophytes; this has implications for the evolution of the embryophytes in the high atmospheric CO₂ environment of the late Lower Palaeozoic. The differences in biochemistry between charophycean algae and embryophytes can to some extent be related functionally to the structure and physiology of embryophytes. Examples of components of embryophytes, which are qualitatively or quantitatively different from those of charophytes, are the water repellent/water resistant extracellular lipids, the rigid phenolic polymers functional in water-conducting elements and mechanical support in air, and in UV-B absorption, flavonoid phenolics involved in UV-B absorption and in interactions with other organisms, and the greater emphasis on low *M_r* organic acids, retained in the plant as free acids or salts, or secreted to the rhizosphere. The roles of these components are discussed in relation to the environmental conditions at the time of evolution of the terrestrial embryophytes. A significant point about embryophytes is the predominance of nitrogen-free extracellular structural material (a trait shared by most algae) and UV-B screening components, by contrast with analogous components in many other organisms. An important question, which has thus far been incompletely addressed, is the extent to which the absence from bryophytes of the biochemical pathways which produce components found only in tracheophytes is the result of evolutionary loss of these functions.

Keywords: carbon dioxide; cutin; lignin; oxygen; RUBISCO; UV-B

1. INTRODUCTION

Biochemistry *per se* currently seems to be the poor relative of molecular biology. Certainly the nucleotide sequence of genes with the appropriate nucleotide substitution rate for the taxon under investigation is preferable to the epiphenomenon of the biochemical outcome of the genetic code for the purpose of phylogenetic reconstruction, provided that an appropriate analysis of the sequence data has been performed. However, studies of biochemistry do have some significance in functional terms, which cannot be predicted from the currently known nucleotide sequences.

To a considerable extent this is a result of the absence of complete nucleotide sequences for any embryophytes or for any of their closest relatives on the basis of nucleotide sequence evidence, as well as much other evidence, which are members of the Class Charophyceae of the Division Chlorophyta. Accordingly, we cannot predict the entire suite of enzymes that could be expressed by any embryophyte. Even when the *Arabidopsis* genome has been completely sequenced there will still be a need for complete nucleotide sequences of embryophytes of the pteridophyte and bryophyte grades of evolution, as well as for the Charophyceae. Accordingly, we do not know which enzymes can potentially be expressed by the

embryophytes and the closest living relatives of the ancestors of embryophytes, i.e. the charophycean green algae.

A further problem with predicting biochemical outcomes from nucleotide sequences occurs even when we know the nucleotide sequence for the gene encoding the appropriate enzyme. As we shall see in our consideration of ribulose biphosphate carboxylase-oxygenase (RUBISCO), functionally very significant differences in kinetic properties of different RUBISCOs can occur with very small differences in activation energy (Lorimer *et al.* 1993). Such activation energy differences may not relate to differences in nucleotide sequence at the active centre of the enzyme (Maynard Smith & Szathmáry 1995). Accordingly, even the complete nucleotide sequence of the genome may not tell us everything about the functional roles of the encoded enzymes, at least with our present predictive ability in deducing catalytic function from the primary structure (and deduced higher-order structure) of proteins.

A third problem with the molecular biological approach is that it is rarely, if ever, applicable to fossil material, whereas the organic chemical end-products of the activity of the genes and the proteins which they encode are more likely to survive, albeit with more or less chemical modification. The question of whether DNA can survive for more than a few thousand years at most in a

Table 1. *Comparison of kinetic properties of the cyanobacteria-green plant RUBISCO lineage^a*

source of RUBISCO	selectivity factor for CO ₂ over O ₂	<i>K_m</i> (CO ₂) (mmol CO ₂ m ⁻³)	specific reaction rate (mol CO ₂ mol ⁻¹ active site s ⁻¹) at	
			saturating CO ₂	1 mmol CO ₂ m ⁻³
Chlorophyta (aquatic with CCM)	61–63	29–38	—	—
Chlorophyta (terrestrial without CCM)	83	12	—	—
Euglenophyta (aquatic with CCM)	54	25	—	—
Cyanobacteria (aquatic with CCM)	35–36	105–185	11–12	0.06–0.07
Embryophyta (terrestrial C ₃)	82–90	10–11	2.9–3.0	0.27–0.29
Embryophyta (terrestrial C ₄)	78	32	4.2	0.13

^aBased on Badger *et al.* (1998) and Raven *et al.* (2000).

form which can be amplified and sequenced to give the nucleotide sequence of the original DNA is still contentious (Logan *et al.* 1993; Poinar *et al.* 1996; Austin *et al.* 1997; Lindahl 1997); clearly it suits my purposes here to side with the sceptical majority.

Having mentioned three reasons for looking at biochemical evidence in considering the phylogeny and evolution of the bryophytes in relation to that of other embryophytes and of the algal ancestors of the embryophytes, it must be admitted that there are problems with using biochemical markers of phylogeny. A major problem is clearly that very similar, or identical, chemical end-products could arise in different taxa using dissimilar metabolic sequences, and even if the chemical intermediates are similar, the enzymes involved may be dissimilar (see Chapman & Ragan 1980). Examples from O₂-evolving photolithotrophs of non-homologous catalysts using the simplest substrate and product, i.e. electrons at various redox potentials in other proteins, are the pairs of redox catalysts plastocyanin/cytochrome *c*₆ and ferredoxin/ flavodoxin (Falkowski & Raven 1997). However, the Charophyceae and the embryophytes only express plastocyanin and ferredoxin of these alternative redox catalysts (Falkowski & Raven 1997).

The exploration of the potential for using biochemical data in casting light on the evolution of bryophytes in relation to that of the Charophyceae and of other embryophytes (Bateman *et al.* 1998; Doyle 1998; Edwards *et al.* 1998; Graham 1993; Kenrick & Crane 1997) will first consider the molecular genetics and kinetics of the main carboxylase of all O₂-evolvers, RUBISCO, in land plants and their ancestors in relation to the environment in which the organisms evolved. We then briefly consider the pathways that could be involved in the synthesis of the unique end-products of some or all embryophytes, i.e. water-repellent/water-resistant extracellular lipids, and the phenolics and their polymers which are involved in lignin synthesis, in UV-B absorption and in interactions among organisms, the additional synthesis of organic acids, in relation to the environmental conditions at the time of evolution of embryophytes. Particular attention will be given to the nitrogen-free status of structural and UV-B-absorbing compounds relative to the condition in many non-embryophytes. Finally, we consider the likelihood that certain embryophyte biochemical pathways which are absent from bryophytes have been lost in the course of evolution.

2. RUBISCO: MOLECULAR PHYLOGENETIC AND FUNCTIONAL STUDIES IN RELATION TO ENVIRONMENTAL CONDITIONS AT THE TIME OF ORIGIN OF EMBRYOPHYTES

RUBISCO catalyses not only the conversion of CO₂ (and H₂O) and ribulose biphosphate to two molecules of phosphoglycerate, but also the competing reaction in which ribulose biphosphate and O₂ form one molecule of phosphoglycerate and one of phosphoglycolate. The known RUBISCOs have a wide range of selectivity factors for CO₂ over O₂, and of affinity for CO₂, as well as for the maximum specific rate of carboxylation (mol CO₂ fixed per mol enzyme per second), with a generally inverse relationship between the selectivity factor and CO₂ affinity on the one hand and the maximum specific rate of carboxylation on the other (Badger & Andrews 1987; Badger *et al.* 1998; Watson & Tabita 1997; Raven *et al.* 2000). These variations in the kinetics of RUBISCO can be broadly related to the molecular phylogeny of this enzyme (Badger *et al.* 1998; Watson & Tabita 1997). Here we focus on the RUBISCO clade which encompasses the enzyme from the embryophytes, the Chlorophyta (and the plastids of euglenoids and chlororachniophytes derived from secondary endosymbioses involving unicellular green algae) and the cyanobacteria from which the green algal plastids were derived by primary endosymbiosis (Badger *et al.* 1998; Bhattacharya & Medlin 1998; Kaplan *et al.* 1998; Price *et al.* 1998; Watson & Tabita 1997). The highest specific reaction rates for carboxylation, the lowest selectivity of CO₂ over O₂ and the lowest affinity for CO₂ in this RUBISCO clade are found in the cyanobacteria (table 1). A greater selectivity for CO₂ over O₂, and a higher affinity for CO₂, is found in aquatic members of the Chlorophyta and Embryophyta (Yeoh *et al.* 1981; Uemura *et al.* 1996; Badger *et al.* 1998; tables 1 and 2). In general, terrestrial C₄ and crassulacean acid metabolism (CAM) embryophytes have an even higher CO₂/O₂ selectivity and CO₂ affinity than do the aquatic plants, but less than the selectivity and affinity values for terrestrial C₃ plants, regardless of whether these are algae such as the lichen symbiont *Coccomyxa*, or terrestrial bryophytes, pteridophytes, gymnosperms and angiosperms (Badger *et al.* 1998; Bird *et al.* 1982; Palmqvist *et al.* 1995, 1997; Rintamäki & Aro 1985; tables 1 and 2).

What is the significance of these kinetic differences? In the context of CO₂ and O₂ concentrations at the active

Table 2. K_m (CO_2) values for RUBISCO in vitro for C_3 embryophytes

organism	terrestrial (T) or aquatic (A)	K_m (CO_2) (N_2) ^a	K_m (CO_2) (air O_2)	references
Musci				
<i>Funaria</i> sp.	T	23	—	Yeoh <i>et al.</i> (1981)
<i>Ceratodon purpureus</i> protonemata	T	22.3	—	Rintamäki & Aro (1985)
shoots	T	19.4	—	
<i>Fissidens rigidulus</i>	A	42	—	Yeoh <i>et al.</i> (1981)
Pteropsida	T	16–23 (4)	—	Yeoh <i>et al.</i> (1981)
	T	15.4–15.7 (2)	20.4–24.5	Bird <i>et al.</i> (1982)
Sphenopsida	T	15.9	25.5	Bird <i>et al.</i> (1982)
Lycopsidea	T	18	—	Yeoh <i>et al.</i> (1981)
Cycadopsida	T	14	—	Yeoh <i>et al.</i> (1981)
Ginkgopsida	T	23	—	Yeoh <i>et al.</i> (1981)
		17.9	25.1	Bird <i>et al.</i> (1982)
Coniferopsida	T	24	—	Yeoh <i>et al.</i> (1981)
		16.8	24.8	Bird <i>et al.</i> (1982)
Magnoliophyta	T	12–25 (27)	—	Yeoh <i>et al.</i> (1981)
	T	11.1–14.0 (10)	16.4–20.4 (10)	Bird <i>et al.</i> (1982)
	T	14.8–20.4 (5)	—	Rintamäki & Aro (1985)
	A	30–49 (7)	—	Yeoh <i>et al.</i> (1981)

^aThe K_m (CO_2) values in N_2 are lower than those in air-equilibrium O_2 concentrations due to the competitive effects of O_2 (via RUBISCO oxygenase activity) and CO_2 (via RUBISCO oxygenase activity). The anoxia in the (N_2) data of Bird *et al.* (1982) and Rintamäki & Aro (1985) is probably greater than that of Yeoh *et al.* (1981).

site of RUBISCO in extant organisms, the CO_2/O_2 ratio around RUBISCO in cyanobacteria is ten or more times that in air-equilibrium solution as a result of the activity of an inorganic carbon pump at the plasmalemma delivering HCO_3^- to the cytosol, and generation of CO_2 by carbonic anhydrase activity in the carboxysomes which house most of the RUBISCO activity in the cells (Kaplan *et al.* 1998; Price *et al.* 1998). This essentially saturates the carboxylase activity of cyanobacterial RUBISCO and minimizes the oxygenase activity.

