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THE ROYAL SOCIETY

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 bisphosphate 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 CO_2 affinities and of CO_2/O_2 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 CO_2 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

iochemistry *per se* currently seems to be the poor relative f molecular biology. Certainly the nucleotide sequence of enes with the appropriate nucleotide substitution rate for ne taxon under investigation is preferable to the epiphe-omenon of the biochemical outcome of the genetic code or the purpose of phylogenetic reconstruction, provided nat an appropriate analysis of the sequence data has een performed. However, studies of biochemistry do ave some significance in functional terms, which cannot e predicted from the currently known nucleotide equences.

To a considerable extent this is a result of the absence f complete nucleotide sequences for any embryophytes or or any of their closest relatives on the basis of nucleotide equence evidence, as well as much other evidence, which re members of the Class Charophyceae of the Division hlorophyta. Accordingly, we cannot predict the entire uite of enzymes that could be expressed by any embryo-hyte. Even when the *Arabidopsis* genome has been ompletely sequenced there will still be a need for omplete nucleotide sequences of embryophytes of the teridophyte and bryophyte grades of evolution, as well s for the Charophyceae. Accordingly, we do not know hich 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 bisphosphate 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	K_m (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

[a]Based 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 O_2-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 O_2-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 CO_2 (and H_2O) and ribulose bisphosphate to two molecules of phosphoglycerate, but also the competing reaction in which ribulose bisphosphate and O_2 form one molecule of phosphoglycerate and one of phosphoglycolate. The known RUBISCOs have a wide range of selectivity factors for CO_2 over O_2, and of affinity for CO_2, as well as for the maximum specific rate of carboxylation (mol CO_2 fixed per mol enzyme per second), with a generally inverse relationship between the selectivity factor and CO_2 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 CO_2 over O_2 and the lowest affinity for CO_2 in this RUBISCO clade are found in the cyanobacteria (table 1). A greater selectivity for CO_2 over O_2, and a higher affinity for CO_2, 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 C_4 and crassulacean acid metabolism (CAM) embryophytes have an even higher CO_2/O_2 selectivity and CO_2 affinity than do the aquatic plants, but less than the selectivity and affinity values for terrestrial C_3 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 CO_2 and O_2 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				
Funaria sp.	T	23	—	Yeoh *et al.* (1981)
Ceratodon purpureus				
protonemata	T	22.3	—	Rintamäki & Aro (1985)
shoots	T	19.4	—	
Fissidens rigidulus	A	42	—	Yeoh *et al.* (1981)
Pteropsida	T	16–23 (4)	—	Yeoh *et al.* (1981)
	T	15.4–15.7 (2)	20.4–24.5	Bird *et al.* (1982)
Sphenopsida	T	15.9	25.5	Bird *et al.* (1982)
Lycopsida	T	18	—	Yeoh *et al.* (1981)
Cycadopsida	T	14	—	Yeoh *et al.* (1981)
Ginkgopsida	T	23	—	Yeoh *et al.* (1981)
		17.9	25.1	Bird *et al.* (1982)
Coniferopsida	T	24	—	Yeoh *et al.* (1981)
		16.8	24.8	Bird *et al.* (1982)
Magnoliophyta	T	12–25 (27)	—	Yeoh *et al.* (1981)
	T	11.1–14.0 (10)	16.4–20.4 (10)	Bird *et al.* (1982)
	T	14.8–20.4 (5)	—	Rintamäki & Aro (1985)
	A	30–49 (7)	—	Yeoh *et al.* (1981)

[a] The 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; Tarnawaski *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; Tarnawaski *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 Tarnawaski *et al.* (1992), the vascular plant source is less likely especially for mainland Antarctica (Tarnawaski *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

