Biochem. J. (1981) **195**, 615–622
Printed in Great Britain

L-Lactate dehydrogenase from leaves of higher plants

Kinetics and regulation of the enzyme from lettuce (*Lactuca sativa* L.)

Thomas BETSCHE
Botanisches Institut der Universität, Schlossgarten 3, D 4400 Münster, Federal Republic of Germany

(*Received 1 December 1980/Accepted 5 February 1981*)

1. L-Lactate dehydrogenase from lettuce (*Lactuca sativa*) leaves was purified to electrophoretic homogeneity by affinity chromatography. 2. In addition to its NAD(H)-dependent activity with L-lactate and pyruvate, the enzyme also catalyses the reduction of hydroxypyruvate and glyoxylate. The latter activities are not due to a contamination of the enzyme preparation with hydroxypyruvate reductase. 3. The enzyme shows allosteric properties that are markedly affected by the pH. 4. ATP is a potent inhibitor of the enzyme. The kinetic data suggest that the inhibition by ATP is competitive with respect to NADH at pH 7.0 and 6.2. The existence of regulatory binding sites for ATP and NADH is discussed. 5. Bivalent metal cations and fructose 6-phosphate relieve the ATP inhibition of the enzyme. 6. A function of leaf L-lactate dehydrogenase is proposed as a component of the systems regulating the cellular pH and/or controlling the concentration of reducing equivalents in the cytoplasm of leaf cells.

Lactate dehydrogenase is an enzyme common in bacteria and animal tissues. The enzyme from these sources has been studied intensively, whereas there are only few reports on lactate dehydrogenase from plants (Everse & Kaplan, 1973; Garvie, 1980). In higher plants the enzyme has been found in achlorophyllous tissues such as potato tubers, roots and seedlings (Davies & Davies, 1972; Davies *et al.*, 1974; Rothe, 1974; Oba *et al.*, 1977; Barthova *et al.*, 1977; King, 1970). There lactate dehydrogenase is assumed to be related to anaerobic metabolism. Considering photosynthetically active cells, the enzyme has been found in green algae and a few lower land plants, but could not be detected in the green tissues of higher plants (Gruber *et al.*, 1974). More recently the occurrence of L-lactate dehydrogenase has been demonstrated for leaves of shepherd's purse (*Capsella bursa-pastoris*), lettuce (*Lactuca sativa*) and a number of other higher plants (Betsche *et al.*, 1979). In order to elucidate the function of L-lactate dehydrogenase in leaves of higher plants, the enzyme was purified and its kinetic properties and possible regulatory mechanisms were studied.

Abbreviations used: Mes, 4-morpholine-ethanesulphonic acid; Tes, 2-{[hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino}ethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Materials and methods

Purification of lactate dehydrogenase

Leaf lettuce (*Lactuca sativa* L., var. longifolia) was purchased locally. Only the outer leaves and the green part of inner leaves were used for enzyme preparation. Leaves (500g) were carefully washed with water and their main veins were removed. The leaf material was put in a cold blender and 500ml of ice-cold homogenization medium was added. The homogenization medium was 50mM-Tris/acetate buffer, pH 7.4, containing 5mM-dithiothreitol and 40g of insoluble polyvinylpyrrolidone (1-ethenyl-pyrrolidin-2-one polymers), previously soaked with water. Before homogenization the mixture was bubbled with N_2 for 5min. The homogenate was filtered through cheesecloth and the filtrate centrifuged at 5000*g* for 40min. From the resulting supernatant (crude extract) protein precipitated between 30 and 50% saturation of $(NH_4)_2SO_4$, was collected by centrifugation (5000*g*, 40min) and redissolved in 30ml of homogenization buffer. This solution could be stored frozen for several weeks without loss of enzyme activity. After centrifugation (15000*g*, 20min) 25ml of the solution was applied to a column (100cm × 12cm^2) of Sephadex G-25 previously equilibrated with 50mM-phosphate buffer, pH 6.2, and the protein was eluted with the same buffer. The fractions were pooled, and ATP-adipoyl-

0306-3275/81/060615-08$01.50/1

dihydrazo-Sepharose, prepared from 1g of Sepharose 4B as described by Kuntz *et al.* (1978), was added. The suspension was stirred gently for 15min at room temperature, then the suspension was transferred into a column (20cm × 1cm²). The column was rinsed with 100ml of 50mM-phosphate buffer, pH6.2, at a flow rate of 30ml/h (Jervis & Schmidt, 1977). An NaCl gradient was used to elute the enzyme from the column (0–300mM in 50mM-phosphate buffer, pH6.2; 100ml total volume; 30ml/h). Those fractions were pooled in which the enzymic activity with pyruvate (0.7mM) and hydroxypyruvate (2.5mM) was inhibited by ATP (62μM) to the same extent (pH7.0, 0.125mM-NADH). In some experiments the enzyme solution was concentrated with an ultrafiltration membrane (Diaflo PM 10; Amicon, Lexington, KY, U.S.A.).