C_4 and CAM terrestrial vascular plants have CO_2 pumps which maintain a CO_2/O_2 ratio and CO_2 concentration around RUBISCO in steady-state photosynthesis, which may be lower than that in cyanobacteria. However, these CO_2 pumps in eukaryotes are still adequate to essentially saturate RUBISCO carboxylase and minimize RUBISCO oxygenase activity, granted the RUBISCO kinetics in these organisms (table 1).

The C_3 terrestrial green eukaryotes, with diffusive CO_2 supply to RUBISCO, include lichen algae such as *Coccomyxa*, all bryophytes except certain hornworts, and vascular plants other than those with C_4 and CAM (see Palmqvist *et al.* 1995, 1997; Raven 1995; Raven *et al.* 1998). Despite having the highest CO_2/O_2 selectivities and CO_2 affinity of any of the enzymes in this clade of RUBISCOs (Badger *et al.* 1998; Palmqvist *et al.* 1995, 1997; Raven *et al.* 1998; tables 1 and 2), the diffusive resistance to CO_2 entry and O_2 exit means that the steady-state CO_2/O_2 ratio and CO_2 concentration at the active site of RUBISCO only yields half saturation of the RUBISCO carboxylase activity, and gives very significant rates of O_2 uptake by RUBISCO oxygenase activity, during photosynthesis at light saturation at the present bulk atmospheric CO_2 concentration (see Raven *et al.* 1998).

The use of bulk atmospheric CO_2 levels as the basis of the estimation of the CO_2 concentration and CO_2/O_2

ratio at the site of RUBISCO activity *in vivo* in C_3 plants could be challenged on the basis of the measured CO_2 concentrations in the atmosphere around the photosynthetic cells of some low-growing terrestrial C_3 embryophytes, e.g. many bryophytes (see Sonesson *et al.* 1992; Tarnawski *et al.* 1992). The measured CO_2 concentrations in some moss canopies can be higher than those in the bulk atmosphere during photosynthetic CO_2 fixation (Sonesson *et al.* 1992; Tarnawski *et al.* 1992), i.e. the opposite of the situation in the canopies of taller plants with CO_2 supply from the bulk atmosphere to the leaf surface down a concentration gradient. A higher CO_2 concentration around photosynthesizing tissues of bryophytes than that in the bulk atmosphere requires a net CO_2 flux from the bryophytes plus their substratum to the bulk atmosphere rather than vice versa. In the general case in which geological sources of CO_2 are absent, the net CO_2 flux to the atmosphere derives from ecosystem respiration exceeding bryophyte photosynthesis even in the light, and the organic carbon substrate for this respiratory CO_2 must ultimately have been derived from atmospheric CO_2 by ecosystem photosynthesis (occasionally with allochthonous organic inputs). Where vascular plants taller than the bryophytes are (or had been) present at the site, then photosynthesis by these plants can provide the additional carbon, e.g. by respiration of weakly photosynthetic or non-photosynthetic stems of vascular plants in the case of stem epiphytes, or respiration by roots and other soil biota for ground-dwelling bryophytes. However, in the high-latitude bryophytes examined by Sonesson *et al.* (1992) and Tarnawski *et al.* (1992), the vascular plant source is less likely especially for mainland Antarctica (Tarnawski *et al.* 1992) with only two species of vascular plant (Fogg 1998). Here the simplest assumption is that the CO_2 from the substrate comes from bryophyte photosynthate produced from atmospheric CO_2 at some earlier

time, although earlier photosynthesis by terrestrial algae and cyanobacteria, or allochthonous inputs (e.g. via penguins) must also be considered. This discussion suggests that the generality of high CO_2 levels around photosynthesizing bryophyte shoots is not invariant in either space or time, and that examination of the CO_2 environment for terrestrial bryophytes should proceed on a case-by-case basis until sufficient data are available for better generalizations. At all events it appears that there is considerable evidence for many terrestrial bryophytes photosynthesizing for at least some of their life cycle, or in some localities, with CO_2 concentrations in the gas phase surrounding their photosynthetic tissues, which are lower than bulk atmosphere values, thus providing a potential rationale in natural selection for the high CO_2 affinity and high CO_2/O_2 selectivity of RUBISCO from terrestrial C_3 bryophytes (tables 1 and 2).

Finally, we come to the aquatic eukaryotic green plants. Here RUBISCO has a lower CO_2/O_2 selectivity and lower CO_2 affinity than that of C_4 and CAM plants, and especially of terrestrial C_3 plants, but higher than that of cyanobacteria. Most of the (primarily) aquatic green algae, and many of the (secondarily) aquatic embryophytes, have inorganic carbon pumps, which maintain a higher CO_2/O_2 and absolute CO_2 concentration around RUBISCO than in the natural medium. There are a number of aquatic green plants (a few algae; all bryophytes; some vascular plants) which rely on diffusive CO_2 entry followed by C_3 biochemistry; these are mainly freshwater organisms, where the CO_2 concentration is often higher than that found at air equilibrium (Raven 1991a, 1997c). This leads to a higher external CO_2/O_2 ratio than would be expected at air equilibrium, and thus increases the carboxylase activity and lowers the oxygenase activity of RUBISCO relative to that found in air-equilibrium solutions, granted the same diffusive restrictions and RUBISCO activity and kinetics in plants growing in air-equilibrium solutions as are found in plants in their natural CO_2 and O_2 levels.

However, there seem to be some marine ulvophycean green algae which lack a CO_2 -concentrating mechanism, and rely on diffusion of CO_2 from air-equilibrium seawater to RUBISCO (e.g. some species of *Caulerpa*; Raven 1997b). The diffusion boundary layer around the macroalgae restricts CO_2 diffusion more than does the gaseous diffusion boundary layer around a similar-sized terrestrial plant; it is not clear if this is in any way offset by a higher CO_2 affinity and CO_2/O_2 selectivity by the RUBISCO in these *Caulerpa* spp. in terms of allowing a higher rate of photosynthesis than would a 'standard' ulvophycean RUBISCO (Raven 1997b).

An area of ignorance about the kinetics properties of a RUBISCO in green plants which has more relevance to the evolution of the bryophyte grade of green plant organization is that of those anthocerototes which have pyrenoids (Smith & Griffiths 1996a,b). The pyrenoid is found in many (but by no means all) algae with CO_2 -concentrating mechanisms, and seems to play a role analogous to that of the carboxysome in cyanobacteria (Badger *et al.* 1998; Raven 1997b,c). Smith & Griffiths (1996a,b) showed that those hornworts they tested that had pyrenoids had a CO_2 -concentrating mechanism, as shown by CO_2 accumulation, decreased photorespiration

and $^{13}\text{C}/^{12}\text{C}$ discrimination, while those without pyrenoids relied on diffusive CO_2 entry. By analogy with the kinetic properties of RUBISCO in all of the (primarily) aquatic green algae and (secondarily) aquatic embryophytes, it could be predicted that RUBISCO from those hornworts with pyrenoids would have lower CO_2/O_2 selectivity than that from C_3 terrestrial plants.

Regardless of what further research shows about the kinetic characteristics of RUBISCO from pyrenoid-containing hornworts, the range of RUBISCO kinetic properties in the Chlorophyta and embryophytes is wide enough to allow a considerable range of RUBISCO oxygenase activities *in vivo* during C_3 physiology photosynthesis in terrestrial organisms in the present atmosphere, and hence a considerable range of ratios of photorespiratory carbon oxidation cycle activity to photosynthetic carbon reduction cycle activity.

The trends noted here for *in vitro* RUBISCO kinetics for the cyanobacterial, green algal and embryophytic clade of RUBISCOs is for an inverse relationship between CO_2 affinity and CO_2/O_2 selectivity of the enzyme from a given organism and the CO_2 concentration and the CO_2/O_2 concentration ratio to which the enzyme is exposed in steady-state photosynthesis (see Badger & Andrews 1987; Badger *et al.* 1998). This trend is best seen when cyanobacteria (with inorganic carbon-concentrating mechanisms which maintain very high CO_2 concentrations around RUBISCO) are compared with many aquatic green algae and tracheophytes which have inorganic carbon-concentrating mechanisms, and with C_4 and CAM plants with rather lower CO_2 concentrations around RUBISCO, and with C_3 terrestrial plants which have the lowest steady-state CO_2 concentration and CO_2/O_2 ratio around RUBISCO. The situation for aquatic eukaryotes lacking inorganic carbon-concentrating mechanisms, and with large diffusion limitations on CO_2 supply to RUBISCO, is unclear even when they are living in naturally CO_2 -enriched waters, as is that for (terrestrial) hornworts with inorganic carbon-concentrating mechanisms but using atmospheric CO_2 .

The rationalization of these findings in terms of natural selection involves the observed trade-off between high affinity for CO_2 and high CO_2/O_2 selectivity and a high specific reaction rate, which economizes on nitrogen use in the photosynthetic apparatus when CO_2 concentration and CO_2/O_2 ratio are high around RUBISCO, even when N costs of inorganic carbon pumps and N costs of the photorespiratory carbon oxidation cycle are taken into account (Badger & Andrews 1987; cf. Raven *et al.* 1985). Inorganic carbon-concentrating mechanisms can contribute to a lower transpiratory water cost of photosynthesis in plants obtaining CO_2 from air even when these plants lack stomata (bin Surif & Raven 1990). There may also be energetic advantages in operating an inorganic carbon-concentrating mechanism in conjunction with a RUBISCO of high specific reaction rate relative to diffusive CO_2 entry and a RUBISCO with lower specific reaction rate but higher CO_2 affinity and CO_2/O_2 selectivity, and the photorespiratory carbon oxidation cycle, both in terms of synthetic and running costs (Raven 1997b,c).

How can the evolution of RUBISCO in the cyanobacterial-green algal-embryophyte clade be related to the changes in the atmospheric levels and the gases

CO₂ and O₂? These changes have been quantitatively modelled by Berner (1997, 1998) and Berner & Canfield (1989), refining earlier models (Budyko *et al.* 1985).