ime, although earlier photosynthesis by terrestrial algae nd cyanobacteria, or allochthonous inputs (e.g. via enguins) must also be considered. This discussion uggests that the generality of high CO_2 levels around hotosynthesizing bryophyte shoots is not invariant in ither space or time, and that examination of the CO_2 nvironment for terrestrial bryophytes should proceed on case-by-case basis until sufficient data are available for etter generalizations. At all events it appears that there s considerable evidence for many terrestrial bryophytes hotosynthesizing for at least some of their life cycle, or n some localities, with CO_2 concentrations in the gas hase surrounding their photosynthetic tissues, which are ower than bulk atmosphere values, thus providing a otential rationale in natural selection for the high CO_2 ffinity and high CO_2/O_2 selectivity of RUBISCO from errestrial 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 ower CO_2 affinity than that of C_4 and CAM plants, and specially of terrestrial C_3 plants, but higher than that of yanobacteria. Most of the (primarily) aquatic green lgae, and many of the (secondarily) aquatic embryo- hytes, have inorganic carbon pumps, which maintain a igher CO_2/O_2 and absolute CO_2 concentration around UBISCO than in the natural medium. There are a umber of aquatic green plants (a few algae; all bryo- hytes; some vascular plants) which rely on diffusive CO_2 ntry followed by C_3 biochemistry; these are mainly reshwater organisms, where the CO_2 concentration is ften higher than that found at air equilibrium (Raven 991*a*, 1997*c*). This leads to a higher external CO_2/O_2 atio than would be expected at air equilibrium, and thus ncreases the carboxylase activity and lowers the oxygen- se activity of RUBISCO relative to that found in air- quilibrium solutions, granted the same diffusive restric- ions and RUBISCO activity and kinetics in plants rowing in air-equilibrium solutions as are found in lants in their natural CO_2 and O_2 levels.

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

An area of ignorance about the kinetics properties of RUBISCO in green plants which has more relevance o the evolution of the bryophyte grade of green plant rganization is that of those anthocerotes which have yrenoids (Smith & Griffiths 1996*a*,*b*). The pyrenoid is ound in many (but by no means all) algae with CO_2- oncentrating mechanisms, and seems to play a role nalogous to that of the carboxysome in cyanobacteria Badger *et al*. 1998; Raven 1997*b*,*c*). Smith & Griffiths 1996*a*,*b*) showed that those hornworts they tested that ad pyrenoids had a CO_2-concentrating mechanism, as hown by CO_2 accumulation, decreased photorespiration and $^{13}C/^{12}C$ 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 1997*b*,*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

CO_2 and O_2? 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 O_2 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). CO_2 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 CO_2 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 CO_2 levels than in present-day freshwaters, if the high CO_2 levels in the atmosphere offset the lower CO_2 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 CO_2 concentrations at the time of diversification of eukaryotic algae and the lower O_2 levels.

The earliest terrestrial (or at least amphibious) charophyceans and embryophytes would equally have been exposed to higher CO_2 levels than present atmospheric values, but O_2 levels no greater than the present levels. Any inputs of CO_2 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 CO_2 levels. Even if the early embryophyte RUBISCOs had CO_2/O_2 selectivities and CO_2 affinities that were lower than those for present-day C_3 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 CO_2 fixation than in present C_3 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 C_3 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, C_3 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 anthocerotes 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 C_3 physiology achieved their present high CO_2 affinity and high CO_2/O_2 selectivity. Such kinetic changes might be expected to relate to the low CO_2 level and high CO_2/O_2 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 CO_2 and O_2 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 CO_2 affinity, high CO_2/O_2 selectivity RUBISCOs, consideration of the small activation energy differences involved in these kinetic properties relative to the ancestral, low CO_2 affinity, low CO_2/O_2 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 CO_2 affinity, high CO_2/O_2 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 O_2-evolvers and that show similarities between embryophytes and charophytes are the enzymes

hat deal with active oxygen species. One example is the orm of superoxide dismutase that is expressed. The Cu–Zn uperoxide dismutase of the embryophytes and charohytes is not found in other green algae or in cyanoacteria; these use the Fe and Mn forms of the enzyme. Elsewhere the Cu–Zn enzyme occurs in the peridininontaining dinoflagellates, but in no other algae (see Raven *et al*. 1999), as well as in some bacteria, in 'true' ungi and metazoa (Chapman 1985). Some horizontal ene transfer must have occurred to explain the distribuion of Cu–Zn superoxide dismutase.

