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Activity of cytoplasmic NAD$^+$-dependent dehydrogenases in the cotyledons of soaked Leguminous seeds

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Abstract

The activity of three NAD$^+$-dependent dehydrogenases: malic (MDH), alcohol (ADH), and lactic (LDH), were compared in seeds of seven species of Leguminous plants soaked for 24 hours. The total activity of three dehydrogenases varies at most about twice, while the ratio of activity of aerobic (MDH) to anaerobic (ADH + LDH) dehydrogenases varies about seven times. LDH activity was significantly lowest. The activity of the enzymes was thoroughly investigated in seeds of peas (*Pisum sativum* L.) and beans (*Phaseolus vulgaris* L.) soaked in aerated or nitrogenated water during 48 hours. Some differences in dehydrogenases activity in two species occurred, especially in MDH and ADH. In both species the activity of all enzymes under study was, after a certain time, always lower in the seeds soaked in nitrogenated than in those soaked in aerated water. Peas proved significantly more sensitive to the deficiency of oxygen than beans.

INTRODUCTION

The activity of cytoplasmic NAD$^+$-dependent dehydrogenases appears to result from different metabolic pathways and is affected by the supply of oxygen to the cells. In the initial stage of germination seeds grow in a natural anaerobiosis, considering the weak permeability of testa to oxygen. In this period the accumulation of ethanol takes place in all seeds as a rule (e.g. Davies 1956, Cossins and Turner 1959, Wager 1959, Cossins 1964), and in some cases of lactic acid also (Schneider 1941, Philips 1947, Oota et al. 1955, Wager 1961, Cos-

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sins 1964, Effer and Ranson 1967, Sherwin and Simon 1969, Davies and Davies 1972), both products resulting from the activity of NAD^+-dependent dehydrogenases of anaerobic pathway: alcohol (ADH) and lactic (LDH). Many investigators assume that the activity of the enzymes first increases, but after rupture of the testa by the germ the enzymes decline (Cossins and Turner 1959, Castelfranco et al. 1969, Kollöffel 1970, Ovczarov and Achmedov 1972). In view of the role of dehydrogenases in the germination of seeds periodic current investigations on the subject have been performed, but they are still few and knowledge of the problem is inadequate. In particular the simultaneous activity of ADH and LDH in the same seeds has not been subjected to close scrutiny, although some mechanism controlling the pathways leading to the production of ethanol or lactate seems to be evident (Sherwin and Simon 1969).

EXPERIMENTAL

Materials

Chemicals: NADH, oxalacetic acid, sodium pyruvate, and Tris (Trizma) were obtained from Sigma Chemical Co., St. Louis, Mo., USA. 2-mercaptoethanol was from Koch-Light Labs., Colnbrook, Buchs, England. Acetaldehyde and maleic acid were purchased from Sojuzchimeksport, Moskwa, USSR. Other chemicals were of Polish origin of the purest obtainable grade.

Seeds. Seeds of seven Leguminous species were used throughout the investigations: pea (*Pisum sativum* L.), field pea (*Pisum arvense* L.), bean (*Phaseolus vulgaris* L.), broad bean (*Vicia faba* L. *maior*), horse bean (*Vicia faba* L. *minor*), soya bean (*Glycine hispida* Max.), and yellow lupin (*Lupinus luteus* L.), all from the 1970 crop.

Methods

Cultures. Cultures were grown in thermostat 23 ± 1°C, in dark.

a) For comparative assay in seven species 10 g of respective seeds were sterilized by 10 min. immersion in 0.2% of sublimate, then washed and submerged in a beaker into 200 ml of distilled water. The culture was aerated with air by means of an aquarium pump. After 6 hours the seeds were transferred into a Petri dish on wet filter paper. Analyses were performed after 24 hours of culture.

b) For comparison of enzymes activity in peas and beans two different cultures of 48 hours were performed.

b-1) aerobic: Sterilized seeds were submerged in aerated water (as above).

b-2) anaerobic: Sterilized seeds were submerged into distilled and boiled water in a glass rinsing bowl. The water was aerated with nitrogen (0.005 atm., 99.8% of purity), refined by passing through washers with pyrogallol and concentrated sulphuric acid.