We deal mainly with the atmospheric composition from the Ordovician onwards, since this is the time for which the embryophytes have existed. The O₂ content of the atmosphere over this time has varied from not less than half the present value to not more than twice (in the Carboniferous) the present content (Berner & Canfield 1989). CO₂ has been considerably more variable, with values of 12–22 times the present value in the Ordovician, Silurian and Early Devonian, followed by a decrease to a value similar to that found at present in the Carboniferous (Berner 1997, 1998). The early Mesozoic had about four times the present CO₂ level, with a downward trend through most of the Cretaceous and Tertiary to near-present (pre-industrial) levels in the interglacial episodes in the Pleistocene, i.e. a sea-level partial pressure of 280 Pa, and as little as 180 Pa in the glacial episodes (Berner 1997, 1998; Pearson & Palmer 1999; Petit *et al.* 1999).

The charophycean ancestors of the embryophytes growing in freshwaters before and during the early evolution of embryophytes may have been exposed to higher dissolved CO₂ levels than in present-day freshwaters, if the high CO₂ levels in the atmosphere offset the lower CO₂ inputs from soil respiration in the catchment as a result of the lower productivity on the pre-embryophyte land surface (Raven 1998). Extant charophyceans generally have inorganic carbon-concentrating mechanisms; this is certainly the case for *Coleochaete*, the living alga most closely related to the embryophytes, which has pyrenoids (Graham 1993). Raven (1997*b,c*) has argued for the ancestral nature of pyrenoids among algae, despite the relatively high CO₂ concentrations at the time of diversification of eukaryotic algae and the lower O₂ levels.

The earliest terrestrial (or at least amphibious) charophyceans and embryophytes would equally have been exposed to higher CO₂ levels than present atmospheric values, but O₂ levels no greater than the present levels. Any inputs of CO₂ to the low-growing plants from soil respiration by earlier, non-embryophytic phototrophs (see above) would have had less significance in the early Palaeozoic with the high atmospheric CO₂ levels. Even if the early embryophyte RUBISCOs had CO₂/O₂ selectivities and CO₂ affinities that were lower than those for present-day C₃ embryophytes, the atmospheric composition in the Ordovician, Silurian and earliest Devonian, combined with the anatomy and morphology of the embryophyte fossils, would have permitted the photosynthesis of those plants to occur with less diffusive restriction on net CO₂ fixation than in present C₃ plants, with occurrence of a higher fraction of the potential maximum carboxylation rate and a lower fraction of the potential maximum oxygenation rate than in extant C₃ embryophytes (Raven 1977, 1984*a*, 1998). This line of argument reasonably assumes that the earliest terrestrial embryophytes had, like the great majority of extant terrestrial embryophytes, C₃ physiology (Raven 1977, 1984*a*, 1993, 1998). An alternative view is that the pyrenoid-based inorganic carbon-concentrating mechanism found in *Coleochaete* and many hornworts was widespread among early embryophytes, with some anthocrotes as the only remaining pyrenoid-containing

embryophytes (Raven 1997*b,c*; Raven *et al.* 1998). This latter view cannot readily be tested on the basis of extant organisms since there are no known molecular markers for pyrenoids other than the enzymes that they are known to contain, which are RUBISCO and its activase in green algal pyrenoids, since these also occur in other green algae and embryophytes (Badger *et al.* 1998; Raven 1997*b,c*). This means that monophyly of pyrenoids in the green algal and embryophyte clade cannot currently be examined on the basis of genetic evidence.

We are also unable to make any definitive judgement on when the RUBISCOs of *Coccomyxa*-like green algae and of embryophytes with C₃ physiology achieved their present high CO₂ affinity and high CO₂/O₂ selectivity. Such kinetic changes might be expected to relate to the low CO₂ level and high CO₂/O₂ ratio of the Carboniferous or in the Late Tertiary to Pleistocene; the former seems more likely. In any case, it seems that such evolutionary changes in relation to atmospheric CO₂ and O₂ levels must have occurred independently in the non-charophycean green algal lichen photobiont *Coccomyxa* and in the charophycean-derived embryophytes. In the absence of molecular genetic data on the *Coccomyxa* RUBISCO, its phylogenetic status cannot be determined, and horizontal gene transfer cannot be eliminated as a means of acquisition of similar kinetics in *Coccomyxa* and in embryophyte RUBISCO. Even if molecular genetic data reveal the phylogeny of these two high CO₂ affinity, high CO₂/O₂ selectivity RUBISCOs, consideration of the small activation energy differences involved in these kinetic properties relative to the ancestral, low CO₂ affinity, low CO₂/O₂ selectivity means that the molecular genetic evidence may only with difficulty be used to indicate what amino acid sequence(s) in RUBISCO relate to the high CO₂ affinity, high CO₂/O₂ selectivity condition (Lorimer *et al.* 1993; Maynard Smith & Szathmáry 1995).

A related phylogenetic question is that of the enzymes which catalyse the metabolism of the phosphoglycolate generated in RUBISCO oxygenase activity. The enzyme in the photorespiratory carbon oxidation cycle which is known to have phylogenetic variability is that which catalyses the conversion of glycolate to glyoxylate. In the organisms with the cyanobacterial-green algal-embryophyte RUBISCO, the cyanobacteria and all green algae except the Charophyceae have glycolate dehydrogenase, while the Charophyceae and the embryophytes have glycolate oxidase (see Raven 1997*b,c*). These two enzymes seem to have had a common ancestry, at least as far as some component polypeptides are concerned, since the cyanobacterium *Synechocystis* PCC 6803 has genes encoding some subunits of glycolate oxidase, yet cyanobacteria express only glycolate dehydrogenase activity. (Raven 1997*c*; cf. Kaneko *et al.* 1996). The other (eukaryotic) algal Divisions and Classes have either the dehydrogenase, or the oxidase, or both (Raven 1997*b,c*). The presence of both activities in some algae means that a search for the genes encoding the missing subunit(s) in algae which express either one enzyme activity or the other would be worthwhile: are the genes more widespread than their expression?

Other examples of enzymes that relate to universal processes in O₂-evolvers and that show similarities between embryophytes and charophytes are the enzymes

that deal with active oxygen species. One example is the form of superoxide dismutase that is expressed. The Cu–Zn superoxide dismutase of the embryophytes and charophytes is not found in other green algae or in cyanobacteria; these use the Fe and Mn forms of the enzyme. Elsewhere the Cu–Zn enzyme occurs in the peridinin-containing dinoflagellates, but in no other algae (see Raven *et al.* 1999), as well as in some bacteria, in ‘true’ fungi and metazoa (Chapman 1985). Some horizontal gene transfer must have occurred to explain the distribution of Cu–Zn superoxide dismutase.

A further example of an enzyme that is involved in metabolism of active oxygen species is glutathione peroxidase. This enzyme is universal in metazoa and occurs in some algae, i.e. diatoms and in *Chlamydomonas* in the Chlorophyta, Class Chlorophyceae; these algae accordingly have a Se requirement, at least when this enzyme is expressed as a supplement to ascorbate peroxidase (see Raven *et al.* 1999). However, the Charophyceae and Embryophyta always use ascorbate peroxidase rather than glutathione peroxidase, this resembling other eukaryotic algae and most cyanobacteria (Raven *et al.* 1999). Some flowering plants have a lipoperoxidase, which does not use Se, and has a very low specific reaction rate; essentially all hydrogen peroxide in plastids and cytosol is destroyed by ascorbate peroxidase (Raven *et al.* 1999).

A final example concerns metabolism of urea. Urea metabolism is initiated in charophytes and in embryophytes by urease; this contrasts with other green algae, which, like some fungi, have urea amidolyase (see Raven 1977). All other organisms that can metabolize urea have urease.

3. COMPONENTS OF EMBRYOPHYTES WHICH DIFFER QUALITATIVELY OR QUANTITATIVELY FROM THOSE OF CHAROPHYCEAE IN RELATION TO THE FUNCTIONING OF THE ORGANISMS

(a) *Qualitative and quantitative biochemical differences between embryophytes and charophytes and their functional significance*

Major biochemical features of embryophytes that are not shared by charophytes that we shall consider in more detail in functional terms are the lipid materials of the cuticle and its wax, the diversity of phenolics in lignin and flavonoids, and the much greater accumulation and secretion of low M_r organic acids and their anions (Raven 1977, 1984b, 1991b, 1993, 1995; Raven *et al.* 1980; Chapman 1985; Graham 1993; Edwards *et al.* 1996). The cuticle and its wax are not present, at least in a chemically verified form, in the characean algae (but are also lacking in some bryophytes): Graham (1993). Lignin and flavonoids seem to be restricted, among extant plants, to embryophytes, with lignin limited to (eu)tracheophytes, although lignans are found in bryophytes (Graham 1993; Raven 1993; Kenrick & Crane 1997). We note that earlier suggestions that charophytean algae can synthesize flavonoids are not currently well-supported (Graham 1993; Raven 1993). Finally, it is clear that low M_r organic acid and organic anion accumulation and secretion is much more widespread in embryophytes than in algae, including charophytean and other green algae (Raven *et al.* 1980; Raven 1991b, 1995).

(b) *Functional significance of biochemical differences between embryophytes and charophytes*

The cuticle and its associated wax layer function ecophysiologically in embryophytes in water resistance and water repellence, i.e. in reducing the permeability (conductance) of the plant surface to H_2O (and, indeed, to substances which can dissolve in water) and in limiting or preventing the occurrence of liquid water on the plant surface, respectively (Raven 1977; Edwards *et al.* 1996). Water resistance is largely a function of the wax layer, and is crucial to homoiohydry in greatly restricting water loss from plant shoots when stomata are closed (Raven 1977). Water repellence, which is also largely a function of the wax layer, is important on the plant outer surface in facilitating runoff of liquid water from the surface, thus favouring CO_2 diffusion from the atmosphere to the stomata in the gas phase, wherein CO_2 diffuses 10 000 times faster than in identical conditions in aqueous solution (Raven 1977). Water repellence is also important in producing and maintaining intercellular gas spaces and stomata, which are also crucial aspects of homoiohydry (Raven 1977, 1984a, 1996, 1997a; cf. Jarvis 1998). The cuticle and its wax layer can also function in the attenuation of UV radiation, including the particularly dangerous UV-B, and are also significant in restricting biophagy (Raven 1977; Edwards *et al.* 1996). Extracellular lipids are present in charophytes, although the extent to which they are chemically related to cutin and wax is not clear (Graham 1993). These extracellular lipids are apparently related to UV-B attenuation and to restriction of biophagy (Raven 1977).