Electrophoresis and isoelectric focusing

Analytical electrophoresis was performed on 7.5% (w/v)-polyacrylamide-gel slabs. The gels were prepared and the electrophoresis was performed (400V, 5h, 2°C) essentially as described by Loeschke & Stegemann (1966). To locate enzyme activity on the gels, the applied samples contained 50–400pkat of pyruvate reductase activity. The activity was raised to 12–24nkat for staining of protein. Isoelectric focusing was done on polyacrylamide gels as described by Maurer (1971), system 1 with wide-range ampholytes (Serva) being used. To determine the pI of lactate dehydrogenase, that portion of several unstained gels where according to stained gels the enzyme was located was transferred into 1ml of distilled water. After incubation for 10h at 20°C the pH of the solution was measured. Staining for lactate dehydrogenase activity was performed as described by Shaw & Prasad (1970) with L-lactate as substrate. The gels were stained for protein with Coomassie Brilliant Blue G 250.

Assays

Lactate dehydrogenase and hydroxypyruvate reductase activities were measured photometrically at 340nm and 23°C. The buffers used were: pH6.2, 50mM-Mes; pH7.0, 50mM-Tes; pH7.8, 50mM-Hepes. The concentrations of substrates and co-substrates varied. Freshly prepared NAD(P)(H) solutions were always used. Protein was measured by the Lowry procedure.

Unit of enzyme activity

The SI unit, kat, is used, which is defined as the removal of 1mol of NADH/s. Specific activity is defined as units of enzyme activity (nkat)/g of protein.

Results

Purity and substrate specificity

L-Lactate dehydrogenase from green lettuce leaves was purified by affinity chromatography on ATP–Sepharose (Table 1). Electrophoresis and isoelectric focusing of the purified enzyme resulted in only one band of enzyme activity. A pI of pH5.0 was determined for leaf lactate dehydrogenase. If the gel slabs were stained for protein, apart from a barely detectable band, only one intensively stained band matching the activity band was observed. On the basis of these results the enzyme preparation was concluded to consist essentially of one protein only.

The affinity of leaf lactate dehydrogenase for D-lactate (5mM) was negligible. By using pyruvate (2mM) as substrate and NADPH (0.3mM) as co-substrate, 13% as much activity as with NADH (0.3mM) was observed (pH7.0). Since the lactate dehydrogenase was purified to electrophoretic homogeneity, the activity with NADPH is unlikely to be due to the presence of $NADP^+$-dependent malic enzyme in the enzyme preparation. Apart from that, $NADP^+$-dependent malic enzyme needs Mg^{2+} or Mn^{2+} for activation, but no bivalent metal cations were added to the standard assays (Johnson & Hatch, 1970).

In addition to its activity with pyruvate and L-lactate, the purified lactate dehydrogenase also catalysed the reduction of hydroxypyruvate and glyoxylate, as it has been reported for the enzyme from potato (*Solanum tuberosum*) tubers and animals (Davies & Davies, 1972; Poerio & Davies,

Table 1. *Summary of the purification of L-lactate dehydrogenase from lettuce leaves*
The assay conditions were: pH7.0; 0.7mM-pyruvate; 0.3mM-NADH.

Fraction	Volume (ml)	Protein (mg)	Enzyme activity (nkat)	Specific activity (nkat/g)	Purification	Yield (%)
Crude extract	415	1297	320	247	—	100
27–50%-satd. $(NH_4)_2SO_4$	35	245	203	829	3.4	63
ATP–Sepharose eluate	10	0.19	96	505000 (1010000*)	2045	30

* Specific activity of the enzyme completely saturated with substrate ($V_{max.}$/g of protein).