In both cultures the water was changed every twelve hours to remove the compounds passing from the seeds into water during soaking (Barton and McNab 1956, Amoros and Durand 1964, Mazurowa and Schramm 1969). The assays were performed after 3, 6, 12, 24, and 48 hours respectively.

Enzyme preparation

Enzyme assay were performed in crude extracts.

Five grams of cotyledons (fresh weight, without testa and germ) were homogenized in 20 ml of buffer mixture tested as proper for the respective enzyme. They were: for MDH and ADH — 0.05 M phosphate buffer pH 7.0 + 0.01 M 2-merkaptoethanol, for LDH — 0.05 M Tris-maleate buffer pH 6.0 + 0.01 M 2-merkaptoethanol. The homogenate was clarified by centrifugation at 900 g for 10 min. and twice at 15.000 g for 15 min.

For assay of enzyme activity in dry seeds, 10 g of seeds were powdered in an electric mill and then ground in a cold mortar with an equal weight of fine acid-washed sand and ice-cold buffer. The homogenate was clarified as above.

All operations were performed in 4°C.

Assay of enzyme activity

Assays were carried out at 25°C with the VSU-2P Zeiss spectrophotometer by measuring the decrease in E_{340} associated with NADH oxidation. The specific activity of enzymes was expressed in international enzyme activity units EU/mg of protein. The unit is defined as the removal of 1.0 μmol of NADH/min. The reaction was performed immediately after sample preparation and initiated by the addition of respective substrate to the mixture verified as most favourable for individual enzyme, the final volume being filled up with distilled water to 2.0 ml.

The mixtures consisted of: For MDH — phosphate buffer pH 7.4 100 mM, extract 0.02 ml, NADH 0.1 mM, oxalacetic acid 1.0 mM. For ADH — phosphate buffer pH 7.2 100 mM, extract 0.02 ml, NADH 0.1 mM, acetaldehyde 10 mM. For LDH — Tris-maleate buffer pH 6.8 50 mM, extract 0.1 ml, NADH 100 mM, pyruvate 5 mM.

Protein assay

Soluble protein was determined by the Lowry method (1951).

RESULTS

Activity of dehydrogenases in several species

Individual species of Leguminous differ in activity of NAD^+-dependent dehydrogenases in cotyledons as shown in Fig. 1.

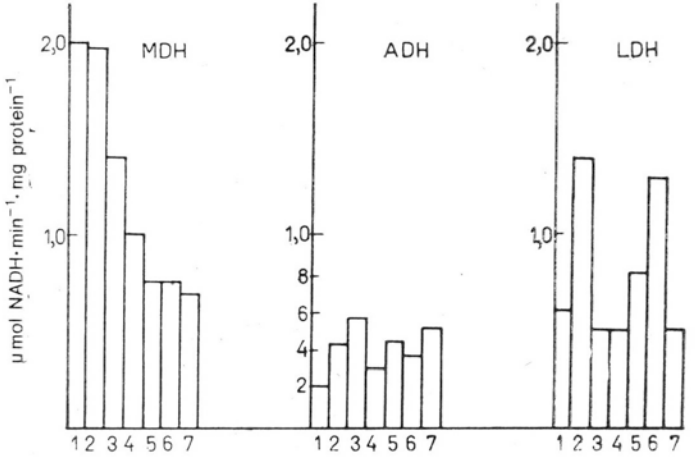

Fig. 1. Activity of MDH, ADH and LDH in seven species of Leguminous plants seeds soaked for 24 hours. For details see text. 1 — bean (*Phaseolus vulgaris* L.), 2 — soya-bean (*Glycine hispida* Max.), 3 — broad bean (*Vicia faba* L. *maior*), 4 — yellow lupine (*Lupinus luteus* L.), 5 — horse been (*Vicia faba* L. *minor*), 6 — pea (*Pisum sativum* L.), — 7 field pea (*Pisum arvense* L.)

MDH is always the most active, the special activities vary from EU about 2 in bean and in soya-bean, to 0.65 in peas. The ADH activity usually evolves one fourth to one third of the respective MDH activity, varying from EU 0.56 in broad bean to 0.20 in beans. The LDH activity is of three value orders lower, though always measurable, varying about EU 0.01.

