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The Potential effect of Low Cell Osmolarity on Cell Function through decreased concentration of enzyme substrates

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Highlight

Algae with low intracellular osmolarities and high specific growth rates have lower metabolite concentrations than are modelled to occur for these growth rates. The paper suggests possible explanations for this.

Abstract

Some freshwater algae have lower (< 130 osmol m^{-3}) intracellular osmolarities than most others (> 180 osmol m^{-3}). Low osmolarities are related to the presence of flagella and low energy cost of active water efflux following downhill water influx unconstrained by cell walls covering the plasmalemma, and low resource cost of cell-wall synthesis with the same mechanical degree of safety. One consequence of low intracellular osmolarity is limitation on the concentration of metabolites, i.e. substrates and products of enzyme activity. Models of the flux through metabolic pathways, and hence specific growth rate, using steady-state concentration of enzymes and metabolites, have involved organisms with intracellular metabolites > 280 osmol m^{-3} where the metabolite concentrations are much greater than the total osmolarity of some freshwater algae. Since the protein concentration (mol m^{-3}) in the cells and the specific growth rates of

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freshwater cells with low and with higher intracellular osmolarity are closely similar, the models of tradeoffs between enzyme and metabolite concentrations for cells with high intracellular osmolarity needs modification for cells with low intracellular osmolarity. The soluble free radical scavenger ascorbate can be as little as 0.2% of the low intracellular metabolite concentration (mol m^{-3}) of low intracellular osmolarity cells.

Keywords: active water transport; ascorbate; enzyme concentration; metabolite concentration; osmolarity; trade-offs; UV screens

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Introduction

There is a wide range of intracellular osmolarities of photosynthetic organisms. The highest values are found in organisms living in seawater or, especially, hypersaline habitats, e.g., *Dunaliella* (Chlorophyceae) (Borowitzka and Brown 1974, Ehrenfeld and Cousins 1982, Katz and Avron 1985) and other algae (and certain terrestrial flowering plants (Kirst 1977,1989; Munns and Tester 1982). The lowest values (< 130 osmol m^{-3}) are found for four (three chlorophycean and one chrysophycean) freshwater flagellates and a freshwater giant-celled (chlorophycean) alga (Raven 1982, 1984, 1995; Komsic-Buchman et al. 2014; Raven and Doblin 2014). Much more emphasis has been given to the upper end and the middle than to the lower end of the range of osmolarities. After discussing the derived nature, and the rationale(s), of low intracellular osmolarities, this paper discusses possible constraints of a low intracellular osmolarity, and particularly the proposed trade-offs of metabolite (enzyme substrate) and protein (enzyme) concentrations in achieving the observed metabolic fluxes (Veneklaas et al. 2012; Tepper et al. 2013; Mettler et al. 2014; Lambers et al. 2015; Barenholz et al. 2016; Davidi et al. 2016; Noor et al. 2016; Park et al. 2016; Davidi and Milo 2017), with implications for RNA content (Flynn et al. 2010; Loladze and Elser 2011; Veneklaas et al. 2012; Raven 2013a; Raven 2013b; Lambers et al. 2015) and hence specific growth rate (Flynn and Raven 2017). Further contributors to 'metabolite load' are the terminal metabolic products that act as $^{\bullet}OH$ radical scavengers and as soluble intracellular UV screens (Raven 1995); these are also discussed.

Occurrence of, and possible rationale for, low intracellular osmolarity.

Some freshwater algae , and other organisms have very low intracellular osmolarity relative to other freshwater, and marine, cells (Raven 1982, 1984, 1995; Komsic-Buchmann et al. 2014; Raven and Doblin 2014). The low intracellular osmolarity algae (with values rounded to two significant figures) are the chlorophycean algae *Chlamydomonas reinhardtii* (62-130 osmol m^{-3}), *Scherffelia dubia* (93 osmol m^{-3}) and *Hydrodictyon africanum* (59 - ≥79 osmol m^{-3}), the charophycean *Mesostigma viride* (85 osmol m^{-3}) and the chrysophycean *Poteriochromonas malhamensis* (75 osmol m^{-3}) (Raven 1982, 1995; Komsic-Buchmann et al. 2014). Some components of the osmolarity are relatively well known for *Hydrodictyon africanum*, since the higher value (≥79 osmol m^{-3}) for osmolarity is derived from the content of major inorganic ions (Raven and DeMichelis 1979; Raven 1995). It therefore is a lower limit; the lower, directly determined, value for intracellular osmolarity for *Hydrodictyon africanum* was obtained under different growth conditions.

