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LACTATE DEHYDROGENASE IN PLANTS: DISTRIBUTION AND FUNCTION

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Key Word Index—Lactate dehydrogenase; alcohol dehydrogenase; plant distribution.

Abstract—Contrary to previous reports, lactate dehydrogenase (LDH; EC 1.1.1.27) occurs in all green land plants ranging from flowering plants to mosses. Conditions must be carefully designed and monitored, however, to achieve detection and extraction, and even then the specific activity is always orders of magnitude lower than that typically encountered in animal tissues. Green algae contain even lower levels of an NAD-dependent LDH activity not clearly identified as EC 1.1.1.27. Red and brown algae seem to lack LDH activity entirely. A striking feature of all the flowering plants investigated was a prominent peak of expression of LDH in stem tissue, at, or immediately above, soil level which was paralleled by a similar peak of alcohol dehydrogenase (ADH; EC 1.1.1.1) activity. The possible functional significance of this, and the function(s) of plant LDH generally, are discussed.

INTRODUCTION

The relatively low levels of lactate dehydrogenase activity (LDH) in plants has been the subject of some excellent investigations, notably by Davies [1, 2], Betsche [3], Hanson and co-workers [4–7], Jervis [8] and their associates, but its function and certain of its properties remain poorly understood by comparison with the equivalent activities of many bacteria, invertebrate animals and certainly by comparison with the lactate dehydrogenases of vertebrates, which rank among the most investigated of biocatalysts. One of the barriers to more detailed study of the plant enzyme is the relatively low level of the activity in plants. In our attempts to develop purification tactics allowing more efficient isolation of these low levels of activity, we have noted some interesting features that seem to have previously gone unnoticed. In particular, we find a marked and seemingly general elevation of the enzyme in certain parts of flowering plants – the base of the main stem particularly – and we feel that this activity differential must have a bearing on the poorly resolved question of the function(s) of plant LDH. We also demonstrate the presence of this enzyme in lower plants previously thought to be devoid of such activity.

RESULTS AND DISCUSSION

General/phylogenetic distribution

Table 1 and Figs. 1 and 2 summarize representative results of a survey we have carried out of the occurrence of LDH and ADH in various plant groups and species and in various tissues of higher plants. We include some data on levels of LDH in some animal tissues for comparative purposes (Table 1, top). The angiosperm (flowering plant) tissues listed in Table 1 are chosen as typically representative of the range of LDH levels we have encountered in this major group of plants.

In our preliminary work [9] we calculated and represented specific activities on the basis of units of enzyme activity per g of tissue (a method that has been used, in various forms, by other groups of workers in this field). But, fearing that activity profiles based on such a criterion might be distorted by factors such as differing degrees of lignification or water content of different tissues, we subsequently changed to specific activity calculations based on units of enzyme activity per mg of protein, using a special protein estimation method developed by Bensadoun and Weinstein [10] to avoid interference from secondary metabolites often encountered in plant extracts. This methodological change did not, however, cause any significant qualitative change in the profiles we have already reported [9], and in all the plants we have since examined the enzyme distribution profiles represented by either of these specific activity criteria remained qualitatively very similar. One representative example of this parallelism is shown in the first two sections of Fig. 2.

It can be seen from Table 1 that the levels of LDH activity in plants are usually order(s) of magnitude lower than those encountered in animals (and in lactate-forming bacteria, which have LDH levels comparable

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Table 1. Levels of LDH and ADH activities in the major plant groups, with comparative values for selected animal tissues. The activities were measured from the pyruvate reductase and aldehyde reductase direction respectively. A unit of activity (nkat) is that which oxidizes 1 nmol of NADH sec^{-1} at 30°.* Extraction procedures are described in the Experimental section; in the case of each moss, liverwort and algal species, the entire plant was extracted. The reproducibility of analysis was about ±2% as regards the enzyme assays, but variability of about ±10% was introduced by the extraction procedures

