Phytochemistry, Vol. 28, No. 2, pp. 349–354, 1989.
Printed in Great Britain.

0031–9422/89 $3.00 + 0.00

NITROGEN SUPPLY AND LIGHT INTENSITY ON PROPERTIES OF GLUTAMATE DEHYDROGENASE AND GLYCOLLATE OXIDASE IN *LEMNA*

RICARDO M. B. FERREIRA* and DAVID D. DAVIES

School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

(*Received* 16 *May* 1988)

Key Word Index—*Lemna minor*; Lemnaceae; glycollate oxidase; purification; properties; glutamate dehydrogenase; nitrogen source; effect of light.

Abstract—The properties of glycollate oxidase and glutamate dehydrogenase were studied in relation to the conditions used for the growth of *Lemna* from which the enzymes were extracted. Glycollate oxidase is an octameric protein (M_r 300 000) with an absorption spectrum indicating the presence of FMN and another chromophore. The enzyme could neither be detected nor activated in etiolated fronds, but the activity in green fronds was promoted by light. Contrary to earlier reports, we found no differences in the properties of glycollate oxidase isolated from *Lemna* grown in nitrate or ammonium. Light intensity and the form of inorganic nitrogen markedly affect the relative activity of glutamate dehydrogenase with NADH and NADPH. Our data are consistent with the presence of an enzyme whose coenzyme specificity is modified in response to environmental conditions.

INTRODUCTION

Although glycollate oxidase (EC 1.1.3.1), a key enzyme of photorespiration [1] was first isolated from tobacco leaves more than 30 years ago [2, 3], its quaternary structure is still controversial, with diversity of structure being reported between and within species [4–7]. Some of the reported differences could be artefactual or due to growth conditions. Thus, Emes and Erismann [8] reported that *Lemna* glycollate oxidase had different molecular properties depending on whether the fronds were grown on nitrate or ammonium.

The physiological role of glutamate dehydrogenase (EC 1.4.1.2, EC 1.4.1.3, EC 1.4.1.4) in plants is now somewhat uncertain. Joy [9] has suggested that *Lemna* contains two forms of glutamate dehydrogenase, one specific for NADH, the other for NADPH, in which the relative activity is affected by the balance between nitrate and ammonium present in the growth medium. On the other hand, Ehmke and Hartmann [10] argued that *Lemna* contains a single glutamate dehydrogenase active with both NADH and NADPH, and failed to detect any changes in enzyme activity levels in response to nitrogen source, the ratio of the relative activities remaining constant during various treatments.

In this paper we report the effect of various growth conditions on the forms of glycollate oxidase and glutamate dehydrogenase present in *Lemna*.

RESULTS AND DISCUSSION

Glycollate oxidase

Purification of glycollate oxidase

The purification of glycolate oxidase from *Lemna* is summarized in Table 1. The enzyme was purified to homogeneity (1120-fold), as shown by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE). The purified enzyme had a specific activity of 559.7 nKat/mg protein. The addition of FMN to the pure enzyme did not alter its specific activity, indicating that FMN was not depleted during the purification procedure. An apparent increase in glycollate oxidase activity following the $(NH_4)_2SO_4$ step (Table 1) was observed in all preparations. A similar effect was previously reported [11].

The purification was achieved by exploiting the rather alkaline isoelectric point of the enzyme and by the use of an affinity chromatography step. Indeed, at pH 8.3, glycollate oxidase did not bind to the DEAE cellulose, whereas 95% of the extract proteins did. A similar step was used in the purification of the enzyme from *Pisum sativum* leaves [5]. Affinity chromatography was performed with sepharose aminohexyl oxamate [11], since *N*-octyl oxamate is a reversible inhibitor of glycollate oxidase [12].

Absorption spectrum

The absorption spectrum of glycollate oxidase from *Lemna minor* is shown in Fig. 1, Fig. 1A shows the absorption spectrum of the oxidized and reduced forms of the enzyme. The oxidized spectrum shows a typical dominance of the flavin chromophore, with maxima at 275, 345 and 445 nm, and shoulders at 410 and 480 nm. The ratio absorbance due to the protein peak in the near UV/A_{445} may be taken as an index for glycollate oxidase purity: the lower the ratio, the purer the enzyme. For the spectrum shown in Fig. 1A, the calculated ratio is 6.0, as compared with other values reported for the enzyme from rat liver (7.0; [11]), lettuce leaves (7.3; [13]), pumpkin cotyledons (6.7; [14]) and human liver (6.1; [12]). The characteristic peaks of FMN disappeared when the enzyme was reduced anaerobically by glycollate.