Other freshwater algae, e.g., the multicellular Charales (Charophyceae) that are largely made up of giant coenocytic cells, have osmolarities of at least 190 – 320 osmol m^{-3}, based on the measured vacuolar ion concentration (Table 8.2. of Raven 1984) and an osmotic coefficient (osmol m^{-3} for a given mol m^{-3} solution) of 0.9 (Appendix 10 of Milburn 1979). More comparable to the four low intracellular osmolarity algal flagellates in terms of cell size and the absence of a large central vacuole (Table 8.1 of Raven 1984) is the walled non-flagellate alga *Chlorella pyrenoidosa* (Treboxiophyceae) (Scott 1943; Schaedle and Jacobson 1965; Barber and Shieh 1972). Based on the intracellular cations that were measured, the intracellular osmolarity of *Chlorella pyrenoidosa* is at least 150-330 osmol m^{-3} (Supplementary Information 1).

Taking 'average' seawater as a typical high-osmolarity environment (1100 osmol m^{-3}), a number of organisms living in seawater have lower osmolarity than does seawater, e.g., teleost fishes (toadfish 400 osmol m^{-3}) as do their freshwater (210-250 osmol^{-3}) and

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terrestrial (310-330 osmol $^{-3}$) descendants (Takei 2000). The lower osmolarity of these extant marine organisms than that of surrounding seawater has been attributed to the origin of life in seawater more than 3.5 billion years ago when seawater had a salinity less than half of the present value (Takei 2000). However, more recent approaches suggest that Archaean seawater had an osmolarity 1.5 – 2.0 times the present value (Knauth 2005), so some other explanation is needed for the lower than extant seawater osmolarity of some extant marine organisms. Regardless of the osmolarity, the inorganic ion concentration in the cytosol and (in eukaryotes) mitochondrial matrix and plastid stroma of organisms other than certain archaeans is only about half or less that of seawater (e.g., Raven 2017). Recent evidence is consistent with a freshwater origin of photosynthetic eukaryotes (Blank 2013; Brasier 2013; Sánchez-Baracaldo et al. 2017a, 2017b), although it is not clear how this relates to intracellular osmolarity of the earliest photosynthetic eukaryotes.

The null hypothesis for freshwater algae with low intracellular osmolarities is that the contribution to osmolarity of the cytosol, mitochondrial matrix and chloroplast stroma of the bulk of metabolites is similar to that of cells with higher intracellular osmolarity, and the decreased intracellular osmolarity comes from decreased concentrations of the major solutes such as K^+ and glutamate$^-$. For K^+, the dominant cation in concentration and charge terms, in compartments of high protein diversity, with osmolarity can be made for *Chlamydomonas reinhardtii*, although with different growth conditions and algal strains for the different measurements. There are data on the K^+ (and Na^+) concentration in *Chlamydomonas reinhardtii*: Ronkin and Buretz (1960) found 20 mol K^+ m^{-3} (and 1.4 mol Na^+ m^{-3}), while Malhotra and Glass (1995a,b) found 65 mol K^+ m^{-3} in the cytosol and 64 mol m^{-3} in the chloroplast for cells grown in 0.1 mol K^+ m^{-3}. The difficulties of measuring osmolarity and ion concentration in *Chlamydomonas reinhardtii*, and possibly strain differences, are shown by the concentration of K^+ of 20 – 65 mol m^{-3} (uncorrected for the osmotic coefficient, and requiring inorganic or organic counter-ions) compared with the osmolarity of 62-130 osmol m^{-3}). A large number of enzymes require K^+ to activation of catalysis; in a few cases (none from algae) full activation of the enzyme needs more than 100 mol K^+ m^{-3} (Evans and Sorger 1966; Evans and Wildes 1971). There seem to be no data on the K^+ affinity for enzyme activation in algae with low intracellular osmolarities (Raven 1995). Other contributors to intracellular osmolarity are low molecular mass organic compounds, including

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metabolic intermediates as well as end-products such as UV-screening and reactive oxygen-scavenging compounds (Raven 1982, 1984, 1987, 1995,1997).