	LDH activity*		ADH activity
Group, Species and Tissue	nkat g^{-1} of fr. wt	nkat mg^{-1} of protein	nkat mg^{-1} of protein
Selected Animal Tissues:			
Mammalian skeletal (thigh) muscle (rat, Sprague-Dawley)	8420	183	0.0
Crustacean tail muscle (*Nephrops norvegicus*)	2090	6.2	0.0
Gastropod 'foot' (*Littorina littorea*)	121†	3.3†	0.0
Plants:			
Angiosperms: selected tissues			
Potato tuber	1.93	1.42	3.22
Lettuce leaf	1.50	0.42	trace
Turnip 'tap root' (*Brassica rapa*-)	2.12	0.95	115
Leek bulb (*Allium porrum*)	1.25	0.35	7.83
Ferns (stems only):	(see footnote‡)		
Phyllitis scolopendrium	2.90	1.60	0.0
Dryopteris filix-mas	3.10	1.33	0.0
Liverwort:			
Conocephalum conicum	0.33	0.30	0.08
Mosses:			
Brachythecium rivulare	5.78	0.80	0.0
Isothecium myosuroides	0.48	0.28	0.43
Calliergon cuspidatum	1.45	0.80	0.25
Green Algae:			
Enteromorpha linza	0.20	0.03	0.0
Monostroma grevillei	0.18	0.02	<0.02
Cladophora rupestris	0.23	0.03	0.0
Red algae:			
Rhodymenia palmata	0.0	0.0	0.0
Porphyra umbilicalis	0.0	0.0	0.0
Polysiphonia elongata	0.0	0.0	0.0
Brown algae:			
Fucus serratus	neither activity detectable in either species.		
Pelvetia canaliculata	but see footnote‡		

*In the case of LDH from angiosperms (and most of the other organisms listed above) the assay conditions used here (0.5 mM pyruvate and 0.2 mM NADH at pH 7.4) yield velocities of about one third V_{max}. These activities should be approximately doubled to make them comparable with specific activities measured at pH 6.5–7.0 or with pyruvate concentrations of about 3 mM (assay conditions used by some other workers).

†D-LDH (i.e. catalyses the formation of D- rather than L-lactate).

‡Ferns and brown algae (but not red algae) seem to contain factor(s) that may inactivate or 'sequester' ADH activity unless special extraction conditions are used which then reveal LDH activity in ferns but not in the brown algae (see 'Extraction Procedures' in the Experimental section, and 'Distribution' in the Result and Discussion section).

to those found in mammalian tissues). This is a point worth emphasizing, as it may account for some reports of the absence of LDH from certain plants. The differing perceptions of what constitutes significant levels of LDH activity may be illustrated by contrasting the observation of Davies [2], from the perspective of a plant biochemist: 'potato tubers are particularly rich in lactate dehydrogenase', with that of Mayr *et al.* [11], more familiar with bacterial LDH-levels: 'L-lactate dehydrogenase activity in ... potato tubers was very weak'. The LDH activity in potato tubers is, in fact, high by 'plant standards' (see Table 1). Since many of the plant LDH activities we have been dealing with are a further order of magnitude lower than those found in potato tubers, they could easily be overlooked by biochemists accustomed to regarding much higher levels of activity as marginal. A serious additional problem is caused by the pervasive background or 'endogenous' NADH oxidising activity [12]. As explained in the Experimental section (see 'Enzyme Assays'), we concluded that a high proportion of this 'background activity' is caused by LDH itself and if it is indiscriminately subtracted, LDH levels that are already low may 'disappear' completely. (We have, indeed, been guilty of this error ourselves in our preliminary publication [9], fortunately without serious distortion of the particular activity profiles presented there).

Possibly as a result of these and other problems mentioned below, information on the general distribution of LDH in plants is sparse and conflicting. Only two such investigations seem relevant here: those of Gruber *et al.* [12] and of Betsche *et al.* [13]. Gruber *et al.* [12] reported scattered occurrence of LDH activity in some 'lower' land plants (some mosses and liverworts) and a D-lactate specific LDH in some green micro-algae, but no activity in higher plants (angiosperms) or in ferns. Betsche *et al.* [13] later reported LDH activity (although low by any standards) in some higher plants, but not in others. It appears that both of these groups concentrated entirely on green photosynthesising tissue – green leaves and their equivalents in the 'lower' plants.

