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Spectral Dependence of Photoregulation of Inorganic Nitrogen Metabolism in *Chlamydomonas reinhardii*[1]

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ABSTRACT

The utilization of NO_3^- by green algae growing photoautotrophically under air, which are growth conditions close to their more habitual situations in nature, is associated with the excretion of NO_2^- and NH_4^+ to the culture medium. The entire process is promoted by blue light and depends on photosynthetically active radiation for the required reducing equivalents. The stimulation of NO_3^- utilization and of its associated NO_2^- and NH_4^+ excretions saturated at very low quantum fluxes of blue light (15 microequivalents per square meter per second) in *Chlamydomonas reinhardii* cells sparged with CO_2-free air and irradiated with 50 microequivalents per square meter per second background red light. The wavelength dependence data of this stimulation correlated closely with the *in situ* photoactivation of nitrate reductase and also with the light induced increase in its biosynthesis and/or assembly.

These results indicate that the photoregulation of inorganic N metabolism in *C. reinhardii* is mainly due to the blue light modulation of nitrate reductase. Although flavins are the most suitable candidates to act as physiological photoreceptors, the wavelength dependence data only show a major peak in the blue region between 400 and 500 nanometers.

In eukaryotic photosynthetic organisms, the reduction of NO_3^- to NH_4^+ takes place in two well defined steps: (a) the reduction of NO_3^- to NO_2^- catalyzed by the NAD(P)H-nitrate reductase wherein $2e^-$ are involved and (b) the reduction of NO_2^- to NH_4^+ catalyzed by the Fd-nitrite reductase wherein $6e^-$ are involved (13).

In green algae, the best characterized regulatory step concerns the activity and biosynthesis and/or assembly of nitrate reductase. This enzyme, that contains FAD, Cyt b_{557}, and a pterin molybdenum cofactor, exhibits two interconvertible forms, active and inactive (13, 21). Factors leading to *in vitro* inactivation are low potential reductants (including physiological electron donor NAD(P)H) and potassium cyanide among other chemicals (17). *In vivo*, several environmental conditions lead to nitrate reductase inactivation, NH_4^+ and darkness being the most significant (12). Activation can be achieved *in vitro* by strong oxidants like ferricyanide or trivalent manganese (16, 21). Recently, blue light has emerged as a physiological agent that reactivates not only *in vitro* but also *in vivo* nitrate reductase in a photoreaction more likely sensitized by flavins. Excited flavins that become strong oxidants may activate nitrate reductase similarly as ferricyanide does (3). Moreover, as shown recently, other photoexcited nonphysiological pigments absorbing red light like methylene blue also activate nitrate reductase *in vitro* (18).

Although in green algae the reduction of nitrate has been currently considered mainly an assimilatory process, recent studies have shown that these organisms growing under the prevailing air-CO_2 tensions excrete high amounts of NO_2^- and NH_4^+ to the culture medium in the light. Under these conditions, the cells utilize NO_3^- and NO_2^- as electron sinks probably to unload photosynthetically generated reducing power. Recently, it was also shown that this process was sustained much more efficiently by blue than by red light (4, 6). When the CO_2 tension in the gas phase was increased to 2%, NH_4^+ and NO_2^- excretions ceased and previously excreted NO_2^- and NH_4^+ became rapidly assimilated, most probably as a consequence of the increase in the availability of carbon skeletons for incorporating NH_4^+ (7).

As shown in this paper, to achieve high rates of NO_3^- utilization and NO_2^- and NH_4^+ excretions, either high irradiance of blue light or combined high irradiance of red light with small quantum fluxes of blue light are required. High irradiances of PAR radiations would generate the necessary reducing equivalents, while blue light has the additional role of activating *in situ* nitrate reductase.

A close correlation was found between the blue light-induced increase in nitrate reductase activity and the blue light stimulation of the NO_2^- and NH_4^- excretion rates.

MATERIALS AND METHODS

Chlamydomonas reinhardii 11-32b from the Alga Collection of Göttingen University was grown synchronously in a NO_3^- containing medium as previously described (6) but using a photoperiod of 14 h light and 10 h dark. The cells were removed from the growing cultures in the middle of the dark interval, when they had low levels of active nitrate reductase (6), and suspended in standard pH 8 culture medium containing 10 mM NO_3^- or 0.5 mM NO_2^- according to the particular experiment.

