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THE REGULATION OF NITRATE ASSIMILATION IN LOWER PLANTS: A CRITIQUE

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ABSTRACT

Nitrate assimilation requires the function and regulation of three processes, namely (1) the uptake and translocation of nitrate from the environment into the cell, (2) the reduction of the anion to ammonium via nitrate and nitrite reductases, and (3) the incorporation of the reduced nitrogen into amino acid precursors. All three processes are anabolic and endothermic and, consequently, must be thermodynamically coupled to catabolic and exothermic reactions. While most of the recent research on nitrate assimilation has focused on one or the other of these processes individually, less attention has been given to describing the energy sources required for their function and the regulation of the processes by thermodynamic coupling.

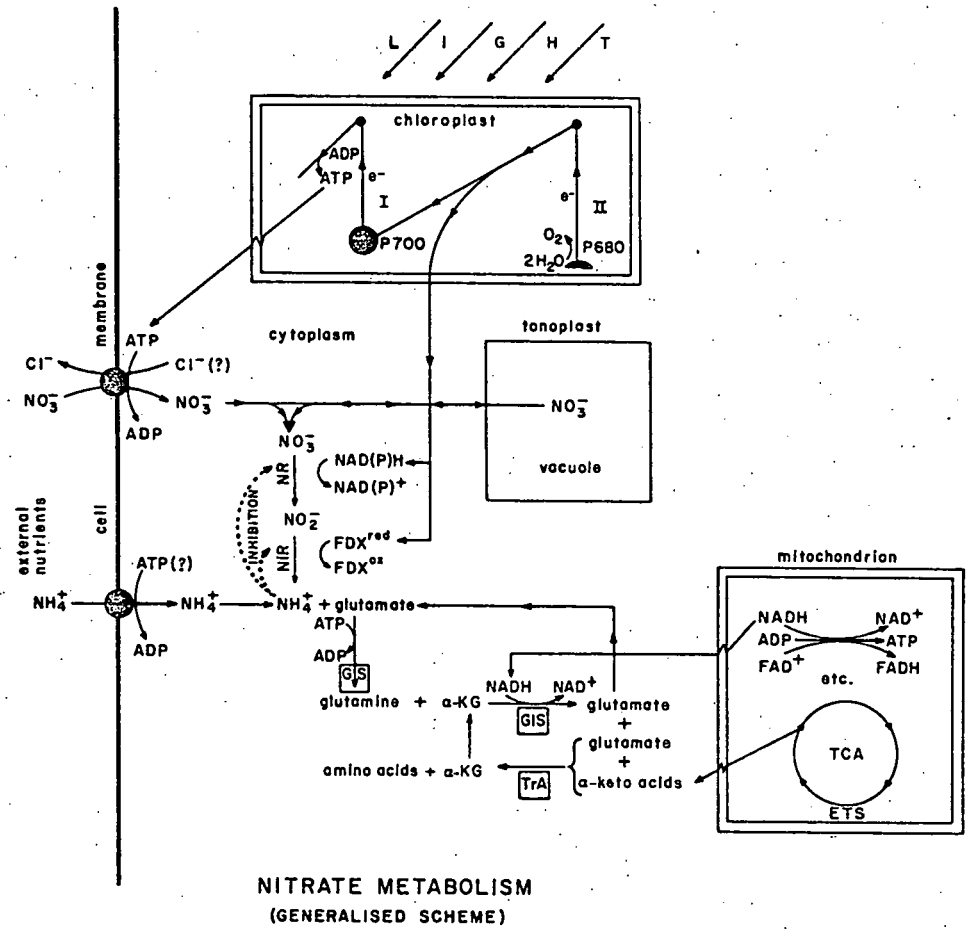
I. NITRATE UPTAKE

In order to utilize nitrate as a substrate for cellular anabolism, plants must transport the ion across the plasmalemma, against the negative chemical potential of the ion. The mechanism of passage of nitrate across cell membranes has been hypothesized by some investigators. Allusions to "permeases" and facilitated diffusion have been made by Packard and Blasco and Platt and Subba Rao;[1,2] however, no direct evidence has been published to support these speculations. The phospholipid bilayer, thought to be the major constituent of most plasma

membranes, is not very permeable to charged molecules like nitrate, so absorption of the ion must follow other routes into the cell. An alternative possibility might be a membrane-bound protein in the phospholipid bilayer that mediates the translocation of nitrate. This "ionophore" might simply allow diffusion, in which case it would be a true permease, following the original definition of the term by Monod;[3] or it may "pump" nitrate against the negative gradient of the chemical potential of the ion.