In general we find LDH activity in all the plant groups we have studied, with the exception of the previously uninvestigated red algae (*Rhodophyta*) and brown algae (*Phaeophyta*) – see Table 1. As indicated in the footnote of Table 1, we also failed initially to detect any LDH activity whatever in ferns, but this seems to have been due to some form of 'sequestration' of the activity that was overcome by modification of the extraction procedure. We had already encountered a similar problem with one flowering plant – *Iris pseudacorus* L. (Common flag). At first we could detect no LDH or ADH activity in extracts of any part of this plant, although zymological staining procedures suggested the presence of LDH activity in cross sections of the stem/leaf base region. Subsequent work (see under 'Extraction Procedures' in the Experimental section) suggested that the absence of activity in the extracts was due to some form of adsorption of the enzymes onto the discarded centrifugation 'pellet', and that this adsorption might be overcome by the mild detergent, Triton, in conjunction with high salt con-

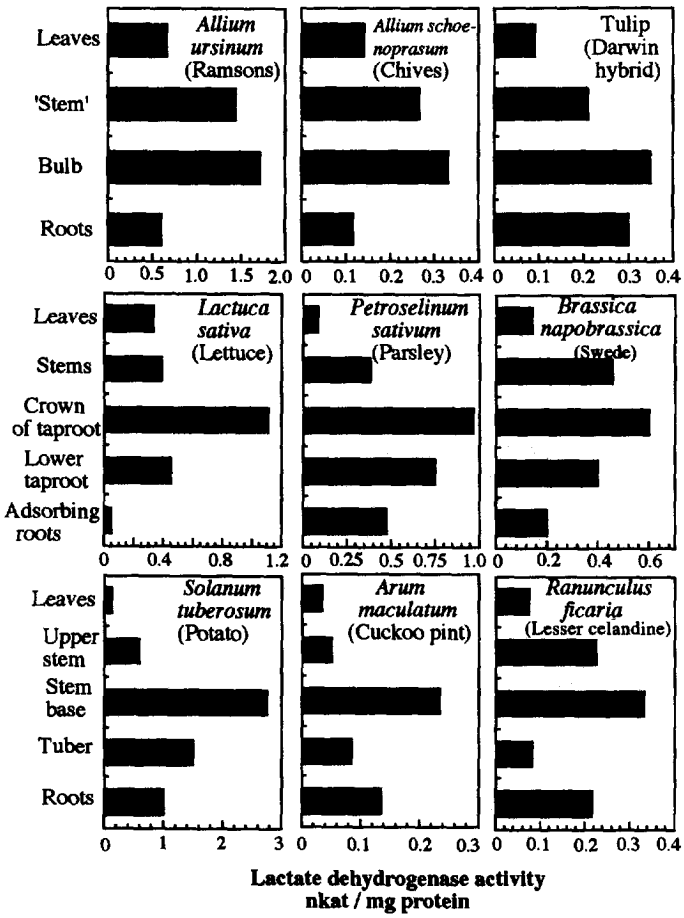

Fig. 1. Distribution of lactate dehydrogenase (LDH) in the various sections of some selected angiosperms. Triplicate assays of enzyme activity for extracts of segments from individual plants varied by less than 2%. Activities for segments from different individual plants of the same species, grown under identical conditions, sometimes varied by as much as 10%, but this applied throughout the plant so that the overall pattern of LDH distribution did not deviate significantly from that shown above.