For each experiment, 13-ml aliquots of cell suspensions were sparged with CO_2-free air and either kept in the dark or illuminated with different monochromatic lights. Other experimental conditions were as reported elsewhere (7). Measurements of irradiance and/or quantum flux were carried out with a Yellow Springs Radiometer and with a Licor LI-1888 Integrating Quantum Photometer, respectively. The values obtained with both instruments were sufficiently congruent.

Nitrate reductase activity before and after ferricyanide incubation was estimated as previously described (6). One unit of nitrate reductase corresponds to 1.0 μmol of NO_2^- formed/min. NO_2^- was measured by diazotization (20), and NH_4^+ using glutamate dehydrogenase (10).

RESULTS

It has been shown elsewhere (7) that the NO_3^- utilization and NO_2^- and NH_4^+ excretions that green algae carry out in the light

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under air-CO_2 tensions become enhanced under CO_2-free air resulting in the disappearance of NO_3^- from the cultures stoichiometric with the amounts of NO_2^- and NH_4^+ released. Recently (4) it was also demonstrated that NO_3^--dependent NO_2^- excretion saturated under CO_2-free air at much lower irradiances under 460 nm blue radiation (60 μE m^{-2} s^{-1}) than under 630 nm red radiation (well over 100 μE m^{-2} s^{-1}), both photosynthetically active.

The wavelength dependence of the NO_3^--dependent NO_2^- excretion using independent radiations of equal quantum flux shows in the visible range a remarkable peak in the blue region and a small peak in the red region (4). This later peak was attributed to the contribution of the photosynthetic apparatus to the process. A clear synergistic effect on this process between 404 nm blue and 630 nm red lights (60 μE m^{-2} s^{-1} each) was also previously described (7). These radiations, when assayed separately, were mostly ineffective in sustaining NO_3^- dependent NO_2^- excretion. We concluded that 404 nm blue light, which more likely activates nitrate reductase, was unable to raise the required reducing equivalents for the reduction of NO_3^- as other blue radiations of longer wavelength did.

Figure 1 shows similar synergistic effects between blue radiations, at irradiances low enough that became mostly inoperative in the process (see Fig. 7 in Ref. 4), and sufficiently high irradiances of photosynthetically active red light of 690 nm. Low quantum fluxes (15 μE m^{-2} s^{-1}) of 480 nm blue light combined with 50 μE m^{-2} s^{-1} of 690 nm red light sustained similar rates of NO_2^- excretion as those obtained under high quantum fluxes (65 μE m^{-2} s^{-1}) of 460 nm blue light, while 65 μE m^{-2} s^{-1} of 690 nm red light were much less effective.

Figure 2 shows the 460 nm blue light saturation curve for NO_2^- and NH_4^+ excretions in cells irradiated with background 50 μE m^{-2} s^{-1} of 690 nm red light under CO_2-free air. Under these conditions, the saturating quantum flux of the blue radiation was 15 μE m^{-2} s^{-1}.

To study the wavelength dependence of both the activation of nitrate reductase and the photostimulation of its biosynthesis and/or assembly in relation to the NO_2^- and NH_4^+ excretion rates, the selected illuminating conditions were precisely 50 μE m^{-2} s^{-1} for 690 nm red light and 15 μE m^{-2} s^{-1} for radiations of

