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L-LACTATE DEHYDROGENASE FROM LEAVES OF HIGHER PLANTS. OCCURRENCE AND METABOLISM OF LACTATE UNDER AEROBIC CONDITIONS

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Abstract—Leaves from three higher plant species (*Lactuca sativa*, *Capsella bursa-pastoris*, *Glycine max*) are shown to contain substantial amounts of L-lactate under normal aerobic conditions. No D-lactate is detected in these leaves. Lactate formation occurs under aerobic conditions, in the light and in the dark. Combined labelling and inhibition experiments, using the glycolate oxidase inhibitor α-hydroxybutynoic acid, show that glycolate oxidase oxidizes the bulk of applied L-[^{14}C]lactate in whole leaves. However, it appears that endogeneously formed lactate may not be oxidized by glycolate oxidase, but by another unknown enzyme.

INTRODUCTION

In many bacteria and animal tissues, anaerobic conditions give rise to lactate formation which is catalysed by lactate dehydrogenase (LDH). This process constitutes a mechanism to remove excess NADH [1]. In higher plants, LDH activity and lactate formation were detected in achlorophyllous tissues, such as tubers, roots and cotyledons [2–7]. The role of the enzyme in these tissues has been connected with anaerobic metabolism which occurs under flooding stress [8, 9]. This view, however, has recently been criticized [10, 11]. Furthermore, LDH has been suggested to function in pH regulation [2, 3]. LDH activity has also been detected in photosynthetically active cells, such as green algae and a few lower land plants [12, 13]. However, in these studies the enzyme was not detected in green tissues of higher plants. In contrast to these results, a recent study has reported the occurrence of L-LDH in leaves from several higher plants [14]. It was later suggested that the leaf LDH is involved with pH regulation and/or with the regulation of the level of reducing equivalents (NADH) in the leaf cell [15]. The latter function is essentially that suggested for LDH in animals or bacteria kept under anaerobic conditions, yet in leaves of land plants anaerobic conditions are most unlikely to occur. Nevertheless, studies on the regulation of leaf L-LDH suggest that the enzyme forms lactate *in vivo* and under aerobic conditions [15]. Furthermore, there are several reports on the isolation of an organic acid from leaves of higher plants that was identified as L-lactate by chemical analysis [16]. Traces of lactic acid of undefined configuration were detected in *Rhododendron* leaves, presumably using an enzymic assay with LDH [17]. The aim of the present studies was to demonstrate whether leaves of plants, which have been shown to have L-LDH, contain larger amounts of lactic acid. Lactate production is examined in leaves held under normal aerobic conditions, which are the natural conditions for leaves of land plants. The metabolism of exogeneous and endogeneous L-lactate in leaves is also examined.

RESULTS AND DISCUSSION

Proof and quantitative determination of lactate in leaves

The addition of L-LDH to a reaction medium containing NAD^+ and aliquots of the alcohol extracts from leaves of Shepherd's Purse (*Capsella bursa-pastoris*), lettuce and soybean resulted in the formation of NADH. Unfortunately, L-LDH is a rather unspecific enzyme. Besides L-lactate, it also catalyses the NAD^+-dependent oxidation of other organic acids, such as α-hydroxybutyrate, and glyoxylate [1, 18]. Therefore, the observed reaction with crude extracts is not proof for the presence of L-lactate in leaves. In order to avoid the interferences of other organic acids with the lactate determination, lactate was purified from aliquots of the leaf extracts by TLC. α-Hydroxybutyrate, glycerate, glycolate and glyoxylate had R_f values different from that of lactate. There was no loss of L-lactate during the purification procedure. When L-LDH was substituted by D-LDH only a negligible reaction occurred, regardless of whether alcohol extract or purified lactate were used in the assay. These experiments demonstrate that leaves of the three plants studied contain substantial amounts of L-lactate, but no D-lactate (Table 1). Bourne and Ranson [17] determined lactate in

Table 1. Content of L-lactate in leaves from lettuce, Shepherd's Purse (*Capsella bursa-pastoris*) and soybean

	L-Lactate	
Species	μmols/g fr. wt	μmols/mg chlorophyll
Lettuce	1.28	3.68
Shepherds Purse	1.83	1.45
Soybean	0.86	1.05

The leaves were harvested after a 7 hr illumination period.