A rationale for the low intracellular osmolarity of the freshwater flagellates *Chlamydomonas reinhardtii*, *Mesostigma viride*, *Poteriochromonas malhamensis* and *Scherffelia dubia* is that there is plasmalemma area not surrounded by a rigid cell wall, and the resulting osmotic water entry requires active water efflux to prevent bursting of the protoplast (Raven 1982, 1984,1995; Komsic-Buchmann et al. 2014; Raven and Doblin 2014). The osmotic water entry is directly proportional to the water potential difference across the plasmalemma, and the energy cost of active water efflux is also directly proportional to the water potential difference (Raven 1982, 1984, 1995, Raven and Doblin 2014). For the walled vegetative cells such as those of *Hydrodictyon africanum*, the thickness of the cell wall needed to confine turgor with a defined safety margin, a given wall composition and cell shape and dimensions, is directly proportional to the osmolarity difference across the plasmalemma (Raven 1987, 1997). The low intracellular osmolarity of all five algae can therefore be rationalised in terms of decreasing resource costs of cell volume regulation.

Not considered previously in the context of low intracellular osmolarities are the constraints imposed on intracellular osmolarities by the requirements of the intracellular metabolite concentrations, acting as substrates for enzymes. These are now considered, with updates on the previously considered roles of metabolites as intracellular $^{\bullet}OH$ radical scavengers and soluble UV screens.

Constraints on metabolite concentrations: effects of concentration of enzymes and other proteinaceous catalysts

The specific growth rate of a cell at a given temperature is directly related to the metabolic flux, which in turn depends on the concentration of proteins (mol m^{-3} cell volume). For each enzyme protein, the *in vivo* specific reaction rate depends on the maximum, substrate-saturated, specific reaction rate *in vivo*, the substrate concentration dependence (= substrate affinity) of the enzyme activity *in vivo*, and the steady-state

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substrate concentration at the enzyme active site *in vivo*. *Chlamydomonas reinhardtii* has similar maximum specific growth rates to non-flagellate freshwater green algae of similar size and at the same temperature but with a higher intracellular osmolarity (Table 1).

While the concentration of proteins is not directly related to enzyme activity, it is worth considering the protein concentration in low and high osmolarity green microalgae. There is a paucity of relevant data on the protein concentration in cells with low intracellular osmolarity. Kliphuis et al. (2012) showed that there is 0.376 – 0.425 g protein per g dry matter in chemostats with growth rates in the range 0.018 – 0.064 h^{-1} in *Chlamydomonas reinhardtii* CC1690. Zuñiga et al. (2018) found that there is 0.48 g protein per g dry matter in N-replete cultures which decreases to 0.14 g protein per g dry matter in N-depleted cultures in *Chlorella vulgaris* UTEX 395. Other data are for protein per cell. Schmollinger et al. (2014) found 3 pg protein per *Chlamydomonas reinhardtii* cell, while John et al. (1982) found 8 pg protein per *Chlorella* strain 211-8p. Before concluding that the higher-osmolarity *Chlorella* than the lower-osmolarity *Chlamydomonas* has higher protein per unit dry matter, it must be remembered that a spherical cell only needs to have a diameter 1.34 times greater and hence a 8/3 greater volume, and a similar dry matter per unit volume, to have the same protein per unit dry matter as in the smaller cell. Figure 6A of Hsieh et al. (2013) gives the quantities, in zmol (10^{-21} mol) per cell of a very large range of proteins in *Chlamydomonas reinhardtii*: however, since some proteins may not have been measured, the sum of these values, after conversion from mol to mass for each individual protein would under-estimate the mass of total protein per cell or per unit dry mass. In the absence of measurements of cell size the data of Hsieh et al. (2013) cannot be used to calculate the intracellular concentrations of proteins.

