Phytochemistry, 1976, Vol. 15, pp. 1611–1617. Pergamon Press. Printed in England.

PROPERTIES OF GLUTAMATE DEHYDROGENASE FROM *LEMNA MINOR*

ADELHEID EHMKE and THOMAS HARTMANN
Botanisches Institut der Universität Bonn, D-5300 Bonn, BRD

(*Revised received* 25 *March* 1976)

Key Word Index—*Lemna minor*; Lemnaceae; duck weed; nitrogen metabolism; glutamate dehydrogenase; nitrogen source and enzyme level; coenzyme specifity; kinetic properties; metal ion activation.

Abstract—Sterile cultures of *Lemna minor* grown in the presence of either nitrate, ammonium or amino acids failed to show significant changes in glutamate dehydrogenase (GDH) levels in response to nitrogen source. Crude and partially purified GDH preparations exhibit NADH and NADPH dependent activities. The ratio of these activities remain *ca* 12:1 during various treatments. Mixed substrate and product inhibition studies as well as electrophoretic behaviour suggest the existence of a single enzyme which is active in the presence of both coenzymes. GDH activity was found to be localized mainly in mitochondria. Kinetic studies revealed normal Michaelis kinetics with most substrates but showed deviations with NADPH and glutamate. A Hill-coefficient of 1.9 determined with NADPH indicates positive cooperative interactions, whereas a Hill-coefficient of 0.75 found with glutamate may be interpreted in terms of negative cooperative interactions. NADH dependent activity decreases rapidly during gel filtration whereas the NAD^+ and NADPH activities remain unchanged. GDH preparations which have been pretreated with EDTA show almost complete loss of NADH and NAD^+ activities. NADPH activity again remains unaffected. NAD^+ activity is fully restored by adding Ca^{2+} or Mg^{2+}, whereas the NADH activity can only be recovered by Ca^{2+} but not at all by Mg^{2+}. Moderate inhibition of GDH reactions observed with various adenylates are fully reversed by adding Ca^{2+}, indicating that the adenylate inhibition is due solely to the chelating properties of these compounds.

INTRODUCTION

Glutamate dehydrogenase (GDH) occupies a central position in nitrogen metabolism of N-autotrophic organisms since it catalyzes the interconversion of amino nitrogen and ammonium, and serves as a link between nitrogen and carbohydrate metabolism. Some microorganisms have been shown to contain distinct NAD^+ and $NADP^+$ specific GDHs. The $NADP^+$ specific enzyme is considered to have a biosynthetic function whereas the NAD^+ specific enzyme may have a catabolic function [1–3]. In various Ascomycetes the individual levels of both enzymes are controlled by induction and repression. Phycomycetes possess only one NAD^+ specific GDH which has complex regulatory properties permitting it to serve both anabolic and catabolic functions [4–8].

Since glutamate synthase has recently been demonstrated in some higher plants [9–13] it seems likely that ammonium assimilation via the glutamine synthetase/glutamate synthase pathway, which has been well established in bacteria [14] may also operate in plants. But it still remains obscure whether this pathway accounts for all the glutamate produced during N-assimilation. Our knowledge about function and control of GDH in plants is still too limited to decide whether it is concerned in glutamate formation or merely acting catabolically. A $NADP^+$ specific GDH has been reported in chloroplasts and this enzyme presumably plays a role in relation to photosynthetic electron transport [15,16]. Outside chloroplasts plants possess a GDH which is active with NAD^+ and $NADP^+$. Some authors suggest the existence of distinct NAD^+ and $NADP^+$ specific enzymes [3,17,18] whilst others [19–21] propose a single GDH using unspecifically both coenzymes.

GDH has been purified and characterized to various degrees from several plant sources. These studies clearly show that the plant enzyme has complex kinetic and regulatory properties [20,22,23]. In addition there are some reports on ammonium dependent induction of total GDH activity or individual isoenzymes [17,24–26].

The present work describes the influence of various nitrogen sources upon GDH activity in *Lemna minor*, its cellular localisation, coenzyme specificity and some kinetic properties.

RESULTS

Growth conditions with different nitrogen sources

Sterile cultures of *Lemna minor* are known to utilize nitrates, ammonium salts, or amino acids as sole sources of nitrogen [27]. However, preliminary studies showed that growth with ammonium is somewhat critical. Ammonium sulfate or chloride at various concentrations caused slow growth rates, small leaflets and marked chlorosis, even when $CaCO_3$ was added to the medium to prevent pH drifts. Satisfactory growth was achieved when the inorganic ammonium sources were replaced by organic ammonium salts. In agreement with results obtained with plant tissue cultures [28], ammonium citrate or succinate proved to be excellent ammonium sources. As shown in Table 1 these ammonium salts support plant growth even better than KNO_3. In order to

