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REGULATION OF THE ASSIMILATION OF NITRATE IN *CHLAMYDOMONAS REINHARDII*

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Key Word Index—*Chlamydomonas reinhardii*; Chlorophyceae; regulation; nitrate assimilation; nitrate reductase inactivation; ammonia assimilation.

Abstract—In *Chlamydomonas*, The assimilation of ammonia proceeds through the glutamine synthetase–glutamate synthase pathway. The primary target in the regulation of nitrate assimilation by this alga appears to involve the nitrate uptake system. Evidence is presented which shows that its operation is immediately inhibited by ammonia, darkness, or uncouplers of photophosphorylation. In addition, when nitrate was removed from the cell cultures, or its uptake was blocked, the nitrate reductase activity became inactive. The original level of enzyme activity was recovered when the nitrate uptake by the cells was restored. Cyanate prevents the uptake of nitrate by *Chlamydomonas* cells, but *in vivo* it protects the nitrate reductase against the inactivation produced by the nitrate uptake interruption. Moreover, *in vivo*, cyanate promotes the nitrate reductase activation. In *Chlamydomonas*, the nitrate reductase activity appears to be controlled by the intracellular nitrate concentration through the nitrate uptake system.

INTRODUCTION

The reduction of nitrate to ammonia has been extensively studied in green algae, and it is mediated by the consecutive action of two enzymes, NAD(P)H-nitrate reductase (EC 1.6.6.2) and ferredoxin-nitrite reductase (EC 1.7.7.1). At present, there is considerably less information available concerning the mechanism of nitrate uptake and the pathway of ammonia assimilation (see [1, 2]).

The nitrate uptake system of green algae is an energy-requiring process [3, 4] which is stimulated by CO_2 or other carbon sources, and light [3, 5], or by nitrogen starvation [6, 7]. The system is inhibited by ammonia [4, 8, 9], chloride, or high concentrations of nitrate [6]. It would now appear that the assimilation of ammonia by green algae operates through the glutamine synthetase–glutamate synthase pathway [2, 10], although a significant role for glutamate dehydrogenase had been proposed [1].

Most of the attention paid to the regulation of nitrate assimilation in green algae has been focused on the activity level of nitrate reductase. However, the nitrate uptake system also seems to play a significant role in the control of the available nitrate; this has been shown in *Chlorella* [4, 5, 9] and *Ankistrodesmus braunii* [6]. The nitrate reductase from green algae is an interconvertible enzyme, the intracellular level of which depends on nutritional and environmental conditions [11, 12]. Of particular interest is the ammonia-promoted inactivation of nitrate reductase *in vivo*, and the *in vitro* reactivation of the inactive enzyme by treatment with ferricyanide, since this appears to be a general behaviour for the nitrate reductases from different species of green algae including *Chlamydomonas* [12]. Inactivation of *Chlamydomonas* nitrate reductase has been shown to take place also after transfer of cells to darkness [13, 14].

In this paper we have studied the regulation of nitrate assimilation in *Chlamydomonas reinhardii* with respect to the nitrate uptake and nitrate reductase activity. An important innovation was the assay of the enzyme activity *in situ*, using cells whose permeability was altered by treatment with toluene. This method limits possible alterations originated during cell collection and crude extract preparation. The results reported here show that: (a) the nitrate uptake system is the primary point of control of the nitrate assimilation in this alga, and (b) the intracellular nitrate reductase activity, which may represent another point of control for this pathway, depends on the availability of nitrate, which protects the enzyme *in vivo* against its reversible inactivation, and restores the activity once inactivation has occurred. Some of the results shown here have been already presented in abstract form [14, 15].

RESULTS

Nitrate assimilation pathway in Chlamydomonas

Table 1 shows the activities of the enzymes involved in the nitrate assimilation by *Chlamydomonas* under different culture conditions. It should be noted that (a) in cells grown with nitrate as sole nitrogen source, the activity level of nitrate reductase is lower than that shown by the other enzymes. Nitrite reduc-

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Table 1. Activities of the enzymes involved in the assimilation of nitrate in *Chlamydomonas* cells under different culture conditions