1980; Everse & Kaplan, 1973). A contamination of the lactate dehydrogenase preparation with hydroxypyruvate reductase, present in high activity in leaves, could be excluded again from the electrophoresis experiment and, additionally, by the finding that the activity with hydroxypyruvate as well as with pyruvate was inhibited by ATP to the same degree. Inhibition by ATP has been shown for leaf lactate dehydrogenase and the enzyme from other sources, whereas there are no reports on an inhibitory effect of ATP on hydroxypyruvate reductase (Betsche *et al.*, 1979; Wittenberger, 1968; Davies & Davies, 1972). Insensitivity of hydroxypyruvate reductase to ATP was demonstrated for the enzyme from lettuce leaves. Some hydroxypyruvate reductase bound to the ATP–Sepharose presumably in an unspecific manner could be eluted from the column with very low concentrations of NaCl. No enzymic activity with pyruvate (lactate dehydrogenase) was detected in these fractions. In contrast with the hydroxypyruvate reductase activity in the fractions containing lactate dehydrogenase, the hydroxypyruvate reductase activity of the fractions free from lactate dehydrogenase was not influenced by ATP.

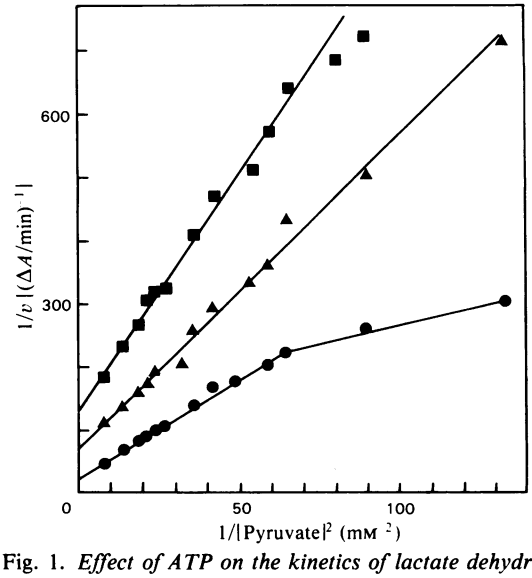

Fig. 1. *Effect of ATP on the kinetics of lactate dehydrogenase at pH 6.2 with pyruvate as varied substrate*
Assay conditions: 0.2 mM-NADH; ●, no ATP; ▲, 30 μM-ATP; ■, 50 μM-ATP.

Kinetics

At pH 7.0 and pH 7.8, normal Michaelis–Menten kinetics were observed for the substrates pyruvate, glyoxylate, L-lactate, hydroxypyruvate (pH 7.0) and NAD^+ (pH 7.8). Plotting the kinetic data obtained at pH 6.2 with pyruvate or glyoxylate as varied substrates ($1/v$ versus $1/[S]^2$) resulted in straight lines and a Hill coefficient (h) for pyruvate at pH 6.2 of 2.0 was calculated from a Hill plot (Fig. 1). It should be mentioned that, in the present paper, Hill coefficients (Segel, 1975) refer to the slopes of the curves in Hill plots at those velocities given in the Figures.

The kinetics of leaf lactate dehydrogenase with respect to NADH were found to be sigmoidal in plots v versus [S] at pH 6.2 as well as at pH 7.0 (Fig. 2 and Fig. 3). Hill coefficients for NADH of 3.9 (pH 6.2) and 1.9 (pH 7.0) were calculated from the data.

The kinetic properties of leaf lactate dehydrogenase with respect to its various substrates are summarized in Table 2.