If the nitrate-accepting protein simply allowed diffusion of nitrate, the internal concentration of the ion should approximate the external concentration, providing no other process intervened. This hypothesis is difficult to test directly because in the steady-state, intracellular nitrate is continuously being reduced to ammonium, diminishing the intracellular nitrate concentrations. This type of diffusion has been called "trapping diffusion,"[4] the driving force being provided by the biochemical reduction of nitrate within the cell. This model implies that the nitrate uptake rate is a function of nitrate reduction, or specifically the enzyme(s) nitrate reductase (E. C. 1.6.6.1 and E. C. 1.6.6.3).

Nitrate uptake by marine unicellular algae can be described adequately by Michaelis-Menten kinetics, or the Lamgmuir adsorption isotherm.[5,6] If trapping diffusion were responsible for nitrate uptake by the cell, it should follow that the half-saturation constant for nitrate uptake should be of a similar order of magnitude as the half-saturation constant (K_m) for nitrate reductase. Determinations of K_m values for nitrate reductase indicate that these are usually in excess of nitrate concentrations present in natural seawater by about a hundred-fold.[7] Thus, it is improbable that nitrate reductase serves to provide a chemical gradient across the cell membrane; in fact, the cell must "pump" nitrate into the cytoplasm in order to reach effective substrate concentrations for a high degree of reducing efficiency (Fig. 1).



NITRATE METABOLISM
(GENERALISED SCHEME)

Fig. 1. A generalized scheme of primary nitrate metabolism. Nitrate uptake is facilitated by a (NO_3^-, Cl)-activated ATPase, located in the plasma membrane. Once translocated into the cytoplasm, NO_3^- may be stored in a vacuole, or reduced via nitrate reductase to NO_2^-. Subsequently, NO_2^- is reduced to NH_4^+ via nitrite reductase. The primary source of reductant cofactors for these processes in the light is probably from photosystems I and II. The incorporation of NH_4^+ into amino acids appears to be mediated by glutamine synthetase and glutamate synthase. NH_4^+ may inhibit NiR and NR through an unknown intermediary.

Another possible mechanism for nitrate uptake is the so-called "primary active transport." This process requires that nitrate uptake be directly coupled to an exergonic reaction. Here the internal nitrate concentration may be relatively insignificant; nitrate may be accumulated against its concentration gradient, providing that a suitable energy source is available to drive the ion across the membrane. The obvious candidate for this chemical energy is ATP, which, through hydrolysis, liberates enough energy for the translocation of nitrate. In this model the nitrate accepting protein is an adenosine triphosphatase, coupling the hydrolysis of ATP to the uptake of nitrate from the medium. An analagous situation is the $(Na^+ + K^+)$-activated transport adenosine triphosphatase (E. C. 3.6.1.3), first described by Skou in 1957,[8] and found in many animal and plant tissues.[9-12]

The results of laboratory studies with membranes isolated from six species of marine phytoplankton indicate the presence of an enzyme that hydrolyzes ATP in the presence of Mg^{2+}, Cl^-, and NO_3^-. The physiological function of the ATPase cannot be inferred from the biochemical studies alone because the protein is no longer oriented *in vitro* in the plasmalemma, and none of the activating ions are truly substrates for the reaction (i.e., the true substrate is ATP). Mainly because of the difficulty in understanding the mechanisms of transport ATPases (or, more specifically, the difficulty in explaining the coupling between the scalar energy liberated from ATP hydrolysis and the vectoral flux of ions), the roles of membrane-bound ATPases in regulating intracellular ion concentrations has to be determined by an examination of both physiological and biochemical properties, since neither is adequate to serve this purpose alone.