Table 1. Growth of *Lemna minor* on different nitrogen sources

N-Source	Concn of N (mM)	Fr wt after 25 d growth (g)
K-nitrate	5	4.5
	10	6.3
Ammonium citrate	5	6.0
	10	7.9
Ammonium succinate	5	6.6
	10	9.6
Hydrolyzed casein	5	5.8
	10	8.8

Results are means of two experiments

Table 2. Influence of nitrogen source upon GDH activity in *Lemna minor*

N-Source	Concn of N	Specific activity (mU/mg protein) NADH reaction	NAD^+ reaction	NADPH reaction	Ratio NADH/NADPH
K-nitrate	5 mM	82	7.1	6.5	13
Ammonium succinate	5 mM	151	12.7	9.3	16
Hydrolyzed casein	5 mM	155	13.3	10.0	16

Growth period 25 days. Acetone powder extracts dialyzed against tap water were used for enzyme assays. Results are means of two experiments.

exclude any toxic effect of the nitrogen source the concentrations chosen as standard for further experiments were 5 mM in the case of NO_3^- and NH_4^+, respectively, and 0.05% with hydrolyzed casein.

Influence of nitrogen source upon GDH activity

Examination of various extraction procedures revealed that crude enzyme preparations as used by Joy [17] in his experiments were disadvantageous since only poorly reproducible results were obtained. The acetone powder method was found to be the best for enzyme level determinations in *Lemna*. Using this method nearly equal levels of GDH were detected in plants grown on ammonium nitrogen and amino acids. The GDH level in nitrate cultures was somewhat lower (Table 2). It should be noted that the activity measured with the three coenzymes (NADH, NAD^+, NADPH) remained unchanged by the nitrogen source. The ratio of NADH:-NADPH dependent GDH activity is approximately constant (Table 2), although variations between ratios of 11 and 16 were found in different experiments.

Intracellular location of GDH

About 60–65% of total GDH activity are found in the particulate fraction. Particulate and soluble fractions showed the same ratio of NADH:NADPH dependent activity. This ratio does not change when mitochondria are separated from chloroplasts by differential centrifugation. Only traces of GDH activity could be detected in the chloroplast fraction. In addition heterotrophically grown *Lemna* having no mature chloroplasts shows the same activity ratio as autotrophic cultures. This ratio does not change when heterotrophic cultures are transferred from the dark into light and become green. These results suggest that NADH and NADPH dependent activities have the same distribution within the cell and may be located in the mitochondria as it is in many other plant tissues [20–22,29,30]. GDH from both particulate and soluble fractions were found to be identical in coenzyme dependency and electrophoretic behaviour. Therefore in agreement with other authors [21,31] it seems likely that some if not all GDH activity present in the soluble fraction may be leached from the mitochondria during extraction.

Electrophoretic behaviour of GDH

Crude GDH preparations of *Lemna minor* were separated by disc polyacrylamide gel electrophoresis. Only one band with GDH activity could be detected using the tetrazolium technique. The band position was identical with enzyme material derived from plants grown on different nitrogen sources. In addition analysis of the coenzyme dependency revealed that NAD^+ and $NADP^+$ stained exactly the same band.

Partial purification and general properties

Purification. The enzyme was partially purified in order to obtain GDH preparations of sufficient purity for kinetic studies. Table 3 shows the main steps of the purification procedure when GDH was purified starting with acetone dry powder extracts grown on ammonium succinate as nitrogen source. The relatively poor degree of purity to some extent depends on the fact that acetone powders were used as starting material for enzyme extraction instead of fresh plants [32]. As shown in Table 3 the ratio of NADH:NADPH dependent activity remains approximately constant during purification. Both activities were eluted in exactly the same fractions during gel filtration on Sephadex G_{200} and Sepharose 6B.

pH and temperature. Under standard assay conditions the pH optima for the aminating reactions were found to be 8.15 with NADH and 7.2 with NADPH, respectively. The pH optimum for the deaminating reaction with NAD^+ was found to be 8.8. The enzyme was stable

Table 3. Partial purification of GDH from *Lemna minor* (ammonium culture)

Fraction	Volume (ml)	Total activity (U) NADH	NADPH	Specific activity (U/mg) NADH	NADPH	Purification NADH	NADPH	Ratio NADH/NADPH
Acetone powder extract	230	67.5	6.15	0.25	0.023	—	—	11
$(NH_4)_2SO_4$ precipitation (40–70%)	16	53.5	7.40	0.6	0.084	2.4	3.6	7.2
Sephadex G 200	80	37.5	7.75	1.2	0.156	4.6	6.8	7.4
Sepharose 6 B	35	34.4	3.50	3.5	0.360	14.0	15.6	9.7

Table 4. Michaelis constants for *Lemna* GDH

Substrate	K_m (mol/l.)		
	NAD^+ reaction	NADH reaction	NADPH reaction
NADH	—	1.1×10^{-4}	—
NADPH*	—	—	1.3×10^{-4}
NAD^+	4.6×10^{-4}	—	—
Glutamate*	1.2×10^{-2}	—	—
NH_4^+	—	2.7×10^{-2}	1.5×10^{-2}
2-oxoglutarate	—	3.3×10^{-3}	2.1×10^{-3}

* K_m was calculated from Hill plots. All other K_m values were obtained from double reciprocal Lineweaver–Burk plots.

for short periods at 55°, but the activity rapidly decreased above this temperature.