Enzyme	Specific activity (nkat/mg)				
	No nitrogen	NO_3^-	$NO_3^- + NH_4^+$	$NO_3^- + MSX$	NH_4^+
Nitrate reductase	0.01	0.34	0.03	0.03	0.03
Nitrite reductase	0.02	1.34	0.17	0.14	0.03
Glutamine synthetase	2.86	2.16	2.35	0	2.16
Glutamate synthase	0.34	0.39	0.39	0.31	0.39

Cells growing at the logarithmic phase on nitrate as nitrogen source were harvested, washed, and resuspended in fresh media (20 μg of chl/ml) without nitrogen source, and where indicated, 8 mM nitrate, 8 mM ammonia, and 0.2 mM MSX were added. In the culture with a mixture of nitrate plus ammonia, these compounds were added at 4 mM each. After 8 hr of growth in the light, the cells were harvested, washed and the enzymatic activities were measured as indicated in the Experimental, using aliquots of the corresponding crude extract prepared by freezing the cells.

tase and glutamine synthetase activities appear to be in large excess; (b) in the presence of ammonia, nitrate and nitrite reductase levels are negligible, but glutamine synthetase and glutamate synthase are essentially unaltered compared to growth of the algae on nitrate; (c) in the presence of L-methionine-DL-sulfoximine (MSX), a specific inhibitor of the glutamine synthetase activity, the nitrate reductase and nitrite reductase activities are very low, even in the presence of nitrate as nitrogen source. Glutamate synthase does not show significant change under these conditions.

Other activities which have been considered to participate in ammonia assimilation, such as glutamate dehydrogenase and alanine dehydrogenase are usually present at levels about 0.02 nkat/mg and 1.29 nkat/mg respectively, when ammonia was used as nitrogen source. These values did not show significant changes under the culture conditions used in Table 1. When ammonia was used as sole nitrogen source, its utilization rate by the cells was 6.8 μmol/mg chl/hr. However, when the cells were preincubated with MSX (0.2 mM) or azaserine (5 mM) for 20 min, the ammonia utilization was completely blocked (not shown), evidence consistent with the view that the glutamate synthase cycle functions as the sole pathway of ammonia assimilation in *Chlamydomonas*.

Nitrate assimilation by Chlamydomonas *cells*

Table 2 shows the effect of different compounds or metabolic situations on the nitrate utilization by *Chlamydomonas*. The addition of ammonia, or inhibitors of the ammonia assimilation, such as MSX or azaserine, which lead to intracellular ammonia accumulation, immediately blocks the nitrate consumption by the cells, and also induces the nitrate reductase inactivation. Similar effects were observed when the cells were placed in the darkness, or if uncouplers of the photophosphorylation, such as carbonylcyanid-*p*-trifluoromethoxy-phenylhydrazone (FCCP), methylamine or arsenate, were added to the culture.

The nitrate utilization by the cells and the normal level of nitrate reductase activity were achieved by restoring the initial culture conditions (Florencio and Vega, unpublished observations).

Antagonistic effect of ammonia in the nitrate assimilation by Chlamydomonas

The assimilation of nitrate in *Chlamydomonas* is highly sensitive to the addition of low concentrations of ammonia [1, 11]. In order to ascertain if this effect was due to ammonia itself or to any metabolite formed from the ammonia assimilation, we studied the effect of ammonia on the nitrate utilization by *Chlamydomonas* in the presence or in the absence of MSX. The results shown in Fig. 1 indicate that ammonia blocks the nitrate utilization by the cells in both cases, independently of whether assimilation of ammonia is inhibited or not.