A partial biochemical characterization of the nitrate-activated ATPase from *Skeletonema costatum*, a marine diatom, indicate (1) a pH optima at 7.8 - 8.2, (2) a break in the Arrhenius plot at *ca.* 2^oC, (Fig. 2) corresponding to a change in the energy

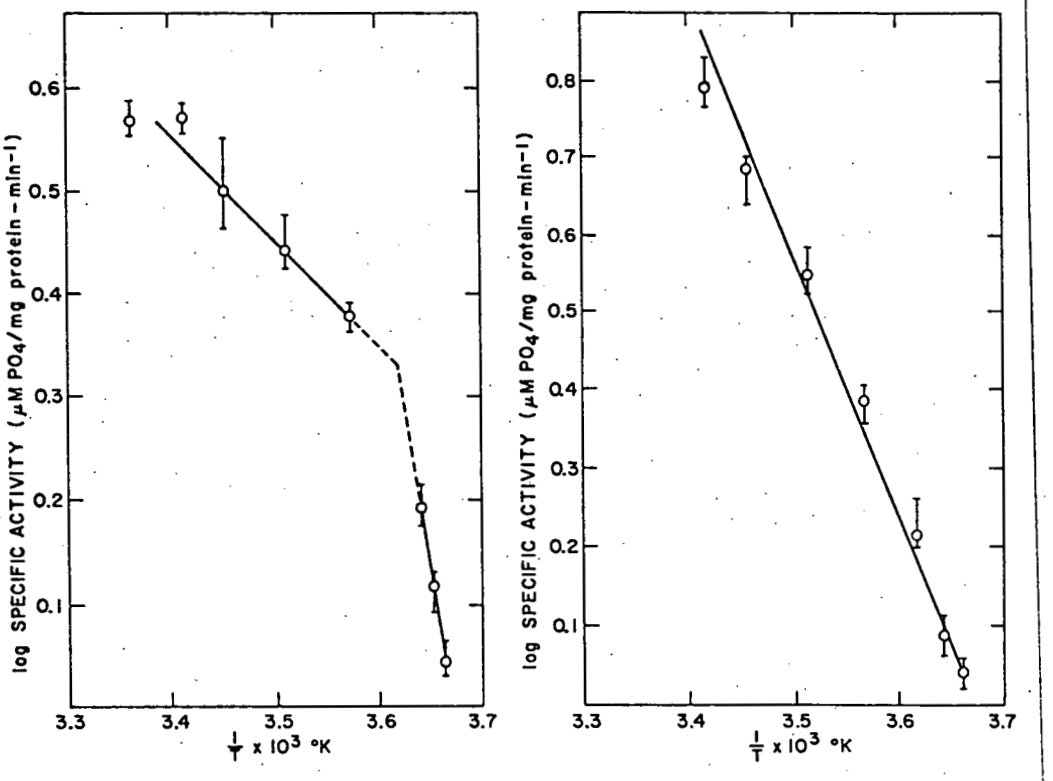


Fig. 2. Arrhenius plots of enzyme activity for the (NO_3^-, Cl^-)*-activated ATPase from* Skeletonema costatum. *In Fig. 2A, no detergents were added to the enzyme preparation and a break in the plot occurs at* ca *2°C, thought to correspond to a change in the liquid-crystal structure of the phospholipid moiety of the lipo-protein. In Fig. 2B, detergents were added that reduced the lipid phase transition, resulting in a log-normal enzyme profile. The change in the energy of activation in Fig. 2A is 31 Kcal/mole.*

of activation of 31 Kcal/mole, and (3) a half-saturation constant for nitrate activation of 0.5 μM NO_3. Upon addition of extracellular nitrate to intact cells, in the light, a transient decline in intracellular ATP pools is observed.[13,14] Addition of CCCP decreases nitrate uptake markedly, and changes the uptake profile from a rectangular hyperbolae to a linear function. Taken together, these results imply that nitrate transport is active, the enzyme responsible for the process is membrane bound, the energy source *in vivo* is ATP, and the major source of energy for the enzyme in the light is derived from cyclic phosphorylation.