A further example of an enzyme that is involved in netabolism of active oxygen species is glutathione peroxiase. This enzyme is universal in metazoa and occurs in ome algae, i.e. diatoms and in *Chlamydomonas* in the Chlorophyta, Class Chlorophyceae; these algae accordngly have a Se requirement, at least when this enzyme is xpressed as a supplement to ascorbate peroxidase (see Raven *et al*. 1999). However, the Charophyceae and Embryophyta always use ascorbate peroxidase rather han glutathione peroxidase, this resembling other eukarotic algae and most cyanobacteria (Raven *et al*. 1999). ome flowering plants have a lipoperoxidase, which does ot use Se, and has a very low specific reaction rate; ssentially all hydrogen peroxide in plastids and cytosol is estroyed by ascorbate peroxidase (Raven *et al*. 1999).

A final example concerns metabolism of urea. Urea netabolism is initiated in charophytes and in embryohytes by urease; this contrasts with other green algae, which, like some fungi, have urea amido-lyase (see Raven 977). All other organisms that can metabolize urea have rease.

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 ot shared by charophytes that we shall consider in more etail in functional terms are the lipid materials of the uticle and its wax, the diversity of phenolics in lignin nd flavonoids, and the much greater accumulation and ecretion of low M_r organic acids and their anions Raven 1977, 1984*b*, 1991*b*, 1993, 1995; Raven *et al*. 1980; Chapman 1985; Graham 1993; Edwards *et al*. 1996). The uticle and its wax are not present, at least in a chemially verified form, in the characean algae (but are also acking in some bryophytes): Graham (1993). Lignin and avonoids seem to be restricted, among extant plants, to mbryophytes, with lignin limited to (eu)tracheophytes, lthough lignans are found in bryophytes (Graham 1993; Raven 1993; Kenrick & Crane 1997). We note that earlier uggestions that charophycean algae can synthesize flavoloids are not currently well-supported (Graham 1993; Raven 1993). Finally, it is clear that low M_r organic acid nd organic anion accumulation and secretion is much nore widespread in embryophytes than in algae, ncluding charophycean and other green algae (Raven *et l*. 1980; Raven 1991*b*, 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, 1984*a*, 1996, 1997*a*; 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 chlorophycean 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 1984*b*). 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 charophycean algae of the family Characeae produced flavonoids

ppear 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 1991*c*,*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 C6 (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 1985*b*, 1989, 1991*b*, 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 1985*b*). The assimilation of NO_3^- in the shoots is generally associated with vacuolar accumulation of the organic anion ($malate^{2-}$, $oxalate^{2-}$) 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 1985*b*). 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 1985*b*). 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 1991*b*; 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 1985*b*). 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)*

rocess	H^+ flux	references
et 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)
et 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 $m^{-2} s^{-1}$	Raven & Wollenweber (1992), assuming K^+ influx involves K^+–H^+ symport (Maathuis & Sanders 1993) rather than a passive K^+ uniport
	230 nmol $m^{-2} s^{-1}$	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 $m^{-2} s^{-1}$

rocesses has a significant direct impact on intracellular cid–base balance.

The processes described so far increase or decrease hizosphere pH. The assimilation of NO_3^- with OH^- xcretion increases rhizosphere pH. NO_3^- assimilation ith organic anion efflux slightly increases rhizosphere H if the external pH is initially lower than the highest pK_a of the organic anion that is secreted. NH_4^+ assimilation and symbiotic N_2 assimilation lead to rhizosphere cidification by direct H^+ excretion. Additional organic cid synthesis, with H^+ excretion in exchange for some ther cation, or organic acid excretion *per se*, lowers xternal pH, exacerbating the pH decrease associated ith NH_4^+ assimilation or symbiotic N_2 fixation or miting or reversing the pH increase associated with NO_3^- assimilation (Raven & Wollenweber 1992; table 3).

Before considering the impact of these processes on utrient acquisition from the soil, it is necessary to introuce a process which causes pH increase at the apex of rowing roots and root hairs (and, probably, bryophyte hizoids and the rhizoids of free-living gametophytes of ree-sporing vascular plants), i.e. the external and nternal (to the plant cell or organ) circulation of electric urrent carried by H^+ (mainly, in the internal and xternal aqueous phases of the pathway, as buffered H^+) see Raven 1991*b*, 1995; table 2). The circulation of H^+ an include a symplasmic flux in the opposite direction of rganic anions which can be metabolized to consume H^+ t the sink just as their synthesis generated H^+ (Raven 991*b*, 1995). A modification of this process presumably nderlies the secretion of organic anions (malate) around he apex of roots in response to challenge by soluble xternal Al (Kochian 1995). The likely processes here are nalic acid synthesis in more basal root regions, with H^+ xcretion and K^+ uptake, and two K^+ malate^{2-} transfer o the apex, followed by two K^+ malate^{2-} efflux.

