Planta (Berl.) 128, 73–80 (1976)

Planta

Reversible Inactivation of Nitrate Reductase in *Chlorella vulgaris in vivo*

Elfriede K. Pistorius, Hans-Siegfried Gewitz, Helga Voss, and Birgit Vennesland

Forschungsstelle Vennesland der Max-Planck-Gesellschaft, Harnackstraße 23, D-1000 Berlin 33 (Dahlem)

Summary. The NADH-nitrate oxidoreductase of *Chlorella vulgaris* has an inactive form which has previously been shown to be a cyanide complex of the reduced enzyme. This inactive enzyme can be reactivated by treatment with ferricyanide *in vitro*. In the present study, the activation state of the enzyme was determined after different prior *in vivo* programs involving environmental variations. Oxygen, nitrate, light and CO_2 all affect the *in vivo* inactivation of the enzyme in an interdependent manner. In general, the inactivation is stimulated by O_2 and inhibited by nitrate and CO_2. Light may stimulate or inhibit, depending on conditions. Thus, the effects of CO_2 and nitrate (inhibition of reversible inactivation) are clearly manifested only in the light. In contrast, light stimulates the inactivation in the presence of oxygen and the absence of CO_2 and nitrate. Since the inactivation of the enzyme requires HCN and NADH, and it is improbable that O_2 stimulates NADH formation, it is reasonable to conclude that HCN is formed as the result of an oxidation reaction (which is stimulated by light). The formation of HCN is probably stimulated by Mn^{2+}, since the formation of reversibly-inactivated enzyme is impaired in Mn^{2+}-deficient cells. The prevention of enzyme inactivation by nitrate *in vivo* is in keeping with previous *in vitro* results showing that nitrate prevents inactivation by maintaining the enzyme in the oxidized form. A stimulation of nitrate uptake by CO_2 and light could account for the effect of CO_2 (prevention of inactivation) which is seen mainly in the presence of nitrate and light. Ammonia added in the presence of nitrate has the same effect on the enzyme as removing nitrate (promotion of reversible inactivation). Ammonia added in the absence of nitrate has little extra effect. It is therefore likely that ammonia acts by preventing nitrate uptake. The uncoupler, carbonylcyanide-m-chloro-phenylhydrazone, causes enzyme inactivation because it acts as a good HCN precursor, particularly in the light. Nitrite, arsenate and dinitrophenol cause an enzyme inactivation which can not be reversed by ferricyanide in crude extracts. This suggests that there are at least two different ways in which the enzyme can be inactivated rather rapidly *in vivo*.

Introduction

The nitrate reductase (NADH-nitrate oxidoreductase EC 1.6.6.1.) of several species of algae has been shown to have an inactive as well as an active form. Losada and his associates have studied the inactivation and activation of nitrate reductase in the algae *Chlorella fusca* (Losada *et al.*, 1970; Moreno *et al.*, 1972; Maldonado *et al.*, 1973) and *Chlamydomonas reinhardi* (Herrera *et al.*, 1972; Losada *et al.*, 1973). They reported that addition of ammonia to cells growing in the light on CO_2 and nitrate led to a rapid inactivation of the enzyme, and that subsequent removal of ammonia led to reactivation. Added arsenate behaved like ammonia. These effects were light- and CO_2-dependent. In the dark, the enzyme was inactivated under anaerobic conditions, and activated under aerobic conditions (Losada *et al.*, 1973). *In vitro*, inactivation of the enzyme occurred on addition of reduced pyridine nucleotides, and the inactive enzyme could be reactivated by ferricyanide. Furthermore, the inactivation by NADH was potentiated by ADP (Maldonado *et al.*, 1973). These and other results were interpreted to mean that inactivation required NADH and ADP, and occurred *in vivo* when NAD(P)H and ADP accumulated, due to uncoupling by ammonia or arsenate (Moreno *et al.*, 1972; Maldonado *et al.*, 1973; Losada *et al.*, 1973).

Rigano *et al.* (1974) have likewise shown that ammonia has a strong inactivating effect *in vivo* on the nitrate reductase of the algae *Cyanidium caldarium*,

though the *in vitro* behavior of this enzyme was rather different from that of the enzymes from *Chlorella fusca* and *Chlamydomonas reinhardi*. Heat activation of a latent enzyme has been described for the nitrate reductases from *Cyanidium caldarium* (Rigano and Violante, 1972) and *Dunaliella parva* (Heimer, 1975).