Substrate kinetics. Apparent K_m values for the various substrates and coenzymes are listed in Table 4. With the exception of NADPH and glutamate the enzyme showed normal Michaelis–Menten kinetics for all substrates. However high concentrations of NADH in excess of 0.1 mM and NH_4Cl in excess of 0.1 M produced pronounced inhibition. This inhibitory effect is not altered when NH_4Cl is replaced by $(NH_4)_2SO_4$ as substrate. On the other hand the aminating reaction with NADH is inhibited by NaCl and Na_2SO_4 which indicates that the inhibition by high concentrations of ammonium salts is a non-specific effect, presumably brought about by competitive inhibition of the anion with respect to 2-oxoglutarate. With freshly prepared enzyme sometimes slightly biphasic double reciprocal plots were recorded for NAD^+ (see Fig. 4). This biphasic feature is absent in older preparations.

Saturation kinetics with NADPH always produced sigmoidal curves with both crude and purified GDH preparations. From Hill plots (Fig. 1) with NADPH a slope of 1.9 was calculated.

Glutamate also showed non-Michaelis–Menten kinetics. The Hill plot is given in Fig. 2. The complex nature of the saturation kinetics with glutamate may change during enzyme storage to give normal Michaelis kinetics.

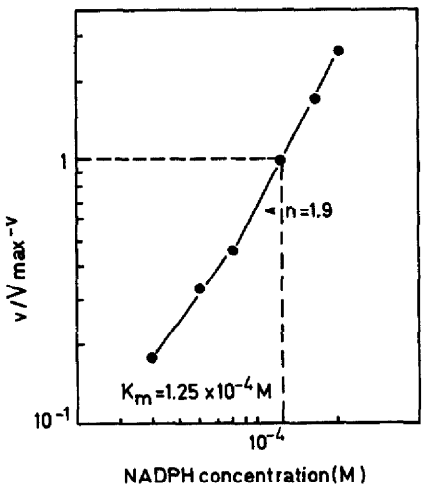

Fig. 1. Hill plot (log v/V − v vs log S) for the data obtained from GDH saturation kinetics with NADPH. Assays were carried out under standard conditions.

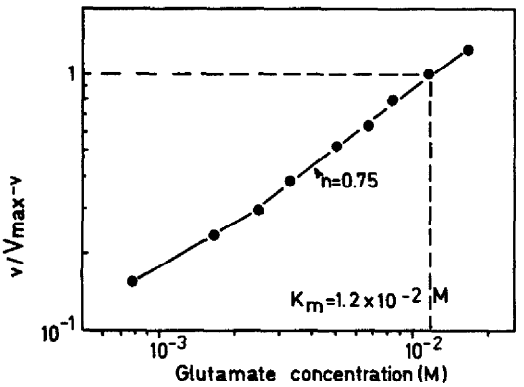

Fig. 2. Hill plot (log v/V − v vs log S) for the data obtained from GDH saturation kinetics with glutamate (NAD^+ dependent reaction). Assays were carried out under standard conditions.

This change is accompanied by a decrease in K_m from 1.2×10^{-2} M to 3.5×10^{-3} M.

Coenzyme specifity

The foregoing results support the assumption that *Lemna* may possess a single GDH which uses either NADH or NADPH in the aminating reaction. To obtain more evidence on this further studies were carried out. Figure 3 shows the result of a mixed substrate experiment in which saturation kinetics for NADH in the presence and absence of two defined NADPH concentrations were performed. No additive effects were obtained. The total rate of reaction is clearly less than the sum of the reaction rates measured separately. Furthermore product inhibition studies of NAD^+ reduction in the presence of NADPH were accomplished. Double reciprocal plots of velocity against NAD^+ concentration at various but fixed levels of NADPH showed clear competitive inhibition (Fig. 4), indicating that NADPH and NAD^+ bind to the same site.

Effects of bivalent metal ions (Me^{2+})

When GDH was purified from crude acetone powder extracts it was noted that about 90% of NADH dependent activity was lost during gel filtration on Sephadex G_{200} or simple desalting with Sephadex G_{50}, whereas the NAD^+ and NADPH dependent activity remained unaffected. However this inactivation could be fully reversed by adding Ca^{2+} (Table 5). As further shown in the Table, addition of EDTA to the assay system caused complete inactivation of both NADH and NAD^+ dependent activity, but left the NADPH activity nearly unchanged. In contrast to NADH activity which is not reactivated by Mg^{2+} the NAD^+ activity could be fully restored with either Ca^{2+} or Mg^{2+}.