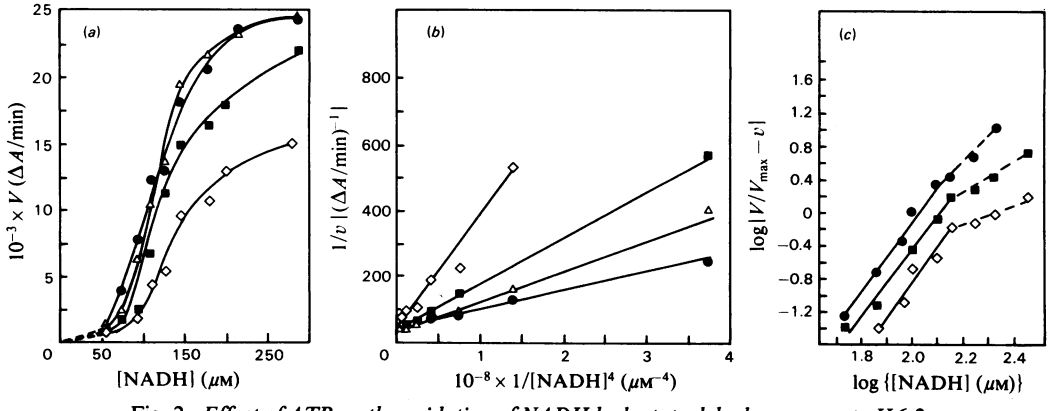

Fig. 2. *Effect of ATP on the oxidation of NADH by lactate dehydrogenase at pH 6.2*
Assay conditions: 2 mM-pyruvate; ●, no ATP; △, 7.5 μM-ATP; ■, 12.5 μM-ATP; ◇, 25 μM-ATP.

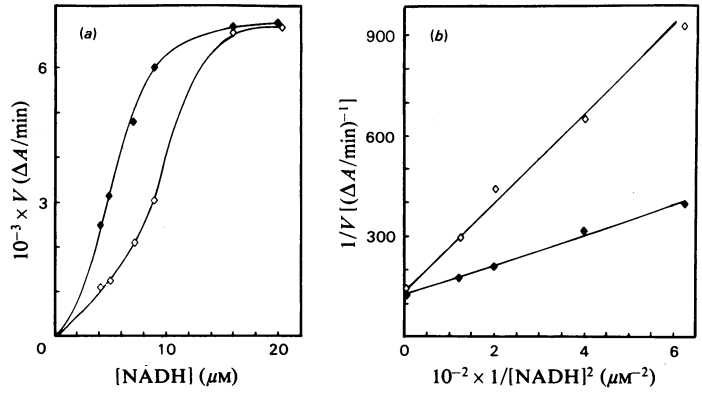

Fig. 3. *Inhibitory effect of NAD$^+$ on the oxidation of NADH by lactate dehydrogenase*
Assay conditions: pH 7.0; 2 mM-pyruvate; ♦, no NAD$^+$; ◇, 0.4 mM-NAD$^+$.

Table 2. *K_m and $V_{max.}$ of leaf L-lactate dehydrogenase for its different substrates and the effect of pH on these parameters*
The data for $V_{max.}$ are shown as percentages of the $V_{max.}$ with pyruvate as substrate at pH 7.0. Assay conditions: for varied substrates pyruvate, hydroxypyruvate or glyoxylate, 0.2 mM-NADH; for L-lactate, 3 mM-NAD; for NADH, 2 mM-pyruvate; for NAD, 20 mM-L-lactate.

pH ...	6.2		7.0		7.8	
Substrate	K_m (mM)	$V_{max.}$ (%)	K_m (mM)	$V_{max.}$ (%)	K_m (mM)	$V_{max.}$ (%)
Pyruvate	0.35*	12	0.72	100	0.86	42
Hydroxypyruvate	—	—	3.0	240	—	—
Glyoxylate	158*	58	62	110	43	17
L-Lactate	—	—	25	10	9.5	17
NADH	0.11*	—	0.010*	—	—	—
NAD	—	—	—	—	0.90	—

* The substrate concentration at $\frac{1}{2}V_{max.}$ (see the text) is shown.

Inhibitors

As judged from a double-reciprocal plot, NADH is a competitive inhibitor of the enzyme with respect to NAD$^+$ (pH 7.0). The reverse reaction was inhibited by NAD$^+$, apparently also in a competitive manner (Fig. 3).

Table 3 shows that leaf lactate dehydrogenase was inhibited by a great number of metabolic compounds. ATP was found to be the most potent inhibitor of the enzyme. At extremely low concentrations, however, ATP stimulated the activity of lactate dehydrogenase (see Fig. 4). ADP and AMP also inhibited the enzyme, but the inhibitory effects were considerably less than that of ATP (Table 3). As can be seen from Table 4, bivalent metal cations or fructose 6-phosphate relieved the ATP inhibition of leaf lactate dehydrogenase. The effect of Ba^{2+} and fructose 6-phosphate, applied together, on the ATP inhibition of the enzyme is shown in Fig. 4.