The nitrate reductase of *Chlorella vulgaris* has been purified and extensively characterized (Solomonson *et al.*, 1975; Solomonson and Vennesland, 1972a). In crude extracts, this enzyme appears to behave rather similarly to the nitrate reductase of *Chlorella fusca*, i.e., crude enzyme preparations are inactivated by added NAD(P)H, and reactivated by ferricyanide (Jetschmann *et al.*, 1972; Solomonson *et al.*, 1973). On purification of this enzyme, however, Solomonson (1974) found that added NAD(P)H alone (with or without ADP) caused no inactivation, unless a second factor, such as cyanide, was added with the NADH. Subsequently, Gewitz *et al.* (1974) identified cyanide as a constituent of *Chlorella vulgaris* cells. Lorimer *et al.* (1974) showed further, with purified enzyme, that the inactivation process is associated with the firm binding to the protein of $H^{14}CN$, which is released when the enzyme is activated. An equivalent amount of HCN was also released when enzyme which had been inactivated *in vivo*, was purified and activated.

The present study describes the *in vivo* inactivation (and activation) of the nitrate reductase of *Chlorella vulgaris*. Emphasis has been placed on the elucidation of the conditions required for the formation *in vivo* of an inactive enzyme which can be activated *in vitro* by ferricyanide. The results are rather different from those described for *Chlorella fusca* by Losada and his associates.

Materials and Methods

Growth of Algae

Chlorella vulgaris cells were grown autotrophically in continuous white light in a stream of 5% (v/v) CO_2 in air, at 21 °C to 22 °C, in standard mineral salts medium plus microelements with nitrate as the only nitrogen source, as previously described (Vennesland and Jetschmann, 1971; Solomonson and Vennesland, 1972b). The amount of nitrate reductase obtained in extracts from these cells varied with the treatment of the cells prior to harvesting. Our standard procedure for culture maintenance involved inoculation of 60 µl cells per 250 ml culture medium, and illumination with white light from a metal filament lamp giving a light intensity of 16,000–20,000 lux at the surface of the container facing the light. After 48 h, the cells had grown to a concentration of 5.5–6.5 µl/ml, and were harvested by centrifugation. Alternatively, 400 µl of cells were inoculated into 500 ml medium and illuminated with about 40,000 lux, to give the same cell density after 24 h growth. Cell extracts prepared directly from such cells contained about 2 to 2.5 units of nitrate reductase per ml (one ml extract represents 250 µl cells). Treatment with ferricyanide generally resulted in an activity increase of 15 to 40%. For the present experiments, the cells were harvested by centrifugation and resuspended in fresh growth medium with microelements, to a concentration of 7µl cells per ml medium, and illuminated with 5% CO_2 in air, for a period of 60 to 90 min. This "refresher" treatment resulted in an increased yield of enzyme, which was now almost entirely active, that is, there was little increase in activity on treatment with ferricyanide. The extracts usually contained about 3 units of enzyme per ml, though lower values were sometimes seen. Values up to 4 units per ml have also been observed occasionally.

To obtain manganese deficient cells, 60 µl of 2-day old cells grown under normal conditions were inoculated into 250 ml growth medium from which Mn^{2+} had been omitted. The cells used for the inoculum were not washed since only a partial deficiency was desired. After 48 h the culture reached a cell density of 2.0 to 2.5 µl cells/ml, and the cells were harvested by centrifugation.

Experiments with Intact Cells

The experiments with intact cells were carried out in 500 or 250 ml culture bottles with a light intensity of 50,000 lux, unless otherwise indicated. The conditions were those used for "refresher" treatment, except as specified for each experiment. The nitrate-containing medium was the normal growth medium (20 *mM* $MgSO_4$, 20 *mM* KH_2PO_4, 35 *mM* NaCl, 2 *mM* $Ca(NO_3)_2$, 20 *mM* KNO_3 plus microelements). The nitrate-free medium had the same composition, except that the nitrate salts were omitted. Cells were washed once with this medium and resuspended in the same medium to give the original cell density. When ammonium chloride was added, the pH was adjusted to 6.1 (instead of 4.3).

French Press Extract for Enzyme Assay

The cells were harvested by centrifugation, washed once with 10 *mM* potassium phosphate buffer, pH 7.6, and resuspended to give a cell concentration of 250 µl cells per ml buffer. This cell suspension was disrupted with a French Pressure Cell Press (Aminco), precooled in ice, at 10,000 p.s.i. The French press extract was centrifuged at $10{,}000 \times g$ for 10 min. The deep green supernatant was decanted and tested immediately.

Previously, we have usually disrupted the cells by sonication. It was found, however, that sonication leads to the formation of HCN from an unidentified precursor(s) present in the cells (Gewitz, unpublished). So far as we can ascertain, disruption of the cells with a French press or Ribi cell fractionator does not lead to extra HCN generation (Lorimer *et al.*, 1974).