Sporopollenin is an extracellular lipoidal substance (with significant phenolic content) which is ubiquitous in embryophytes but is restricted to their spores (see Edwards *et al.* 1996). Functions probably include water resistance and repellence, UV-B attenuation and restriction of biophagy. The lack of a clear chemical definition of sporopollenin means suggestions of its occurrence in taxa other than embryophytes cannot necessarily be subject to rigorous testing, but it is clearly present in the oospore walls of the Characeae (Charophyceae) as well as in vegetative cell walls of certain members of the Chlorophyceae (Raven 1977; Graham 1993). The chlorophytean sporopollenin is in a trilaminar (in the transmission electron microscope with heavy-metal staining) layer, and its hydrophobicity is clearly not expressed at the cell wall surface to such an extent as to render it unwettable. Furthermore, pores in the sporopollenin permit water and hydrophilic solute with an M_r less than 800 to cross the cell wall, much as in the outer membrane of Gram-negative (eu)bacteria (Raven 1984b). Such sporopollenin layers in vegetative cell walls are presumably involved in restriction of biophagy and in UV-B attenuation (Raven 1977). We do not know the chemical and structural basis for an analogous M_r cut-off for diffusion through the walls of extant arbuscular mycorrhizal (zygomycete) fungi, which were significant in establishing an embryophyte flora on land (see Smith & Read 1997; Read *et al.*, this issue).

We have already mentioned phenolics in the context of sporopollenin. Specifically, embryophytic phenolics include the flavonoids and lignin; reports that charophytean algae of the family Characeae produced flavonoids

appear to be incorrect, and lignin is confined to (eu)tracheophytes (Graham 1993; Kenrick & Crane 1997). Lignin's role as a rigid cell wall component (at least when in association with cellulose) in preventing implosion of xylem elements whose contents are under tension, and in providing mechanical support for plant organs more than a few tens of centimetres in vertical extent, is too well known to require detailed exposition here. Flavonoids, via their UV and visible absorption bands, function in advertising plants to (sighted) animals, which interact with plants in ways that are beneficial (e.g. pollination, seed/fruit dispersal) or detrimental (biophagy) to the plant. Furthermore, flavonoids, with other phenolics (including high polymers such as lignin) have a role in UV-attenuation (see Raven 1991c,d). Flavonoids also function in interactions between plants and symbiotic (e.g. N_2 -fixing) bacteria. Attractant (warning) pigments, which are also confined to embryophytes but with a much more restricted taxonomic range (i.e. to the Centrospermae or Caryophyllales), are the N-containing betalains (betacyanin and betaxanthin). We shall discuss the preponderance of N-free structural radiation-absorbing and semiochemicals in embryophytes (and, in many cases, in algae of a range of higher taxa) relative to many other organisms such as fungi and metazoa. Precursors of these flavonoids and lignins in the algae (including the Charophyceae) function in resisting biophages and UV absorption. The phenylpropanoid nucleus, from which flavonoids and lignins are derived, comes from the protein amino acid phenylalanine via phenylalanine ammonia-lyase, which generates the nitrogen-free C_6 (aromatic)- C_3 (aliphatic) skeleton. Phenylalanine ammonia-lyase is found in all organisms that can synthesize ubiquinone (i.e. all photolithotrophic eukaryotes, as well as many others), but the actual form of the enzyme used in phenylpropanoid synthesis may differ from that used in ubiquinone synthesis (Raven 1997; Graham 1993).

Turning now to the higher production of organic acids by embryophytes than by charophyceans, and by the great majority of other algae, the argument developed here relates to the regulation of intracellular pH and the manipulation of extracellular pH (Raven *et al.* 1980, 1998; Raven 1985b, 1989, 1991b, 1995). The argument will be developed here in the context of the best-investigated, i.e. vascular, plants, with subsequent consideration of possible applications to the nutrition of bryophytes. As regards intracellular acid-base regulation, the synthesis of organic acids such as malic and oxalic acids can be used to neutralize the OH^- generated in NO_3^- assimilation, although when cells (aquatic algae, embryophyte rhizoids, roots) are surrounded by an extended aqueous phase the OH^- can be disposed of by OH^- efflux (or H^+ efflux). The use of organic acids to neutralize OH^- permits NO_3^- reduction to occur in cells isolated from a large aqueous phase, i.e. in aerial shoots of the larger terrestrial embryophytes which lack a continuous water film over the shoot when growing, i.e. they are endohydric rather than ectohydric (Raven *et al.* 1980), with possible benefits in terms of energy and water cost of NO_3^- assimilation (Raven 1985b). The assimilation of NO_3^- in the shoots is generally associated with vacuolar accumulation of the organic anion (malate²⁻, oxalate²⁻)

with the cation which accompanied the NO_3^- up the xylem (and hydrome?). However, in a few cases, some or all of the organic acid salt moves back to the roots in the phloem (and leptome?), where metabolism of the organic anion regenerates OH^- , which is then excreted (Raven & Smith 1976; Raven 1985b). This latter option gives a similar effect of NO_3^- assimilation on rhizosphere pH (i.e. an increase) as does NO_3^- assimilation in the roots with direct OH^- excretion, but has different effects on photon and water costs of growth (Raven & Smith 1976; Raven 1985b). The final alternative for acid-base regulation in NO_3^- assimilation involves synthesis of organic acids in OH^- neutralization, with organic anion efflux in exchange for NO_3^- (Loss *et al.* 1993, 1994).

The use of external NH_4^+ or (in symbioses) N_2 as sole N sources in terrestrial embryophytic plants is very generally confined to below-ground organs, or organs (e.g. of the N_2 -fixing *Phaeoceros*, *Anthoceros* and *Blasia*) in direct contact with the soil. For NH_4^+ as N source this relates to the need for a sink for the one H^+ produced per NH_4^+ assimilated into neutral organic N using neutral organic carbon generated in photosynthesis (Raven & Smith 1976); H^+ cannot be disposed of biochemically in the quantities generated in NH_4^+ assimilation (Raven 1986). N_2 fixation into neutral organic N compounds does not involve H^+ production or consumption. However, conversion of neutral organic C compounds plus further neutral organic C generated in photosynthesis into the whole plant involves production of organic anions (proteins, free amino acids with net negative charge; cell wall uronic acids which occur *in vivo* mainly as the uronate form) and H^+ . When the latter is generated in the shoot it cannot be neutralized biochemically (Raven 1986), and shoot acid-base regulation demands organic anion transport to the shoot from the roots where organic acids are synthesized from neutral photosynthate and CO_2 , followed by H^+ excretion and uptake of a non- H^+ cation. This H^+ excretion increases the net H^+ excretion paralleling NH_4^+ assimilation to more than the basal one H^+ per NH_4^+ , and involves net H^+ excretion during whole-organism growth with N_2 as the sole N source (Raven & Smith 1976; Raven & Wollenweber 1992; table 3).

It is important to note that these conclusions about the excretion of more than one H^+ per N assimilated into the whole plant from exogenous NH_4^+ , and of a finite quantity of H^+ for each N assimilated into the whole plant from exogenous N_2 , do not qualitatively depend on the production of more low M_r organic acids per unit plant N than are found in the majority of algae with their minimal low M_r organic acid content, since it depends on net negative charge on proteins and on cell wall uronates (Raven 1991b; Raven & Wollenweber 1992).

Turning from the effects of organic acid synthesis on the regulation of intracellular pH to the effect of organic acid synthesis on extracellular (rhizosphere) or mycorrhizosphere pH, organic acid synthesis in excess of what is needed to neutralize OH^- from NO_3^- assimilation, or when NH_4^+ is the N source, can lower extracellular pH in one of two ways. One way involves the excretion of H^+ , with uptake of a (non- H^+) cation from the medium and accumulation of the organic salt (Raven & Smith 1976; Raven 1985b). The other process involves excretion of the organic acid as such (see Jones 1998). Neither of these

Table 3. Comparison of H⁺ fluxes at root surface (whole root surface area basis)

process	H ⁺ flux	references
net H ⁺ efflux in acid–base regulation with N ₂ as N source	94–141 nmol m ^{−2} s ^{−1} (efflux)	table 1 of Raven & Wollenweber (1992)
net H ⁺ influx or efflux in acid–base regulation with NO ₃ [−] as N source	15–131 nmol m ^{−2} s ^{−1} (efflux)	table 1 of Raven & Wollenweber (1992)
H ⁺ influx/efflux associated with cotransport; currents typically circulate over 10–100 nm along membranes	75 nmol m ^{−2} s ^{−1} (influx) 52 nmol m ^{−2} s ^{−1} (efflux)	table 1 of Raven & Wollenweber (1992)
H ⁺ influx/efflux associated with currents circulating over > 1 mm	400 nmol m ^{−2} s ^{−1} 230 nmol m ^{−2} s ^{−1}	Raven & Wollenweber (1992), assuming K ⁺ influx involves K ⁺ –H ⁺ symport (Maathuis & Sanders 1993) rather than a passive K ⁺ uniport Raven & Wollenweber (1992), who note that the apical H ⁺ influx is over a much smaller area than the basal H ⁺ efflux, so that the apical H ⁺ influx is 20–300 nmol m ^{−2} s ^{−1} while the basal H ⁺ efflux is about 2–30 nmol m ^{−2} s ^{−1}

processes has a significant direct impact on intracellular acid–base balance.

The processes described so far increase or decrease rhizosphere pH. The assimilation of NO₃[−] with OH[−] excretion increases rhizosphere pH. NO₃[−] assimilation with organic anion efflux slightly increases rhizosphere pH if the external pH is initially lower than the highest pK_a of the organic anion that is secreted. NH₄⁺ assimilation and symbiotic N₂ assimilation lead to rhizosphere acidification by direct H⁺ excretion. Additional organic acid synthesis, with H⁺ excretion in exchange for some other cation, or organic acid excretion *per se*, lowers external pH, exacerbating the pH decrease associated with NH₄⁺ assimilation or symbiotic N₂ fixation or limiting or reversing the pH increase associated with NO₃[−] assimilation (Raven & Wollenweber 1992; table 3).

Before considering the impact of these processes on nutrient acquisition from the soil, it is necessary to introduce a process which causes pH increase at the apex of growing roots and root hairs (and, probably, bryophyte rhizoids and the rhizoids of free-living gametophytes of free-sporing vascular plants), i.e. the external and internal (to the plant cell or organ) circulation of electric current carried by H⁺ (mainly, in the internal and external aqueous phases of the pathway, as buffered H⁺) (see Raven 1991b, 1995; table 2). The circulation of H⁺ can include a symplasmic flux in the opposite direction of organic anions which can be metabolized to consume H⁺ at the sink just as their synthesis generated H⁺ (Raven 1991b, 1995). A modification of this process presumably underlies the secretion of organic anions (malate) around the apex of roots in response to challenge by soluble external Al (Kochian 1995). The likely processes here are malic acid synthesis in more basal root regions, with H⁺ excretion and K⁺ uptake, and two K⁺ malate^{2−} transfer to the apex, followed by two K⁺ malate^{2−} efflux.