To check the effect of pH on the spectrum of glycollate oxidase, a preliminary experiment was performed, in

*Present address: Departamento de Botânica, Instituto Superior de Agronomia, P-1399 Lisboa Codex, Portugal.

Table 1. Purification of *Lemna* glycollate oxidase

Purification step	Total protein (mg)	Total activity (nkat)	Specific activity (nkat/mg) protein)	Recovery (%)	Purification factor	
					In step	total
(A) Crude homogenate	2883	1421.7	0.50	100.0	1	1
(B) 0–55% $(NH_4)_2SO_4$ fractionation	1480	1938.3	1.33	136.3	2.7	2.7
(C) DEAE + CM-cellulose chromatography	71	2893.3	40.83	203.6	30.6	81.7
(D) Affinity chromatography	4.1	1681.7	410.2	118.3	10.0	820.3
(E) FPLC Mono Q column	1.9	1063.3	559.7	74.8	1.4	1119.3

Lemna fronds were grown in complete medium containing nitrate as the only nitrogen source.

which the absorption spectrum of free FMN was determined at three different pH values (Fig. 1B). The peak at 372 nm seems to shift gradually to shorter wavelengths as the pH increases, which is probably due to an ionization of FMN with a pK of 10.3 [15]. *Lemna* glycollate oxidase does not have a peak at 372 nm—rather, it has a peak at 345 nm, presumably due to the effect of the protein on the 372 nm FMN peak.

The dependence of glycollate oxidase absorption spectrum on pH is shown in Fig. 1C and D. The main features are: (i) the gradual shift to shorter wavelengths of the peak at 345 nm as the pH increases, probably due to the ionization of FMN (Fig. 1B); (ii) the relative height of the two peaks at 345 and 445 nm: as the pH increases from 8.6 to 10.2, the ratio A_{345}/A_{445} gradually increases from 0.95 to 1.25; (iii) as the pH increases from 8.6 to 10.2, the peak at 445 nm gradually gives rise to two peaks: one at 450 nm and another at 425 nm. This result suggests that the peak at 450 nm is due to the FMN molecule, whereas the peak at 425 nm may be due to a second chromophore, reported in the pig liver enzyme to have an absorption maximum at 425 nm [15], and whose structure was found to be 6-OH-FMN [16].

Molecular structure of glycollate oxidase

When subjected to SDS-PAGE, purified preparations of glycollate oxidase from *Lemna* gave a single protein band, suggesting that the enzyme consists of only one type of subunit. The M_r of the subunit was estimated to be *ca* 38 000 (Table 2). The subunit size reported for glycollate oxidase from a wide variety of species is generally within the range 37 000–48 000. Allowing for the error in SDS-PAGE, which is $ca \pm 10\%$ [17], this suggests a similar subunit size regardless of the source of the enzyme [6].

Reports on M_r and structure studies conducted with native glycollate oxidase obtained from various animal and plant sources have yielded widely divergent results—M_rs from 93 000 [*Spartina anglica*, 6] to 700 000 [cucumber cotyledons; 18] have been reported, with the number of subunits varying from two [pea leaves; 5] to 16 [cucumber cotyledons; 18]. However, most reports suggest either a tetrameric or octameric structure for glycollate oxidase. The reason for this apparent diversity of structure is unclear, but several hypotheses have been suggested [6]: (i) instability during the early stages of purification with an intrinsic tendency to form aggregates [18]; (ii) different molecular forms of the enzyme may have been selected during the isolation procedure; [iii] the enzyme may have real diversity in its molecular structure in different species.

Purified glycollate oxidase from *Lemna* has an estimated molecular weight of *ca* 300 000 (Table 2), consistent with an octameric structure. This is in close agreement with the data for spinach leaves [19] and pumpkin cotyledons [14]. Lindqvist and Branden [7] recently reported the high resolution structure of the spinach leaf enzyme by X-ray crystallography, showing that the enzyme has an octameric form in the crystal. However, it contrasts with other studies [6, 12], including the reported M_rs of 250 000 and 500 000 for *Lemna* glycollate oxidase (8).