The changes in pH, and organic acid (and organic nion) concentration of the rhizosphere which are nduced by land plants have effects on nutrient acquisition Raven *et al* 1990; Marschner 1995) and on limiting amage 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 Fe^{2+} 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 O_2-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 1991*b*; 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 1991*b*; 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 NH_4^+ and NO_3^-.

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

hosphates, has not apparently been investigated for bryohytes with rhizoids/rhizomes/mycorrhizas in aerobic oils. However, the hypothesis suggested here (Raven 991*b*; Raven *et al*. 1980, 1990) gives a role along these lines o bryophytes containing high levels of low M_r organic nions in P and Fe acquisition. The finding that free-oating (secondary aquatic) heterosporous fern sporohytes, with a corresponding inability to influence P and Fe vailability from sediments via H^+ excretion and organic cid excretion from their roots, have relatively low contents f low M_r organic acid anions (Raven *et al*. 1991; Martins-ouçao *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, n the more basal parts of the root system or of root hairs, re amplified by the active H^+ efflux from the symplasm n the longitudinal circulation of electric current carried y H^+ (free, buffered or released/absorbed by biohemical changes to organic molecules) (Raven 1991*b*, 995; Raven & Wollenweber 1992). However, there is an bservable alkalization of the rhizosphere around the pices of roots and root hairs, even in root systems with a arge net efflux of H^+ (as H^+ or as undissociated organic cids which dissociate at the rhizosphere pH value) from ne root systems as a whole in NH_4^+ or N_2 assimilation Raven 1991*b*, 1995; table 3) related to the H^+ circulation. his has been related to the acquisition of Mo (see Raven 988*b*, 1991*b*, 1995; Raven *et al*. 1990). This essential lement is required in especially large amounts by mbryophytes that are using NO_3^- as their N source (due o 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 (1988*b*). lants growing on NO_3^- generally cause an overall ncrease in rhizosphere pH, although this can be reversed n plants with very high contents of anions of low M_r rganic acids (Raven & Smith 1976; Raven *et al*. 1980, 990; Raven 1985*a*,*b*; Raven & Farquhar 1990), so that his would favour Mo acquisition. However, even here ne root apex is the most alkaline region of the rhizoohere (Raven & Wollenweber 1992), so that this could e the most important region of the root surface for Mo ptake; this has yet to be tested. By contrast with growth n NO_3^-, the growth of (symbiotic) plants with N_2 as heir N source invariably causes a net acidification of the hizosphere (averaged over the whole root surface), so hat these plants are doubly disadvantaged compared ith plants growing on NO_3^- with respect to Mo supply: heir need for Mo on a biomass basis is greater if the ame specific growth rate is to be achieved, and the vailability of Mo from the rhizosphere is lower, granted milar soil bulk phase mean pH and Mo concentrations or growth on the two N sources. Accordingly, it might be xpected that the apical zone of rhizosphere alkalinizaon of roots and root hairs (and, perhaps, mycorrhizas) ight be important in Mo acquisition. Again, this seems ot to have been tested. The lowest rhizosphere Mo vailability might be expected for NH_4^+-grown plants, hich cause the greatest overall net acidification of the hizosphere. However, the Mo requirement (e.g. for such ow-content Mo enzymes as xanthine dehydrogenase) is ery small for NH_4^+-grown plants and is presumably satisfied by apical rhizosphere alkalinization in the face of large bulk rhizosphere acidification.