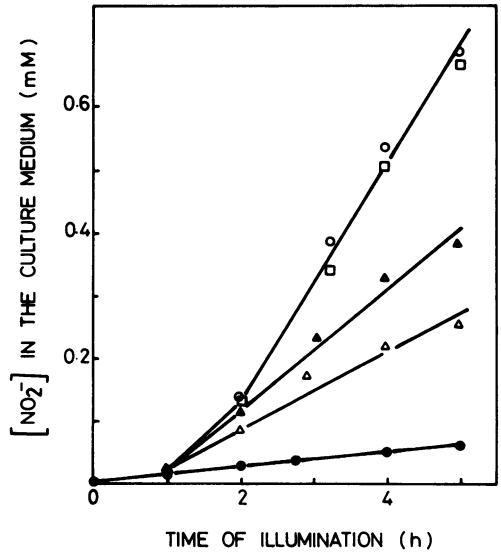

FIG. 1. Stimulating effect of low irradiance blue light on excretion of NO_2^- carried out by *Chlamydomomas* cells kept under high irradiance red light. Dark (●), 50 μE m^{-2} s^{-1} 690 nm red light (△), 65 μE m^{-2} s^{-1} 690 nm red light (▲), 15 μE m^{-2} s^{-1} 480 nm blue light plus 50 μE m^{-2} s^{-1} 690 nm red light (○), 65 μE m^{-2} s^{-1} 460 nm blue light, (□).

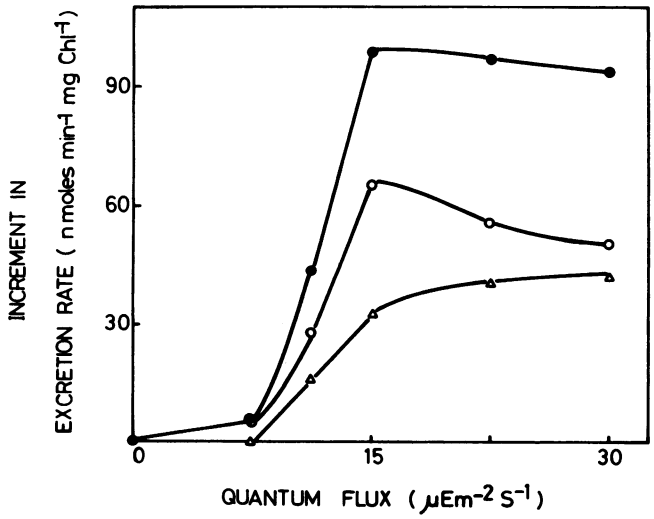

FIG. 2. 460 nm light saturation curves of the rates of NO_2^- and NH_4^+ excretions by cells under 50 μE m^{-2} s^{-1} 690 nm red light. NO_2^- plus NH_4^+ (●), NO_2^- (○), NH_4^+ (△). Zero values of excretion rates obtained under background red light comprised from 30 to 50 and 10 to 25 nmol min^{-1} mg Chl^{-1} for NO_2^- and NH_4^+, respectively.

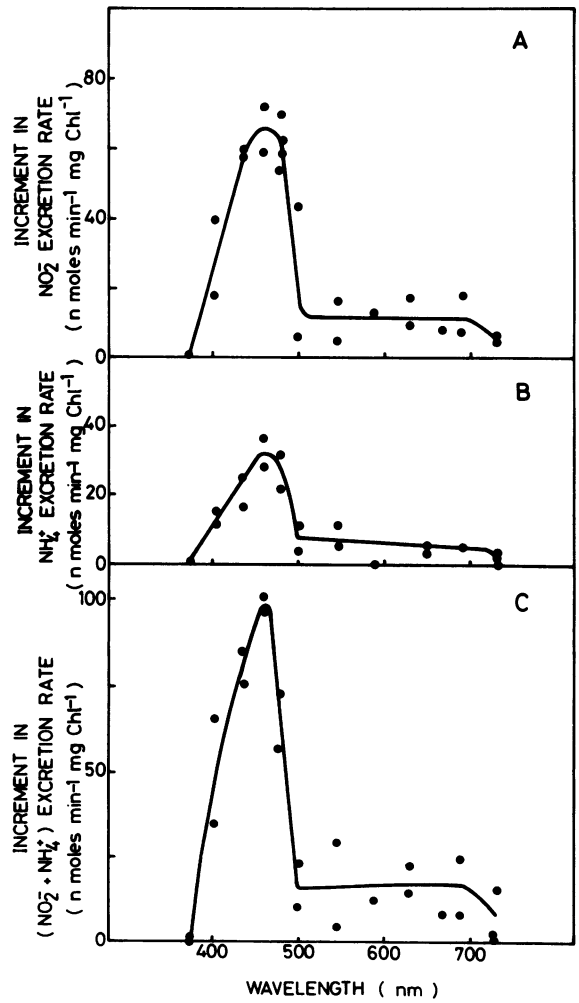

FIG. 3. Wavelength dependence of the increments of NO_2^- and NH_4^+ excretion rates induced by 15 μE m^{-2} s^{-1} quantum flux of different monochromatic lights by cells under 50 μE m^{-2} s^{-1} 690 nm red light. Other conditions were as in Figure 2.