The effect of nitrate or ammonium in the growth medium on the properties of glycollate oxidase

Emes and Erismann [8] reported that glycollate oxidase is influenced by the form of nitrogen supplied to *Lemna minor* in such a way that the enzyme from plants grown on ammonium had a M_r weight of 500 000, whereas the enzyme from *Lemna* grown on nitrate had a M_r of 250 000. Our experiments have not confirmed this report. Table 2 shows a comparison of several molecular characteristics of purified glycollate oxidase from *Lemna* grown on either nitrate or ammonium. All characteristics investigated were similar, suggesting that only one form of glycollate oxidase is present in *Lemna*.

Lemna glycollate oxidase was found to have a pH optimum of 8.3 and an isoelectric point of *ca* 8.8. These findings are in good agreement with those obtained for the enzyme from other species (5, 11, 13, 14, 18). The rather alkaline isoelectric point was further illustrated by the fact that the enzyme did not migrate at pH 9.3 in polyacrylamide (5.5% w/v) gel electrophoresis—a similar situation to that previously reported for the pumpkin cotyledon enzyme [14].

The effect of light

Lemna fronds were grown under several irradiances. For each light condition, the soluble protein fraction was isolated and assayed for glycollate oxidase, chlorophyll *a* and *b* and protein, the results are presented in Fig. 2. Because of the absence of glycollate oxidase in dark grown fronds, extracts from dark grown fronds were