Assay of Nitrate Reductase Activity

Nitrate reductase activity in the crude extract was assayed at 20° C by determining the rate of nitrite formation as follows: 100 µl extract was added to 2.9 ml of a solution containing 200 µmol potassium phosphate, pH 7.6, 20 µmol KNO_3, and 1.7 µmol NADH. After a time interval of two min, 0.5 ml of the reaction mixture was added to 2.2 ml of 0.1 *M* $ZnSO_4$ which had been heated to 97°C in a water bath. After cooling and addition of 0.2 ml of 1.0 *N* NaOH, the solution was clarified by centrifugation. Nitrite was determined on an aliquot of the supernatant solution (Nicholas and Nason, 1957). The initial nitrite content of the extract was also determined and subtracted. A unit of enzyme is the amount which catalyzes the reduction by NADH of 1 µmol of nitrate to nitrite per minute under the standard conditions. Nitrite formation is linear with time for the period of the assay. For activation of the enzyme in crude extracts, 0.05 ml of 0.05 or 0.025 *M* $K_3Fe(CN)_6$ was added to 0.2 ml of extract cooled in an ice bath. After 5 min,

the enzyme activity was determined by measuring the rate of nitrite production as previously described.

Results

In vivo Inactivation of Nitrate Reductase

Effect of Nitrate, Light, O_2 and CO_2. All of these agents affect the activation state of the nitrate reductase in an interdependent manner. After a large number of preliminary observations, a set of four experiments were designed to illustrate these effects. Table 1 shows the results of two experiments, performed in the presence of nitrate, one experiment in the light and one in the dark. Table 2 shows the results of two analogous experiments performed in the absence of nitrate. In each case, the effects of air, oxygen and argon, in the presence and in the absence of 5% CO_2, were tested. Ideally, all of these experiments should have been made with the same cell suspension, but this was impossible in practice. Fig. 1 shows a graph of the data obtained after 180 min incubation, with the results calculated as percent of the initial total enzyme level (i.e., units of enzyme found after ferricyanide activation), to facilitate comparison. The upper row shows the results of the two experiments made in the light, one in the presence of nitrate and one in its absence. The lower row shows the results of the two analogous experiments in the dark.

Table 1. Change of activity of nitrate reductase in normal growth medium with nitrate. Experiments were done with intact *Chlorella* cells in normal growth medium with nitrate plus microelements in culture bottles in white light (50,000 lux) or in the dark. The bottles were gassed as indicated. At the indicated times, the cells were harvested by centrifugation, disrupted in a French Pressure Cell Press, and the nitrate reductase activity of the extract was determined before and after ferricyanide treatment. For further details, see Materials and Methods. The numbers are nitrate reductase, units per ml extract (250 µl cells). Values after activation with ferricyanide are in parenthesis

Incubation time	Air 5% CO_2	Air	O_2 5% CO_2	O_2	Argon 5% CO_2	Argon
0	3.1 (3.3)					
90′ light	3.4 (3.5)	1.3 (2.1)	2.9 (3.2)	0.8 (2.5)	3.3 (3.5)	1.5 (2.5)
180′ light	3.5 (3.7)	1.4 (2.0)	2.8 (3.1)	0.7 (2.1)	3.6 (3.7)	1.3 (2.1)
0	3.6 (3.8)					
90′ dark	1.3 (2.4)	2.6 (3.2)	1.3 (1.9)	2.0 (2.5)	2.2 (2.7)	2.5 (2.7)
180′ dark	1.5 (2.2)	1.4 (2.4)	1.6 (2.2)	1.5 (2.4)	2.0 (2.6)	2.2 (2.8)

Table 2. Change of activity of nitrate reductase in mineral salts medium without nitrate. Conditions and procedures as described for Table 1, except that nitrate was omitted from the medium. The numbers are nitrate reductase, units per ml extract (250 µl cells). Values after activation with ferricyanide are in parenthesis

Incubation time	Air 5% CO_2	Air	O_2 5% CO_2	O_2	Argon 5% CO_2	Argon
0	2.9 (3.1)					
90′ light	1.1 (2.7)	0.8 (1.8)	1.1 (2.8)	0.4 (2.2)	1.6 (2.6)	1.0 (2.0)
180′ light	0.6 (2.3)	0.7 (2.3)	0.6 (2.6)	0.2 (2.4)	1.4 (2.5)	0.8 (1.7)
0	2.9 (3.1)					
90′ dark	1.5 (2.4)	1.9 (2.7)	1.6 (2.4)	1.3 (2.3)	2.6 (2.9)	2.5 (2.9)
180′ dark	1.3 (2.1)	1.5 (2.2)	1.4 (2.0)	1.1 (2.2)	2.8 (2.9)	2.4 (2.7)