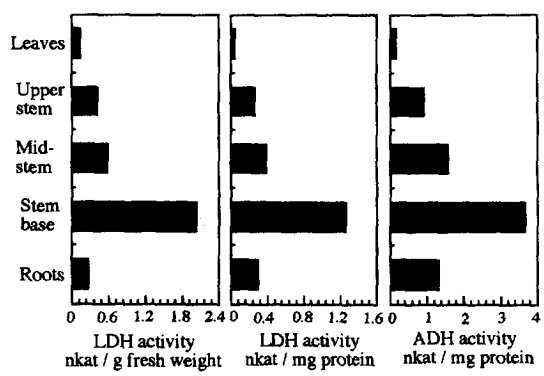

Fig. 2. Distribution of lactate dehydrogenase (LDH) and alcohol dehydrogenase (ADH) in groundsel (*Senecio vulgaris*). Triplicate assays of enzyme activity for extracts of segments from individual plants varied by less than 2%. Activities for segments from different individual plants of the same species, grown under identical conditions, sometimes varied by as much as 10%, but this applied throughout the plant so that the overall pattern of LDH and ADH distribution did not deviate significantly from that shown above.

centrations. Extraction of various segments of *I. pseudacorus* in this manner then yielded a tissue-distribution profile analogous to that obtained with a range of other plants using the 'normal' extraction procedure.

Such problems, in addition to those mentioned above, probably contributed to some of the negative reports of Gruber *et al.* [12] and Betsche *et al.* [13]. A major additional factor, however, was undoubtedly the emphasis in these previous studies on green leaves to the exclusion of other tissues. As can be seen from Fig. 1, we find much higher activity generally in non-photosynthesising parts of higher plants though not, generally, in absorbing roots (which have been the other major focus of attention in this field of study).

It seems strange that the strikingly elevated expression of LDH in tissues anatomically intermediate between the leaves and the absorbing roots of angiosperms should have previously gone unnoticed. However, apart from the 'leaf-oriented' studies just mentioned, the major plant materials that have been previously investigated for their LDH activity have been potato tubers (usually described as having been purchased from local markets) and barley seedlings (the

malt of the brewing industry). These sources are commercial food products, and such factors may possibly have limited the range of investigation.

Our studies reveal not only characteristic tissue distribution patterns of LDH activity in plants (discussed below), but also show that absolute levels of activity vary widely from one plant to another, even among closely related species, such as the members of the genus *Allium* – the levels in *A. ursinum*, for example, being about fivefold higher than those in *A. porrum* or *A. schoenoprasum* (Fig. 1). The highest LDH specific activity we have encountered in a plant tissue is that in the stem base (rather than in the tubers) of the potato plant (2.83 nkat mg protein^{-1}).

The general levels in ferns and mosses are comparable to those in higher plants, the activity in these lower plants being L-lactate specific (when assayed form the 'back' lactate directions) and strongly inhibited by oxamate, a highly-specific competitive inhibitor of the 'classic' mammalian types of LDH [14].

The levels of LDH activity in green algae are very much lower (Table 1), so low that we were unable to establish the specificity with regard to D- or L-lactate, the assay from the lactate direction being a further order of magnitude slower and not readily interpretable, even when the activity was concentrated over twenty-fold by ammonium sulphate precipitation. The LDH activity of these green algae, while strongly inhibited by 30 mM oxamate, was only marginally inhibited by 8 mM oxamate, a property characteristic of D-lactate dehydrogenases of invertebrate animals, but not of L-lactate dehydrogenases, while are almost totally inhibited by 2 mM oxamate [15]. The only algal plant (macro-alga) that seems to have been previously examined for LDH activity was the green alga *Codium*, reported to be devoid of such activity by Gruber *et al.* [12], who, however, reported NAD-dependent LDH activity in some microalgae, which seemed to be specific for D-lactate.

In all the higher plants tested and in the ferns and mosses, the LDH activity displayed the classic (EC 1.1.1.27) specificity for L-lactate when assayed from the lactate direction and was strongly inhibited by oxamate.

As already mentioned, we can find no trace of LDH activity in any of the red or brown algae species we have examined (Table 1).