The Me^{2+} dependency of *Lemna* GDH is very stable and could be demonstrated at all stages of enzyme purity. "Storage activation" as has been reported for Zn^{2+} on GDH of *Pisum sativum* roots [23] was not observed with the *Lemna* enzyme (Table 6). The slight increase of activity in Sephadex G_{50} effluents without added Ca^{2+} during storage over 10 days may be due to partial reactivation by means of unspecifically bound Me^{2+} ions which were not completely removed by gel filtration. Such effects were not observed with EDTA treated preparations. During storage, NADH dependent activity in the presence of Ca^{2+} shows the same degree of decrease

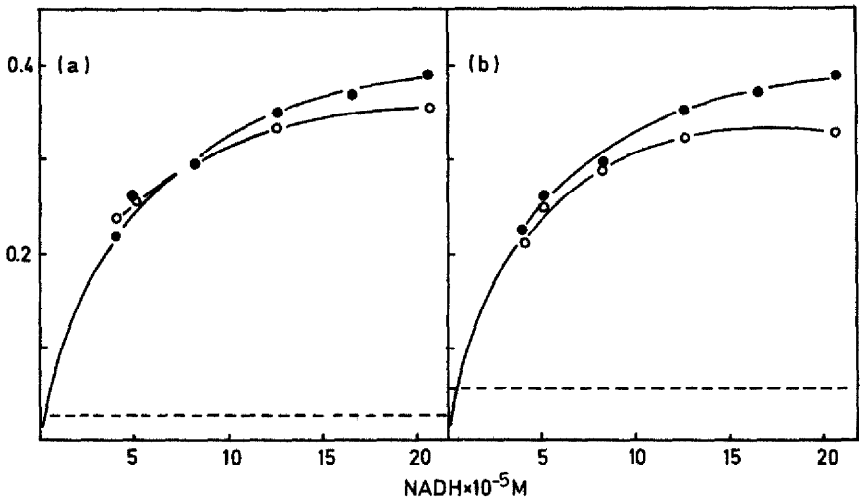

Fig. 3. Rate of the reductive amination of 2-oxoglutarate catalyzed by *Lemna* GDH as a function of NADH concentration in the absence (●) or presence (○) of (a) 0.08 mM NADPH and (b) 0.16 mM NADPH. (---) rate of NADPH oxidation in the absence of NADH. Assays were carried out under standard conditions at pH 7.5.

in total activity as already fully activated NAD^+ dependent activity, demonstrating that this loss of activity is solely due to time dependent normal decay of enzyme activity.

Table 7 shows that the three GDH activities respond quite differently to treatments with various metal binding agents. The inhibitory effects on NADH reaction were much greater than on NAD^+ reaction whereas the NADPH reaction remained relatively unaffected in the presence of the chelators.

Joy [33] observed that the NADH dependent GDH activity from *Lemna minor* grown with amino acids or organic ammonium salts as sole sources of nitrogen is extremely sensitive to EDTA inhibition whereas the activity from plants grown with inorganic ammonium salts or nitrate is not. Reinvestigating these experiments we never observed any difference between GDH preparations obtained from plants grown with the nitrogen sources used in our experiments. NADH dependent GDH activity is always completely inhibited in the presence of 1 mM EDTA and can be fully restored by addition of Ca^{2+}.

In preliminary studies a large number of potential effectors had been tested for their ability to influence the GDH catalyzed reactions. During these studies a moderate inhibition of *Lemna* GDH by purine nucleotides had been found (Table 8). Further studies revealed that this inhibition is solely due to the metal chelating properties of these compounds. In the presence of excess Ca^{2+} any inhibitory effect of purine nucleotides is absent.

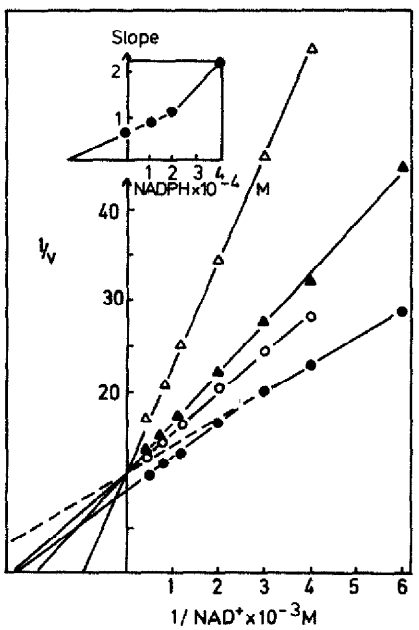

Fig. 4. Product inhibition of *Lemna* GDH by NADPH with NAD^+ as the variable substrate. (●) no NADPH; (○) 0.1 mM NADPH; (▲) 0.2 mM NADPH; (△) 0.4 mM NADPH. Inset: replot of slope against NADPH concentration.