In order to elucidate the type of inhibition caused by ATP, the inhibitor was studied for its effect on leaf lactate dehydrogenase with respect to the

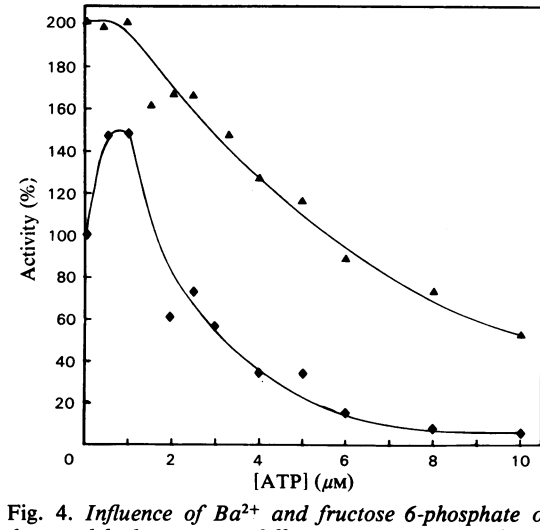

Fig. 4. *Influence of Ba^{2+} and fructose 6-phosphate on lactate dehydrogenase at different concentrations of ATP*
Assay conditions: pH 7.0; 2 mM-pyruvate; 18 μM-NADH; ♦, no addition; ▲, Ba^{2+} (0.5 mM) and fructose 6-phosphate (0.5 mM) added.

different substrates of the enzyme. Double-reciprocal plots of the kinetic data obtained with pyruvate, hydroxypyruvate or glyxoylate as varied substrates indicated a non-competitive inhibition by ATP with respect to these substrates at pH 7.0 and pH 6.2 (Fig. 1; others not shown). The observation that the maximal velocity of the enzyme with regard to NADH was not diminished by ATP, at pH 7.0 as well as at pH 6.2, suggests that the ATP inhibition is competitive with respect to NADH (Fig. 2). A Dixon plot for ATP, obtained with different NADH concentrations at pH 7.0, did not result in straight lines, whereas in a plot $1/v$ versus $1/[ATP]^2$, these data fit straight lines reasonably well (Fig. 5). The intersection point of the extrapolated linear part of the lines in the Dixon plot was found to be located above the abscissa (K_i for ATP 0.7 μM). This finding also indicates competition between NADH and ATP for binding sites (pH 7.0). Corresponding results have been reported for lactate dehydrogenase from other sources (Davies & Davies, 1972; Everse & Kaplan, 1973).

The kinetic behaviour of leaf lactate dehydrogenase with respect to its inhibitor, ATP, was found to be highly pH-dependent. Fig. 6 shows that with decreasing pH the kinetic response of the enzyme towards ATP became substantially more sigmoidal. At pH 6.2, a sharp decline of the enzyme activity occurred once a certain ATP concentration had been reached. As can be seen from Fig. 6, this critical ATP concentration was dependent on the NADH concentration in the medium. The following Hill coefficients for ATP were obtained from the recalculated data of Fig. 6: at pH 7.8, $h = 1.8$; at pH 7.0, $h = 1.9$; at pH 6.2 and high ATP concentration, $h = 2.1$; at pH 6.2 within the range of sharp decline of enzyme activity, $h = 10$.

Table 3. *Inhibition of leaf* L-*lactate dehydrogenase by metabolic compounds*

The assay conditions were: pH 7.0; 2 mM-pyruvate; 0.1 mM-NADH

Compound	Inhibition (%)
ATP (40 μM)	92
ADP (40 μM)	31
AMP (40 μM)	24
Phosphate (0.5 mM)	0
Phosphate (5.0 mM)	0
Fructose 6-phosphate (0.1 mM)	0
Fructose 1,6-bisphosphate (0.1 mM)	39
Fructose 1,6-bisphosphate (0.2 mM)	60
Glucose 6-phosphate (0.2 mM)	8
Glucose 6-phosphate (1.0 mM)	42
α-Glycerophosphate (2.0 mM)	0
α-Glycerophosphate (20.0 mM)	0
3-Phosphoglycerate (2.0 mM)	21
Phosphoenolpyruvate (0.1 mM)	0
Citrate (0.2 mM)	23
Citrate (1.0 mM)	33
Isocitrate (0.1 mM)	33
Glycolate (1.4 mM)	0
Oxalate (.10 mM)	45
Glycine (1.0 mM)	16
Serine (1.0 mM)	18
Alanine (1.0 mM)	11

Table 4. *Effect of bivalent metal cations and fructose 6-phosphate on leaf* L-*lactate dehydrogenase and its inhibition by ATP at various NADH concentrations*

The assay conditions were: pH 7.0; 2 mM-pyruvate. The concentrations of NADH were in the experiments of group (*a*) 200 μM, in those of group (*b*) 18 μM and in those of group (*c*) 100 μM.