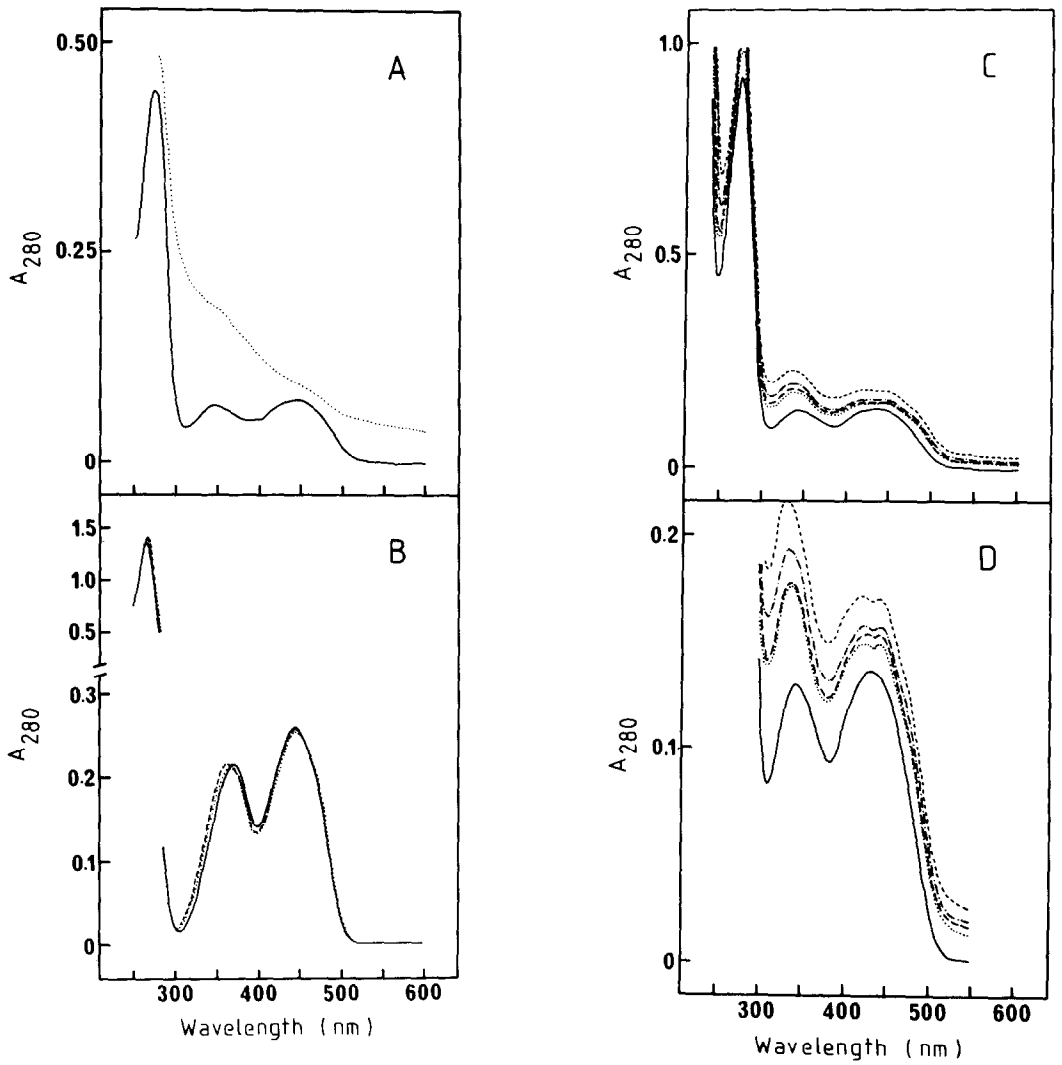

Fig. 1. Absorption spectrum of *Lemna* glycollate oxidase. Purified glycollate oxidase was extensively dialysed against 1 mM piperazine buffer pH 9.4. Enough 1.0 M piperazine buffer (with the required pH) was added to obtain a 70 mM piperazine buffer protein solution. The absorption spectrum of the enzyme was determined in a 1 ml silica cuvette at 10°, in a Pye-Unicam SP8-200 spectrophotometer. The absorption spectrum of 70 mM piperazine buffer was checked at several pH values, but no significant differences were observed. All readings were made against a 70 mM piperazine buffer solution. (A) (——): Absorption spectrum of oxidised glycolate oxidase dissolved in 70 mM piperazine buffer, pH 8.6; (· · · · ·): Absorption spectrum of anaerobically reduced glycolate oxidase (by addition of glycolate) dissolved in the same buffer. (B) Absorption spectrum of FMN dissolved in 70 mM piperazine buffer, at three different pH values: 8.6 (——), 10.1 (· · · · ·), and 10.7 (------). (C) Absorption spectrum of oxidized glycolate oxidase dissolved in 70 mM piperazine buffer, at five different pH values: 8.6 (——), 9.3 (· · · · ·), 9.6 (– – –), 9.9 (-.-.-.), and 10.2 (------). (D) As in (C) but using an expanded scale.

mixed with extracts from plants grown in the light and assayed for glycollate oxidase. In all cases, the measured activities indicated the absence of inhibitors in the etiolated fronds.

Light has long been known to promote the development of glycollate oxidase activity in leaves [20, 21]. In *Lemna minor*, glycollate oxidase activity increases with increasing irradiance, paralleling the increase in the growth rate of *Lemna*. (Fig. 2A).

Tolbert and coworkers [21–23] have shown that glycollate oxidase is present in green tissue, but little activity is found in the leaves of plants germinated and grown in total darkness. However, when etiolated leaves grown in total darkness are placed in the light, the activity of glycollate oxidase increases dramatically during the first 6 hr of illumination, and its activity continues to increase for at least 48 hr [21, 22]. Furthermore, when the leaves of etiolated plants were sprayed with substrate (glycollate, lactate, α-hydroxybutyrate or glyoxylate), glycollate oxidase increased during the following 24 hr [21, 23]. Additionally, incubation of a cell-free extract of etiolated leaves with glycollate or FMN for 18 hr also resulted in a sharp

Table 2. Molecular characteristics of glycollate oxidase from *Lemna* grown on either nitrate or ammonium as the source of nitrogen

Enzyme characteristics	NO_3^-	NH_4^+
Enzyme purity	pure*	pure*
K_m for glycollate (mM)	0.22–0.22†	0.26–0.23†
K_m for O_2 (mM)	0.15–0.16†	0.21–0.17†
Native M_r	304 000	301 000
Subunit M_r	38 000	38 500
Number of subunits	8	8
Isoelectric point	8.8	8.7

Two batches of *Lemna* fronds were grown in complete medium containing either nitrate or ammonium as the only source of nitrogen. Glycollate oxidase from each batch was extracted and purified as described in the Experimental section. K_m values were estimated using two BASIC computer programs [38]. To estimate this parameter for glycollate and O_2, glycollate oxidase was assayed using the 2,6-dichlorophenol indophenol method and the oxygen electrode method, respectively. The enzyme native M_r was estimated by gel filtration on Sephacryl S-300 superfine, whereas the subunit molecular weight was by SDS-PAGE. The isoelectric point was measured by chromatofocusing on PBE-94.

*As judged by SDS-PAGE (10% w/v acrylamide).

†Estimates obtained by the use of two different methods: best fit (least squares method) to the Michaelis–Menten equation and direct linear plot, respectively.

increase in enzyme activity. However, it should be noted that although a very active glycollate oxidase was always found in extracts from green leaves, its activity could also be increased two to fivefold by *in vitro* incubation with FMN or glycollate [23]. Tolbert and coworkers have suggested that glycollate oxidase proenzyme exists in etiolated leaves, but in amounts insufficient to account for all the active enzyme present in the corresponding green tissue.