DISCUSSION

Our observations clearly demonstrate that the GDH level in *Lemna minor* grown in the presence of either nitrates, ammonium salts or amino acids as sole nitrogen source, is not changed significantly in response to the nitrogen source. Furthermore the enzyme extracted from various nitrogen cultures appears to be identical in terms of disc electrophoresis and coenzyme dependency. These findings, which are similar to those obtained from studies with aseptically grown *Medicago sativa* [34], seem to be in contrast to reports on stimulatory effects of ammonium upon GDH activity in various plant species. A 5-fold increase in GDH activity was detected when *L. gibba* was transferred from a medium containing glutamate to one in which ammonium was the sole source of nitrogen [25]. The rise in activity was shown to be due to *de novo* protein synthesis. Furthermore stimulatory ammonium effects on GDH activity were claimed for roots of soybean and sunflower [35], rice roots [24], oat leaves [26], lupin seedlings [36], and cucumber leaves [37].

Table 5. Inactivation and Me^{2+} dependent reactivation of the GDH reactions in various enzyme preparations

Enzyme preparation	Total GDH activity in %								
	NADH reaction			NAD^+ reaction			NADPH reaction		
	–	$+Ca^{2+}$	$+Mg^{2+}$	–	$+Ca^{2+}$	$+Mg^{2+}$	–	$+Ca^{2+}$	$+Mg^{2+}$
Crude extract (acetone powder)	83	100	84	100	100	100	100	100	100
Sephadex G 50 eluate	4.5	95	5.0	125	121	121	115	118	118
Sephadex G 50 eluate + EDTA	< 1	93	< 1	< 1	117	119	112	115	118

* EDTA was added to the assay system to give a final concentration of 0.1 mM. $+Ca^{2+}$ and $+Mg^{2+}$ mean addition of $CaCl_2$ or $MgSO_4$, respectively, to the assay system at a final concentration of 1 mM.

In general these observations have been interpreted in two ways, (a) as evidence for enzyme induction as a regulatory mechanism in control of GDH function in ammonium assimilation [17, 36], (b) in respect to ammonium detoxification within the cells [25, 26, 37]. From our results the first possibility seems to be most unlikely since otherwise marked differences in enzyme level should have been observed at least between ammonium and amino acid cultures. On the other hand stimulatory effects of ammonium upon GDH activity may indeed offer a mechanism whereby the plant can increase the rate of removal of ammonium to avoid toxic effects [25]. But it should be mentioned that, for instance, isolated young pea shoots show a 3- to 10-fold increase in GDH activity in combination with a total change in isoenzymatic patterns within 16–20 hr, even in the absence of ammonium (Hartmann, unpublished). This result reflects the very sensitive response of GDH activity to any disturbance of the physiological conditions within the plant, making precise interpretations extremely difficult.

Our observations on coenzyme specifity strongly suggest that NADH and NADPH dependent activity found in enzyme preparations are catalyzed by only one enzyme. The evidence may be summarized as follows, (a) both activities were shown to be identical in terms of disc electrophoretic behaviour, (b) the activity ratio remained approximately constant during enzyme purification or particle fractionation. (c) Results obtained from product inhibition experiments as well as studies using the mixed substrate method indicate that both coenzymes bind to the same site on the enzyme.

In contrast to these findings Joy [17] suggested the existence of separate NADH and NADPH specific enzymes in *Lemna minor* as a result of detecting differences in response to nitrate and ammonium of NADH and NADPH activities and their changing ratios in crude extracts. This result may be explained by the differential response of the two activities to activation by bivalent metal ions (discussion below). Separate NADH and NADPH specific enzymes were also claimed to exist in etiolated barley leaves [18], and root nodules of *Trifolium repens* [38]. However NADH and NADPH dependent activity observed in peas [20], pumpkin cotyledons [39], and lettuce leaf mitochondria [21] was found to be due to only one enzyme.

Further studies were focused on the kinetic and regulatory properties of the *Lemna* enzyme. The apparent K_m values were comparable to those from other plant sources [20, 22, 29]. Deviations from normal Michaelis–Menten kinetics were observed with glutamate and NADPH. The latter shows sigmoidal saturation curves indicating positive cooperative interactions between substrate and enzyme. In the case of glutamate, a Hill coefficient of 0.75 was determined, which may be interpreted in terms of negative cooperativity with respect to substrate binding and positive cooperativity to catalysis [40, 41]. Similar characteristics have been reported for the mitochondrial GDH of soybean cotyledons [22].