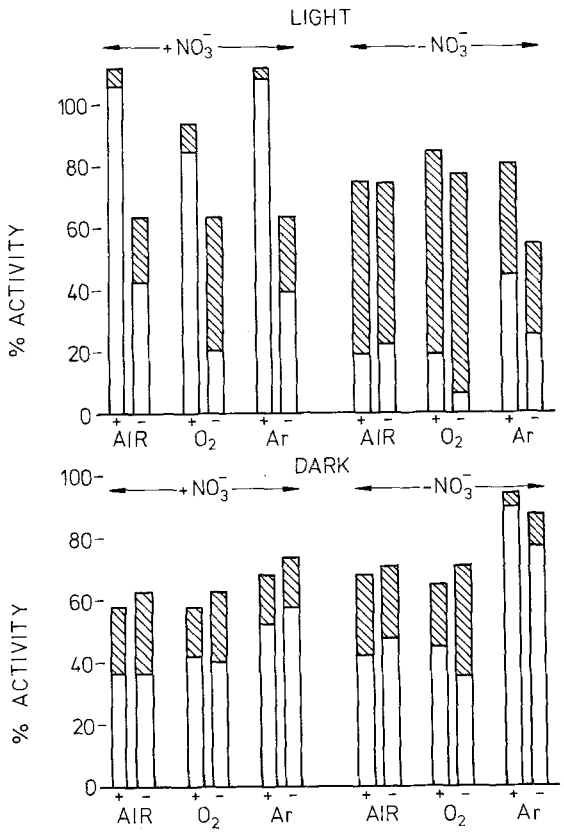

Fig. 1. Effects of CO_2, O_2, nitrate and light on reversible inactivation. The data employed are those given in Tables 1 and 2 for 180 min. In each bar, the cross-hatched area represents the amount of reversibly-inactivated enzyme. The symbol "+" under the bars indicates the presence of 5% CO_2 added to gas; the symbol "−" under the bars indicates no CO_2 added to gas

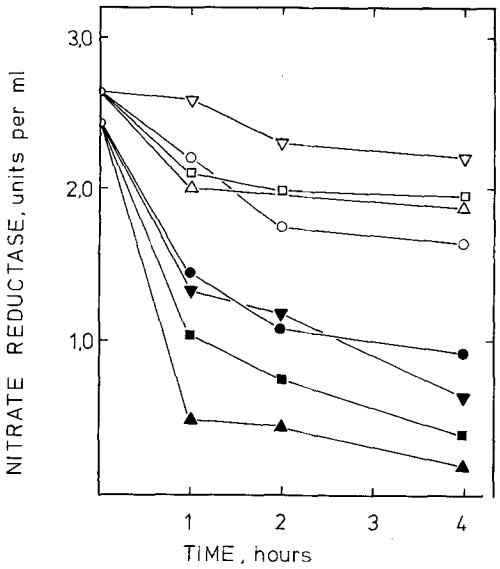

Fig. 2. Effect of light of different intensities on reversible inactivation. Intact *Chlorella* cells were incubated in mineral salts medium without nitrate, with 5% CO_2-air, in the dark and at three different light intensities. The closed symbols represent the enzyme units per ml extract prior to ferricyanide treatment; the open symbols represent the values after activation by ferricyanide. ○ and ● = dark; ▽ and ▼ = 5,000 lux; □ and ■ = 40,000 lux; △ and ▲ = 95,000 lux

The enzyme was maintained at the high initial level with little inactivation when the cells were illuminated with nitrate and CO_2, in argon or air. Use of CO_2 in O_2 under these conditions sometimes led to a small drop in enzyme level (not always seen). There was little reversible inactivation as long as CO_2 was present. Surprisingly, a similar maintenance of enzyme level was observed in the dark in the *absence* of nitrate, under argon. Under all other conditions, the total level of enzyme declined, and appreciable amounts of reversibly-inactive enzyme were formed. Illumination of the cells in O_2 in the absence of CO_2 and nitrate led to a maximal reversible inactivation.

Generalizing, we can say: The formation of reversibly inactive enzyme is promoted by O_2 and light, and inhibited by CO_2, nitrate and light. Thus, light has two effects: it stimulates the formation of reversibly inactive enzyme, particularly in the absence of nitrate and CO_2. In contrast, it prevents the inactivation in the presence of nitrate and CO_2. Oxygen stimulates the reversible inactivation both in light and dark. CO_2 prevents the reversible inactivation in the presence of nitrate and light. Or we can say: nitrate prevents the reversible inactivation, particularly in the presence of light and CO_2.

The light intensity employed in these experiments (50,000 lux) was not sufficient to maximize the formation of reversibly-inactive enzyme. Fig. 2 shows the time course of inactivation in the dark, and at 3 different light intensities in 5% CO_2-air in the absence of nitrate. It is likely that still higher light intensity than the highest employed, will give still faster inactivation rates.

Effect of Ammonia. The results of two experiments showing the effect of ammonia addition in the light, on the activation state of nitrate reductase are given in Table 3. One experiment was carried out in 5% CO_2 in air, the other 5% CO_2 in O_2. Both experiments lead to the same conclusion. The added ammonia has relatively little effect on the enzyme inactivation in the absence of nitrate. Added nitrate prevents the reversible inactivation of the enzyme; and this effect of nitrate is prevented by the simultaneous addition of ammonia with the nitrate. The simplest interpretation of these results is that the added ammonia prevents the uptake of nitrate by the cells.