II. NITRATE REDUCTION

The regulation of nitrate assimilation through the reduction processes is well documented. In many algae and higher plants, nitrate reductase (NR) is induced by exogenous nitrate ions and light. The effect of nitrate ions usually predominates light effects, but the relative importance of the two variables is species specific, and in the case of higher plants dependent on location of the redycing enzymes (i.e., root vs. shoot). In some algae (e.g., *Cyanidium caldarium*), total NR activity may be greatly enhanced by nitrogen starvation,[15] however, it is not clear that this is a universal feature that occurs in all plant species. The regulation of NR activity *in vivo* is complex; exogenous ammonium (above *ca.* 0.5 - 1.0 μM) may repress NR activity *in vivo*, but isolated, purified NR does not appear to be markedly inhibited by ammonium. Sorger, et al.,[16] suggested that NO_3^- ions may stabilize NR *in vivo*, thus preventing the apparent rapid decay of the enzyme. This hypothesis does not immediately preclude direct genetic induction-type regulation, although it would appear that these authors do not favor genetic regulation as the primary influence of NR activity.

Less work has been done on the regulation of nitrite reductase (NiR) than nitrate reductase. Like NR, NiR may be repressed

in vivo by ammonium. Both NR and NiR may undergo diel periodicity in many species of algae, reaching a plateau around midday. In some unusual instances, nitrite may accumulate as a result of a transient unbalance between NR which produces nitrate and NiR which removes it. This feature has been demonstrated in some grasses and cereals when nitrate supplies are high.[17] Under more usual circumstances however, intracellular nitrite pools are often so small as to be experimentally impossible to determine by colorimetric techniques. These results may be interpreted as suggesting that NiR, in particular, is not the usual rate-limiting step in the assimilation of nitrate by most plant cells.

Experimental evidence based on N^{15} uptake studies and extractable NR activity often suggests a poor correlation between nitrate uptake and reduction. This discrepancy may be due to the poor relationship between extractable NR activity and nitrate reduction *in vivo*,[18] or the fact that N^{15} uptake studies are really N^{15} assimilation studies, i.e., the uptake of N^{15} represents the integrated result of uptake *per se*, reduction and incorporation.

III. INCORPORATION

Increasingly, evidence is accumulating that the incorporation of ammonium into the amino acid pool in some species of plants, at ecological nitrate concentrations, is *via* glutamine synthetase (E. C. 6.3.1.2) and glutamate synthase (E. C. 2.6.1.53).[19] Glutamine synthetase, isolated from *Skeletonema costatum*, exhibits 3.5 times more activity than glutamate dehydrogenase at saturating substrate concentrations. Furthermore, at similar pH and temperature, the K_m values for ammonium were three orders of magnitude lower for GS than GDH.[20] However, GS requires one mole of ATP for each mole of ammonium incorporated into glutamate. When extracellular nitrogen levels are high, therefore, GDH activity is markedly increased, suggesting that the GDH pathway is energetically more favorable, and perhaps more responsive to changes

in intracellular ammonium concentrations, especially when initial ammonium levels are high.

A. *The Effect of Light on Nitrate Assimilation*

Grant and Turner[21] and MacIssac and Dugdale[6] have indicated that nitrate uptake velocities are a function of light intensities. In particular, the latter two authors have reported that nitrate uptake velocities are related to light intensities by rectangular hyperbolae (specifically Michaelis-Menten kinetics). Consequently, two independent half-saturation constants have been described to affect the uptake velocity, namely, K_s (for extracellular nitrate concentrations) and K_L, the light intensity supporting half the maximum uptake velocity. It is possible to incorporate light into a simple model of nitrate uptake based on bisubstrate enzyme kinetics, thus:

$$K_{LN} = -LN \left(1 + \frac{K_L}{L} + \frac{K_s}{N} - \frac{V_{max}}{v}\right)$$

where: L and N are light and extracellular nitrate respectively,
K_L and K_s are the half saturation constants for light and nitrate respectively, determined when the counter-substrate is saturating;
V_{max} is the maximum uptake velocity determined when both light and nitrate are simultaneously saturating;
V is the velocity of nitrate uptake,
and K_{LN} represents a physiological parameter inversely proportional to the affinity of the plant for nitrate, but also dependent on light intensity. It is suggested that values of K_{LN} may be useful in describing the integrated effects of the two substrates on nitrate uptake kinetics.

B. *Regulation of Nitrate Assimilation by Adenylate Nucleotides*

A primary feature of intracellular regulation of nitrogen assimilation appears to be through allosteric interaction with adenylate nucleotides (ATP, ADP, AND AMP). Eaglesham and

Hewitt[22] suggested that nitrate reductase from spinach is non-competitively inhibited by ADP without the presence of thiols; upon addition of thiols, the inhibition became mixed and non-linear. These authors inferred that nitrate reductase is a hysteretic enzyme, obeying ping-pong kinetics, and that ADP may be a physiological regulator of enzyme activity.

In 1976, Weissman[23] suggested that glutamine synthetase from sunflower roots may be regulated by adenylate nucleotides, or specifically, the relative proportion of nucleotides. Similar data are shown in Fig. 3. At energy charge values greater than *ca.* 0.45, the incorporation of ammonium into glutamate by glutamine synthetase is greatly enhanced. Like nitrate reductase, glutamine synthetase is inhibited by ADP, despite the presence of saturating levels of ATP (a substrate for the enzyme).

While it is clear that the adenylate nucleotides may regulate at least two enzymes important in the intermediary metabolism of nitrate, the evidence required for modeling the interaction of the nucleotide pools and nitrogen fluxes is scant. Nitrogen assimilation processes utilize, either directly or indirectly, the energy available in the adenylate pool. Catabolic processes (e.g., the oxidation of glucose in higher plant roots) and photoreactions in leaves and singled celled plants, provide increased energy fluxes via the adenylate pool.[24] Relative adenylate nucleotide concentrations, representing energy charge values between 0.4 and 0.8 may be critical inflection points for the regulation of the anabolic enzymes of nitrogen assimilation. While not all nitrogen assimilating enzymes necessarily regulate nitrogen flux, it is clear that, those which are may be regulated not only by classical substrate induction mechanisms, but on a finer level, mutually affected by energy supply and demand. In this general sense, energy supply is not only manifested in the adenylate pool but is provided by the redox potential of enzyme cofactors. Such cofactors as NAD(P)H and ferredoxin are obviously important, especially in the reduction processes. It has