The most striking property of *Lemna* GDH is its differential activation by bivalent metal ions. The overall situation is summarized in Fig. 5. Gel filtration causes nearly complete inactivation of only the NADH activity whereas further treatment with EDTA inactivates both NADH and NAD^+ reactions leaving the NADPH activity essentially unaffected. In contrast to the inhibition of NADH activity which is fully reversed by addition

Table 6. Effect of storage at 4° upon inactivated NADH dependent GDH activity

Age of preparations (days)	Total GDH activity (%)					
	Sephadex G 50 eluate			EDTA treated preparation		
	NADH reaction		NAD^+ reaction	NADH reaction		NAD^+ reaction
	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$
0	9.5	100	100	< 1	81	84
1	11.6	97	96	< 1	78	82
5	14.6	89	90	< 1	74	76
8	15.7	84	83			
10	14.5	78	76			

Sephadex G 50 eluate: on Sephadex desalted acetone powder extract. EDTA treated preparation: Sephadex G 50 eluate treated with 1 mM EDTA and dialyzed for 24 h at 4° against 0.05 M Tris–HCl pH 8.0 in presence of 0.5 mM $MgSO_4$. $+Ca^{2+}$ means addition of $CaCl_2$ to the assay system at a final concentration of 1 mM.

Table 7. Effect of metal binding agents on *Lemna* GDH

Agents (final concn in assay = 1 mM)	Total GDH activity (%)		
	NADH reaction	NAD^+ reaction	NADPH reaction
Untreated enzyme	100	100	100
EDTA	<1	<1	65
EDTA (0.1 mM)	<1	<1	100
Nitrilotriacetic acid	5	40	78
o-Phenanthroline	65	82	97
Sodium citrate	53	87	
Sodium oxalate	82	100	

Table 8. Effect of purine nucleotides on *Lemna* GDH

Agents (final concn in assay = 1 mM)	Total GDH activity (%)		
	NADH reaction	NAD^+ reaction	NADPH reaction
Untreated enzyme	100	100	100
AMP	92	105	109
ADP	91	93	100
ATP	35	62	100
ATP + 1 mM Ca^{2+}	100	92	
GTP	58	89	100
GTP + 1 mM Ca^{2+}	98	101	

As enzyme source Sephadex G 50 eluate Ca^{2+} saturated by addition of 0.1 mM $CaCl_2$ was used.

of Ca^{2+}, NAD^+ activity is completely restored by both Ca^{2+} and Mg^{2+}.

In order to explain the Me^{2+} activations, we propose the existence of two mechanisms, (a) a Ca^{2+} specific mechanism which controls exclusively the NADH reaction and which is further characterized by relatively weak bonds between Ca^{2+} and the enzyme. (b) a less specific mechanism dependent on either Ca^{2+} or Mg^{2+} controlling the NAD^+ reaction in which the Me^{2+} obviously binds more firmly to the enzyme.

Inhibition of plant GDH by EDTA and other chelating agents and its reversal by various Me^{2+} ions have been reported by several authors [20, 23, 42]. Although not as differentiated as reported here, the inhibitory effects of chelating agents appeared always to be pronounced in the direction of reductive amination with NADH.

Finally we must mention the fact that the observed inhibition of *Lemna* GDH by purine nucleotides which can be fully reversed by adding Ca^{2+}, is solely due to the chelating properties of these compounds. On the other hand King and Wu [22] presented kinetic data which suggest that ATP affects the activity of soybean GDH by binding to a non-active site. However since ATP inhibition is only observed at high concentrations (above 1 mM) the authors presume, in contrast to results published most recently [32], that the ATP binding site may not be physiologically important. Thus it appears unlikely that purine nucleotides are concerned in control of plant GDH.

Certainly the observed differential activation of the GDH catalyzed reactions by bivalent metal ions offers a mechanism for the control of the direction of the reversible reaction. Further information about the mechanism of Ca^{2+} dependent control of the NADH reaction obtained from kinetic studies will be provided in a later paper [43].

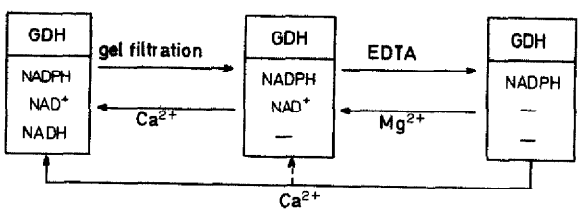

Fig. 5. Scheme showing the gradual inactivation of *Lemna* GDH and its differential reactivation by Ca^{2+} and Mg^{2+}.

EXPERIMENTAL

Culture conditions. Lemna minor was grown aseptically at 22–24° in the continuous light (1200 lx), on 600 ml of a modified medium according to Ref. [44]. containing 0.5% sucrose in 1.5 l. Fernbach-flasks. Nitrogen being supplied either as KNO_3, 5 mM (nitrate medium), ammonium succinate, 5 mM in respect to ammonium (ammonium medium), or hydrolyzed casein (Hycase-SF, Serva), 0.05% (amino acid medium). pH was adjusted to 4.6–4.8 (nitrate medium), 5.2–5.4 (ammonium medium) and 5.1–5.3 (amino acid medium). Heterotrophic cultures were grown in the dark at 23–25°. Culture medium was the same as above, but contained 2% sucrose and as nitrogen source 5 mM KNO_3, 0.08% Hycase-SF, and 40 mg/l. yeast extract.