Activity of dehydrogenases in peas and beans soaked in aerobic and anaerobic conditions

MDH. Figs. 2A and 2B show the changes in activity of MDH which in dormant seeds is more than twice that in beans than in peas; however, the changes in activity are more distinct in peas. By soaking in aerated

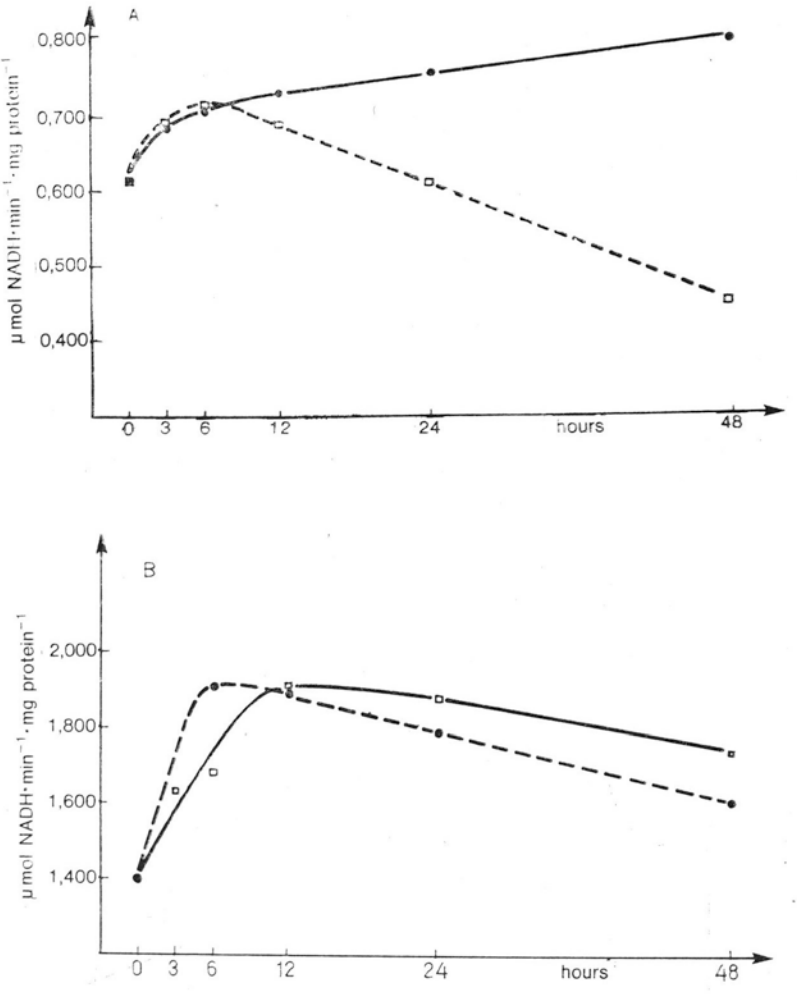

Fig. 2. Changes in MDH activity in cotyledons soaked in aerated (—) and nitrogenated (---) water

A — Pisum sativum; B — Phaseolus vulgaris

water, MDH in the pea cotyledons varies slightly; after six hours in nitrogenated water, however, the enzyme activity significantly decreases to a level below the starting value in dry seeds. The differences in the bean cotyledons are somewhat conspicuous.

ADH. ADH activity (Figs. 3A and 3B) in dormant seeds is almost at the same level both in peas and in beans. However, the changes in the soaked seeds are distinctly expressed, especially in peas, where the enzyme activity rises rapidly, reaching maximum in six (nitrogenated