To test this hypotheses for *Lemna minor*, fronds were grown in complete darkness. Glycollate oxidase activity could not be detected in extracts prepared from etiolated fronds (Fig. 2A). Furthermore, we could not determine enzyme activity when etiolated fronds were: (i) transferred to light for up to 18 hr; (ii) sprayed in the dark with 0.1 M glycollate 24 hr before extraction; (iii) extracted and incubated *in vitro* for up to 24 hr in the presence of either 10 mM glycollate or 10 mM FMN. Incubation of an extract prepared from etiolated fronds with an extract prepared from light grown fronds failed to show any increase in enzyme activity.

The greening of etiolated seedling leaves is a programmed development involving the activation of glycollate oxidase. The failure to detect the activation of glycollate oxidase in etiolated *Lemna* suggests a modified programmed greening in this species.

Glutamate dehydrogenase

Coenzyme specificity in relation to growth on nitrate or ammonium

Whilst some authors suggest the existence of distinct NADH and NADPH specific enzymes [24, 25], others proposed a single glutamate dehydrogenase using unspecifically both coenzymes [26–28]. Furthermore, multiple forms of this enzyme have been reported in plants, and the pattern of isoenzymes appears to vary with the part of the plant or the growth conditions [29–32].

In *Lemna minor*, Joy [9] suggested the existence of separate NADH and NADPH specific glutamate dehydrogenases, as a result of detecting changes in the ratio of NADH to NADPH activity in response to changes in the nitrogen source of the growth medium. Subsequently, Ehmke and Hartmann [10] failed to detect any changes in glutamate dehydrogenase levels in response to nitrogen source, and the ratio of the NADH/NADPH-dependent activities remained constant (*ca* 12:1) during the various treatments. Consequently, these authors suggested the existence of only one enzyme in *Lemna minor*.

Our experiments showed that the nitrogen source affects differentially the NADH and NADPH-dependent activities of *Lemna* glutamate dehydrogenase. The results presented in Table 3 show that when *Lemna* fronds were grown in a medium containing ammonium as the only source of nitrogen, the NADH-dependent activity was *ca* 70% higher than when the fronds were grown in a medium containing nitrate as the only source of nitrogen. On the other hand, the NADPH-dependent activity was unaffected by the nitrogen source. These results are in good agreement with those reported by Joy [9] and at variance with those reported by Ehmke and Hartmann [10]. One possible explanation of the discrepancy lies in the use of acetone [10] to precipitate the enzyme before assay. It should be noted that the NADH and NADPH activities are not additive (Table 3) and the data is therefore consistent with the presence of a glutamate dehydrogenase whose coenzyme specificity is modified by the presence of nitrate or ammonium in the growth medium.

Coenzyme specificity in relation to light intensity

The activity of glutamate dehydrogenase with NADH and NADPH varies with the light conditions (Fig. 2B). Thus, the NADH-dependent activity doubled from the dark condition to a light intensity of *ca* 0.4 W/m^2, but then gradually declined with further increases in irradiance, being 15 times lower under the highest irradiance used. The NADPH-dependent activity followed a totally different pattern, being highest in the fronds grown in the dark. This activity gradually decreased to 30% with increasing irradiance, before starting to increase once more. Although the ratio NADH to NADPH activity varied greatly (over 22-fold), depending on the light supplied to the fronds, the two activities were never additive, suggesting that a single glutamate dehydrogenase is involved. If this explanation is valid then the coenzyme specificity of glutamate dehydrogenase must be modified not only by the nitrogen source but also by the light intensity. We have no explanation for how these changes are affected, but failure to separate the NADH and NADPH activities of glutamate dehydrogenase by FPLC is evidence in support of the single enzyme hypothesis.

EXPERIMENTAL

Plant material and growth conditions. Lemna minor L., grown under sterile conditions at 25° under continuous light (17 W/m^2), was used as the source of plant material in all the experiments.