Mettler et al. (2014) determined the content of 644 proteins, including photosynthesis-related enzymes, in *Chlamydomonas reinhardtii* growing at a limiting photon flux density (41 μmol m^{-2} s^{-1}, 400-700 nm) and in cells transferred to a higher photon flux density (145 μmol m^{-2} s^{-1}, 400-700 nm). As discussed below for metabolite concentrations, most enzymes of the Calvin-Benson cycle are substrate-limited in vivo, although for three, ribulose bisphosphate carboxylase/oxygenase (Rubisco), fructose-1,6-bisphosphate-1-phosphatase and sedoheptulose-1,7-bisphosphate-1-phosphatase,

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were close to substrate saturation in vivo (Mettler et al. 2014). Interestingly, these three enzymes have the greatest control strength in the Calvin-Benson cycle of terrestrial C_3 plants (Raines 2003; Zhu et al. 2007). It is also clear that the affinity of most enzymes are lower in vivo than in vitro, and that enzymes involved in photosynthesis and downstream reactions of growth are present in excess of the requirements for growth under light-limited conditions since increased light increased photosynthetic rate and then growth rate before there was an increased rate of protein synthesis (Mettler et al. 2014).

While the number of proteins encoded in the nuclear genome cannot be related to the protein number in, or the mass in the proteome, it is of interest that the flagellate *Chlamydomonas* has significantly more encoded proteins than the similarly sized non-flagellate green algae *Chlorella* and *Coccomyxa* (Table 2).

Constraints on metabolite concentrations: intermediary metabolites

For metabolite concentrations, the detailed analyses are for cells with relatively high intracellular osmolarities (≥ 280 osmol m^{-3}: Martinez de Mařanon et al. 1996; Table 3 of Cayley et al. 2000; Takei 2000). Park et al. (2016) determined the total intracellular concentration of measured metabolites in three cell cultures: *Escherichia coli* (240 mol m^{-3}), *Saccharomyces cerevisiae* (240 mol m^{-3}) and mammalian iBMK cells (180 mol m^{-3}); see also Bennett et al. (2009), Bar-Even et al (2011), Tepper et al. (2013) and Yang et al. (2017) for additional data and analyses on *Escherichia coli* and *Saccharomyces cereviseae*. These values are clearly in excess of what can be accommodated in any of the five freshwater algae with low osmolarities (59-130 osmol m^{-3}, especially granted the fraction of osmolarity occupied by just one of the inorganic ions, K, in *Chlamydomonas reinhardtii* considered above.

Despite the large body of metabolomic data available for *Chlamydomonas reinhardtii* (Bölling and Fiehn 2005; Lee and Fiehn 2008; Timmins et al. 2009; Renberg et al. 2010; Lee and Fiehn 2013; Kleessen et al. 2015; Park et al. 2015; Williamme et al. 2015; Lee et al. 2016) , the only measurements of intermediary metabolites that are in units of the

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intracellular concentrations of metabolites are those of Mettler et al. (2014). This paper compares cells growing at a limiting photon flux density (41 μmol $m^{-2} s^{-1}$, 400-700 nm) with cells transferred to a higher photon flux density (145 μmol $m^{-2} s^{-1}$, 400-700 nm). While120 intermediary metabolites were measured, concentration values are only given for 16. For these 16 intermediary metabolites the combined intracellular concentration is 6.2 mmol m^{-3} for the low light controls, and 12.4 mmol m^{-3} for cells transferred to the higher irradiance. While these values are readily accommodated in the range of measured intracellular osmolarities of *Chlamydomonas reinhardtii*, there are hundreds (at least) more intermediary metabolites in the cell. The concentration of metabolites is usually lower than that of the corresponding enzyme active site in *Chlamydomonas reinhardtii* (Mettler et al. 2014).