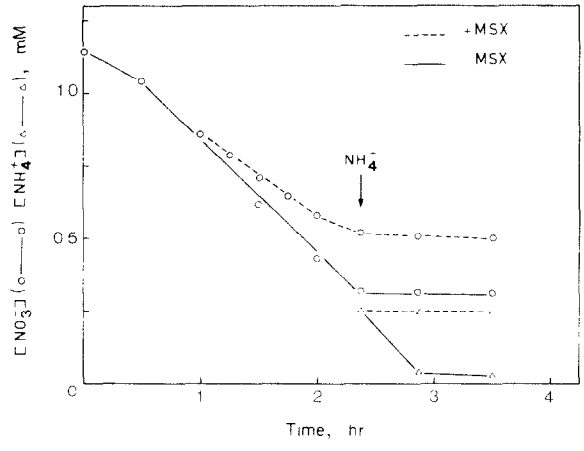

Fig. 1. Effect of ammonia on the nitrate utilization by *Chlamydomonas* in the presence or absence of MSX. A cell suspension containing 25 μg chl/ml, and prepared as indicated in the Experimental for *in vivo* experiments was used. At zero time, 1 mM nitrate was added and the culture was continuously shaken under illumination. After 1 hr, the culture was divided in two fractions and 0.2 mM MSX was added to one of them. When glutamine synthetase activity was negligible (arrow) in the culture with MSX, 0.25 mM ammonia was added to each culture. At the indicated times, nitrate and ammonia were determined in the cultures after removing cells by centrifugation.

Effect of darkness on nitrate assimilation by Chlamydomonas

When cells growing with nitrate as sole nitrogen source were darkened, the utilization of nitrate was immediately blocked and the nitrate reductase activity slowly inactivated (Fig. 2). A similar inhibition pattern of nitrate assimilation was observed after addition of ammonia or uncouplers of the photophosphorylation to cell cultures (not shown). Fig. 2 also shows that light promotes the nitrate reutilization by the cells, and the recovering of the nitrate reductase activity.

Nitrate reductase inactivation in vivo

As seen in Table 2, when nitrate utilization by the cells was interrupted, the nitrate reductase activity level became low after 1 hr of treatment. The enzyme inactivation was reversible in all cases, and the original nitrate reductase activity was recovered by restoring the initial culture conditions, or *in vitro* by treating with ferricyanide the toluenized cells, or the corresponding crude extracts which contained the inactive nitrate reductase (not shown).

In order to determine if the enzyme inactivation *in vivo* followed the interruption of the nitrate uptake, and consequently a lacking of nitrate inside the cells, the nitrate reductase activity was studied in a culture of *Chlamydomonas* in which the nitrate of the medium was consumed. In these conditions, the nitrate reductase activity was inactivated, its level being negligible 40 min after the depletion of nitrate in the medium (Fig. 3).

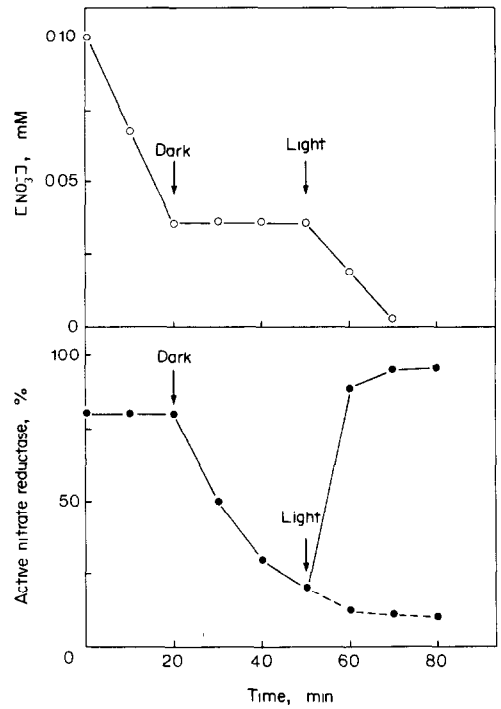

Fig. 2. Effect of darkness on nitrate utilization and nitrate reductase activity in *Chlamydomonas*. At zero time, 0.1 mM nitrate was added to a cell suspension containing 30 μg chl/ml and placed under illumination and continuous shaking in a bath at 25°. Dark–light transition period is indicated by the arrows. Nitrate was determined in the culture medium after removing the cells. Total and active nitrate reductase activities were determined *in situ* as described in the Experimental. 100% nitrate reductase activity in each determination represents the value obtained for total activity (after activation with ferricyanide).

Table 2. Effect of different compounds which inhibit the nitrate utilization by *Chlamydomonas* cells on the nitrate reductase activity

Addition	Concentration (mM)	Nitrate utilization (%)	Nitrate reductase activity (%)
None	—	100	100
NH_4^+	1	0	22
MSX	0.5	5	27
Azaserine	5	10	20
Darkness	—	0	10
FCCP	0.01	0	7
Methylamine	6	20	22
Arsenate	30	10	25

Cells suspensions, containing 25 μg chl/ml, were prepared for *in vivo* experiments with 0.2 mM nitrate as sole nitrogen source, and placed in separate flasks. After 20 min under illumination (100 W/m^2), different compounds were added to each culture at the indicated concentrations, placing one of the cultures in the darkness. Nitrate in the media was measured at 5 min intervals, and BVH-nitrate reductase was measured *in situ* after 60 min of the addition. 100% of nitrate utilization rate corresponds to 9 μmol/mg chl/hr, and 100% BHV-nitrate reductase to 0.42 nkat/mg. Other conditions in the Experimental.