different wavelengths. Under these illuminating conditions, wavelength interference on the rates of NO_2^- and NH_4^+ excretions due to the photosynthetic apparatus would be greatly minimized. For the experiments in Figures 2 and 3, two parallel cell suspensions were illuminated with either 50 μE m^{-2} s^{-1} 690 nm background red light or with the same background red light plus 15 μE m^{-2} s^{-1} of light of different wavelengths. In these figures, the increments of the rates of NO_2^- and NH_4^+ excretions induced by the supplementary light as a function of wavelength are indicated. Several experiments were conducted for each wavelength and the results of Figure 1 can be considered representative in this respect. Blue light between 404 and 500 nm clearly stimulated NO_2^- (Fig. 3A) and NH_4^+ (Fig. 3B) excretions, while other wavelength lights in the visible and near UV regions were much less effective. Figure 3C shows for each wavelength the resulting rates after adding the increments of NO_2^- excretion rates plus those of NH_4^+ shown in Figure 3, A and B, respectively. These obtained values become immediately comparable with the increments of nitrate reductase activity induced during light exposure (see below).

In the above described experiments, nitrate reductase activity levels were also monitored at each wavelength. After 3 h light exposure, an aliquot of the cell suspension was sonicated and the enzyme activity was measured before and after ferricyanide incubation, to obtain information about, respectively, the active enzyme and the total enzyme present in the cells. Figure 4 shows the increments of nitrate reductase activity induced by 15 μE m^{-2} s^{-1} of the different wavelength radiations. The increments in activity corresponding to the active enzyme show a broad peak in the blue region with a maximum at 460 nm (Fig. 4A). These increments of activity correlate closely both in magnitude and wavelength with the added increments of NO_2^- and NH_4^+ excretion rates shown in Figure 3C. Figure 4B shows that the greatest increments of the total amount of enzyme also occurred in cells illuminated with the additional 15 μE m^{-2} s^{-1} blue radiations. However, the increments in total enzyme were significantly smaller than those corresponding to the photoactivation of the previously present inactive nitrate reductase. Thus, the *in vivo* effect of blue light appears to be more remarkable in promoting the activation of nitrate reductase than in stimulating its biosynthesis and/or assembly.