The changes in pH, and organic acid (and organic anion) concentration of the rhizosphere which are induced by land plants have effects on nutrient acquisition (Raven *et al.* 1990; Marschner 1995) and on limiting damage from high concentrations of soluble Al species. In

all vascular plants examined, except the Poaceae (Gramineae), a response to Fe deficiency in aerobic soils is the induction of a plasmalemma-located Fe(III) reductase, which generates soluble Fe(II) which can be taken up by Fe²⁺ transporters at the plasmalemma. The Fe(III) reductase requires soluble (chelated) Fe(III), which is generated from the plentiful insoluble Fe(III) chelation by secreted organic acids (especially citrate), with low pH favouring Fe(III) chelation and reduction. The Poaceae use an iron-acquisition system involving phytosiderophores, i.e. specific Fe-chelaters such as hydroxamic acids, which are taken up by the plants as Fe(III)-siderophore complexes, a mechanism known elsewhere among O₂-evolving photolithotrophs in the cyanobacteria. The Fe(III) reduction mechanism is found in all of the (few) eukaryotic algae that have been tested, but does not involve organic acid excretion or large-scale H⁺ excretion, probably because the organisms tested (*Chlamydomonas*, *Laminaria*) obtain Fe from a bulk water phase lacking solid Fe(III) phases contiguous with the algal surface on which citric acid and H⁺ could act (see Jones 1998; Raven 1991b; Raven *et al.* 1990).

Phosphorus deficiency, at least in non-mycorrhizal dicotyledons (such as the naturally non-mycorrhizal Brassicaceae), increases organic acid synthesis and both H⁺ efflux (and organic anion salt accumulation) and organic acid efflux. These secretions help to release P (as orthophosphate) from insoluble calcium phosphate deposits and phosphate–Fe(III) (if not from phosphate–Al(III)) complexes (Jones 1998; Raven 1991b; Raven *et al.* 1990). These effects may be less important in grasses (Raven *et al.* 1990; Logan *et al.* 2000; cf. Kirk *et al.* 1999). The involvement of H⁺ or organic acid excretion by (arbuscular) mycorrhizal fungi in phosphate acquisition awaits clarification (see Raven *et al.* 1978), as does their role in organic acid synthesis and H⁺ fluxes related to assimilation of exogenous NH₄⁺ and NO₃[−].

These iron and phosphate acquisition ‘advantages’ of H⁺ and organic acid excretion to oxidized media containing Fe mainly as Fe(III) and P mainly as orthophosphates bound to Fe(III), Al(III) and as calcium

phosphates, has not apparently been investigated for bryophytes with rhizoids/rhizomes/mycorrhizas in aerobic soils. However, the hypothesis suggested here (Raven 1991b; Raven *et al.* 1980, 1990) gives a role along these lines to bryophytes containing high levels of low M_r organic anions in P and Fe acquisition. The finding that free-floating (secondary aquatic) heterosporous fern sporophytes, with a corresponding inability to influence P and Fe availability from sediments via H^+ excretion and organic acid excretion from their roots, have relatively low contents of low M_r organic acid anions (Raven *et al.* 1991; Martins-Loucao *et al.* 1993) is also consistent with this notion.

The discussion thus far on rhizosphere acidification by H^+ and organic acid excretion concerns processes which, in the more basal parts of the root system or of root hairs, are amplified by the active H^+ efflux from the symplasm in the longitudinal circulation of electric current carried by H^+ (free, buffered or released/absorbed by biochemical changes to organic molecules) (Raven 1991b, 1995; Raven & Wollenweber 1992). However, there is an observable alkalization of the rhizosphere around the apices of roots and root hairs, even in root systems with a large net efflux of H^+ (as H^+ or as undissociated organic acids which dissociate at the rhizosphere pH value) from the root systems as a whole in NH_4^+ or N_2 assimilation (Raven 1991b, 1995; table 3) related to the H^+ circulation. This has been related to the acquisition of Mo (see Raven 1988b, 1991b, 1995; Raven *et al.* 1990). This essential element is required in especially large amounts by embryophytes that are using NO_3^- as their N source (due to the Mo requirement for NO_3^- reductase) and, particularly those relying on symbiotic N_2 fixation (using the 'conventional' Mo-requiring nitrogenase rather than the Mo-independent V- or Fe-nitrogenases): Raven (1988b). Plants growing on NO_3^- generally cause an overall increase in rhizosphere pH, although this can be reversed in plants with very high contents of anions of low M_r organic acids (Raven & Smith 1976; Raven *et al.* 1980, 1990; Raven 1985a,b; Raven & Farquhar 1990), so that this would favour Mo acquisition. However, even here the root apex is the most alkaline region of the rhizosphere (Raven & Wollenweber 1992), so that this could be the most important region of the root surface for Mo uptake; this has yet to be tested. By contrast with growth on NO_3^- , the growth of (symbiotic) plants with N_2 as their N source invariably causes a net acidification of the rhizosphere (averaged over the whole root surface), so that these plants are doubly disadvantaged compared with plants growing on NO_3^- with respect to Mo supply: their need for Mo on a biomass basis is greater if the same specific growth rate is to be achieved, and the availability of Mo from the rhizosphere is lower, granted similar soil bulk phase mean pH and Mo concentrations for growth on the two N sources. Accordingly, it might be expected that the apical zone of rhizosphere alkalization of roots and root hairs (and, perhaps, mycorrhizas) might be important in Mo acquisition. Again, this seems not to have been tested. The lowest rhizosphere Mo availability might be expected for NH_4^+ -grown plants, which cause the greatest overall net acidification of the rhizosphere. However, the Mo requirement (e.g. for such low-content Mo enzymes as xanthine dehydrogenase) is very small for NH_4^+ -grown plants and is presumably

satisfied by apical rhizosphere alkalization in the face of large bulk rhizosphere acidification.

A further possible role of apical alkalization of the rhizosphere relates to restriction of toxicity due to soluble Al compounds. This Al toxicity is exacerbated at low pH, and is particularly related to the zone of cell division in the root apex (Kochian 1995). Offsetting this Al toxicity could, then, be one of the functions of alkalization of the rhizosphere by the circulating current carried by H^+ (Raven 1991b; Raven & Wollenweber 1992). Again, this has not yet been tested. The secretion of malate²⁻ from apical regions of roots of certain relatively Al-tolerant genotypes of flowering plants is an important part of the Al-tolerance syndrome (Kochian 1995). Such malate²⁻ secretion into an acid medium (such as favours Al toxicity) will itself raise rhizosphere pH due to protonation of one of the malate²⁻ carboxylate anions if the medium pH is close to, or below, the pK_{a2} of malic acid (Kochian 1995).

At the outset it must be acknowledged that many extant bryophytes obtain some or even most of their nutrients other than carbon from precipitation, with or without augmentation from leaf leachates from vascular plant canopies above them. However, there is also evidence for significant rhizoid-based nutrition in some bryophytes (Richardson 1981). The relevance of this apical alkalization phenomenon to bryophytes can only be conjectured. The very occurrence in bryophytes of H^+ -mediated current circulation over hundreds of micrometres and more between symplasm and apoplasm is as yet incompletely established (Raven *et al.* 1998). Bryophytes can (as a level of organization) use NO_3^- as N source, although some aquatics eschew significant NO_3^- concentrations in their natural environment in favour of less abundant reduced (NH_4^+ , organic) N sources (see MacFarlane & Raven (1990) for discussion of data on freshwater liverworts and a red alga). Two groups of bryophytes (certain hornworts such as *Anthoceros* and *Phaeoceros*; the liverwort *Blasia*) have symbiotic N_2 fixation involving the cyanobacterium *Nostoc*. While it is likely that this symbiotic *Nostoc* uses Mo-nitrogenase, the use of alternative (V-, or Fe-only) nitrogenases cannot be ruled out. The use of non-Mo nitrogenases could be tested in two ways. One is by the reduction of C_2H_2 to C_2H_6 as well as the normal (C_2H_4) product of Mo nitrogenase, which is diagnostic of the other two nitrogenases (Rowell *et al.* 1998). The other test is the measurement of natural abundance $^{15}N/^{14}N$ ratios, since the alternative nitrogenases discriminate against ^{15}N relative to ^{14}N much more than does Mo nitrogenase (Rowell *et al.* 1998). The anatomy of these *Nostoc*-bryophyte symbioses means that Mo transfer from the soil to *Nostoc* obligatorily involves movement of Mo through the bryophyte, so that apical alkalization of the rhizosphere around rhizoids (or mycorrhizas) would be helpful to Mo acquisition. However, the possibility of Mo acquisition from rain or dust cannot be ruled out. The high Fe need for diazotrophy (Fe content per unit biomass for a given specific growth rate increases in the following order for different N sources: $N_2 > NO_3^- > NH_4^+$) would be favoured in aerobic soils by acidification of some part of the rhizosphere around rhizoids (or mycorrhizas), i.e. net acidification averaged over the whole rhizosphere could be achieved by net accumulation of low M_r organic acid

anions in the bryophyte as well as by any circulating current carried by H^+ . Al toxicity in bryophytes has been very little investigated, so nothing can be said about the role of rhizosphere alkalization at growing tips where cell division occurs.

Having examined the possible nutritional roles of the accumulation of organic acid anions by embryophytes, and its relationship to circulating current carried by H^+ , it remains to consider the evolutionary aspects of these phenomena: are the biophysical properties of extant Charophyceae in any way uniquely suited to the ancestors of these algae being the progenitors of the embryophytes? It is prudent to warn the reader that no unequivocal answer can currently be given, but that there are indications that the Charophyceae are appropriate ancestors.

Raven (1991b, 1995) has considered the significance of circulating currents carried by H^+ in embryophytes in relation to nutrient acquisition (acidification, alkalization of parts of the rhizosphere) and in relation to other modes of rhizosphere acidification (organic acid synthesis with organic acid excretion or H^+ excretion in exchange for some (non- H^+) cation) in an evolutionary context. Circulating currents probably carried by H^+ (and Ca^{2+}) have been reported for planktonic desmids (Charophyceae sensu lato), while the Characeae (Charophyceae) clearly have circulating currents, carried by H^+ , around their shoots. This has been especially investigated in the context of the acid–base ‘banding’ of ecorticate internodes, which play an important role in acquisition of inorganic C from HCO_3^- in alkaline waters, and which are functionally analogous to the acid abaxial surface/alkaline adaxial surface polarity of the leaves of some (secondarily aquatic) freshwater submerged flowering plants (Raven 1991b, 1995). Both the Characeae and these submerged flowering plants are rhizophytes (i.e. have roots or rhizoids in sediments which commonly have higher nutrient concentrations than does the bulk aqueous phase in which the photosynthetic shoots occur), although some freshwater and marine benthic flowering plants are haptophytes, i.e. are attached to rock surfaces (e.g. the freshwater Podostemaceae; some seagrasses such as *Phyllospadix* spp.) (Raven 1984b). The sediments in which parts of these rhizophytes are embedded can be anoxic, in which case the requirement for acidification of, and release of chelating organic acids to the rhizosphere is less obviously important in terms of P and Fe acquisition. This is because the bulk sediment has Fe in the Fe(II) form, which is at once available to the rhizoid and unable (contrast Fe(III)) to bind inorganic phosphate. These early rhizophytes (rhizoids of *Palaeonitella* are very well-preserved in the Lower Devonian Rhynie Chert, some 395 Myr old) might then not have used circulating currents or net H^+ and organic acid secretion into the rhizosphere in Fe and P nutrition (Raven 1984b, 1991b; Raven & Wollenweber 1992). They may not even have used apical rhizoid alkalization in Mo acquisition as Mo could be obtained from the bulk waterbody. Furthermore, Mo may in any case have not been very important for assimilation of at least rhizoid-derived N, since these anoxic sediments do not permit nitrification of mineralized (ammonified) organic N, and denitrify such NO_3^- as invades from the bulk water above, so that the

predominant form of nitrogen is reduced as NH_4^+ or organic N. Shoot-supplied NO_3^- from the bulk water phase would, however, need Mo for its assimilation.