Effect of Nitrite. The effect of nitrite addition on the nitrate reductase is shown in Table 4. In this experiment nitrite was added to a cell suspension growing in the presence of nitrate, 5% CO_2 in air. The nitrite caused a prompt decline in enzyme activity, but this inactivation could not be reversed by ferricyanide *in vitro*. When the nitrite had been consumed by the cells, the enzyme level slowly returned to the original higher level.

Effect of Manganese Deficiency. The formation of reversibly-inactivated enzyme requires cyanide. In unpublished studies (Gewitz *et al.*) of model systems for cyanide generation by illuminated grana, it had been found that Mn^{2+} sometimes strongly stimulated

Table 3. Effect of ammonia and nitrate on nitrate reductase activity. Experiments were done with intact Chlorella cells in mineral salts medium without nitrate in culture bottles in white light. The bottles were gassed with 5% CO_2 in air or with 5% CO_2 in O_2. Nitrate was added to 20 *mM*, NH_4Cl to 50 *mM*, and the pH was adjusted to 6.1. Otherwise conditions were as in Table 1. The numbers are nitrate reductase, units per ml extract (250 µl cells). Values after activation with ferricyanide are in parenthesis

Gas	Incubation time (min)	Additions to medium			
		NO_3^-	$NO_3^- + NH_4^+$	---	NH_4^+
Air 5% CO_2	0	3.0 (3.4)			
	90	2.8 (3.0)	1.9 (2.7)	1.2 (2.4)	2.0 (3.0)
	180	3.2 (3.4)	0.8 (2.4)	0.9 (2.3)	1.1 (2.5)
O_2 5% CO_2	0	2.7 (3.1)			
	90	3.2 (3.5)	0.8 (2.6)	0.7 (2.3)	0.6 (2.5)
	180	2.8 (2.9)	0.8 (2.1)	0.5 (2.3)	0.1 (2.3)

Table 4. Effect of nitrite on nitrate reductase activity. Experiments were done with intact Chlorella cells in growth medium with nitrate in light and in 5% CO_2 in air. Nitrite was added to give a final concentration of 10^{-3} *M*. Other conditions as in Table 1

Incubation time (min)	Nitrate reductase, units per ml extract	
	before activation	after activation
0	2.1	2.4
5	0.8	1.1
10	0.8	1.0
60	1.1	1.3
180	1.8	1.8

Table 6. Inactivation of nitrate reductase by the uncoupler CCCP. Experiments were done with intact Chlorella cells in growth medium with nitrate in white light and 5% CO_2 in air. Other conditions as in Table 1

Additions	Incubation time (min)	Light/ dark	Nitrate reductase, units per ml extract	
			before activation	after activation
–	0		1.7	2.0
10^{-5} *M* CCCP	15	light	0.4	2.1
10^{-5} *M* CCCP	45	light	0.3	2.1
–	0		2.3	2.5
10^{-5} *M* CCCP	20	dark	1.3	1.9

Table 5. Effect of Mn^{2+} deficiency on inactivation of nitrate reductase. Normal Chlorella cells or cells grown under Mn^{2+} deficiency were suspended in salts medium plus microelements, without nitrate. For the Mn^{2+} deficient cells no Mn^{2+} was added. Experiments were performed in white light and in 5% CO_2 in air, or in O_2. The numbers are nitrate reductase, units per ml extract (250 µl cells). Values after activation with ferricyanide are in parenthesis

Incubation time (min)	Mn^{2+}-deficient cells		Normal cells	
	Air 5% CO_2	O_2	Air 5% CO_2	O_2
0	1.9 (2.1)		2.2 (2.3)	
45	1.8 (2.3)	2.0 (2.5)	1.7 (2.5)	0.9 (2.2)
120	1.9 (2.1)	1.1 (2.0)	0.8 (2.4)	0.3 (2.0)