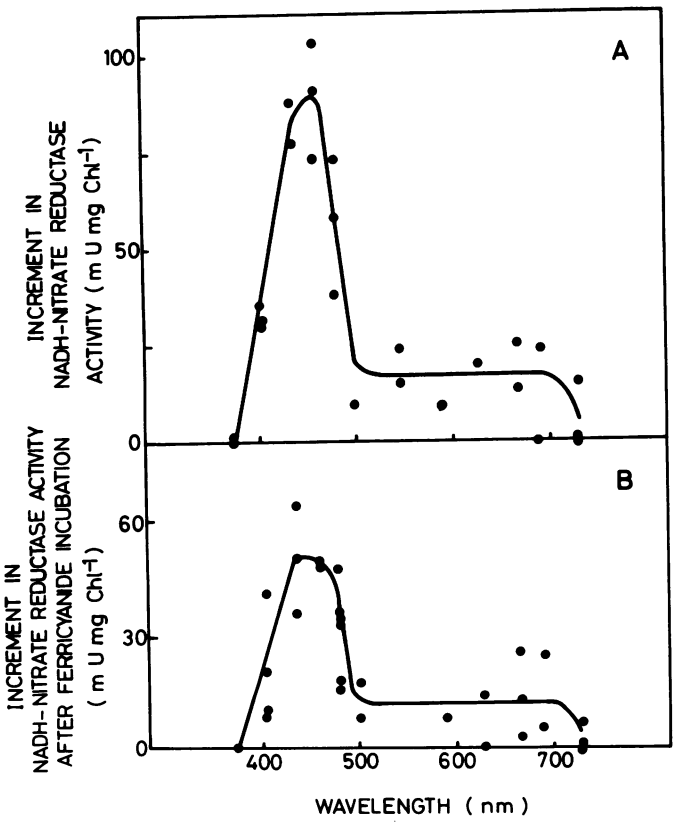

FIG. 4. Wavelength dependence of the increments in enzyme activity expressed by the active form of nitrate reductase (A) and by total enzyme (B). Experimental conditions were as in Figure 3. The activity zero values under background red light ranged from 40 to 70 and 150 to 200 milliunits mg Chl^{-1} for active and total enzyme, respectively.

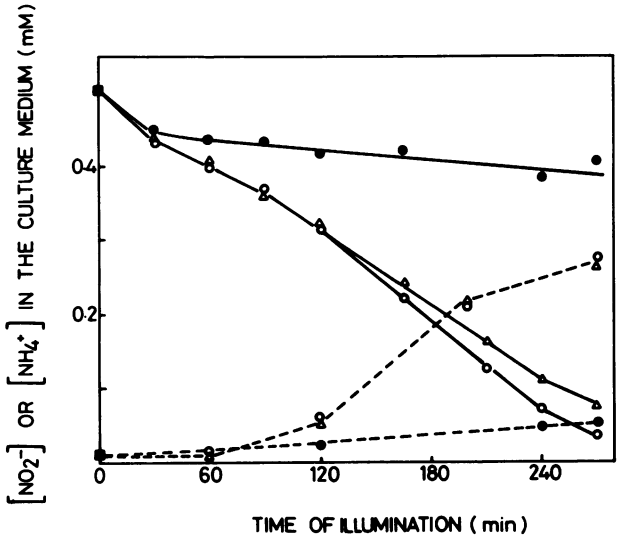

FIG. 5. Absence of stimulating effect of low irradiance blue light on NO_2^- consumption and its associated NH_4^+ excretion by cells under high irradiance red light. Cells kept in darkness (●) or illuminated with either 65 μE m^{-2} s^{-1} 690 nm red light (Δ) or 15 μE m^{-2} s^{-1} 460 nm blue light plus 50 μE m^{-2} s^{-1} 690 nm red light (○). NO_2^- consumption (———) and NH_4^+ excretion (- - -).

The correlation found between NO_2^- and NH_4^+ excretions shown in Figure 3 suggests that nitrite reductase activity was mostly dependent on NO_2^- concentration and mainly unaffected by light quality other than PAR. To confirm this point, both NO_2^- uptake and its associated NH_4^+ release using 65 μE m^{-2} s^{-1} 690 nm monochromatic red light, and 15 μE m^{-2} s^{-1} 460 nm blue light plus 50 μE m^{-2} s^{-1} 690 nm red light were studied under the same experimental conditions. The data of Figure 5 show that low quantum fluxes of blue light had no effect either on NO_2^- reduction nor on NH_4^+ release when compared with similar fluxes of red light. The difference observed between the amounts of NO_2^- incorporated and NH_4^+ excreted may be due to some N storage capacity of the cells since the rate of NH_4^+ excretion eventually reached NO_2^- uptake rate.

DISCUSSION

Inorganic N metabolism of green algae growing under unlimited carbon supply, *i.e.* high CO_2 tensions in the gas phase, is predominantly of assimilatory nature since NO_3^- uptake is not associated with the release of NO_2^- and NH_4^+. Nevertheless, under these conditions it was observed that NO_3^- uptake was much higher in blue than in red light of nonsaturating light intensities (9). However, under air CO_2 tensions, NO_3^- utilization become associated with a significant NO_2^- and NH_4^+ release and the whole process showed a clear dependency on both light intensity and quality, blue light being required to achieve maximal rates (4). These later conditions should indeed be considered highly physiological since they are much closer to the prevailing environmental situations in nature. As described elsewhere, green