	Effectors	Inhibition or activation (%)
(*a*)	ATP (100 μM)	−98
	$MgCl_2$ (2.5 mM)	+10
	$BaCl_2$ (2.5 mM)	+4
	ATP (100 μM) + $MgCl_2$ (2.5 mM)	+11
	ATP (100 μM) + $BaCl_2$ (2.5 mM)	−20
(*b*)	ATP (20 μM)	−100
	$MgCl_2$ (2.5 mM)	−14
	$BaCl_2$ (2.5 mM)	0
	ATP (20 μM) + $MgCl_2$ (2.5 mM)	+23
	ATP (20 μM) + $BaCl_2$ (2.5 mM)	−40
(*c*)	ATP (25 μM)	−54
	Fructose 6-phosphate (0.1 mM)	0
	Fructose 6-phosphate (1.0 mM)	0
	ATP (25 μM) + fructose 6-phosphate (0.1 mM)	−46
	ATP (25 μM) + fructose 6-phosphate (0.5 mM)	−15
	ATP (25 μM) + fructose 6-phosphate (1.0 mM)	−12

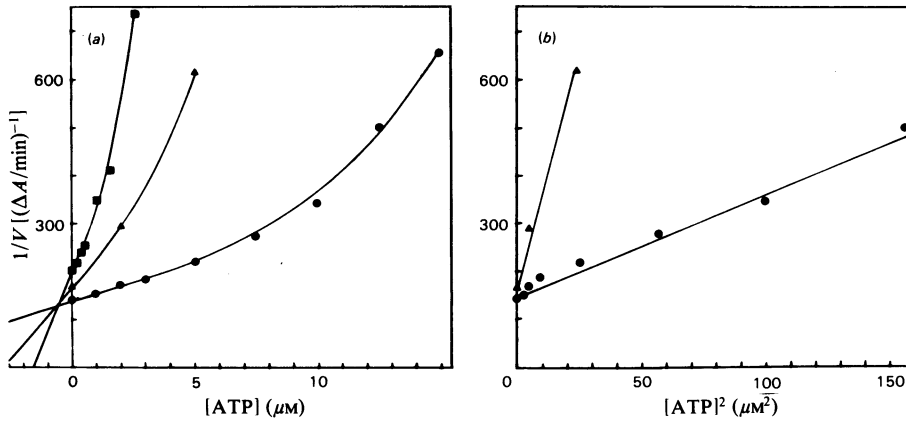

Fig. 5. *Inhibition of lactate dehydrogenase by ATP at pH 7.0 and different concentrations of NADH (Dixon plot)*
Assay conditions: 0.2 mM-pyruvate; ■, 20 μM-NADH; ▲, 35 μM-NADH; ●, 60 μM-NADH.

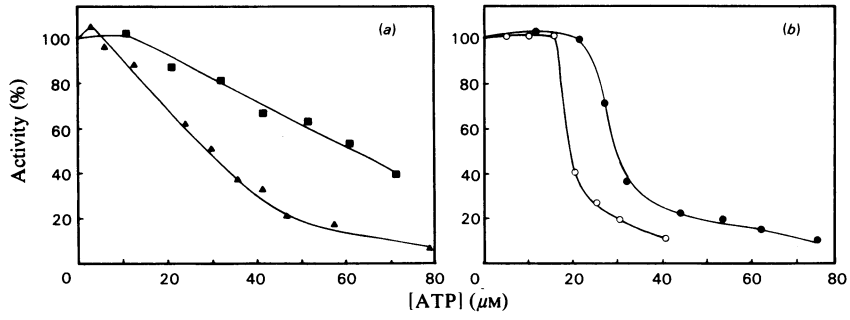

Fig. 6. *Dependence of the kinetics of lactate dehydrogenase with respect to ATP on the pH and the concentration of NADH*
Assay conditions: 2 mM-pyruvate. (*a*) 0.11 mM-NADH; ■, pH 7.8; ▲, pH 7.0. (*b*) pH 6.2; ○, 0.09 mM-NADH; ●, 0.11 mM-NADH.