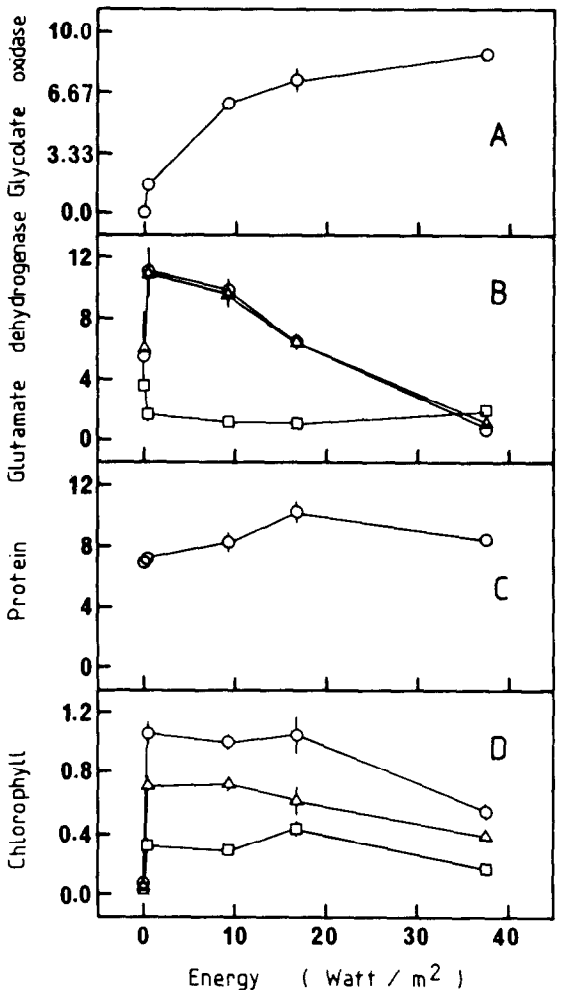

Fig. 2. Effect of light on the activity of glycollate oxidase and glutamate dehydrogenase from *Lemna minor*. *Lemna* fronds were grown under several irradiances, and harvested in batches of 3.0 g fr. wt. For each light condition, the soluble protein fraction from each of three batches of fronds was isolated and assayed for enzyme activities as described in the Experimental section. Vertical bars represent ± the standard deviation, shown when bar is bigger than symbol. (A) Glycollate oxidase activity (nkat/g fr. wt). (B) Glutamate dehydrogenase activity (nKat/g fr. wt); O: NADH-dependent activity; □: NADPH-dependent activity; Δ: NADH + NADPH-dependent activity. (C) Protein concentration (mg/g fr. wt). (D) Chlorophyll concentration (mg/g fr. wt); O: total chlorophyll; Δ: chlorophyll *a*; □: chlorophyll *b*.

The medium, containing nitrate as the only source of nitrogen, was the same as the one described by Ferreira and Davies [33] except that it contained 10 mM sucrose.

Where appropriate, the medium was modified in order to grow the fronds in the presence of ammonium as the only source of nitrogen. The modification was as follows: no KNO_3 was used; instead, 5 mM NH_4HCO_3 was used and the concentration of KH_2PO_4 was increased from 2 mM to 10 mM, to prevent the drop in pH which occurs when *Lemna* is grown in the presence of NH_3 as the only N source [34].

Where appropriate, illumination was also varied, and the following irradiances were used: (i) complete darkness; (ii) 0.34 W/m^2; (iii) 9.25 W/m^2; (iv) 16.75 W/m^2; (v) 37.5 W/m^2.

Table 3. NADH and NADPH-dependent activities of glutamate dehydrogenase from *Lemna* grown on either nitrate or ammonium as the source of nitrogen

Enzyme activity (nkat/g fr.wt)	NO_3^-	NH_4^+
NADH-dependent	6.4	10.9
NADPH-dependent	1.1	1.1
NADH + NADPH-dependent	5.9	10.3

Lemna fronds were grown in complete medium containing either nitrate or ammonium as the only nitrogen source. Protein was extracted and assayed for glutamate dehydrogenase activities as described in the Eperimental Section.

Irradiance reaching the fronds was measured with a radiometer LI-COR, model LI-185, with the sensor parallel to the surface of the medium.

Extraction of soluble protein. Lemna fronds (3 g fr. wt) were frozen in liquid N_2, then ground to a fine powder and extracted (4 ml per g fr. wt) in 100 mM Tris–HCl buffer pH 8.1, containing 1 mM DTT and 1 mM PMSF. The homogenate was squeezed through two layers of muslin and clarified by centrifugation at 30 000 g for 20 min at 1°. The supernatant was desalted at 1° on a PD-10 prepacked Sephadex G-25 M column, previously equilibrated with 10 mM Tris–HCl buffer pH 8.1, and the eluate assayed for enzyme activities.