Table 2. Formation of lactate from [^{14}C]pyruvate in leaves from Shepherd's Purse (*Capsella bursa-pastoris*)

Conditions	Light				Dark
Labelling time	10 min		45 min		10 min
Inhibitor	None	HBA	None	HBA	None
Lactate (%)	1.1	1.0	0.4	0.5	1.0
Alanine (%)	48.3	53.5	25.3	26.3	42.9
Radioactivity incorporated per leaf (cpm) × 10^3	30.8	80.1	109.4	184.0	42.1

The data are shown as percent of the total incorporation of radioactivity.

Rhododendron leaves. Assuming that the lactate in *Rhododendron* leaves is also of L-configuration, their results show that *Rhododendron* leaves contain less than 3% of the amount of L-lactate found in leaves from Shepherd's Purse or lettuce. It is possible that this difference is a result of the absence of LDH in *Rhododendron* leaves. The presence of this enzyme in leaves of Shepherds' Purse and lettuce has previously been demonstrated [14], but no LDH-activity could be detected in an extract from *Rhododendron* leaves. LDH is the only enzyme in higher organisms known to be capable of synthesizing lactate.

Formation and metabolism of L-*lactate*

The occurrence of L-LDH and L-lactate in leaves suggests that lactate synthesis also occurs. However, lactate formation is generally a result of oxygen deficiency. In the present experiments the plants containing large amounts of L-lactate in their leaves were grown under aerobic conditions (air). One explanation for the occurrence of lactate in leaves is that the lactate from leaves has its origin in the root where oxygen deficiency may occur. On the other hand, studies on the regulation of leaf L-LDH from lettuce indicate that the enzyme may be active in the leaf under aerobic conditions [15]. In order to determine whether leaves synthesize lactate under normal aerobic conditions (air), [^{14}C]pyruvate or $^{14}CO_2$ were fed as precursors for lactate formation to leaves from Shepherd's Purse and lettuce. Table 2 shows that these leaves form lactate from applied [^{14}C]pyruvate in both the light and the dark. Lactate is also formed from photosynthetically fixed $^{14}CO_2$ under normal, aerobic conditions (Table 3). Contamination of the isolated [^{14}C]lactate by other compounds is unlikely considering the extensive purification and identification procedure. The findings that leaves from several air-grown plants contain high amounts of L-lactate and that lactate is produced in these leaves support the suggestion that, in higher plants, L-LDH does not play a role in anaerobic metabolism.

With regard to the oxidation of L-lactate in leaves, two enzymes can be considered candidates for catalysing such a reaction. Obviously the cytoplasmic L-LDH is able to oxidize L-lactate [14]. However, previous work suggests that leaf L-LDH may produce lactate instead of oxidizing it [15]. A more likely candidate would be glycolate oxidase. This enzyme, present with high activities in leaf peroxisomes, *in vitro* catalyses the oxidation of glycolate, as well as that of L-lactate. V_{max} is roughly the same for both substrates, while the K_m of glycolate oxidase for L-lactate (6.6 mM) is much higher than that for glycolate (0.25 mM) [19].

To study whether glycolate oxidase is involved in lactate metabolism, leaves were treated with α-hydroxybutynoic acid (HBA), a strong inhibitor of glycolate oxidase [20]. Labelling experiments using [^{14}C]pyruvate or $^{14}CO_2$ as precursors for lactate synthesis were also conducted with these leaves. Tables 2 and 3 show that the label in lactate did not increase as a result of the inactivation of glycolate oxidase. Furthermore, extention of the labelling time from 10 to 45 min (pyruvate as precursor) did not result in a higher percentage label being incorporated in lactate. This was independent of whether glycolate oxidase was inactivated or not (Table 2). The latter findings indicate that the lactate synthesized in leaves is not just stored in the leaf cell, but is also metabolized. The observation that, as a result of inhibitor treatment, label did not accumulate in lactate does, however, not support the suggestion that lactate synthesized in leaves is metabolized by glycolate oxidase. Evidence for the inactivation of glycolate oxidase by HBA is provided by the observation that a crude extract from