Preparation of Me_2CO *powder.* Mixotrophic cultures were harvested after 24–28 days, heterotrophic cultures after 4–6 weeks growth. Plants were rinsed with H_2O, lightly pressed between pads of filter paper and 10–20 g of fr wt were homogenized in 5 vol of Me_2CO (−18°) for 1 min. Residue was collected by filtration and Me_2CO extraction was repeated twice in the same way. The resulting powder was lyophilized and kept dry and frozen at −18° until used.

Enzyme extraction and partial purification. All procedures were performed at 4°. 300–800 mg Me_2CO powder was extracted with 10 ml 0.1 M Tris–HCl buffer, pH 8.1, for 30 min. The extract was centrifuged at 30 000 *g* for 15 min. Supernatant was made up to 40% sat with solid $(NH_4)_2SO_4$. The ppt was removed by centrifugation and the supernatant was brought to 70% $(NH_4)_2SO_4$. The 40–70% $(NH_4)_2SO_4$ protein fraction, collected by centrifugation was resuspended in 0.1 M Tris–HCl buffer, pH 8.1 and dialyzed for 16–20 hr against running tap H_2O. Protein was then fractionated on a column (2.5 × 100 cm) of Sephadex G_{200}. Eluted fractions containing GDH activity were combined, concentrated by $(NH_4)_2SO_4$ ppn, and further purified by gel filtration on Sepharose 6B. In some expts Me_2CO powder extracts were partially purified by gel filtration on Sephadex G_{50}.

Enzyme assays. GDH activity was assayed by measuring the initial rate of oxidation of NADH, NADPH or reduction of NAD^+ in 2 ml silica cuvettes at 340 nm at 30°. Standard reaction mixture (total vol 1.2 ml) contained the following components (final concn). NADH reaction: 0.15 ml enzyme; 0.6 ml 0.1 M Tris–HCl, pH 8.1; 0.1 ml $(NH_4)_2SO_4$ (0.125 M) or NH_4Cl (0.25 M); 0.25 ml NADH (0.21 mM); 0.1 ml 2-oxoglutarate (16.7 mM). NADPH reaction: 0.15 ml enzyme; 0.6 ml 0.1 M Tris–HCl, pH 7.0; 0.1 ml $(NH_4)_2SO_4$ (0.125 M); 0.25 ml NADPH (0.21 mM); 0.1 ml 2-oxoglutarate (16.7 mM). NAD^+ reaction: 0.15 ml enzyme; 0.7 ml 0.1 M Tris–HCl, pH 8.8; 0.25 ml NAD^+ (2.1 mM); 0.1 ml L-glutamate (16.7 mM). The reaction mixtures were allowed to warm up to 30° for 5 min, then the reactions were started by addition of 2-oxoglutarate and glutamate, respectively. NADH, NADPH and NAD^+ were adjusted to pH 7 with Tris–HCl before use, all other solns were prepared in assay buffer, and if necessary the pH was adjusted with NaOH. Appropriate blank reaction rates were measured omitting individual substrates. Measurable blank activity was found only in crude enzyme preparations. GDH activity is expressed in ΔE_{340}/min or in units. One unit catalyzes the oxidation or reduction of 1 μmol coenzyme per min.

Separation of particulate and soluble GDH. Lemna plants were ground in a chilled mortar in ice cold isolation medium (0.4 M sucrose, 84 mM K_2HPO_4, 8.4 mM $MgSO_4$, 4 mM EDTA, 7.2 mM ascorbic acid, 1 mM mercaptoethanol, 0.04 HEPES, pH 7.8) [45, 46] with small glass beads (ϕ 0.12 mm). Homogenate was centrifuged at 200 *g* for 10 min, and the pellet discarded. Supernatant was then centrifuged at 33 000 *g* for 35 min. Resulting supernatant was designated as "soluble fraction", the pellet as "particulate fraction". The "particulate fraction" was treated with Me_2CO (−18°) and the resulting Me_2CO powder extracted with 0.1 M Tris–HCl buffer, pH 8.4. Both fractions were further purified by gel filtration on Sephadex G_{50} on small columns (1.8 × 15 cm). The

eluted protein was collected and used for assay of GDH activity.

Other methods. Polyacrylamide disc electrophoresis and substrate staining of GDH activity was carried out according to [34]. Protein was determined by the method of [47].

Acknowledgements—We thank Prof. Dr. R. Bornkamm (Berlin) for supplying the sterile culture of *Lemna minor* and the Deutsche Forschungsgemeinschaft for financial support by a grant (Ha 641/6).