Tissue distribution

Our initial tissue distribution studies [9] (which, at that stage, were confined to some *Allium* species and some tap-root vegetables) led us to speculate that high LDH activities might be associated in some way with nutrient storage, since peak LDH activity was observed in the bulbs and tap-roots of these plants. Zymological-histological staining (see Experimental section) showed a qualitatively similar distribution pattern when applied to some of the *Allium* and other species. This pattern seemed to be confirmed by results obtained with many other species, a selection of which are presented in the top and middle rows of Fig. 1. Again maximum specific activity seems to be associated with the nutrient storage organs – bulbs and tap-roots.

However, subsequent extension of the survey cast doubt on this interpretation. As can be seen from the bottom row of Fig. 1, tuber-forming plants do not show an analogous pattern. The root-derived storage tubers of *Arum* and of lesser celandine are not rich in LDH, and even the much-studied potato tuber, while comparatively rich in LDH, has less than half the specific activity of the adjacent stem-base when the growing plant is studied. Some bulb-forming plants we also later found to have no particularly high activity in their bulbs [e.g. the daffodil (*Narcissus* sp.) and bluebell (*Endymion nonscriptus*)]. The nutrient-storing bulbils of the bulbil-forming strain of lesser celandine were also low in LDH activity as were the rhizomes or corns of certain members of the Iris family (e.g. *I. pseudacorus* and *Montbretia/Crocosmia*).

The pattern we now perceive is a general elevation of LDH activity in the bases of stems. This pattern we also find in plants lacking any special nutrient tissue, such as the annual groundsel, illustrated in Fig. 2 and the celery plant (*Apium graveolens*) and is also characteristic of ferns which have high LDH activity in the stems, much lower activity in the fronds (leaves) and no detectable activity in the storage rhizomes. It is relevant in this connection that the bulbs of members of the *Allium* and related genera are formed from swollen stem-plus-leaf bases, that the soil-level crown of the so-called 'tap-root' of swede and turnip are not formed from root tissue, but from stem-base and/or hypocotyl tissue, and that this probably also applies to the top portions of the other tap-root plants we have studied (Fig. 1). Moreover the potato tuber is a modified stem, unlike the root tubers of the other plants represented in the bottom row of Fig. 1 (see e.g. [16] for such plant anatomical information).

Besides illustrating some points already discussed above, Fig. 2 also serves as a typical illustration of the parallelism we find between LDH and ADH activities in angiosperms. Similar parallelism was found for all the species featured in Fig. 1 and for others not included there (a total of over 20 species from 9 families of angiosperms, both dicots and monocots). Although such parallelism of the ADH and LDH distribution profiles seemed a universal feature of angiosperms, the ratio of the two activities varied widely from plant to plant. For example, the ADH/LDH ratio is particularly high throughout turnip plants, but low in lettuce (see Table 1 for representative examples).

We were unable to perceive any similar parallelism of the two activities in lower plants. Ferns had no detectable ADH in their 'leaves' and stems, where LDH is relatively high, nor in their rhizomes which seem to lack LDH also. ADH was detected in their roots, but interpretation of this is problematical, as discussed below. Apparent ADH levels in moss samples, always low by comparison with the higher plants, seemed

variable from sample to sample of the same species and no convincing activity could be demonstrated in green, or other, algae.

While LDH levels in the absorbing roots of plants were usually comparatively low (e.g. Fig. 1), some plants showed relatively high levels in this tissue – see, for example, tulip (Fig. 1, top row), arum and lesser celandine (bottom row). This exceptional 'root activity' was variable from one specimen to another of these particular species and it might be attributable to flooding-induced anoxia which Hoffman *et al.* [6] have shown to cause a dramatic increase in LDH levels in the roots of barley seedlings (some of the *Arum* and celandine plants were collected from the side of a woodland stream after a spell of particularly wet weather). An alternative possibility is that these variable activities might derive from microbial or fungal symbionts which are common in root tissues of some plant species. This might also apply to the high ADH activity in the roots of *A. ursinum*. (None of the plants studied here was grown axenically, and while the roots, in common with all other tissues, were carefully cleaned before extraction, this might not have eliminated such symbionts.)