Purification of glycollate oxidase. All operations were carried out at 2°. Precipitates were separated by centrifuging at 22 500 *g* for 30 min.

Homogenization. Lemna fronds (300 g fr. wt) were homogenized in a Waring blender in 1200 ml of 100 mM K-Pi buffer, pH 8.2. The homogenate was strained through four layers of muslin and centrifuged.

Ammonium sulphate fractionation. The supernatant was taken to 55% saturation $(NH_4)_2SO_4$, centrifuged, and the resulting supernatant redissolved in 18 ml of 25 mM Tris–HCl buffer, pH 8.3.

Ion exchange chromatography on DEAE and CM celluloses. The protein solution was desalted in a Sephadex G-25 M column (2.56 × 34.8 cm), previously equilibrated with 50 mM K-Pi buffer, pH 8.2, and applied to a system of two columns: DEAE-cellulose (2.56 × 30.6 cm) and CM-cellulose (1.22 × 7.2 cm), both equilibrated with 25 mM Tris–HCl buffer, pH 8.3. The eluate was monitored at 280 nm, and the first absorbing band, containing the glycollate oxidase activity, was collected.

Affinity chromatography on Sepharose aminohexyl oxamate. The fractions with the highest enzyme activity were pooled, and the pH of the resulting solution was lowered to 6.6 by the slow and dropwise addition of 1.0 M K-Pi buffer, pH 6.2. The clear solution was applied to a column of Sepharose aminohexyl oxamate (1.2 × 5.2 cm), previously equilibrated with 50 mM K-Pi buffer, pH 6.2. The column was washed with 50 ml of 100 mM K-Pi buffer pH 7.2, and glycollate oxidase was eluted with 70 mM Tris–HCl buffer, pH 9.6.

Anion exchange chromatography on the FPLC Mono Q column (Pharmacia). The concentrated glycollate oxidase extract (4 ml) was desalted on a PD-10 prepacked Sephadex G-25 M column, previously equilibrated with 20 mM piperazine buffer, pH 9.8, and loaded on to the FPLC Mono Q column equilibrated in the same buffer. The bound proteins were eluted with a gradient of NaCl (0 to 1 M). The glycollate oxidase peak was collected and used as the source of pure enzyme.

Enzyme assays. All assays were carried out at 25°. Glutamate dehydrogenase was assayed spectrophotometrically by

measuring the decrease in A_{340} associated with the oxidation of NADH, NADPH or NADH + NADPH. (i) NADH assay: the reaction mixture contained Tris–HCl buffer (100 mM, pH 8.4), NH_4Cl (66.7 mM), NADH (0.18 mM), extract, and 2-oxoglutarate (13.3 mM) in a final vol. of 3 ml. (ii) NADPH assay: identical to the NADH assay, except that NADPH replaced NADH in the reaction mixture. (iii) NADH + NADPH assay: assayed as before, but in the presence of both NADH and NADPH; all extracts were assayed using two different concentrations of pyridine nucleotides: 0.09 mM NADH + 0.09 mM NADPH and 0.18 mM NADH + 0.18 mM NADPH, with identical results being obtained in all the extracts assayed.

Glycollate oxidase was assayed spectrophotometrically, essentially according to the method of ref. [13]. The reaction mixture contained K-Pi buffer (100 mM, pH 8.2), sodium glycollate (10 mM), 2.6-dichlorophenol indophenol (0.003% w/v) and extract in a final vol. of 1 ml. Where appropriate, glycollate oxidase was also assayed using an oxygen electrode, essentially according to the method of ref. [5]. The reaction mixture contained K-Pi buffer (100 mM, pH 8.2), Na glycollate (10 mM) and extract in a final vol. of 3 ml. Chlorophyll was measured by the method of ref. [35], and protein by the method of ref. [36].

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). This technique was carried out by the method described in ref. [37].

Gel filtration on Sephacryl S-300. Gel filtration was performed in a column (2.61 × 40.3 cm) of Sephacryl S-300 superfine (Pharmacia), previously equilibrated with 50 mM K-Pi buffer, pH 8.2. The flow rate was 30 ml/hr. The protein markers used were horse spleen ferritin (M_r = 440 000), bovine liver catalase (M_r = 232 000), yeast alcohol dehydrogenase (M_r = 150 000), pig heart malate dehydrogenase (M_r = 70 000) and horse heart cytochrome *c* (M_r = 12 400).