Table 3. Incorporation of $^{14}CO_2$ into lactate by leaves from Shepherd's Purse and lettuce

Species	Shepherd's Purse			Lettuce	
Fixation time	4.5 min		35 min	35 min	
Inhibitor	None	HBA	None	None	HBA
Lactate (%)	0.040 (0.35)	0.032 (0.10)	0.023 (0.29)	0.021 (0.16)	0.016 (0.21)
Acidic fraction (%)	11.4	32.1	8.6	13.3	8.2
Total incorporation (cpm) × 10^6	0.65	0.17	29.4	32.1	15.2

The data are shown as percent of the total incorporation and, in parentheses, as percent of the acidic fraction.

inhibitor-treated leaves did not reveal any glycolate oxidase activity. Furthermore, percentage incorporation of $^{14}CO_2$ into glycolate increased six-fold, due to the inhibitor treatment, while the incorporation into glycine decreased to less than 10% of the value for untreated leaves [T. Betsche, unpublished work]. These data are evidence for the inactivation of glycolate oxidase in the leaf cell during the experiments. The L-LDH activity of the leaf extracts was not affected by the HBA-treatment.

In order to further study the metabolism of L-lactate in leaves, L-[^{14}C]lactate was fed to leaves of several species of higher plants. The lactate was rapidly metabolized by the leaves, in both the light and the dark, with alanine as the main product (Table 4). Treatment of leaves with HBA severely inhibited the oxidation of the externally supplied lactate. No [^{14}C]alanine could be detected under these conditions (Table 5). However, the CO_2 evolution from L-[^{14}C]lactate was not completely supressed by the glycolate oxidase inhibitor, and a few reaction products were found in the leaf extracts (Table 5).

Table 4. Metabolism of L-[^{14}C]lactate in leaves from Shepherd's Purse

Conditions	Light			Dark		
Leaf No.	1	2	3	4	5	6
Lactate (%)	13.6	39.0	36.5	30.0	31.3	69.0
Alanine (%)	63.3	39.0	54.3	43.8	55.7	21.9
CO_2 released (%)	2.2	1.4	0.3	24.8	10.6	7.4
Others (%)	20.9	20.5	9.0	1.4	2.3	1.9
Radioactivity incorporated per leaf (cpm) × 10^3	53.7	76.6	67.4	44.8	49.0	65.3

The labelling time was 10 min. The data are shown as percent of the total incorporation of radioactivity.

The results of the experiments with L-[^{14}C]lactate demonstrate that glycolate oxidase oxidizes applied L-lactate in the whole leaf. However, the findings that: (1) CO_2 was evolved from externally supplied L-lactate, even though glycolate oxidase was inactivated; and that (2) the inactivation of glycolate oxidase did not lead to the accumulation of lactate produced from [^{14}C]pyruvate or $^{14}CO_2$, indicate that endogeneously produced lactate is not oxidized by glycolate oxidase but by another unknown enzyme.

EXPERIMENTAL

Plants. Leaf lettuce (*Lactuca sativa* L., var. *longifolia*), spinach (*Spinacia oleracea* L.) and soybean (*Glycine max* L.) were cultivated in a growth chamber (soil, 9 hr day). Whole plants of Shepherd's Purse [*Capsella bursa-pastoris* (L.) Med.] were taken from the field and kept in the growth chamber for 1 week before use in the expts.