In a number of angiosperms we examined, there seemed to be no significant radial differential in the activity of LDH. Cross-sections of leek and onion plants and of potato tubers showed no noticeable gradient of activity when stained zymologically for LDH activity. For example, the outer 'rings' of such sections of onion and leek stained as quickly and as darkly as the inner ones, and slices of potato tubers appeared to stain evenly for LDH activity.

Function(s) of plant LDH

We confess to being unable to explain the functional significance of the tissue distribution profiles and some of our other results described above, which seem to add further mystery to an already unclear situation.

In leaves, it seems very unlikely that LDH could play its classic role in anaerobic glycolysis. Davies and Asker [17] have suggested that LDH may act instead on an alternative substrate, glyoxylate, disproportionating it via a complex NAD-dependent oxido-reductive mechanism into oxalate and glycolate. He proposes that this reaction might operate as a pH-stat, counteracting any upward drifts of pH in leaf tissue. Davies and Asker]17] have demonstrated the feasibility of such a process *in vitro*, but we think such a mechanism is unlikely to be metabolically significant. In any case, such a theory would offer no justification for the much higher levels of LDH activity in stems generally and stem bases in particular, nor of the parallel ADH activities, which might, instead, be interpreted as indicative of some connection with anaerobic glycolysis.

Following from other pioneering work of Davies *et al.* [18] it has been well established by NMR [19, 20] and other studies [21–23] that LDH plays an important role in anaerobic glycolysis in anoxic roots, but only as a prelude to alcohol-terminating glycolysis. The lactate initially produced by LDH lowers the pH and this in turn activates pyruvate decarboxylase, switching the glycolytic metabolism, via ADH, to alcohol production, ethanol being much better tolerated than lactate by plant cells. Asker and Davies have proposed an LDH-centered mechanism of control of this 'pH-switch', which some of our other work supports (unpublished data). Such an adaptable mechanism of anaerobic glycolysis almost certainly allows roots to survive periods of anoxia caused by flooding of the soil. The anoxically low levels of oxygen are caused by immobilisation of the water, and hence reduction of the rate of oxygen diffusion, in stagnant mud. It is tempting to propose something similar to account for the high LDH levels (and the correspondingly high ADH levels) in the adjacent tissue at soil-level and immediately above it. However, in above-soil flooding the surface water remains mobile and oxygen diffusion comparatively unimpeded, and this seems unlikely to result in significant anoxia of the stem tissue, certainly hardly enough to justify such 'standby' or constitutive remedial measures not enjoyed by the far more vulnerable underground roots.

EXPERIMENTAL

Biological materials. Wild plants were collected from their native habitats in the field, woods and sea-shores west of Galway City. The marine invertebrates listed in Table 1 were obtained fresh from Galway Bay. Cultivated plants were grown under normal horticultural conditions in local market gardens. The cultivars used in these studies were as follows:

Brassica rapa cv 'Snowball' (White turnip), *B. napobrassica* cv 'Purple-top' (Swede turnip), *Petroselinum sativum* cv 'Moss curled' (Parsley), *Lactuca sativa* cv 'Kweik' (Lettuce), *Solanum tuberosum* cv 'Record' (Potato), *Tulipa* sp. cv 'Darwin hybrid' (Tulip), *A. porrum* cv 'Musselburgh' (Leek). *A. cepa* cv 'White Lisbon' (Onion).

The 'wild' angiosperms used in this study included: *A. ursinum* (Ramsons) L., *A. schoenoprasum* (Chives) L., *Arum maculatum* (Cuckoopint) L., *I. pseudocorus* (Common flag) L., *Ranunculus ficaria* (Lesser celandine) L., *Senecio vulgaris* (Groundsel) L., *Taraxacum* sp. (Common dandelion).