A further possible role of apical alkalinization 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 alkalinization of the rhizosphere by the circulating current carried by H^+ (Raven 1991*b*; Raven & Wollenweber 1992). Again, this has not yet been tested. The secretion of malate^{2-} 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 malate^{2-} secretion into an acid medium (such as favours Al toxicity) will itself raise rhizosphere pH due to protonation of one of the malate^{2-} carboxylate anions if the medium pH is close to, or below, the $pK_{a'2}$ 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 alkalinization 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 alkalinization 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

nions in the bryophyte as well as by any circulating urrent carried by H^+. Al toxicity in bryophytes has been ery little investigated, so nothing can be said about the ole of rhizosphere alkalinization at growing tips where ell division occurs.

Having examined the possible nutritional roles of the ccumulation of organic acid anions by embryophytes, nd its relationship to circulating current carried by H^+, remains to consider the evolutionary aspects of these henomena: are the biophysical properties of extant Charophyceae in any way uniquely suited to the ances- ors of these algae being the progenitors of the embryo- hytes? It is prudent to warn the reader that no nequivocal answer can currently be given, but that here are indications that the Charophyceae are appro- riate ancestors.

Raven (1991*b*, 1995) has considered the significance of irculating currents carried by H^+ in embryophytes in elation to nutrient acquisition (acidification, alkaliniza- ion of parts of the rhizosphere) and in relation to other nodes of rhizosphere acidification (organic acid synthesis with organic acid excretion or H^+ excretion in exchange or some (non-H^+) cation) in an evolutionary context. Circulating currents probably carried by H^+ (and Ca^{2+}) ave been reported for planktonic desmids (Charo- hyceae sensu lato), while the Characeae (Charophyceae) learly have circulating currents, carried by H^+, around heir shoots. This has been especially investigated in the ontext of the acid–base 'banding' of ecorticate inter- odes, which play an important role in acquisition of norganic C from HCO_3^- in alkaline waters, and which re functionally analogous to the acid abaxial surface/ lkaline adaxial surface polarity of the leaves of some secondarily aquatic) freshwater submerged flowering lants (Raven 1991*b*, 1995). Both the Characeae and these ubmerged flowering plants are rhizophytes (i.e. have oots or rhizoids in sediments which commonly have igher nutrient concentrations than does the bulk queous phase in which the photosynthetic shoots occur), lthough some freshwater and marine benthic flowering lants are haptophytes, i.e. are attached to rock surfaces e.g. the freshwater Podostemaceae; some seagrasses such s *Phyllospadix* spp.) (Raven 1984*b*). The sediments in hich parts of these rhizophytes are embedded can be noxic, in which case the requirement for acidification of, nd release of chelating organic acids to the rhizosphere s less obviously important in terms of P and Fe acquisi- ion. This is because the bulk sediment has Fe in the e(II) form, which is at once available to the rhizoid and nable (contrast Fe(III)) to bind inorganic phosphate. hese early rhizophytes (rhizoids of *Palaeonitella* are very ell-preserved in the Lower Devonian Rhynie Chert, ome 395 Myr old) might then not have used circulating urrents or net H^+ and organic acid secretion into the hizosphere in Fe and P nutrition (Raven 1984*b*, 1991*b*; Raven & Wollenweber 1992). They may not even have sed apical rhizoid alkalinization in Mo acquisition as Mo could be obtained from the bulk waterbody. Further- nore, Mo may in any case have not been very important or assimilation of at least rhizoid-derived N, since these noxic sediments do not permit nitrification of mineral- zed (ammonified) organic N, and denitrify such NO_3^- s 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 acidify- ing 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, oxygen- ated 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 alkalinization of the apex of the root/rhizoid system while the rest of the root system may not be greatly acidified or alkalinized (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 1991*b*), while H^+ circulation occurs in freshwater Charophyceae sensu lato and in freshwater *Vaucheria* (Tribophyceae): Raven (1991*b*). Rhizophytic marine green macroalgae have circulating H^+ currents in the case of the eukaryotic char- acean *Lamprothamnium* (and probably the seagrasses, which are flowering plants); these are secondarily marine organisms (Raven 1991*b*). Primarily marine green algal macrophytes in the Ulvophyceae are frequently rhizo- phytic, at least among the acellular organisms in warmer waters, and have circulating currents carried by Cl^- (Raven 1991*b*). Such circulating currents cannot directly lead to acidification or alkalinization of zones around the rhizoids (Raven 1991*b*). While there is evidence that the ulvophycean rhizophyte *Caulerpa* can obtain N and P from sediments via their rhizoids (Raven 1984*a*,*b*, 1991*b*; Chisholm *et al.* 1996), it is thus unlikely that modification of rhizosphere pH by circulating currents is involved, though erosion of calcareous phosphate-containing parti- cles 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 1984*a*,*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 bio- chemical features be related to the Early–Mid-Palaeozoic environment (Chapman 1985; Edwards 1996, 1998;