REFERENCES

1. Sanwal, B. D. and Lata, M. (1961) *Can. J. Microbiol.* **7,** 319.
2. LeJohn, H. B., Suzuki, I. and Wright, J. A. (1968) *J. Biol. Chem.* **243,** 118.
3. Sims, A. P., Folkes, B. F. and Bussey, A. H. (1968) *Recent Aspects of Nitrogen Metabolism in Plants* (Hewitt, E. J. and Cutting, C. V., ed.) p. 91. Academic Press, New York.
4. LeJohn, H. B. (1968) *J. Biol. Chem.* **243,** 5126.
5. LeJohn, H. B., Jackson, S. G., Klassen, G. R. and Sawula, R. V. (1969) *J. Biol. Chem.* **244,** 5346.
6. Price, J. S. and Gleason, F. H. (1972) *Plant Physiol.* **49,** 87.
7. Sanner, T. (1972) *Biochim. Biophys. Acta* **258,** 689.
8. LeJohn, H. B. (1971) *Nature* **231,** 164.
9. Lea, P. J. and Miflin, B. J. (1974) *Nature* **251,** 614.
10. Dougall, D. K. (1974) *Biochem. Biophys. Res. Commun.* **58,** 639.
11. Fowler, M. W., Jessup, W. and Sarkissian, G. S. (1974) *FEBS Letters* **46,** 340.
12. Lea, P. J. and Miflin, B. J. (1975) *Biochem. Biophys. Res. Commun.* **64,** 856.
13. Miflin, B. J. and Lea, P. J. (1975) *Biochem. J.* **149,** 403.
14. Brown, C. M., Macdonald-Brown, D. S., and Meers, J. L. (1974) *Adv. Microbial Physiol.* **11,** 1.
15. Leech, R. M. and Kirk, P. R. (1968) *Biochem. Biophys. Res. Commun.* **32,** 685.
16. Givan, C. V., Givan, A. L. and Leech, R. M. (1970) *Plant Physiol.* **45,** 624.
17. Joy, K. W. (1969) *Plant Physiol.* **44,** 849.
18. Brown, D. H. and Haslett, B. (1972) *Planta (Berl.)* **103,** 129.
19. Yue, S. B. (1969) *Plant Physiol.* **44,** 453.
20. Pahlich, E. and Joy, K. W. (1971) *Can. J. Biochem.* **49,** 128.
21. Lea, P. J. and Thurman, D. A. (1972) *J. Exp. Botany* **23,** 440.
22. King, J. and Wu, W. Y.-F. (1971) *Phytochemistry* **10,** 915.
23. Joy, K. W. (1973) *Phytochemistry* **12,** 1031.
24. Kanamori, T., Konishi, S. and Takahashi, E. (1972) *Physiol. Plantarum* **26,** 1.
25. Shepard, D. V. and Thurman, D. A. (1973) *Phytochemistry* **12,** 1937.
26. Barash, I., Sadon, T. and Mor, H. (1973) *Nature* **244,** 150.
27. Joy, K. W. (1969) *Plant Physiol.* **44,** 845.
28. Gamborg, O. L. and Shyluk, J. P. (1970) *Plant Physiol.* **45,** 598.
29. Davies, D. D. and Teixeira, A. N. (1975) *Phytochemistry* **14,** 647.
30. Miflin, B. J. (1974) *Plant Physiol.* **54,** 550.
31. Bone, D. H. (1959) *Nature* **184,** 990.
32. Pahlich, E. and Hoffmann, J. (1975) *Planta (Berl.)* **122,** 185.
33. Joy, K. W. (1971) *Plant Physiol.* **47,** 445.
34. Hartmann, T., Nagel, M. and Ilert, H.-I. (1973) *Planta (Berl.)* **111,** 119.
35. Weissman, G. S. (1972) *Plant Physiol.* **49,** 138.
36. Kretovich, V. L., Karyakina, T. L., Yazikova, V. V. and Florenskaya, T. G. (1974) *Fiziol. Rast.* **21,** 247.
37. Matsumoto, H., Wakjuchi, N. and Takahashi, E. (1971) *Physiol. Plantarum* **25,** 353.
38. Grimes, H. and Fottrell, P. F. (1966) *Nature* **212,** 295.
39. Chou, K.-H. and Splittstoesser, W. G. (1972) *Plant Physiol.* **49,** 550.
40. Koshland, D. E. Jr., Nemethy, G. and Filmer, D. (1966) *Biochemistry* **5,** 365.
41. Engel, P. C. and Dalziel, K. (1969) *Biochem. J.* **115,** 621.
42. Yamasaki, K. and Suzuki, Y. (1969) *Phytochemistry* **44,** 963.
43. Ehmke, A. and Hartmann, T. (1976) in preparation.
44. Bornkamm, R. (1965) *Flora A* **156,** 139.
45. Gronebaum-Turk, K. and Willenbrink, J. (1971) *Planta (Berl.)* **100,** 337.
46. Nordhorn, G. and Willenbrink, J. (1972) *Planta (Berl.)* **103,** 147.
47. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.