Liverwort: *Conocephalum conicum* (L.) Underw. Mosses: *Brachythecium rivulare* B., S. & G., *Isothecium myosuroides* Brid., *Calliergon cuspidatum* (Hedw.) Kindb. Green Algae: *Enteromorpha linza* (L.) J. Agardh, *Monostroma grevillei* (Thuret) Whittrock, *Cladophora rupestris* (L.) Kutzing. Red Algae: *Rhodymenia palmata* (L.) Greville, *Polysiphonia elongata* (Hudson) Sprengel, *Porphyra umbilicalis* (L.) J. Agardh. Brown Algae: *Fucus seratus* L., *Pelvetia canaliculata* (L.) Decaisne & Thuret. Ferns: *Phyllitis scolopendrium* (L.) Newm., *Dryopteris filix-mas* (L.) Schott.

Plants, when not already growing in containers, were carefully transplanted into pots (or glass containers of sea-water in the case of the algae) and maintained under appropriate growth conditions for short periods until immediately before use.

Extraction procedures. For the enzyme distribution studies, plants were freed of soil, quickly but thoroughly washed under running cold H_2O and immersed in ice-H_2O containing 0.1 M KCl. They were then sectioned, blotted dry, and selected portions were weighed, finely chopped and immediately extracted, all the foregoing operations being completed within 10 min. The temp. at this point and during all subsequent manipulations was maintained at 0–4°. Extraction of most samples was carried out by thorough grinding with sand in a chilled pestle and mortar with appropriate quantities of ice-cold 50 mM KPi buffer (pH 7.4) containing an 8% (w/v) suspension of insoluble polyvinyl-pyrrolidone to minimize interference from phenolic compounds common in plant extracts. Usually one vol. of this extraction medium (equal to the wt of the plant tissue) was used, but owing to the water-absorbent or gelatinous properties of some samples (e.g. the mosses and algae respectively), addition of more of the extraction medium was sometimes required – up to 6 vol. in some cases. The resulting slurries or pastes were centrifuged at 8 000 *g* for 20 min and the supernatants were carefully decanted for immediate assay of the enzyme activities and for other studies. Such supernatants are referred to below as 'clarified extracts'.

One of the angiosperms (*Iris pseudacorus*) and all of the fern, and brown and red algal species studied showed no trace of either LDH or ADH activity in extracts prepared in the standard way described above. Zymological staining, however, suggested that some of these species might contain some LDH activity. When samples of *A. ursinum* bulbs (which contain relatively high levels of both activities – see Fig. 1) were co-extracted with equal quantities of the *Iris*, the fern *Phyllitis*, or of the brown algae *Fucus*, the resulting extracts were also devoid of activity. It thus seemed that these ferns and algae contain substances that inactivated or 'sequestered' LDH and ADH during extraction. 'Sequestration' was suggested because clarified extracts of *Phyllitis*, free of particulate matter, when mixed 1 : 1 with extracts of *Allium* did not inhibit the latter's LDH activity nor cause significant inactivation over periods longer than those required for the co-extractions. A variety of salt concns and amphipathic additives to the standard extraction medium (in addition to insoluble polyvinylpyrrolidone) proved individually ineffective in preventing this putative 'sequestration' of the LDH activity, but a combination of 0.5 M NaCl and 0.1% Triton proved effective. Subsequently, extraction of various regions of *I. pseudacorus* with this modified extraction medium (50 mM KPi buffer (pH 7.4) containing 8% insoluble polyvinylpyrrolidone, 0.5 M KCl and 0.1% Triton) released levels of LDH and ADH activity comparable with those extractable from other angiosperms, and similar extraction of ferns released LDH, but no detectable ADH. Similar extraction revealed neither activity in brown algae or in red algae. (The latter, in any case, had shown no significant tendency to 'sequester' the activities of *A. ursinum* in experiments similar to those mentioned above).