Isolation and determination of lactate. 1–3 g leaves were ground in liquid N_2 to a powder and 10–30 ml hot 90% EtOH was added. The powder was ground again for 3 min. The homogenate was filled into tubes and agitated for 1 hr. After centrifugation (15000 *g*, 10 min), aliquots of the extracts were used directly for the determination of lactate. To purify lactate, other aliquots, to which traces of radioactive L-lactate were added, were chromatographed on cellulose TLC plates with *n*-BuOH–propionic acid–H_2O (30:22:48) as solvent [21]. Lactate was detected by scanning the plates for radioactivity. The lactate was eluted with H_2O and the samples were evaporated (35°) to a small vol. Lactate was then determined. The assay medium for the determination of lactate was 100 mM Tris–HCl buffer, pH 9.0, 3 mM NAD^+ and 75 mM hydrazine (340 nm). The reaction was started with 100 μg commercial L-LDH (bovine heart) or, to determine D-lactate, with 50 μg bacterial D-LDH (Boehringer, Mannheim).

CO_2 fixation experiments. Pieces of stem with 4–6 leaflets were excised from Shepherd's Purse and lettuce plants at the onset of flowering. The plant material was preilluminated (6000 lx, slide projector) for 20 min while standing in 5 mM HBA or in H_2O–HCl of the same pH as the HBA-soln. One stem from each treatment, still standing in the HBA-soln or H_2O–HCl was placed in a photosynthetic chamber (vol. 3 l.) and further

Table 5. Effect of α-hydroxybutynoic acid on the metabolism of L-[^{14}C]lactate in leaves from Shepherd's Purse, lettuce and spinach in the light

Species	Shepherd's Purse		Lettuce		Spinach	
Inhibitor	None	HBA	None	HBA	None	HBA
Lactate (%)	22.8	98.9	57.2	94.4	28.7	95.7
Alanine (%)	57.4	0.0	17.7	0.0	42.5	0.0
CO_2 released (%)	1.0	0.6	1.4	0.7	1.0	0.3
Others (%)	18.9	0.6	23.7	4.9	27.7	4.0
Radioactivity incorporated per leaf (cpm) × 10^3	407.0	432.6	150.6	190.5	126.7	113.4

The labelling time was 10 min. The data are shown as percent of the total incorporation of radioactivity.

illuminated (3500 lx). The chamber was simultaneously flushed with 15 l. CO_2-depleted air. The illumination was then increased to 8000 lx and CO_2 was released into the chamber by acidifying a known amount of [^{14}C]bicarbonate to a normal atmospheric CO_2-concn (sp. act. 2.2 Ci/mol). After the labelling time, the leaves were ground in liquid N_2. Hot 90% EtOH was then added and the homogenate was agitated for 3 hr. After centrifugation (15 000 *g*, 10 min), the extracts were fractionated by ion exchange chromatography to basic, acidic and neutral fractions [22]. To purify lactate, the acidic fraction was chromatographed (× 3) on cellulose plates using PrOH–1% NH_3 in H_2O (2:1) and propionic acid–*n*-BuOH–H_2O (22:30:48) and EtOH–conc. NH_3–H_2O (80:5:15) as solvents [23]. The third TLC revealed only one peak of radioactivity at the position of co-chromatographed lactic acid. The latter was detected by spraying the plates with 0.1% bromocresol green in weak alkaline 90% EtOH. At this purification stage, the incorporation of ^{14}C into lactate was determined. The purity of the lactate was confirmed by additional TLC using various solvents.

Experiments with [^{14}C]*pyruvate and* L-[^{14}C]*lactate.* [1-^{14}C]Pyruvate was purified before use by TLC. L-[U-^{14}C]Lactate was found to be chromatographically pure. Leaves excised from the stem of *Capsella* plants, harvested after the onset of flowering, or from young lettuce plants were pretreated with HBA or H_2O–HCl, as described above. Subsequently, the leaves were transferred into a small photosynthetic chamber (vol. 3 ml), where the leaves stood in a soln of [^{14}C]pyruvate (3 μCi/ml; 0.4 mM) or L-[^{14}C]lactate (25 μCi/ml; 27.5 mM; Na salt). To protect from heat, a H_2O filled chromatography tank was placed between the light source (slide protector, 12 000 lx) and the photosynthetic chamber. Air was pumped through the chamber (6 ml/min) and the evolved CO_2 was captured by two consecutive filter elements consisting of rolls of chromatography paper inserted in Pasteur pipettes and soaked with 0.9 M hyamine hydroxide in 90% MeOH. Metabolites were extracted from the leaves and fractionated to basic, acidic and neutral fractions, as outlined above. With L-[^{14}C]lactate as labelling agent, the metabolites were further separated by 2D-TLC on cellulose using the solvent system of [21]. Radioactivity was detected by autoradiography. Compound identification was verified by separate TLC using various solvents. When [^{14}C]pyruvate was the labelling agent, the separation and identification of basic compounds was performed as described above. Synthesized lactate was purified from the acidic fraction as outlined above.