Edwards *et al.* 1998; Raven 1977, 1984*a*, 1993, 1994*a*,*b*, 1998, 1999; Wellman *et al.* 1998)? The O_2 concentration in the atmosphere was similar to that found today (Berner & Canfield 1989), while CO_2 in the Ordovician–Silurian–Early Devonian was 12–22 times the present value (Berner 1997, 1998). O_2 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 O_2 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 1994*a*,*b*.) This UV-B attenuation by natural waters is not dependent on the presence of dissolved O_2 (Kirk 1994*a*; 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 O_3 screen for UV-B.

Atmospheric O_2 levels such as those found in the Early–Mid-Palaeozoic would have permitted nitrification of NH_4^+ 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 O_2-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 NH_3 concentration in seawater exceeds (and has always exceeded) the NH_3 concentration in the above-ground organs of terrestrial plants in equilibrium with the atmosphere. This higher NH_3 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 NH_3 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 (1988*a*), Raven *et al.* (1992) and Raven & Yin (1998) show that the free NH_3 concentration in the surface layers of the ocean in which photosynthetic growth can occur is less than that in plant shoots interacting with the NH_3 level of 'clean' (i.e. without anthropogenic NH_3 inputs) air, so that if anything, PAL activity to generate cinnamic acid is less constrained by NH_3 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 O_2 as deduced from palaeogeochemical 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 O_2 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 O_2. While these suggestions correlated approximately with the best available reconstructions then available (Budyko *et al.* 1985) of the variation of atmospheric O_2 throughout the Phanerozoic, more recent reconstructions of O_2 in palaeoatmospheres (Berner & Canfield 1989) fail to show the O_2 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* (NH_3 mass action effect on PAL) and the O_2 content of the palaeoatmospheres (O_2 detoxification pathways adapted to biosynthesis of novel metabolites) may not be sustainable in the face of more detailed palaeogeochemical 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 1984*b*; 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

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

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

A further example of N-containing compounds and on-N-containing compounds fulfilling the same function n different organisms is that of UV-B screening ompounds. Marine algae from many Divisions, as well s cyanobacteria, contain mycosporine-like amino acids s quantitatively major UV-B screens, and many near-urface marine invertebrates use these compounds btained directly or indirectly from dietary algae as UV-B screens (Raven 1991*c*; Franklin & Forster 1997; Ehling-chulz & Scherer 1999). However, embryophytes use henolic, non-N-containing compounds (e.g. flavonoids, ignin) as the major UV-B-screens. No data seem to be vailable on the UV-B-screening compounds used by the Charophyceae, which are probably primarily freshwater ith some euryhaline representatives. It must be admitted hat some embryophytes lack this 'N-free' paradigm; thus he Centrospermae (Caryophyllales) have the two N-ontaining betalains (alkaloids) betaxanthin and beta-yanin to give yellow–orange and blue colours (Harborne 982) respectively, while many putative 'defence com-ounds' (e.g. alkaloids) of angiosperms contain N, and re by no means confined to symbiotically N_2-fixing lants. Furthermore, some primarily (algal) and secondarily (vascular plants) halophytic photolithotrophs use N-ontaining compatible solutes such as proline and glycine etaine, while others use N-free linear or cyclic polyols, i- and oligosaccharides and dimethylsulphoniopropionate Raven 1985*b*).

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

4. CONCLUSIONS AND PROSPECTS

Qualitative and quantitative differences in the iochemistry of embryophytes relative to charophytes, nd between bryophytes and tracheophytes in the embryo-hytes, cannot generally be related to the Lower Palaeo-oic environment.

Even complete nucleotide sequences of genes may not ermit in the near future the prediction of all significant inetic values of the enzymes they encode, e.g. CO_2 affinity and CO_2/O_2 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.

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