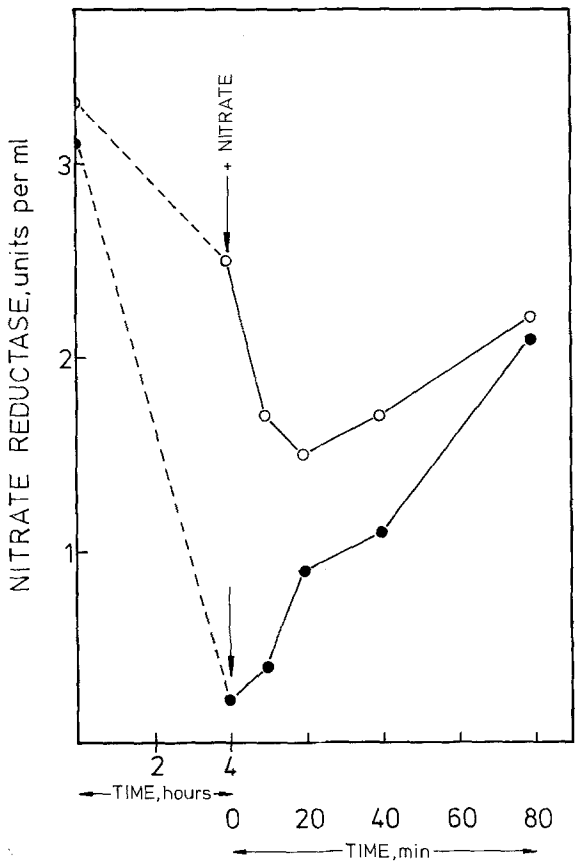

Fig. 3. Reactivation of nitrate reductase in vivo. Intact *Chlorella* cells were incubated in mineral salts medium without nitrate, with 5% CO_2 in air in light for 4 h; then KNO_3 was added to give a final concentration of 20 *mM*. Other conditions as in Table 1. The lower curve represents enzyme units per ml extract prior to activation; the upper curve, enzyme units after ferricyanide activation

cyanide generation. The effect of manganese deficiency on the capacity of the cells to form reversibly-inactivated enzyme was therefore tested. Some representative results, given in Table 5, show that a deficiency of manganese results in a decline of the capacity of the cells to form reversibly-inactivated enzyme.

The cells were illuminated in nitrate-free medium with 5% CO_2 in air or with O_2. In 5% CO_2 in air, there was no reversible inactivation of the nitrate reductase in Mn^{2+} deficient cells. We have never observed this in normal cells, though the rate of formation of inactivated enzyme varies somewhat from one experiment to the next. In O_2, there was a partial inactivation of the enzyme in Mn^{2+}-deficient cells, but clearly less than that seen in normal cells. Manganese-deficient and normal cultures were grown from similar inocula. The yield of manganese-deficient cells was half as great as the yield of normal cells. If manganese was added back to the manganese-deficient cells at the beginning of the experimental illumination period, they behaved like normal cells (not shown).

Effect of Uncouplers. The addition of dinitrophenol or of arsenate to growing cell suspensions led to an enzyme inactivation which could mostly not be reversed by ferricyanide (as observed also on nitrite addition), and was not studied extensively. The addition of the uncoupler, carbonylcyanide-m-chlorophenyl-hydrazone (CCCP), however, led to the rapid formation of inactive enzyme which could be reactivated *in vitro* by ferricyanide. Table 6 shows the results of an experiment in which CCCP was added to a cell culture under normal growth conditions with nitrate and CO_2 (no "refresher" treatment). The results without CCCP are not given, since there is no change in the nitrate reductase level under these conditions. In the light, 15 min after CCCP addition, the inactive enzyme reached a level usually seen only with O_2 in the absence of nitrate after about 90 min. In the dark there was less inactive enzyme formed, but more than was ever observed with such cells in the absence of CCCP.

Reactivation of Nitrate Reductase in vivo

The present study was focussed mainly on the determination of the conditions which favour the formation of reversibly inactive enzyme. It is important to know whether the cells can regenerate active enzyme from inactive enzyme, but studies of the biological reactivation are incomplete. The experiment pictured in Fig. 3 can be used to illustrate some of the questions raised. The amount of nitrate reductase per ml of extract, before activation by ferricyanide is shown in the lower curve, and the amount after activation is shown in the upper curve. In this experiment the cells were first illuminated for 4 h in the absence of nitrate and presence of 5% CO_2 in air, to convert almost all of the enzyme to the inactive form. Then nitrate was added at the time indicated by the arrows, and the cells were illuminated with 5% CO_2 in air.

There was a prompt increase in active enzyme after nitrate addition, but several hours were required to attain the normal level of activity. Curiously, the total amount of enzyme (active plus reversibly-inactive) decreased for the first twenty minutes after nitrate addition, and then increased more slowly. This response to added nitrate has been seen consistently when the cells had a high initial level of enzyme (i.e., after "refresher" treatment). Added cycloheximide causes a partial inhibition of the reactivation of the enzyme after nitrate addition. The significance of this inhibition by cycloheximide is not clear, because this reagent also causes a progressive enzyme inactivation when added to cells under normal growth conditions. These effects of cycloheximide require further study.

Discussion

The present experiments with *Chlorella vulgaris* show that light, O_2 and the absence of nitrate and CO_2 lead to a reversible inactivation of nitrate reductase *in vivo*. The reversible inactivation is known to require HCN and a reducing agent (NADH or NADPH). Nitrate can prevent this inactivation *in vitro*; a similar effect *in vivo* can explain the effect of nitrate.