algae in respect to inorganic N metabolism behave similarly whether growing under air or under CO_2-free air. Under CO_2-free air, the amounts of NO_3^- consumed were almost stoichiometric with the combined amounts of NO_2^- and NH_4^+ excreted and the whole process saturated at a much lower light intensity than under air (7). Hence, the results obtained under CO_2-free air can be mostly extended to physiological conditions. The remarkable effect of the carbon source, *i.e.*, CO_2 tensions, on the inorganic N metabolism, albeit not surprising, again indicates the close relationship between both metabolisms. However, while C metabolism appears to be mainly photoregulated by PAR irradiances (1, 8), N metabolism is additionally blue light dependent (4).

The fact that the stimulation of NO_3^- utilization associated with NO_2^- and NH_4^+ excretions saturated with such low blue light quantum fluxes (15 $\mu E\ m^{-2}\ s^{-1}$) indicates that the regulation of NO_3^- utilization and more probably *in situ* photoactivation of nitrate reductase are very low quantum requirement processes especially taking into account that most blue light would be absorbed by photosynthetic pigments such as Chl and carotenoids.

Although some effect of blue light on the NO_3^- membrane transport system(s) cannot be definitely ruled out (9), the wavelength dependence data strongly suggest that the photoregulation of the reductive NO_3^- utilization depends on the blue light activation of nitrate reductase and on the blue light stimulation of its biosynthesis and/or assembly too. Consistently, light activates nitrate reductase *in vitro* from a variety of organisms such as *Chlamydomonas* (6), *Chlorella* (2), *Ankistrodesmus* (MT Balandin and PJ Aparicio, unpublished) among green algae, wheat (5), maize (11), and spinach (2) in higher plants and *Neurospora* (19) in fungi. It is well documented that flavins act as photosensitizers for *in vitro* activation of nitrate reductase either when nitrate reductase was extracted inactive from the living organisms or when it was previously inactivated *in vitro* (6, 17). Nevertheless, the involvement of flavins as the physiological photosensitizers for the *in vivo* activation of nitrate reductase still remains to be definitively settled, since no peak in the near UV was found in the wavelength dependence experiments with the setup used in this work.

The reduction of NO_2^- to NH_4^+, however, would be photoregulated quite differently, since it appears to depend only on photosynthetic reducing power. The balance between NO_2^- and NH_4^+ excretion rates may be also influenced by compartmentation, since nitrite reductase appears to be located in the chloroplast stroma, while nitrate reductase is more likely in the cytosol (15).

In summary, the most suitable candidates to be the photoreceptors for the photochromic regulation of inorganic N metabolism in green algae are flavins that when excited activate nitrate reductase. Nevertheless, other photoreceptors as phytochrome might be, as in higher plants (14), additionally involved, since significant increments in nitrate reductase activity are observed in cells kept under red light. This fact can be alternatively explained by taking into account the results of Maldonado *et al.* (16) that indicate the possible formation of soluble strong oxidants in spinach thylakoids able to activate nitrate reductase or alternatively assuming that some unknown pigment that might become a strong oxidant upon excitation by red light may activate nitrate reductase as methylene blue does on spinach nitrate reductase *in vitro* (18).