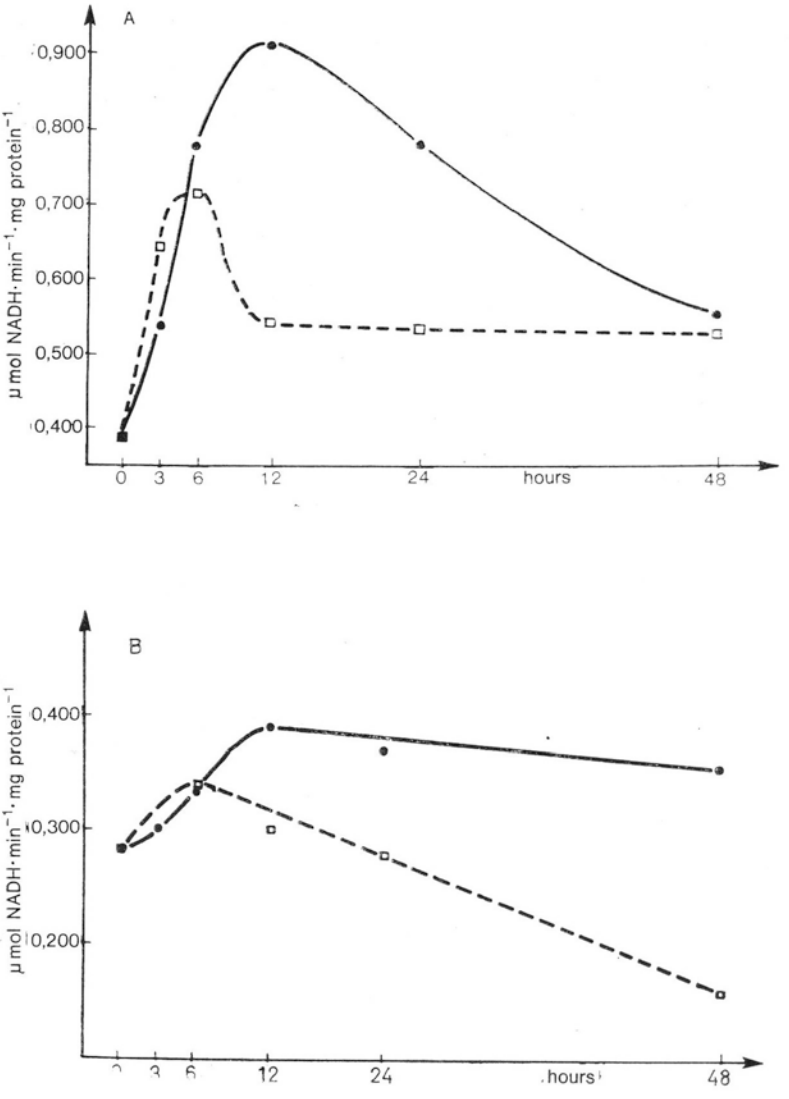

Fig. 3. Changes in ADH activity in cotyledons soaked in aerated (—) and nitrogenated (- - -) water

A — Pisum sativum; B — Phesolus vulgaris

water) or twelve (aerated water) hours, whereafter they significantly decrease, particularly sudden in nitrogen. In beans the changes are fairly reasonable, and in aerated water the increased enzyme activity does not drop much below maximal value after 48 hours.

LDH. LDH activity (Figs. 4A and 4B), although low, found both in dry and soaked peas and beans. When soaked with aeration, the activity of