The earliest (rhizophytic) embryophytes probably inhabited soils that were often waterlogged, so that the considerations above as to Fe, Mo and NH_4^+ acquisition by rhizoids remain valid, although clearly there would be no possibility of significant acquisition of nutrients other than O_2 from the medium surrounding the shoot, except during any periods of induction by free water. While such a waterlogged soil would not necessarily involve H^+ current circulation and organic acid synthesis in acidifying part of the rhizosphere in Fe and P acquisition, such acidification would be important in Fe and P acquisition if the soil should cease to be waterlogged and Fe in the soil becomes oxidized to Fe(III). Furthermore, oxygenated soils permit the occurrence of NO_3^- via nitrification of NH_4^+ produced by mineralization (ammonification), leading to an enhanced requirement for Mo and hence a rationale for alkalization of the apex of the root/rhizoid system while the rest of the root system may not be greatly acidified or alkalized (organic acid synthesis playing a major role in neutralizing OH^- resulting from NO_3^- assimilation).

These nutritional considerations help to rationalize the involvement of high organic acid production rates as a fraction of total carbon assimilated, and of circulation of H^+ along and around nutrient-absorbing structures in the soil, in rhizophytic embryophytes. The additional organic acid production has some precedents in algae, albeit not commonly in the Charophyceae (Raven 1991b), while H^+ circulation occurs in freshwater Charophyceae sensu lato and in freshwater *Vaucheria* (Tribophyceae): Raven (1991b). Rhizophytic marine green macroalgae have circulating H^+ currents in the case of the eukaryotic characean *Lamprothamnium* (and probably the seagrasses, which are flowering plants); these are secondarily marine organisms (Raven 1991b). Primarily marine green algal macrophytes in the Ulvophyceae are frequently rhizophytic, at least among the acellular organisms in warmer waters, and have circulating currents carried by Cl^- (Raven 1991b). Such circulating currents cannot directly lead to acidification or alkalization of zones around the rhizoids (Raven 1991b). While there is evidence that the ulvophycean rhizophyte *Caulerpa* can obtain N and P from sediments via their rhizoids (Raven 1984a,b, 1991b; Chisholm *et al.* 1996), it is thus unlikely that modification of rhizosphere pH by circulating currents is involved, though erosion of calcareous phosphate-containing particles in sediments by the ulvophycean rhizophyte *Caulerpa* may involve secretion of Ca^{2+} -chelating organic acids (see Chisholm *et al.* 1996). These considerations make, on the basis of present evidence, the Charophyceae the only plausible algal ancestors for terrestrial rhizophytes using circulating currents carried by H^+ in nutrition (Raven 1984a,b, 1996).

(c) *Relationship of the evolution of the specific features of embryophytes to the Ordovician–Lower Devonian environment?*

Can the evolution of specific embryophytic biochemical features be related to the Early–Mid-Palaeozoic environment (Chapman 1985; Edwards 1996, 1998;

Edwards *et al.* 1998; Raven 1977, 1984a, 1993, 1994a,b, 1998, 1999; Wellman *et al.* 1998)? The O₂ concentration in the atmosphere was similar to that found today (Berner & Canfield 1989), while CO₂ in the Ordovician–Silurian–Early Devonian was 12–22 times the present value (Berner 1997, 1998). O₂ amounts in the stratosphere were similar to those found today, so land surface UV-B would have been similar to those found today (Raven 1998). The O₂ levels would have permitted synthesis of lignin and other embryophyte-specific compounds involving oxygenases at any time starting not later than the Ordovician onwards (Chapman 1985; Raven 1977, 1998), provided the appropriate enzymes were present.

The UV-B level would have provided a selective constraint on terrestrial plants, i.e. not subject to UV-B screening by a water layer, which invariably attenuates UV-B more than photosynthetically active radiation (Kirk 1994a,b). This UV-B attenuation by natural waters is not dependent on the presence of dissolved O₂ (Kirk 1994a; cf. Falkowski & Raven 1997), so that submerged aquatic plants have always been afforded a limited UV-B flux relative to the flux of photosynthetically active radiation, although this would have still meant relatively high UV-B: photosynthetically active radiation ratios in the sea and freshwater prior to the occurrence of the O₃ screen for UV-B.

Atmospheric O₂ levels such as those found in the Early–Mid–Palaeozoic would have permitted nitrification of NH₄⁺ produced in ammonification of organic N in the soil, and the occurrence of Fe(III) in non-waterlogged soils, thus providing the Mo, N, P and Fe supply and requirement regimes for terrestrial rhizophytes which permit nutrient acquisition to be aided by additional organic acid synthesis and circulating currents carried by H⁺.

Gottlieb (1982, 1989, 1990) has made insightful observations on the role of atmospheric composition at, and later than, the time of the evolution of various grades of organization of O₂-evolving photolithotrophs. However, some of these observations seem to have been premature in terms of what has subsequently been discovered, or deduced, about the composition of the atmosphere at various times. An example is the suggestion (Gottlieb 1982) that the NH₃ concentration in seawater exceeds (and has always exceeded) the NH₃ concentration in the above-ground organs of terrestrial plants in equilibrium with the atmosphere. This higher NH₃ concentration in seawater (and hence in the cells of organisms equilibrated with it) was hypothesized to exert a mass action effect on the enzyme phenylalanine ammonia lyase (PAL) (Gottlieb 1982). PAL is an essential part of the pathway from the essential protein amino acid phenylalanine to cinnamic acid and hence to phenylpropanoids, so that such a constraint on its action in converting phenylalanine to cinnamic acid and NH₃ would act as a constraint on the net production of cinnamic acid, and hence on the development of the phenylpropanoid suite of structural, defence, UV-B-absorbing and inter-organism signalling compounds (Gottlieb 1982). However, data reviewed by Raven (1988a), Raven *et al.* (1992) and Raven & Yin (1998) show that the free NH₃ concentration in the surface layers of the ocean in which photosynthetic growth can occur is less than that in plant shoots inter-

acting with the NH₃ level of 'clean' (i.e. without anthropogenic NH₃ inputs) air, so that if anything, PAL activity to generate cinnamic acid is less constrained by NH₃ mass action effects in marine plants than in terrestrial plants. This argument based on extant plants is likely also to have held in the past (Raven & Yin 1998), so that terrestrial existence *per se* is unlikely to explain the more widespread use of phenylpropanoids in terrestrial than marine (and freshwater) organisms.

A second very interesting point made by Gottlieb concerns the role of atmospheric oxygen concentration on the evolution of phenylpropanoids and cutin. Gottlieb (1989) correlated the evolution of early embryophytic land plants, embryophytes of the pteridophyte and gymnosperm grade of organization, and angiosperms, with peaks in atmospheric O₂ as deduced from palaeo-geochemical evidence. Specifically, Gottlieb (1989) considered the evolution of flavonoids and cutin in the earliest embryophytes, of lignin in pteridophytes and condensed tannins in gymnosperms, and of a wide range of phenolics in angiosperms to be related to the high O₂ level at the time of evolution of these organisms via enzymatic reactions derived from mechanisms for dealing with active oxygen species, which are involved in protection against the high levels of O₂. While these suggestions correlated approximately with the best available reconstructions then available (Budyko *et al.* 1985) of the variation of atmospheric O₂ throughout the Phanerozoic, more recent reconstructions of O₂ in palaeoatmospheres (Berner & Canfield 1989) fail to show the O₂ peaks coincident with the evolution of early embryophytes and of angiosperms, and show a Late Carboniferous peak which is significantly later than the time of evolution of the pteridophyte grade and even that of the gymnosperms.

These two cases show that attempts to correlate the terrestrial habitat *per se* (NH₃ mass action effect on PAL) and the O₂ content of the palaeoatmospheres (O₂ detoxification pathways adapted to biosynthesis of novel metabolites) may not be sustainable in the face of more detailed palaeo-geochemical evidence.

A final point, this time about the chemical nature of many embryophyte-specific compounds (and, indeed, some of those shared with algae) relative to materials which perform similar functions in other higher taxa. This point concerns the greater reliance on N-free than on N-containing compounds in most eukaryotic photolithotrophs than in eukaryotic organisms with other trophic modes, a difference which can influence whole-organism C/N ratios (Raven 1984b; Raven *et al.* 1992; Sprent 1987).

An excellent example here is the nature of structural polysaccharides. Algal (including non-photosynthetic heterokonts in the oomycetes) and embryophyte cell walls usually have structural polysaccharides which are non-aminated. Thus, the chitin of many invertebrates and of true (non-oomycete) fungi contains N, while the polysaccharides of embryophytes and algae rarely contain chitin (cf. certain diatoms in the Heterokontophyta). Furthermore, there is no analogue of metazoan collagen as a major skeletal element in the (quantitatively minor) extracellular proteins of algae and embryophytes. However, the phenolic cross-linking involved in making extracellular polysaccharides more rigid and less deformable is common to lignified (polyphenylpropanoid) walls

of higher embryophytes, which lack aminated polysaccharides and the exoskeleton of insects wherein chitin (N-containing) is cross-linked with phenols (non-phenylpropanoid) in the final stage in tanning.

An additional difference in N requirements in wall components between aerial parts of embryophytes and in fungi concerns the water-repellent components; while embryophytes have N-free cuticle and wax, the fungi use proteinaceous hydrophobins (Wessels 1994).

A further example of N-containing compounds and non-N-containing compounds fulfilling the same function in different organisms is that of UV-B screening compounds. Marine algae from many Divisions, as well as cyanobacteria, contain mycosporine-like amino acids as quantitatively major UV-B screens, and many near-surface marine invertebrates use these compounds obtained directly or indirectly from dietary algae as UV-B screens (Raven 1991c; Franklin & Forster 1997; Ehling-Schulz & Scherer 1999). However, embryophytes use phenolic, non-N-containing compounds (e.g. flavonoids, lignin) as the major UV-B-screens. No data seem to be available on the UV-B-screening compounds used by the Charophyceae, which are probably primarily freshwater with some euryhaline representatives. It must be admitted that some embryophytes lack this 'N-free' paradigm; thus the Centrospermae (Caryophyllales) have the two N-containing betalains (alkaloids) betaxanthin and betacyanin to give yellow–orange and blue colours (Harborne 1982) respectively, while many putative 'defence compounds' (e.g. alkaloids) of angiosperms contain N, and are by no means confined to symbiotically N₂-fixing plants. Furthermore, some primarily (algal) and secondarily (vascular plants) halophytic photolithotrophs use N-containing compatible solutes such as proline and glycine betaine, while others use N-free linear or cyclic polyols, di- and oligosaccharides and dimethylsulphoniopropionate (Raven 1985b).