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LITERATURE CITED

1. ANDERSON LE 1983 Proposed mechanism for photomodulation of carbon metabolism enzyme activity in chloroplasts and cyanobacteria. *In* G Montagnoli, BF Erlanger, eds, Molecular Models of Photoresponsiveness—NATO Advanced Study Institute Series. Plenum Press, New York, pp 153–173
2. APARICIO PJ, JM ROLDAN, F CALERO 1976 Blue light photoreactivation of nitrate reductase from green algae and higher plants. Biochem Biophys Res Commun 70: 1071–1077
3. APARICIO PJ, JM MALDONADO 1979 Regulation of nitrate assimilation in photosynthetic organisms. *In* EJ Hewitt, CV Cutting, eds, Nitrogen Assimilation of Plants, Academic Press, London, pp 207–215
4. APARICIO PJ, MP AZUARA 1984 Wavelength dependence of nitrite release and the effects of different nitrogen sources and CO_2 tensions on *Chlamydomonas reinhardii* inorganic nitrogen metabolism. *In* H Senger, ed, Blue Light Effects in Biological Systems. Springer-Verlag, Berlin, pp 196–206
5. ARYAN AP, RG BATT, W WALLACE 1983 Reversible inactivation of nitrate reductase by NADH and the occurrence of partially inactive enzyme in the wheat leaf. Plant Physiol 71: 582–587
6. AZUARA MP, PJ APARICIO 1983 *In vivo* blue-light activation of *Chlamydomonas reinhardii* nitrate reductase. Plant Physiol 71: 286–290
7. AZUARA MP, PJ APARICIO 1984 Effects of light quality, CO_2 tensions and NO_3^- concentrations on the inorganic nitrogen metabolism of *Chlamydomonas reinhardii*. Photosynth Res 5: 97–103
8. BUCHANAN BB 1980 Role of light in the regulation of chloroplast enzymes. Annu Rev Plant Physiol 31: 341–374
9. CALERO F, WR ULLRICH, PJ APARICIO 1980 Regulation by monochromatic light of nitrate uptake in *Chlorella fusc.* *In* H Senger, ed, The Blue Light Syndrome. Springer-Verlag, Berlin pp 411–421
10. DA FONSECA-WOLLHEIM F, HU BERGMEYER, I GUTMANN 1974 Methoden der Enzymatischen Analyse, Ed 2. Verlag Chemie, Weinheim, pp 1850–1853
11. ECHEVARRIA C, SG MAURIÑO, JM MALDONADO 1984 Reversible inactivation of maize leaf nitrate reductase. Phytochemistry 23: 2155–2158
12. FLORENCIO FJ, JM VEGA 1982 Regulation of the assimilation of nitrate in *Chlamydomonas reinhardii*. Phytochemistry 21: 1195–1200
13. GUERRERO MG, JM VEGA, M LOSADA 1981 The assimilatory nitrate-reducing system and its regulation. Annu Rev Plant Physiol 32: 169–240
14. JONES RW, RW SHEARD 1979 Light factors in nitrogen assimilation. *In* EJ Hewitt, CV Cutting, eds, Nitrogen Assimilation of Plants. Academic Press, London, pp 521–539
15. LOSADA M, MG GUERRERO, JM VEGA 1981 The assimilatory reduction of nitrate. *In* H Bothe, A Trebst, eds, Biology of Inorganic Nitrogen and Sulfur. Springer-Verlag, Berlin, pp 30–63
16. MALDONADO JM, BA NOTTON, EJ HEWITT 1980. The reactivation of nitrate reductase from spinach (*Spinacia oleracea* L.) inactivated by NADH and cyanide, using trivalent manganese either generated by illuminated chloroplasts or as manganipyrophosphate. Planta 150: 242–248
17. MAURIÑO SG, MA VARGAS, PJ APARICIO, JM MALDONADO 1983 Blue-light reactivation of spinach nitrate reductase inactivated by acetylene or cyanide. Effects of flavins, and oxygen. Physiol Plant 57: 411–416
18. MAURIÑO SG, MA VARGAS, G. ECHEVARRIA, PJ APARICIO, JM MALDONADO 1984 Red light effects sensitized by methylene blue on nitrate reductase from spinach (*Spinacia oleracea*, L.) leaves. Z Naturforsch 39c: In press
19. ROLDAN JM, WL BUTLER 1980 Photoreactivation of nitrate reductase from *Neurospora crassa*. Photochem Photobiol 32: 375–381
20. SNELL FD, CT SNELL 1949 Colorimetric Methods of Analysis. D Van Nostrand Company, Inc, New York, pp 802–807
21. VEGA JM, J HERRERA, AM RELIMPIO, PJ APARICIO 1972 NADH-nitrate réductase de *Chlorella*: nouvelle contribution à l'étude de ses propiétés. Physiol Vég 10: 637–652
22. VENNESLAND B, MG GUERRERO 1979 Reduction of nitrate and nitrite. *In* M Gibbs, E Latzko, eds. Encyclopedia of Plant Physiology, New Series, Vol 6. Springer-Verlag, Berlin, pp 425–444