Effect of cyanate on the in vivo *interconversion of nitrate reductase*

Cyanate is a competitive inhibitor with respect to nitrate of the nitrate reductase from the green alga *Chlorella fusca* with a K_i of 1.0 μM [16]. The addition of 40 μM cyanate to cells growing with nitrate produces an interruption of the nitrate consumption by the cells, but the nitrate reductase activity remains high during the experiment (Fig. 4) which indicates that cyanate protects the nitrate reductase activity against its inactivation by the absence of nitrate inside the cells. Furthermore, the addition of cyanate to cells lacking nitrate, and consequently with low level of nitrate reductase, completely restores the nitrate reductase activity after 30 min (Fig. 5).

DISCUSSION

The results shown in this paper confirm that ammonia assimilation in *Chlamydomonas* occurs through the glutamine synthetase–glutamate synthase pathway, as has been reported in green algae [2, 10] and other photosynthetic organisms [17, 18, 19]. The effect of ammonia and other nutritional conditions, such as darkness or uncouplers of the photophosphorylation, on the nitrate utilization and nitrate

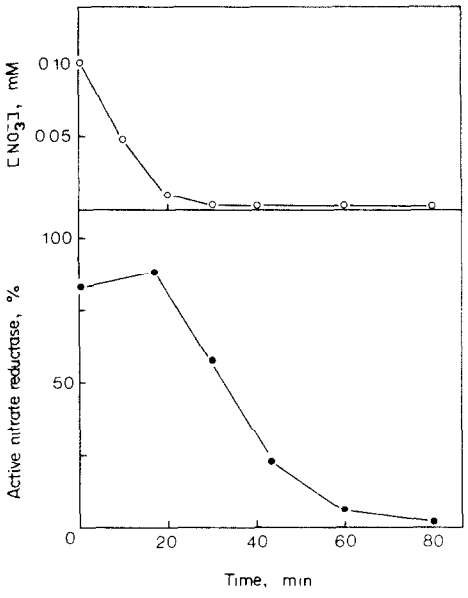

Fig. 3. Inactivation of nitrate reductase in nitrate-lacking cultures of *Chlamydomonas*. A cell suspension containing 40 μg chl/ml was placed in a bath at 25° under illumination. At zero time, 0.1 mM nitrate was added, and at the indicated times, measurements of nitrate in the medium and intracellular nitrate reductase activity were performed as described in Fig. 2.

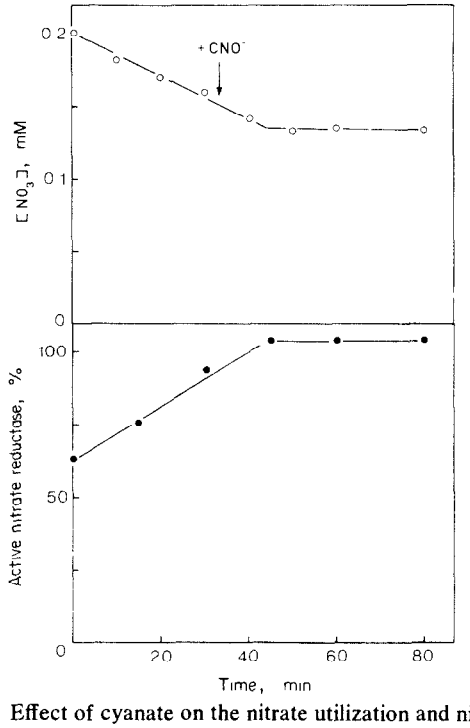

Fig. 4. Effect of cyanate on the nitrate utilization and nitrate reductase activity in *Chlamydomonas*. A cell suspension containing 25 μg chl/ml and 0.2 mM nitrate was placed under illumination in a bath at 25°. When indicated by the arrow, 40 μM cyanate was added and determinations of nitrate in the medium and intracellular nitrate reductase activity were performed as described in Fig. 2.