The increased inactivation caused by O_2, both in light and dark, can hardly be interpreted in terms of an increase in reducing power. It seems reasonable therefore to conclude that O_2 stimulates the generation of HCN. In the presence of O_2, inactivation is greater in the light than in the dark. These results suggest that light stimulates HCN production in O_2. The effect of CO_2 on inactivation of the enzyme is seen only in the light and mainly in the presence of nitrate. That is, CO_2 prevents reversible inactivation of the enzyme, provided light and nitrate are present. Conversely, the largest effect of nitrate (in preventing inactivation) is seen in the presence of CO_2 and light. These effects of CO_2, light and nitrate can be understood in terms of a facilitation of nitrate uptake by CO_2 and light.

Altogether, the greatest difference between our results and those reported for *Chlorella fusca* by Losada's group relate to the effects of anaerobic and aerobic conditions. The Spanish group has found that reversibly-inactive enzyme is formed anaerobically, and in the presence of the uncouplers dinitrophenol and arsenate (in *Chlorella fusca*), whereas we find that oxygen stimulates inactivation (in *Chlorella vulgaris*). With arsenate and dinitrophenol (as well as with nitrite) we observed a large drop in the level of active enzyme present in the extracts, but we were not able to reactivate the preparations with ferricyanide to more than a relatively small extent. It is possible that this kind of "irreversible" inactivation only appears irreversible because the conditions for reactivation have not been found. That is, there may be more than one form of reversibly-inactivated enzyme. We have reported previously that hydroxylamine behaves rather like HCN toward the enzyme, except that higher concentrations of hydroxylamine than of HCN are required (Solomonson and Vennesland, 1972a). Reactivation of hydroxylamine-inactivated enzyme with ferricyanide in crude extracts is only partial (unpublished). Further investigation is required to determine whether a hydroxylamine-inactivated enzyme is formed *in vivo*.

The effect of the "refresher" treatment to which the cells are subjected prior to the actual experiments, is of interest in connection with the nitrite effect. Cultures of *Chlorella vulgaris* grown for two days under the conditions here employed, always contain a little

nitrite in the extracellular medium. The amount is small and variable (0.007 to 0.05 μmol per ml). When the cells are placed in fresh medium, this nitrite is removed, and the cells do not excrete more nitrite rapidly. The lower level of enzyme found in the cell extract made from cells not given "refresher" treatment might be related to the nitrite levels in the medium.

When *Chlorella vulgaris* is grown on ammonia, the nitrate reductase almost disappears (Solomonson and Vennesland, 1972b) just as in the case of many other species (Payne, 1973). It takes some time, however, for the enzyme to disappear, whereas the effect of ammonia addition on nitrate utilization is almost immediate (Jetschmann and Vennesland, unpublished). For *Neurospora crassa*, it has been shown that ammonia inhibits a nitrate permease (Schloemer and Garrett, 1974). It has not been possible to separate nitrate uptake and utilization in *Chlorella vulgaris*, so that no direct evidence for a permease is at hand. The effect of added ammonia on the formation of reversibly-inactivated enzyme strongly suggests, however, that ammonia is inhibiting nitrate uptake, because ammonia added in the presence of nitrate gives the same effect as removal of nitrate, whereas ammonia added in the absence of nitrate has essentially no effect except over longer time periods. The implication of such a conclusion is that the inactivation of the enzyme is not the main controlling mechanism for nitrate utilization, but that the inactivation results when no nitrate is available intracellularly. It is conceivable that the state of activation or inactivation of the enzyme determines the rate of nitrate utilization under some circumstances, but not under others. This requires further study. In the absence of CO_2, nitrate utilization is dependent on the carbohydrate content of the cells (unpublished). The effect of carbohydrate content on the activation state of the enzyme has not yet been determined.

The biological precursor(s) of HCN in *Chlorella vulgaris* has not yet been identified, but the present results help to define the conditions that would be expected to increase the yield of HCN. Two important factors are O_2 and light. The requirement for O_2 may be absolute. Light does not seem to be essential, though it is stimulatory. Manganese is another factor that may play an important role, since the reversible inactivation of nitrate reductase is impaired in manganese-deficient cells.

One of the most effective agents for eliciting rapid reversible inactivation of the enzyme is the uncoupling agent, CCCP. This inactivation has been shown to be due to the generation of relatively large amounts of HCN when CCCP is added to *Chlorella*. The phenomenon is described elsewhere (Pistorius *et al.*, 1975).

It has long been known that Chlorella cells excrete glycolate at high light intensity, high O_2 tension and low CO_2 tension in the absence of nitrate (Warburg and Krippahl, 1960). These are the conditions that have here been shown to cause maximum reversible inactivation of nitrate reductase. We regard the enzyme inactivation as a kind of indicator of HCN generation. If high oxygen tension elicits excess HCN generation, then added HCN might mimic the effects of high O_2 tension. The following paper shows that this is the case (Vennesland and Jetschmann, 1976).