Discussion

Leaf lactate dehydrogenase shows kinetic properties corresponding to those from allosterically regulated enzymes (Segel, 1975). At acidic pH the enzyme binds pyruvate co-operatively ($h = 2.0$), whereas no co-operativity with regard to pyruvate could be detected at neutral pH. Concerning the kinetic behaviour of lactate dehydrogenase with respect to NADH, the co-operativity of the enzyme is also pH-dependent; that is, the allosteric response of lactate dehydrogenase towards NADH, already detectable at neutral pH, increases as the pH is lowered (Figs. 2 and 3). According to the Hill coefficient for NADH of 3.9 at pH 6.2 the enzyme should have a minimum of four catalytic sites for NADH per molecule.

Regarding the type of inhibition by ATP, the kinetics indicate competitive inhibition with respect to NADH at neutral and acidic pH. In good accord with this is the finding that at neutral pH the Hill coefficients for NADH and ATP are identical. The difference of the Hill coefficients for NADH and ATP at acidic pH might be explained as suggested by Davies & Davies (1972), who observed generally similar kinetics for lactate dehydrogenase from potato tubers: NADH and ATP compete for active sites and, in addition, there are regulatory binding sites for ATP to which also NADH can bind. The affinity of the regulatory binding sites is increased for ATP at acidic pH. Another possible explanation is that the enzyme undergoes a conformational change as the pH is lowered and thereby regulatory binding sites, formerly unaccessible, become available for ATP and NADH.

Bivalent metal cations were found to be antagonistic towards ATP (Table 4, Fig. 4). In view of the observation that Mg^{2+} or Ba^{2+} did not significantly affect the enzyme activity unless ATP is present, one can conclude that there is no direct interaction

between lactate dehydrogenase and bivalent metal cations (Me^{2+}). The relief from ATP inhibition is more likely due to the formation of Me^{2+}–ATP complexes that may not be inhibitory to the enzyme.

Considering the activity of leaf lactate dehydrogenase *in vivo*, the antagonism between ATP and bivalent metal cations is certainly of relevance. ATP concentrations sufficient to inhibit the enzyme completely were measured in the cytoplasm of leaf cells, where the enzyme is located (Santarius & Heber, 1965; Betsche *et al.*, 1979; Wirtz *et al.*, 1980). However, since the ATP inhibition is relieved by bivalent metal cations and, additionally, by fructose 6-phosphate, a complete inactivation of the enzyme *in vivo* is unlikely. In view of the physiological function of the enzyme in leaves the question arises as to what is its natural substrate. According to the kinetic properties the enzyme has to be regarded as L-lactate dehydrogenase (pyruvate reductase), even though the enzyme shows quite high activity with other α-oxo acids (Table 2). Glyoxylate, however, is unlikely to serve as natural substrate for the lactate dehydrogenase, since the K_m of the enzyme for glyoxylate is unfavourably high. The affinity of lactate dehydrogenase for hydroxypyruvate would theoretically allow its function as a cytoplasmic hydroxypyruvate reductase. However, one can question whether concentrations of hydroxypyruvate sufficient for a substantial activity of the enzyme as hydroxypyruvate reductase occur in the cytoplasm of leaf cells. In addition, the cell would face the problem of metabolizing L-glycerate, the prospective product of the enzymic reaction with hydroxypyruvate. No enzyme is known in higher plants capable of utilizing L-glycerate as a substrate. Thus L-lactate or pyruvate remain as the substrates most likely for leaf lactate dehydrogenase. The kinetic data do not allow for a clear decision as to whether the enzyme works *in vivo* as L-lactate dehydrogenase or as pyruvate reductase. Nevertheless, a function of the enzyme as pyruvate reductase appears probable, since leaves from lettuce and several other higher plants have been found to contain L-lactate, the synthesis of which in higher organisms only can be catalysed by lactate dehydrogenase (Schneider, 1960; T. Betsche, unpublished work).