However, the trend towards N-free structural materials and defences against abiotic and biotic assaults, and molecules which signal between organisms in both algae and embryophytes is clear. Although this trend is less developed in algae (N-containing UV-B sunscreens) than in embryophytes (N-free UV-B sunscreens), it would appear not to be a response to an improved supply of sunlight and inorganic carbon relative to N, P, K, S, Ca, Mg, Fe, etc., on land as opposed to water, although whole-plant C/N ratios are higher in trees than in aquatic algae or flowering plants (or, indeed, herbaceous embryophytes; Raven *et al.* 1992). In any case, the lower N use in extracellular structures in eukaryotic photolithotrophs than in non-photolithotrophs is not a function of the Lower Palaeozoic environment.

4. CONCLUSIONS AND PROSPECTS

Qualitative and quantitative differences in the biochemistry of embryophytes relative to charophytes, and between bryophytes and tracheophytes in the embryophytes, cannot generally be related to the Lower Palaeozoic environment.

Even complete nucleotide sequences of genes may not permit in the near future the prediction of all significant kinetic values of the enzymes they encode, e.g. CO₂

affinity and CO₂/O₂ selectivity in RUBISCO. However, such efforts are to be encouraged.

A further contribution from genomes might be in helping to determine how much gene loss (or gene inactivation) has occurred in the evolution of extant bryophytes.

Past and present colleagues have greatly aided my thoughts on these matters.

REFERENCES

- Austin, J. J., Smith, A. B. & Thomas, R. H. 1997 Palaeontology in a molecular world: the search for authentic ancient DNA. *Trends Ecol. Evol.* **12**, 303–306.
- Badger, M. R. & Andrews, T. J. 1987 Co-evolution of RUBISCO and CO₂ concentrating mechanisms. In *Progress in photosynthesis research III* (ed. J. Biggins), pp. 501–609. Dordrecht: Martinus Nijhoff.
- Badger, M. R., Andrews, T. J., Whitney, S. M., Ludwig, M., Yellowlees, D. C., Leggat, W. & Price, G. D. 1998 The diversity of coevolution of Rubisco, plastids, pyrenoids, and chloroplast-based CO₂-concentrating mechanisms in algae. *Can. J. Bot.* **76**, 1052–1071.
- Bateman, R. M., Crane, P. R., Di Michele, W. A., Kenrick, P. R., Rowe, N. P., Speck, T. & Stein, W. E. 1998 Early evolution of land plants: phylogeny, physiology and ecology of the primary terrestrial radiation. *A. Rev. Ecol. Syst.* **29**, 263–292.
- Berner, R. A. 1997 The rise of plants and their effects on weathering and atmospheric CO₂. *Science* **276**, 544–546.
- Berner, R. A. 1998 The carbon cycle and CO₂ over Phanerozoic time: the role of land plants. *Phil. Trans. R. Soc. Lond. B* **353**, 75–82.
- Berner, R. A. & Canfield, D. E. 1989 A new model for atmospheric oxygen over Phanerozoic time. *Am. J. Sci.* **289**, 333–361.
- Bhattacharya, D. & Medlin, L. 1998 Algal phylogeny and the origin of land plants. *Pl. Physiol.* **116**, 9–15.
- bin Surif, M. & Raven, J. A. 1990 Photosynthetic gas exchange under emersed conditions in eulitterol and normally submersed members of the Fucales and the Laminariales: interpretation in relation to C isotope ratio and N and water use efficiency. *Oecologia* **82**, 68–80.
- Bird, I. F., Cornelius, M. J. & Keys, A. J. 1982 Affinity of RuBP carboxylases for carbon dioxide and inhibition of the enzymes by oxygen. *J. Exp. Bot.* **33**, 1004–1013.
- Budyko, M. I., Ronov, A. B. & Yanshin, A. L. 1985 *History of the Earth's atmosphere*. Berlin: Springer.
- Chapman, D. J. 1985 Geological factors and biochemical aspects of the origin of land plants. In *Geological factors and the evolution of plants* (ed. B. R. Tiffney), pp. 23–45. New Haven, CT: Yale University Press.
- Chapman, D. J. & Ragan, M. A. 1980 Evolution of biochemical pathways: evidence from comparative biochemistry. *A. Rev. Pl. Physiol.* **31**, 639–678.
- Chisholm, J. R. M., Douga, C., Ageron, M., Grimond, P. A. D. & Joubert, J. M. 1996 'Roots' in mixotrophic algae. *Nature* **381**, 382.
- Doyle, J. A. 1998 Phylogeny of vascular plants. *A. Rev. Ecol. Syst.* **29**, 567–599.
- Edwards, D. 1996 New insights into early land ecosystems: a glimpse of a Lilliputian world. *Rev. Palaeobot. Palynol.* **90**, 159–174.
- Edwards, D. 1998 Climate signals in Palaeozoic land plants. *Phil. Trans. R. Soc. Lond. B* **353**, 141–157.
- Edwards, D., Abbott, G. D. & Raven, J. A. 1996 Cuticles in early land plants: a palaeoecophysiological evolution. In *Plant cuticles* (ed. G. Kierstens), pp. 1–31. Oxford: Bios Scientific Publishers.
- Edwards, D., Wellman, C. H. & Axe, L. 1998 The fossil record of early land plants and interrelationships between primitive embryophytes: too little and too late? In *Bryology for the twenty-first century* (ed. J. W. Bates, N. W. Ashton & J. G. Duckett),

- pp.15–43. *Proceedings of the Centenary Symposium of the British Bryological Society*. Leeds: Maney Publishing and the British Bryological Society.
- Ehling-Schulz, M. & Scherer, S. 1999 UV protection in cyanobacteria. *Eur. J. Phycol.* **34**, 329–338.
- Falkowski, P. G. & Raven, J. A. 1997 *Aquatic photosynthesis*. Malden, MA: Blackwell Science.
- Fogg, G. E. 1998 *The biology of polar habitats*. Cambridge University Press.
- Franklin, L. A. & Forster, R. M. 1997 The changing irradiance environment: consequences for marine macrophyte physiology, productivity and ecology. *Eur. J. Phycol.* **32**, 207–232.
- Gottlieb, O. R. 1982 *Micromolecular evolution, systematics and ecology. An essay into a new botanical discipline*. Berlin: Springer.
- Gottlieb, O. R. 1989 The role of oxygen in phytochemical evolution towards diversity. *Phytochemistry* **28**, 2545–2458.
- Gottlieb, O. R. 1990 Phytochemicals: differentiation and function. *Phytochemistry* **29**, 1715–1724.
- Graham, L. E. 1993 *Origin of land plants*. New York: John Wiley.
- Harborne, J. B. 1982 *Introduction to ecological biochemistry*, 2nd edn. London: Academic Press.
- Jarvis, M. C. 1998 Intercellular separation forces generated by intracellular pressure. *Pl. Cell Environ.* **21**, 1307–1310.
- Jones, D. L. 1998 Organic acids in the rhizosphere—a critical review. *Pl. Soil* **205**, 25–44.
- Kaneko, T. (and 23 others) 1996 Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res.* **3**, 109–136.
- Kaplan, A., Ronen-Tarazi, M., Zer, H., Schwarz, R., Tchernov, D., Bonfit, D. J., Schotz, D., Vardi, A., Hassidim, M. & Reinhold, L. 1998 The inorganic carbon concentrating mechanism in cyanobacteria: induction and ecological significance. *Can. J. Bot.* **76**, 917–924.
- Kenrick, P. & Crane, P. R. 1997 *The origin and early diversification of land plants. A cladistic study*. Washington, DC: Smithsonian Institution Press.
- Kirk, J. T. O. 1994a Optics of UV-B in natural waters. *Arch. Hydrobiol.* **43**, 1–16.
- Kirk, J. T. O. 1994b *Light and photosynthesis in aquatic ecosystems*, 2nd edn. Cambridge University Press.
- Kirk, G. T. O., Santos, E. E. & Santos, M. B. 1999 Phosphate solubilization by organic anion excretion from rice growing in aerobic soil: rates of excretion and decomposition, effects on rhizosphere pH and effects on phosphate solubility and uptake. *New Phytol.* **142**, 185–200.
- Kochian, L. V. 1995 Cellular mechanisms of aluminium toxicity and resistance in plants. *A. Rev. Pl. Mol. Biol.* **46**, 237–260.
- Lindahl, T. 1997 Endogenous damage to DNA. *Phil. Trans. R. Soc. Lond.* **B351**, 1529–1538.
- Logan, G. A., Boon, J. J. & Eglinton, G. 1993 Structural biopolymer preservation in Miocene leaf fossils from the Clarkia site, northern Idaho. *Proc. Natl Acad. Sci. USA* **90**, 2246–2250.
- Logan, K. A. B., Thomas, R. J. & Raven, J. A. 2000 Effect of ammonia and phosphorus supply on H^+ production in gel by two tropical forage grasses. *J. Pl. Nutr.* **23**, 41–54.
- Lorimer, G. H., Chen, Y.-R. & Hartman, F. C. 1993 A role for the ϵ -amino group of lysine-334 of ribulose-1,5-bisphosphate carboxylase in the addition of carbon dioxide to the 2,3-enediol(ate) of ribulose 1,5-bisphosphate. *Biochemistry* **32**, 9018–9024.
- Loss, S. P., Robson, A. D. & Ritchie, G. S. P. 1993 H^+/OH^- excretion and uptake in upper and lower parts of lupin (*Lupinus angustifolius* L.) root systems. *Ann. Bot.* **72**, 315–320.
- Loss, S. P., Robson, A. D. & Ritchie, G. S. P. 1994 Nutrient uptake and organic anion metabolism in lupins and peas supplied with nitrate. *Ann. Bot.* **74**, 69–74.
- Maathuis, F. J. M. & Sanders, D. 1993 Energization of potassium uptake in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA* **91**, 9272–9276.
- MacFarlane, J. J. & Raven, J. A. 1990 C, N and P nutrition of *Lemanea mamillosa* Kutz. (Batrachospermales, Rhodophyta) in the Dighty Burn, Angus, Scotland. *Pl. Cell Environ.* **13**, 1–13.
- Marschner, H. 1995 *Mineral nutrition in higher plants*, 2nd edn. London: Academic Press.
- Martins-Loucao, M. A., Wollenweber, B. & Raven, J. A. 1993 Response of *Salvinia* spp. to different nitrogen sources: the acid–base approach. *Oecologia* **93**, 524–530.
- Maynard Smith, J. & Szathmáry, E. 1995 *The major transitions in evolution*. Oxford University Press.
- Palmqvist, K., Sultemeyer, D., Baldent, P., Andrews, T. J. & Badger, M. R. 1995 Characterization of inorganic carbon fluxes, carbonic anhydrase(s) and ribulose-1,5-bisphosphate carboxylase-oxygenase in the green unicellular alga *Coccomyxa*. Comparisons with low- CO_2 cells of *Chlamydomonas reinhardtii*. *Planta* **197**, 352–361.
- Palmqvist, K., de los Rios, A., Ascaso, C. & Samuelsson, G. 1997 Photosynthetic carbon acquisition in the lichen photobionts *Coccomyxa* and *Trebouxia* (Chlorophyta). *Physiol. Pl.* **101**, 67–76.
- Pearson, P. N. & Palmer, M. R. 1999 Middle Eocene seawater pH and atmosphere carbon dioxide concentrations. *Science* **284**, 1824–1826.
- Petit, J. R. (and 18 others) 1999 Climate and atmospheric history of the past 420 000 years from the Vostok ice core, Antarctica. *Nature* **399**, 429–436.
- Poinar, H. N., Höss, H., Bada, J. L. & Pääbo, S. 1996 Amino acid racemization and the preservation of ancient DNA. *Science* **272**, 264–266.
- Price, G. D., Sultemeyer, D., Klughammer, B. Ludwig, M. & Badger, M. R. 1998 The functioning of the CO_2 concentrating mechanism in several cyanobacterial strains: a review of general physiological characteristics, genes, proteins, and recent advances. *Can. J. Bot.* **76**, 973–1012.
- Raven, J. A. 1977 The evolution of vascular land plants in relation to supracellular transport processes. *Adv. Bot. Res.* **5**, 153–219.
- Raven, J. A. 1984a Physiological correlates of the morphology of early vascular plants. *Bot. J. Linn. Soc.* **88**, 105–126.
- Raven, J. A. 1984b *Energetics and transport in aquatic plants*. New York: A. R. Liss.
- Raven, J. A. 1985a Physiology and biochemistry of pteridophytes. *Proc. R. Soc. Edinb.* **B86**, 37–44.
- Raven, J. A. 1985b Regulation of pH and generation of osmolarity in vascular land plants: costs and benefits in relation to efficiency of use of water, energy and nitrogen. *New Phytol.* **101**, 25–77.
- Raven, J. A. 1986 Biochemical disposal of H^+ in plants? *New Phytol.* **104**, 175–206.
- Raven, J. A. 1987 The role of vacuoles. *New Phytol.* **106**, 357–422.
- Raven, J. A. 1988a Acquisition of nitrogen by the shoots of land plants: its occurrence and implications for acid–base balance. *New Phytol.* **109**, 1–20.
- Raven, J. A. 1988b The iron and molybdenum use efficiencies of plant growth with different energy, carbon and nitrogen sources. *New Phytol.* **109**, 279–287.
- Raven, J. A. 1989 Overview of transport systems in algae and bryophytes. In *Methods in enzymology: biomembranes/biological transport*, vol. 4, *Plant V* (vol. 174) (ed. S. Fleischer & B. Fleischer), pp. 366–390. San Diego: Academic Press.
- Raven, J. A. 1991a Implications of inorganic C utilization: ecology, evolution and geochemistry. *Can. J. Bot.* **69**, 908–924.
- Raven, J. A. 1991b Terrestrial rhizophytes and H^+ currents circulating over at least a millimetre: an obligate relationship? *New Phytol.* **117**, 177–185.