Chromatofocusing on PBE-94. Chromatofocusing was performed in the range of pH 9-7 in a column (1.2 × 26.3 cm) of polybuffer exchanger PBE-94 (Pharmacia), using 25 mM ethanolamine–HCl buffer pH 9.55 as the start buffer and polybuffer 96–HCl pH 7.0 for elution, as described by Pharmacia. The flow rate was 30 ml/hr.

REFERENCES

1. Tolbert, N. E. (1981) *Annu. Rev. Biochem.* **50**, 133.
2. Kenten, R. H. and Mann, P. G. (1952) *Biochem. J.* **52**, 130.
3. Zelitch, I. and Ochoa, S. (1953) *J. Biol. Chem.* **201**, 707.
4. Frigerio, N. A. and Harbury, H. A. (1958) *J. Biol. Chem.* **231**, 135.
5. Kerr, M. W. and Groves, D. (1975) *Phytochemistry* **14**, 359.
6. Hall, N. P., Reggiani, R. and Lea, P. J. (1985) *Phytochemistry* **24**, 1645.
7. Lindqvist, Y. and Branden, C.-I. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6855.
8. Emes, M. J. and Erismann, K. H. (1982) *Plant Sci. Letters* **27**, 103.
9. Joy, K. W. (1969) *Plant Physiol.* **44**, 849.
10. Ehmke, A. and Hartmann, T. (1976) *Phytochemistry* **15**, 1611.
11. Asker, H. and Davies, D. D. (1983) *Biochim. Biophys. Acta* **761**, 103.
12. Schwam, H., Michelson, S., Randall, W. C., Sondey, J. M. and Hirschmann, R. (1979) *Biochemistry* **18**, 2828.
13. Davies, D. D. and Asker, H. (1983) *Plant Physiol.* **72**, 134.
14. Nishimura, M., Akhmedov, Y. D., Strzalka, K. and Akazawa, T. (1983) *Arch. Biochem. Biophys.* **222**, 397.
15. Schuman, M. and Massey, V. (1971) *Biochim. Biophys. Acta* **227**, 500.
16. Mayhew, S. G., Whitfield, C. D. and Ghisla, S. (1974) *Eur. J. Biochem.* **44**, 579.
17. Swank, R. T. and Munkres, K. D. (1971) *Anal. Biochem.* **39**, 462.
18. Behrends, W., Rausch, U., Loffler, H.-G. and Kindl, H. (1982) *Planta* **156**, 566.
19. Nishimura, M., Akhmedov, Y. D. and Akazawa, T. (1983) *Photosyn. Res.* **4**, 99.
20. Gruber, P. J., Becker, W. M. and Newcomb, E. H. (1972) *Planta* **105**, 114.
21. Tolbert, N. E. and Cohan, M. S. (1953) *J. Biol. Chem.* **204**, 639.
22. Tolbert, N. E. and Burris, R. H. (1950) *J. Biol. Chem.* **186**, 791.
23. Kuczmak, M. and Tolbert, N. E. (1962) *Plant Physiol.* **37**, 729.
24. Brown, D. H. and Haslett, B. (1972) *Plant Physiol.* **103**, 129.
25. Grimes, H. and Fottrell, P. F. (1966) *Nature* **212**, 295.
26. Chou, K.-H. and Splittstoesser, W. E. (1972) *Plant Physiol.* **49**, 550.
27. Pahlich, E. and Joy, K. W. (1971) *Can. J. Biochem.* **49**, 127.
28. Lea, P. J. and Thurman, D. A. (1972) *J. Exp. Botany* **23**, 440.
29. Hartmann, T. (1973) *Planta* **111**, 129.
30. Hartmann, T., Nagel, M. and Ilert, H.-I. (1973) *Planta* **111**, 119.
31. Barash, I., Mor, H. and Sadon, T. (1976) *Plant Cell Physiol.* **17**, 493.
32. Brassart, C., Rambour, S. and Bouriquet, R. (1984) *Physiol. Veg.* **22**, 801.
33. Ferreira, R. B. and Davies, D. D. (1987) *Plant Physiol.* **83**, 869.
34. Joy, K. W. (1969) *Plant Physiol.* **44**, 845.
35. Arnon, D. I. (1949) *Plant Physiol.* **24**, 1.
36. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **192**, 265.
37. Ferreira, R. B. and Davies, D. D. (1986) *Planta* **169**, 278.
38. Ferreira, R. M. and Teixeira, A. R. (1985) *Cienc. Biol.* (Portugal) **10**, 15.