Enzyme assays. All the quantitative enzyme assays were performed at 30° in 3 ml final vol, in 1-cm light-path spectrophotometric cuvettes. LDH was mainly assayed as pyruvate reductase by the classic method of ref. [24] but replacing the tris buffer by KPi – this being particularly important here, as we find the LDH of plants even more prone to inhibition by nitrogenous bases than the mammalian enzyme. The assay then comprised 0.5 mM pyruvate, 0.2 mM NADH and 50 mM KPi buffer (pH 7.4) in a final vol. of 3 ml, the enzyme sample being added last. The catalysis is followed in the usual way by following the decrease in *A* at 340 nm, and activity is calculated and expressed in terms of nmol NADH oxidised sec^{-1}, a unit of activity (nkat) being that which oxidised 1 nmol NADH sec^{-1}.

A particular difficulty with plant extracts is that most of them oxidise NADH in the absence of added pyruvate (the 'blank' assay) creating a background that we initially subtracted in the usual way. But we then found that the tissue distribution of this 'background activity' closely paralleled the LDH values remaining after its subtraction, and also that it was partly (usually about 55%) inhibited by oxamate, a highly specific inhibitor of LDH. These and other factors lead us to believe that the background activity is due partly to LDH itself acting on endogenous substrates (which may be 'recycling' in some way, as this background tends to continue while NADH is still present). Subtraction of the total background thus results in subtraction of some of the LDH activity itself and is invalid. This background activity can be removed validly by the cumbersome and rather error-prone procedure of removing the endogenous substrates (e.g. by precipitation of the enzyme with $(NH_4)_2SO_4$, or by gel filtration on Sephadex G-25). A more convenient way, which we tested and used here, is by using a blank assay composed in the usual way (i.e. minus added pyruvate) but with the addition of 30 mM oxamate which effectively and highly specifically suppresses the LDH activity in the blank.

LDH activity was cross-checked by assaying from the lactate and NAD^+ direction by adding the enzyme samples to mixtures consisting of 20 mM lactate (D- or L-) and 0.75 mM NAD^+ in 50 mM KPi buffer (pH 7.4). In this case the background activity was usually slight, showed no evidence of a significant LDH component, and was subtracted in total. As is the case of LDH from mammalian and other sources, the activity from the lactate direction (nmol NAD^+ reduced sec^{-1}) was consistently an order of magnitude slower than the pyruvate reductase activity in the opposite direction.

ADH was assayed in 50 mM KPi buffer (pH 7.4) containing 3 mM acetaldehyde and 0.2 mM NADH.

Qualitative zymological/histochemical staining of sections of plants for LDH activity was carried out by

cutting the sections about 0.5 mm thick, crushing the tissue on glass or plastic plates, and overlaying them with a mixture often used as an immobile overlay for the staining of starch gel electropherograms for LDH. This is made up by dissolving 2.5% (w/v) bacteriological agar (No. 1 Oxoid) in boiling H_2O, cooling to 60° and mixing with an equal vol. of the LDH-staining cocktail of ref. [25] prepared at double strength. This mixture is allowed to cool to 37° and immediately poured over the plant sections. In all cases, two equivalent sections were prepared. One was overlaid with the complete 'cocktail', while the other was overlaid with the same mixture minus the L-lactate, thus acting as a control. These were then incubated at about 25° in the dark (to avoid general darkening of the zymogram mixture by sunlight) and observed visually at intervals, any staining appearing in the control section being 'mentally' subtracted.

Protein assay. The most commonly used sensitive protein assay procedures (e.g. the standard 'Lowry' procedure [26] used by most previous workers in this field) we found to be of very uncertain reliability when applied to plant extracts. With such methods we regularly obtained false results seemingly due to interference by secondary metabolites, particularly phenolic products, commonly encountered in plant extracts. Even the use of insoluble polyvinylpyrrolidone in the extraction procedures (see above) did not always prevent such compounds coming through in the 'clarified extracts' and they could also persist through a number of clean-up operations. However, the more complex modified Lowry procedure of ref. [10], in which protein is precipitated with CCl_3COOH in the presence of deoxycholate before estimation, proved generally reliable and was used by us throughout this work.

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