- Raven, J. A. 1991c Response of aquatic photosynthetic organisms to increased solar UV-B. *J. Photochem. Photobiol. B: Biology* **9**, 239–244.
- Raven, J. A. 1991d Long-term functioning of enucleate sieve elements: possible mechanisms of damage avoidance and damage repair. *Pl. Cell Environ.* **14**, 139–146.
- Raven, J. A. 1993 The evolution of vascular land plants in relation to quantitative functioning of dead water-conducting cells and stomata. *Biol. Rev.* **68**, 337–363.
- Raven, J. A. 1994a Physiological aspects of the functioning of vascular tissue in early vascular plants. *Bot. J. Scotland* **47**, 49–64.
- Raven, J. A. 1994b The significance of the distance from photosynthesising cells to vascular tissue in extant and early vascular plants. *Bot. J. Scotland* **47**, 65–82.
- Raven, J. A. 1995 Symplasmic proton fluxes in photosynthesising and developing plant tissues. *Biol. Rev.* **70**, 189–224.
- Raven, J. A. 1996 Into the voids: the distribution, function, development and maintenance of gas spaces in plants. *Ann. Bot.* **78**, 137–142.
- Raven, J. A. 1997a The vacuole: a cost–benefit analysis. *Adv. Bot. Res.* **25**, 59–86.
- Raven, J. A. 1997b Inorganic carbon acquisition by marine autotrophs. *Adv. Bot. Res.* **27**, 85–209.
- Raven, J. A. 1997c Putting the C in phycology. *Eur. J. Phycol.* **32**, 319–333.
- Raven, J. A. 1998 Extrapolating feedback processes from the present to the past. *Phil. Trans. R. Soc. Lond. B* **353**, 19–28.
- Raven, J. A. 1999 The size of cells and organisms in relation to the evolution of embryophytes. *Pl. Biol.* **1**, 2–12.
- Raven, J. A. & Farquhar, G. D. 1990 The influence of N metabolism and organic acid synthesis on the natural abundance of C isotopes in plants. *New Phytol.* **116**, 505–529.
- Raven, J. A. & Smith, F. A. 1976 Nitrogen assimilation and transport in vascular land plants in relation to intracellular pH regulation. *New Phytol.* **76**, 413–431.
- Raven, J. A. & Wollenweber, B. 1992 Temporal and spatial aspects of acid-based regulation. *Curr. Topics Pl. Biochem. Physiol.* **11**, 270–294.
- Raven, J. A. & Yin, Z.-H. 1998 The past, present and future of nitrogenous compounds in the atmosphere and their interaction with plants. *New Phytol.* **139**, 205–219.
- Raven, J. A., Smith, S. E. & Smith, F. A. 1978 Ammonium assimilation and the role of mycorrhizas in climax communities in Scotland. *Bot. Soc. Edinb.* **43**, 27–35.
- Raven, J. A., Smith, F. A. & Smith, S. E. 1980 Ions and osmoregulation. In *Genetic engineering of osmoregulation: impact on plant productivity for food, chemicals and energy* (ed. D. W. Rains, R. C. Valentine & A. Hollaender), pp. 101–118. New York: Plenum Press.
- Raven, J. A., Osborne, B. A. & Johnston, A. M. 1985 Uptake of CO₂ by aquatic vegetation. *Pl. Cell Environ.* **8**, 417–425.
- Raven, J. A., Franco, A. A., de Jesus, L. L. & Jacob Neto, J. 1990 H⁺ extrusion and organic acid synthesis in N₂-fixing vascular plants. *New Phytol.* **114**, 407–417.
- Raven, J. A., Rothmund, C. & Wollenweber, B. 1991 Acid–base regulation by *Azolla* spp. with N₂ as sole N source and with supplementation with NH₄⁺ or NO₃[−]. *Bot. Acta* **104**, 132–138.
- Raven, J. A., Wollenweber, B. & Handley, L. L. 1992 Ammonia and ammonium fluxes between photolithotrophs and the environment in relation to the global nitrogen cycle. *New Phytol.* **121**, 5–18.
- Raven, J. A., Griffiths, H., Smith, E. C. & Vaughn, K. C. 1998 New perspectives in the biophysics and physiology of bryophytes. In *Bryology for the twenty-first century* (ed. J. W. Bates, N. W. Ashton & J. G. Duckett), pp. 261–275. *Proceedings of the Centenary Symposium of the British Bryological Society*. Leeds: Maney Publishing and The British Bryological Society.
- Raven, J. A., Evans, M. C. & Korb, R. E. 1999 The role of trace metals in photosynthetic electron transport in O₂-evolving organisms. *Photosynth. Res.* **60**, 111–149.
- Raven, J. A., Kübler, J. E. & Beardall, J. 2000 Put out the light, and then put out the light. *J. Mar. Biol. Assoc. UK* **80**, 1–27.
- Richardson, D. H. S. 1981 *The biology of bryophytes*. Oxford: Blackwells.
- Rintamäki, E. & Aro, E.-M. 1985 Photosynthetic and photo-respiratory enzymes in widely divergent plant species with special reference to the moss *Ceratodon purpureus*. *J. Exp. Bot.* **36**, 1677–1684.
- Rowell, P. James, W., Smith, W. C., Handley, L. L. & Scrimgeour, C. M. 1998 ¹⁵N discrimination in molybdenum- and vanadium-grown N₂-fixing *Anabaena variabilis* and *Azotobacter vinelandii*. *Soil Biol. Biochem.* **14**, 2177–2180.
- Smith, E. C. & Griffiths, H. 1996a The occurrence of the chloroplast pyrenoid is correlated with the activity of a CO₂-concentrating mechanism and carbon isotope discrimination in lichens and bryophytes. *Planta* **198**, 6–16.
- Smith, E. C. & Griffiths, H. 1996b A pyrenoid-based carbon-concentrating mechanism is present in terrestrial bryophytes of the Class Anthocerotae. *Planta* **200**, 203–212.
- Smith, S. E. & Read, D. J. 1997 *Mycorrhizal symbiosis*, 2nd edn. San Diego: Academic Press.
- Sonesson, M., Gehrke, C. & Tjus, M. 1992 CO₂ environment, microclimate and photosynthetic characteristics of the moss *Hylocomium splendens* in a subarctic moss. *Oecologia* **92**, 23–29.
- Sprent, J. I. 1987 *The ecology of the nitrogen cycle*. Cambridge University Press.
- Tarnawski, M., Melick, D., Rosen, D., Adamson, E., Adamson, H. & Seppelt, R. 1992 *In situ* carbon dioxide levels in cushions and turf forms of *Grimmia antarctica* at Casey Station, East Antarctica. *J. Bryol.* **17**, 241–249.
- Uemura, K., Suzuki, V., Shikanai, T., Wadano, A., Jensen, R. G., Chmara, W. & Yokota, A. 1996 A rapid and sensitive method for determination of relative specificity of RUBISCO from various species by anion-exchange chromatography. *Pl. Cell. Physiol.* **37**, 325–331.
- Watson, G. M. F. & Tabita, F. R. 1997 Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: a molecule for phyto-genetic and enzymological investigation. *FEMS Microbiol. Lett.* **146**, 13–22.
- Wellman, C. H., Edwards, D. & Axe, L. 1998 Permanent dyads in sporangia and spore masses from the Lower Devonian of the Welsh Borderland. *Bot. J. Linn. Soc.* **127**, 117–147.
- Wessels, J. G. H. 1994 Developmental regulation of fungal cell wall formation. *A. Rev. Phytopathol.* **32**, 439–459.
- Yéoh, H.-H., Badger, M. R. & Watson, L. 1981 Variations in kinetic properties of RuBPC among plants. *Pl. Physiol.* **67**, 1151–1155.