On the basis of the kinetic properties of leaf lactate dehydrogenase the conclusion seems to be justified that the enzyme and, therefore, the synthesis of lactate, is subject to a strict metabolic control. The concentrations of NADH, ATP, and possibly of bivalent metal cations in combined action with the pH of the environment of the enzyme may play the leading roles in the regulation of leaf lactate dehydrogenase. Consequently, the enzyme can be thought to represent a system to buffer the pH in the cytoplasm of leaf cells by forming lactate, and/or to maintain a certain concentration of reducing equivalents (NADH) in the cytoplasm. For lactate dehydrogenase in roots, a function in the field of pH regulation has also been proposed, but more emphasis has been put on malate dehydrogenase, phosphoenolpyruvate carboxylase and malic enzyme as possible pH stabilizers (Davies & Davies, 1972; Davies *et al.*, 1974; Davies, 1979; Smith & Raven, 1979). Support for the other proposed function of lactate dehydrogenase as a part of the system to regulate the concentration of reducing equivalents in the leaf cell is provided by the observation that low concentrations of ATP along with high concentrations of NADH and fructose 6-phosphate are conducive to the enzyme activity. Such conditions would probably occur under oxygen deficiency, resulting in the stimulation of lactate formation and thereby the removal of NADH. Though oxygen deficiency in leaves appears very unlikely, there may still be a need to balance the concentration of reducing equivalents in the cytoplasm of leaf cells. The regulatory properties of the enzyme would be consistent with a function of leaf lactate dehydrogenase as a part of the system to balance the concentration of reducing equivalents as well as with a function of the enzyme in the field of pH regulation. Therefore the two proposed functions of leaf lactate dehydrogenase should not be regarded as alternatives.

This work was supported by the 'Deutsche Forschungsgemeinschaft'. A considerable portion of this work was performed in the laboratory of Professor N. E. Tolbert, Department of Biochemistry, Michigan State University, East Lansing, MI, U.S.A., whose support is gratefully acknowledged. I thank Professor B. Gerhardt and my colleagues at Michigan State University for helpful discussions.

References

Barthova, J., Wilhemova, N. & Leblova, S. (1977) *Biol. Plant.* **19**, 190–195

Betsche, T., Bosbach, K. & Gerhardt, B. (1979) *Planta* **146**, 567–574

Davies, D. D. (1979) *Annu. Rev. Plant. Physiol.* **30**, 131–159

Davies, D. D. & Davies, S. (1972) *Biochem. J.* **129**, 831–839

Davies, D. D., Grego, S. & Kenworthy, P. (1974) *Planta* **118**, 297–310

Everse, J. & Kaplan, N. O. (1973) *Adv. Enzymol. Relat. Areas Mol. Biol.* **37**, 61–133

Garvie, E. I. (1980) *Microbiol. Rev.* **44**, 106–139

Gruber, P. J., Frederick, S. E. & Tolbert, N. E. (1974) *Plant Physiol.* **53**, 167–170

Jervis, L. & Schmidt, C. N. G. (1977) *Biochem. Soc. Trans.* **5**, 1767–1770

Johnson, H. S. & Hatch, M. D. (1970) *Biochem. J.* **119**, 273–280
King, J. (1970) *Can. J. Bot.* **48**, 533–540
Kuntz, W. K., Eber, S., Kessler, W., Krietsch, H. & Krietsch, W. K. G. (1978) *Eur. J. Biochem.* **85**, 493–501
Loeschke, V. & Stegemann, H. (1966) *Z. Naturforsch.* **21b**, 879–888
Maurer, H. R. (1971) *Disc Electrophoresis and Related Technics of Polyacrylamide Gel Electrophoresis*, p. 134, Walter de Gruyter Verlag, Berlin and New York
Oba, K., Murakami, S. & Uritani, J. (1977) *J. Biochem. (Tokyo)* **81**, 1193–1201
Poerio, E. & Davies, D. D. (1980) *Biochem. J.* **191**, 341–348
Rothe, G. M. (1974) *Arch. Biochem. Biophys.* **162**, 17–21
Santarius, K. A. & Heber, U. (1965) *Biochim. Biophys. Acta* **102**, 39–45
Schneider, A. (1960) in *Encyclopedia of Plant Physiology* (Ruhland, W., ed.), vol. 12, part 1, pp. 1009–1022, Springer Verlag, Berlin, Heidelberg and New York
Segel, I. H. (1975) *Enzyme kinetics*, p. 374, Wiley Interscience Publications, New York, London, Syndey and Toronto
Shaw, C. R. & Prasad, R. (1970) *Biochem. Genet.* **4**, 297–320
Smith, A. F., Raven, J. A. (1979) *Annu. Rev. Plant. Physiol.* **30**, 289–311
Wirtz, W., Stitt, M. & Heldt, H. W. (1980) *Plant Physiol.* **66**, 187–193
Wittenberger, C. L. (1968) *J. Biol. Chem.* **243**, 3067–3075