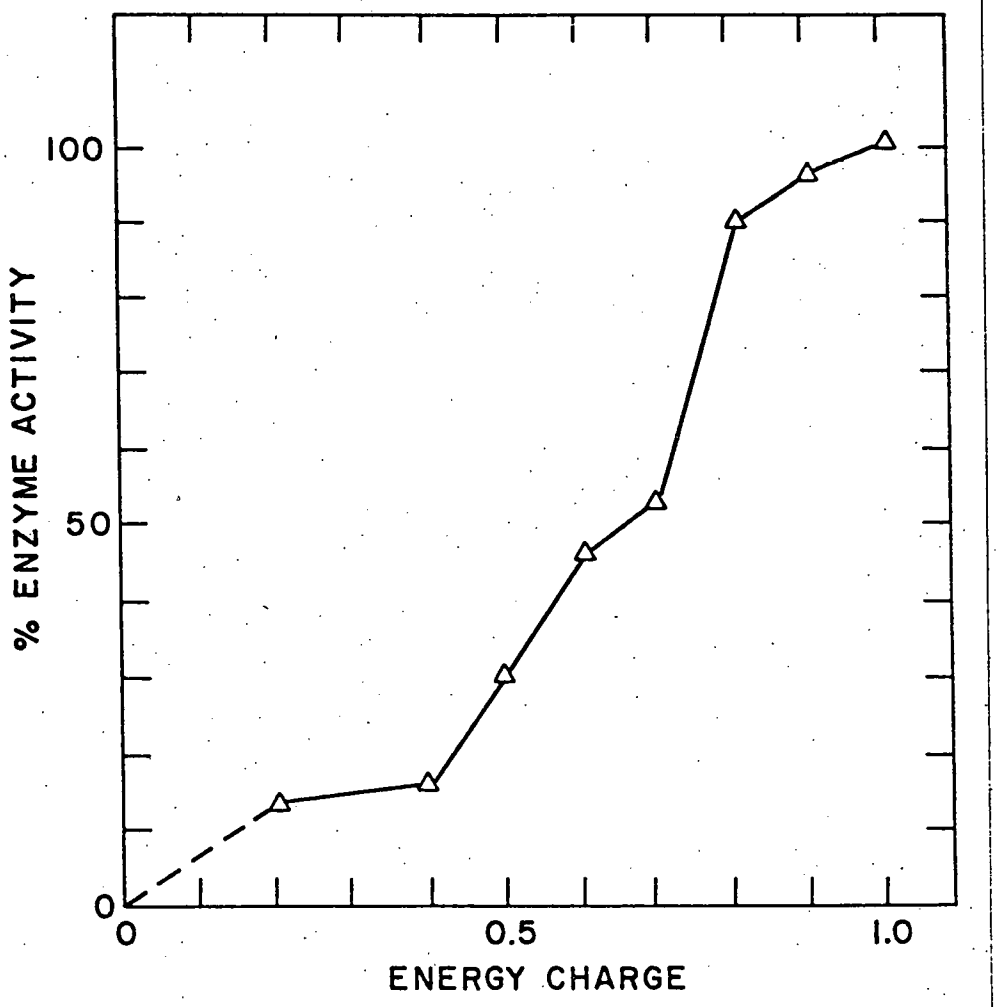


Fig. 3. Effects of the adenylate energy charge on glutamine synthetase from Skeletonema costatum. An inflection is observed at energy charge values greater than 0.4 in vitro.

been suggested that assessment of the molar ratios of NAD(P)H/NAD $(P)^+$ may be useful in understanding the regulation of enzymes requiring these substrates.

C. *Interaction with Carbon Fixation*

In 1975, Falkowski and Stone[13] demonstrated that addition of exogenous nitrate or ammonium to natural phytoplankton populations, growing in seawater containing ample natural nitrogen, may result in an initial decline in net carbon fixation. This phenomenon is thought to be caused by a competition between carbon fixation processes (dark reactions) and nitrogen assimilation processes for a limited supply of energy from the light reactions. Over a 6-8 hr. acclimation period, a steady increase in chlorophyll *a*/cell was observed in those subsamples spiked with additional nitrogen (*ca.* 10% of ambient levels). This "nutrient-adaptation" is suggested to result in greater light trapping ability, and hence, ATP synthesis. These results imply that changes in chlorophyll *a* levels in response to light or external nutrient supply, may be an efficient means of supplying energy for inorganic nitrogen assimilation.

IV. SUMMARY AND CONCLUSIONS

In the past few years, numerous attempts have been made to relate the supply of nitrogen to plants to their net growth rate. For the most part, those models which are marginally adequate to describe the interaction of these processes are built for steady-state conditions, such as chemostat cultures. In part, it appears to this author that the problem of building such models lies in a definition of nutrient limitation (yield vs. rate), but more fundamentally, it appears that plants in general do not always regulate the assimilation of nitrate in relation to nitrate supply in a predictably linear manner. Such common features of nitrogen metabolism as luxury consumption of nitrate, supply of

new nitrogen from the environment vs. utilization of old nitrogen reserves in vacuoles, and pool sizes, compartments, and fluxes of intracellular nitrate and ammonium, still present serious restrictions for our understanding effective crop management, and plant growth kinetics.

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