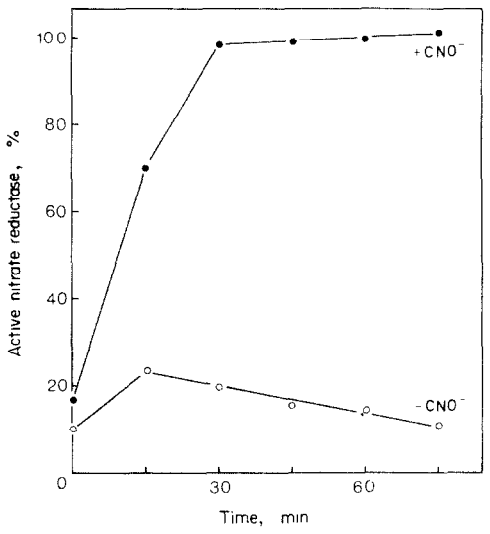

Fig. 5. *In vivo* reactivation by cyanate of nitrate reductase in *Chlamydomonas*. Two cell suspensions containing 40 μg chl/ml each were placed in a bath at 25° without nitrate. When nitrate reductase activity was low, 40 μM cyanate was added (zero time) to one of the cultures. At the indicated times, nitrate reductase activity was determined in each culture. For more details see Fig. 2.

reductase inactivation rates in *Chlamydomonas* have been studied in a series of experiments. The general picture emerging from these studies was of a dramatic and immediate interruption of nitrate utilization by the cells, under conditions where only a relatively slow inactivation of the nitrate reductase occurred. Changes in the activity of nitrate reductase did not appear to be responsible for the rapid interruption of the nitrate utilization and the observations lend support to an important role of the nitrate transport system in the regulation of the nitrate assimilation, see for example references on *Chlamydomonas* [1], other green algae [4–6, 9], diatoms [20, 21], and cyanobacteria [18, 22].

The nitrate uptake system of *Chlamydomonas* appears to show a very high sensitivity to inhibition by ammonia itself, rather than a metabolite formed from ammonia assimilation (Fig. 1). This observation substantially differs from the data reported in this [23] and other algae [1, 17, 18, 24] where the inhibition by ammonia of the nitrate assimilation apparently depended on its assimilation.

The inhibitory effect of darkness on nitrate utilization by *Chlamydomonas* has been related to the interruption of CO_2 assimilation [23]. The results of Fig. 2 are consistent with this inhibition, but they can also be interpreted as if the darkness promoted the inhibition of nitrate utilization by decreasing the intracellular pool of ATP, since a similar pattern of inhibition was observed after the addition to the cell cultures of uncouplers of photophosphorylation (Table 2). In *Chlamydomonas*, the inhibition of nitrate utilization by darkness, which is too rapid to be related with a depletion of carbon reserves in the cell, or with the inactivation of nitrate reductase, is probably located at the nitrate uptake system level, and the observed enzyme inactivation may be a con-

sequence of the nitrate uptake inhibition. Darkness promoted inhibition of nitrate reductase in *Anabaena cylindrica* [22], but did not inhibit the nitrate uptake in the diatom *Skelectonema costatum* [20]. On the other hand, the nitrate uptake system and consequently the nitrate reductase activity are stimulated by light (Fig. 2). Inactivation–reactivation of nitrate reductase following dark–light transitions has been also reported in *Chlorella* [9, 25, 26].

The inactivation of nitrate reductase in *Chlamydomonas* cells is generally observed when the nitrate of the media has been consumed (Fig. 3), or its uptake inhibited (Table 2), which indicates that the absence of nitrate inside the cells promotes the nitrate reductase inactivation. In other words, nitrate, *in vivo*, protects the enzyme against its reversible inactivation. This hypothesis is supported by the results of Fig. 4 where cyanate, competitive inhibitor with respect to nitrate of the nitrate reductase, protects the enzyme against the inactivation derived from the inhibition of the nitrate uptake. These observations are consistent with those reported by Pistorius *et al.* [27] in which addition of ammonia to *Chlorella vulgaris* cells growing with nitrate promoted the reversible inactivation of nitrate reductase to an extent similar to that observed by removing nitrate from the culture media, but they contradict the hypothesis of Syrett and Leftley [1] that ammonia-promoted nitrate reductase inactivation in *Chlamydomonas* was due to the action of a metabolite formed during ammonia assimilation.