Since the total metabolite concentrations must be low enough to be accommodated by the low measured intracellular osmolarity in the four flagellate algae considered, with a similar enzyme concentration and *in vivo* enzyme kinetics the lower metabolite concentration would result in a lower active site occupancy, and hence a lower enzyme activity. This lower metabolic flux would result in a slower growth rate. However, this is not borne out by the specific growth rates shown in Table 1, i.e. there is not a positive correlation between the *total* intermediary metabolite concentration and growth rate. For the best-investigated organisms with high concentrations of intermediary metabolites, i.e. *Escherichia coli* and *Saccharomyces cerevisae*, there is a negative correlation between the concentration of *some* metabolites and growth rate, although other metabolites show a positive correlation (Somsen et al. 2000; Park et al. 2011). This is also the case for *Arabidopsis thaliana* (Sulpice et al. 2009; Pyl et al. 2012). The data of Mettler et al. (2014) show no such negative correlation beween growth rate and (a) metabolite(s) in *Chlamydomonas reinhardtii.*

One possibility for the high growth rate of *Chlamydomonas reinhardtii* despite the low total metabolite concentration is changes in the properties (increased substrate-saturated specific reaction rate, increased substrate increased substrate and activator affinity, decreased inhibitor affinity), or environment (decreased inhibitor concentration, including feedback inhibitors in metabolic sequence) of enzymes and other proteinaceous catalysts. The only demonstrated example of these possibilities is the enzyme glutamine synthetase, where the affinity for glutamate of the enzyme from

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Chlamydomonas reinhardtii is greater than that of the enzyme from other species (Cullimore and Sims 1981); however, the substrate-saturated enzyme activity was not reported.

A further possibility for maintaining a high growth rate is increased microcompartmentation in cells with low intracellular osmolarity. Maintaining the substrate concentration at the enzyme active site despite higher enzyme specific reaction requires a larger diffusive flux of substrate from the producer enzyme to the consumer enzyme. The diffusion coefficient of metabolites in high-protein compartments is less than that in water (Ellis 2001; Verkman 2002; Brangwynne et al. 2008; Novack et al. 2009); for organic compounds with M_r 170-324 Dalton the diffusion coefficient in mammalian cytosol is 1.9 – 2.6 times that in water (Mastro et al. 1984). With a given diffusion coefficient and diffusion distance, this requires a higher mean concentration of the metabolite. With a completely mixed cytosol (or chloroplast stroma or mitochondrial matrix) the diffusion path increases with compartment size. However, there is evidence of metabolic microcompartmentation within a membrane-delimited compartment such as the cytosol, the mitochondrial matrix and the plastid stroma, thus decreasing diffusion distance for metabolites in a given pathway (Conrado et al. 2008; Sweetlove and Fernie, 2013; Angeles-Martinez and Theodoropoulos 2015).

Constraints on metabolite concentrations: terminal metabolites and their roles as UV screens and free radical scavengers

Some terminal, rather than intermediate, soluble metabolites are concerned with damage limitation by screening UV-B radiation and scavenging the very damaging $^{\bullet}OH$ radical (Raven 1995). These functions depend on the concentration of the metabolites.

For UV screening, the prediction is that, for a given screening compound and degree of protection of the nucleus in a given external UV radiation field, there is an inverse relation between effective cell diameter and the required concentration (Garcia-Pichel 1994; Raven, Finkel and Irwin 2005). For water-soluble intracellular UV screening compounds, e.g. mycosporine-like amino acids (MAAs), evenly distributed among cell

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compartments of spherical cells, the modelling of Garcia-Pichel (1994) shows that 'powerful' screening (i.e. decreasing the UV flux at a centrally located nucleus to 10% or less of that in the medium) by MAAs comprising 1% of the dry biomass is only possible for cells with a radius in excess of 200 μm. This size restriction excludes the four flagellates considered here, as well as the multinucleate *Hydrodictyon africanum* giant cells where the nucleii are within 10-30 μm of the cell surface (Raven 1987, 1997). With the wet weight:dry weight ratio given by Raven (1982) and a mean M_r of MAAs of 250 Dalton, the MAA concentration corresponding to 1% of the dry matter would be 5.2 mol m^{-3} or about 4-9% of the total intracellular osmolarity of the low-osmolarity cells, despite their failure to meet Garcia-Pichel's (1994) criterion of 'powerful' screening. Further detail on UV screening in cells with low intracellular osmolarity and comparison with cells of higher osmolarity is given in Supplementary Information 2.