Determination of radioactivity. Radioactivity was determined in a toluene–Triton scintillation mixture.

Assays. Aq. leaf extracts were prepared with 0.1 M Pi buffer (pH 7.4) and centrifuged at 15 000 *g* for 15 min. The supernatant was used in the enzyme assays. Glycolate oxidase was determined as described in ref. [24]. LDH was measured with pyruvate as substrate [25]. Chlorophyll was determined according to ref. [26].

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REFERENCES

1. Everse, J. and Kaplan, N. O. (1973) *Adv. Enzymol.* **37**, 61.
2. Davies, D. D. and Davies, S. (1972) *Biochem. J.* **129**, 831.
3. Davies, D. D., Grego, S. and Kenworthy, P. (1974) *Planta* **118**, 297.
4. Rothe, G. M. (1974) *Arch. Biochem. Biophys.* **162**, 17.
5. Oba, K., Murakami, S. and Uritani, J. (1977) *J. Biochem.* **81**, 1193.
6. King, J. (1970) *Can. J. Botany* **48**, 533.
7. Barthova, J., Wilhelmova, N. and Leblova, S. (1977) *Biol. Plant.* **19**, 190.
8. Wager, H. G. (1961) *J. Exp. Botany* **12**, 34.
9. Crawford, R. M. M. and Tyler, P. D. (1969) *J. Ecol.* **57**, 235.
10. Smith, A. M. and ap Rees, T. (1979) *Phytochemistry* **18**, 1453.
11. Smith, A. M. and ap Rees, T. (1979) *Planta* **146**, 327.
12. Frederick, S. E., Gruber, P. J. and Tolbert, N. E. (1973) *Plant. Physiol.* **52**, 318.
13. Gruber, P. J., Frederick, J. E. and Tolbert, N. E. (1974) *Plant. Physiol.* **53**, 167.
14. Betsche, T., Bosbach, K. and Gerhardt, B. (1979) *Planta* **146**, 567.
15. Betsche, T. (1981) *Biochem J.* **195**, 615.
16. Schneider, A. (1960) in *Encyclopedia of Plant Physiology* (Ruhland, W., ed.) Vol. XII, Part 1, pp. 1009–1022. Springer, Berlin.
17. Bourne, D. T. and Ranson, S. L. (1965) *Plant. Physiol.* **40**, 1178.
18. Poerio, E. and Davies, D. D. (1980) *Biochem. J.* **191**, 341.
19. Kerr, M. W. and Groves, D. (1975) *Phytochemistry* **14**, 359.
20. Jewess, P. J., Kerr, M. W. and Whitaker, D. P. (1975) *FEBS Letters* **53**, 292.
21. Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Hass, V. A. and Stepka, W. (1950) *J. Am. Chem. Soc.* **72**, 1710.
22. Canvin, D. T. and Beevers, H. (1961) *J. Biol. Chem.* **236**, 988.
23. Block, R. J., Durrum, E. L. and Zweig, G. (1958) *A Manual of Paper Chromatography and Paper Electrophoresis.* Academic Press, New York.
24. Gerhardt, B. (1974) *Z. Pflanzenphysiol.* **74**, 14.
25. Bergmeyer, H. U., Gawehn, K. and Graβl, M. (1974) in *Methoden der enzymatischen Analyse* (Bergmeyer, H. U., ed.) Vol. 1, pp. 454–558. Verlag Chemie, Weinheim.
26. Kirk, J. T. O. (1968) *Planta* **78**, 200.