The results reported here indicate that nitrate reductase reactivation occurs when nitrate penetrates into the cells. Nitrate reductase can be also reactivated *in vivo* by cyanate (Fig. 5) which is unable to reoxidize the enzyme [16]. On the other hand, inactive nitrate reductase of *Chlamydomonas* is fully activated *in vitro* by oxidation with ferricyanide. These observations support the hypothesis that nitrate is a good candidate to be the physiological reactivator of nitrate reductase, because it has the ability to bind at the active site of the enzyme (as cyanate does) and also reoxidize it (as ferricyanide does). A mechanism of nitrate reductase reactivation *in vivo* by its own substrate has been also proposed in *Chlorella* [4].

EXPERIMENTAL

Materials. L-Methionine-DL-sulfoximine, azaserine, morpholinopropane sulfonic acid (MOPS), ATP, L-glutamate, L-glutamic dehydrogenase (Type II, from bovine liver), and bovine serum albumin were purchased from Sigma. Benzyl viologen (BV) and methyl viologen (MV) were from Serva. NADH and FCCP were from Boehringer. All other chemicals were reagent grade and used as supplied by Merck.

Organism and culture conditions. Chlamydomonas reinhardii (strain 6145 *c*, a generous gift of Dr. R. Sager, Hunter College, New York) was grown at 25° in 250-ml tubes or 1 l. bottles, using the medium previously described [28] containing 8 mM KNO_3 or NH_4Cl as nitrogen source. Air enriched with CO_2 5% (v/v) was bubbled through the culture, which was illuminated with white fluorescent light (20 W/m^2, at the surface of the tube). For short time *in vivo* experiments, the cells were harvested in the logarithmic phase of growth and resuspended in 50 mM MOPS buffer, pH 7, containing 7 mM $NaHCO_3$, and the indicated nitrogen source. 50 ml of the cell suspension in the 250-ml flask was placed in a bath at 25° with continuous stirring and illumination (100 W/m^2).

Crude extract preparation. The cells were harvested by centrifugation at 12 000 *g* for 10 min, washed with 50 mM MOPS buffer, pH 7, and frozen at −20° until use. They were broken by thawing in MOPS buffer (5 ml/g fr. wt cells), and the suspension was slowly stirred for 20 min at 0°, and then centrifuged at 27 000 *g* for 10 min. The supernatant was used as crude extract.

Enzyme activities. The *in situ* assay of active BVH-nitrate reductase was performed using 1 ml of cell suspension which was vigorously shaken for 1 min in the presence of 20 μl of toluene. Furthermore, 0.9 ml of a mixture containing, Tris–HCl buffer, pH 7.5, 100 μmol; KNO_3, 10 μmol; and BV, 0.2 μmol, were added. The reaction was started by the addition of 1 mg of Na dithionite in 0.1 ml of 0.5 M Tris–HCl buffer, pH 7.5. After 10 min of incubation at 30°, the reaction was stopped by vigorous shaking until oxidation of dithionite was complete, and then the nitrite formed was determined. The total nitrate reductase activity was estimated after reactivation with ferricyanide performed by preincubating the toluenized cell suspensions for 2 min with 50 μl of 6 mM ferricyanide.

BVH-nitrate reductase and MVH-nitrite reductase activities were determined in the crude extracts as previously described [29].

Glutamine synthetase was determined in the crude extract following its synthetic activity as described in ref. [30]. Glutamate synthase activity was measured in the crude extract following the method of ref. [31].

Enzyme activity is expressed in katals which is the amount of activity effecting the conversion of 1 mol of substrate per sec.

Protein determination. Protein was determined by the method of ref. [32] with BSA as standard. Cellular protein was extracted with 10% TCA soln.

Analytical methods. Nitrate was determined by measuring the absorbance at 210 nm as previously described in ref. [33]. Nitrite was estimated by the method of ref. [34] and ammonia, determined either colorimetrically, according to ref. [35] or enzymatically, following the disappearance of NADH in the presence of 2-oxoglutarate and glutamate dehydrogenase [36].

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