References

Gewitz, H.-S., Lorimer, G.H., Solomonson, L.P., Vennesland, B.: Presence of HCN in *Chlorella vulgaris* and its possible role in controlling the reduction of nitrate. Nature **249**, 79–81 (1974)

Heimer, Y.M.: Nitrate reductase of *Dunaliella parva*. Electron donor specificity and heat activation. Arch. Microbiol. **103**, 181–183 (1975)

Herrera, J., Paneque, A., Maldonado, J.M., Barea, J.L., Losada, M.: Regulation by ammonia of nitrate reductase synthesis and activity in *Chlamydomonas reinhardi*. Biochem. biophys. Res. Commun. **48**, 996–1003 (1972)

Jetschmann, K., Solomonson, L.P., Vennesland, B.: Activation of nitrate reductase by oxidation. Biochim. biophys. Acta (Amst.) **275**, 276–278 (1972)

Lorimer, G.H., Gewitz, H.-S., Völker, W., Solomonson, L.P., Vennesland, B.: The presence of bound cyanide in the naturally inactivated form of nitrate reductase of *Chlorella vulgaris*. J. biol. Chem. **249**, 6074–6079 (1974)

Losada, M., Herrera, J., Maldonado, J.M., Paneque, A.: Mechanism of nitrate reductase reversible inactivation by ammonia in *Chlamydomonas*. Plant Sci. Letters **1**, 31–37 (1973)

Losada, M., Paneque, A., Aparicio, P.J., Vega, J.M., Cárdenas, J., Herrera, J.: Inactivation and repression by ammonium of the nitrate reducing system in *Chlorella*. Biochem. biophys. Res. Commun. **38**, 1009–1015 (1970)

Maldonado, J.M., Herrera, J., Paneque, A., Losada, M.: Reversible inactivation by NADH and ADP of *Chlorella fusca* nitrate reductase. Biochem. biophys. Res. Commun. **51**, 27–33 (1973)

Moreno, C.G., Aparicio, P.J., Palacián, E., Losada, M.: Interconversions of the active and inactive forms of Chlorella nitrate reductase. FEBS Letters **26**, 11–14 (1972)

Nicholas, D.J.D., Nason, A.: Nitrite determination by diazotization and coupling reactions. In: Methods in Enzymology. Vol. III, pp. 983–984. Eds.: Colowick, S.P. and Kaplan, N.O., New York: Academic Press 1957

Payne, W.J.: Reduction of nitrogenous oxides by microorganisms. Bact. Rev. **37**, 409–452 (1973)

Pistorius, E.K., Gewitz, H.-S., Voss, H., Vennesland, B.: Cyanide generation from carbonylcyanide. m-chlorophenyl-hydrazone. FEBS Letters (in press)

Rigano, C., Aliotta, G., Violante, U.: Reversible inactivation by ammonia of assimilatory nitrate reductase in *Cyanidium caldarium*. Arch. Microbiol. **99**, 81–90 (1974)

Rigano, C., Violante, U.: Effect of heat treatment on the activity *in vitro* of nitrate reductase from *Cyanidium caldarium*. Biochim. biophys. Acta (Amst.) **256**, 524–532 (1972)

Schloemer, R.H., Garrett, R.H.: Nitrate transport system in *Neurospora crassa*. J. Bact. **118**, 259–269 (1974)

Solomonson, L.P.: Regulation of nitrate reductase activity by NADH and cyanide. Biochim. biophys. Acta (Amst.) **334**, 297–308 (1974)

Solomonson, L.P., Jetschmann, K., Vennesland, B.: Reversible inactivation of the nitrate reductase of *Chlorella vulgaris* Beijerinck. Biochim. biophys. Acta (Amst.) **309**, 32–43 (1973)

Solomonson, L.P., Lorimer, G.H., Hall, R.L., Borchers, R., Bailey, J.L.: Reduced nicotinamide adenine dinucleotide-nitrate reductase of *Chlorella vulgaris*: Purification, prosthetic groups and molecular properties. J. biol. Chem. **250**, 4120–4127 (1975)

Solomonson, L.P., Vennesland, B.: Properties of nitrate reductase of *Chlorella*. Biochim biophys. Acta (Amst.) **267**, 544–557 (1972a)

Solomonson, L.P., Vennesland, B.: Nitrate reductase and chlorate toxicity in *Chlorella vulgaris* Beijerinck. Plant Physiol. **50**, 421–424 (1972b)

Vennesland, B., Jetschmann, C.: The nitrate reductase of *Chlorella vulgaris*. Biochim. biophys. Acta (Amst.) **227**, 554–564 (1971)

Vennesland, B., Jetschmann, K.: The effect of cyanide and some other carbonyl-binding reagents on glycolate excretion by *Chlorella vulgaris*. Planta (Berl.) **128**, 81–84 (1976)

Warburg, O., Krippahl, G.: Glykolsäurebildung in Chlorella. Z. Naturforsch. **15**b, 197–199 (1960)

Received 11 August; accepted 8 September 1975