. By contrast, free radical scavenger concentrations in compartments where free radicals are most damaging (cytosol, nucleoplasm, stroma, matrix) is predicted to be independent of cell size, other things being equal (see Halliwell and Gutteridge 2007). Data in Supplementary Information 3 show that the $^{\bullet}OH$ radical scavenger ascorbate accounts for 0.2-7% of the total intracellular osmolarity of freshwater flagellate algae with almost all the cell volume occupied by compartments prone to free radical damage (i.e. not the aqueous phase of vacuoles). These data do not show what, if any, other $^{\bullet}OH$ scavengers occur, or the residual damage by $^{\bullet}OH$ that must be repaired.

Conclusions and future work

There is a conflict among available data on the low intracellular osmolarity (< 130 osmol m^{-3}) of flagellate and certain large-celled freshwater algae and the high concentration of metabolites required for the enzyme specific reaction rates needed to account for the growth rate, based on data from cells with osmolarities > 280 osmol m^{-3}. Despite this apparent shortfall in metabolite concentrations in cells with low intracellular osmolarity, their specific growth rates are similar to those of cells of higher osmolarity and a similar protein concentration. Further work is needed on the concentration of metabolites (and proteins) in the algae with low intracellular osmolarity, and how the low metabolite concentrations can maintain the metabolic and specific growth rates.

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Table 1

Highest reported specific growth rate of flagellate and non-flagellate chlorophycean and trebouxiohycean green algae in the temperature range 25° C and 28° C. .

Organism	Specific growth rate at 25° C	Specific growth rate at 28° C	Reference
Chlorella pyrenoidosa[1,3] (van Niel)	2.1 d^{-1}		Sorokin & Krauss (1958)
Chlorella pyrenoidosa[1,3] 7-11-05	2.1 f^{-1}		Sorokin & Krauss (1958)
Chlorella pyrenoidoa[1,3]		2.4 d^{-1}	Yang & Gao (2003)
Chlorella vulgaris[1,3] Emerson	1.8 d^{-1}		Sorokin & Krauss (1958)
Chlamydomonas reinhardtii[2,4]	2.6 d^{-1}		Sorokin & Krauss (1958)
Chlamydomonas reinhardtii[2,4]		2.0 d^{-1}	Yang & Gao (2003)
Scenedesmus obliquus[2,3] WH 650	1.5 d^{-1}		Sorokin & Krauss (1958)
Scenedesmus obliquus[2,3]		1.6 d^{-1}	Yang & Gao (2003)

Footnotes

1Trebouxiophyceae

[2]Chlorophyceae

[3]Non-flagellate

[4]Flagellate

Variations in growth rate of microalgae with abiotic environmental conditions are best considered in the context of the definition of stress by Grime (1974) as any abiotic environmental condition that decreases the specific growth rate of which that organism is capable. Stressful conditions include high or low temperatures, high or low photosynthetic photon flux densities, high or low nutrient concentrations, and UV-B.

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Table 2

Organism	Genome size	Chromosome number	Protein coding genes	Reference
Chlamydomonas reinhardtii (Chlorophyceae)[1]	131 Mbp	17	15,143	Merchant et al. 2007
Chlorella vulgaris NC 64A (Trebouxiophyceae)[2]	46 Mbp	12	9, 791	Blanc et al. 2010
Coccomyxa subellipsoidea C-169 (Trebouxiophyceae)[3]	49 Mbp	20	9,831	Blanc et al. 2012

[1]Free-living flagellate; freshwater' cell wall

[2]Free-living or endosymbiotic (in ciliates) non-flagellate; freshwater; cell wall

[3]Free- living non-flagellate freshwater (other species of the genus are symbiotic); cell wall

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