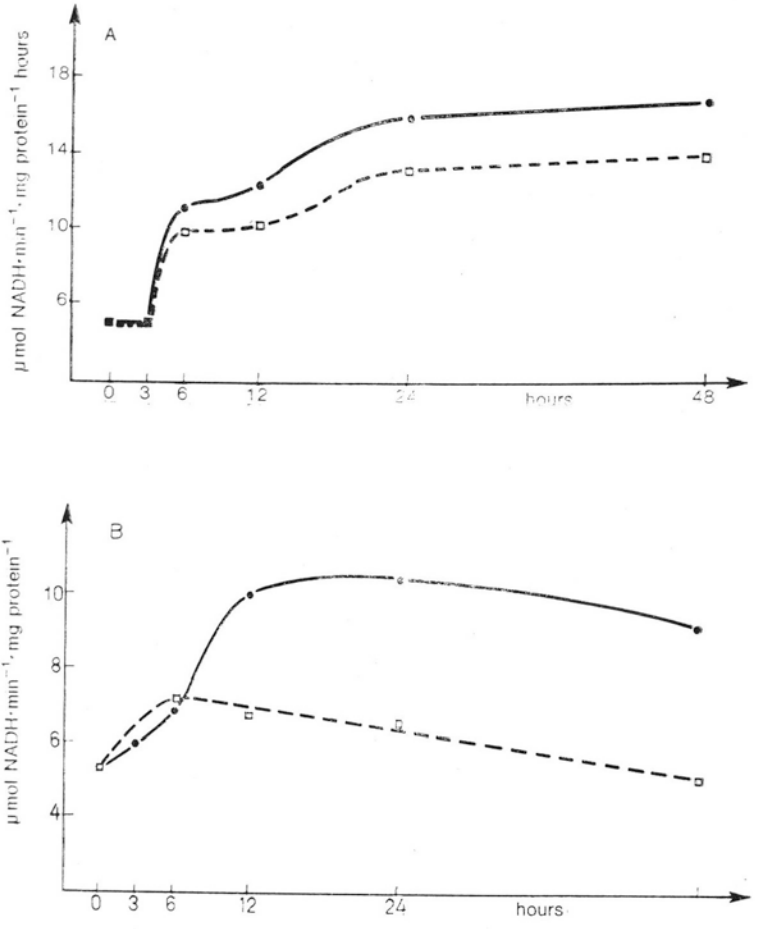

Fig. 4. Changes in LDH activity in cotyledons soaked in aerated (—) and nitrogenated (---) water

A — Pisum sativum; B — Phaseolus vulgaris

the enzyme rises in both species over 24 hours, remaining the next 24 hours on a similar level. In nitrogen the activity is lower, and in beans is additionally depressed by the lack of oxygen.

DISCUSSION

The activity of dehydrogenases in plants is connected both with hydration of tissue and with supply of oxygen. The investigations on flooding tolerance (McManmon and Crawford 1971) showed that plants are either tolerant or intolerant of experimental flooding. In the latter during prolonged flooding the apparent increase of activity of ADH

(but not LDH) occurs. The significantly most intolerant. of flooding and increasing in ADH activity are Leguminous plants; first of all peas. Although the investigations were performed on roots, it seems feasible to perform them on seeds. It is well established that water uptake in seeds occurs in the first phase by inbibition, and water content controlls the start of enzyme activity and respiration rate (Opik and Simon 1963). Individual enzymes differ in water requirement for their activity (Linko and Milner 1959, Linko 1960). However, prolonged soaking causes severe injury to Leguminous seeds (Orphanos and Heydecker 1968).

In our experiments individual species of Leguminous seeds soaked in "normal" conditions (6 hours of imbibition and further soaking on wet blotting paper) differ distinctly in activity of the dehydrogenases under study; the relative values of EU of enzymes varying from 1 to 3, but differ in individual plants (Fig. 1). When comparing the activity of the aerobic pathway dehydrogenase (MDH) to that of glycolytic (anaerobic — ADH + LDH) distinct differences appear in the ratio of activities (aerobic to anaerobic), varying from almost 1 to over 9 (Fig. 5, Table 1).

Table 1

Activity (EU) of NAD^+-dependent cytoplasmatic dehydrogenase of aerobic (MDH) and anaerobic (ADH+LDH) pathways of seven species of Leguminous plants soaked for 24 hours

Species	EU		Ratio aer:anaer
	aer. MDH	anaer. ADH+LDH	
1. *Pisum arvense*	0.65	0.51	1.3
2. *Vicia faba minor*	0.76	0.45	1.7
3. *Pisum sativum*	0.74	0.38	1.9
4. *Vicia faba maior*	1.41	0.56	2.5
5. *Lupinus luteus*	1.05	0.30	3.5
6. *Glycine hispida*	1.98	0.45	4.4
7. *Phaseolus vulgaris*	2.05	0.22	9.3

For comparative assays species differing distinctly from the point of view of activity ratio (aerobic to anaerobic) were chosen, i.e. beans (*Phaseolus vulgaris*) and peas (*Pisum sativum*) as having fairly rich literature on the subject. Since a comparison of simultaneous ADH and LDH formation was aimed at, the conditions as described in "cultures, b" were chosen, in which a distinct increase of anaerobic dehydrogenases as a result of flooding intolerance was anticipated both in aerated and in nitrogenated water. In both specis in the first six hours of soaking no distinc changes occur in the three enzymes activity, what lead to the conclusion that in this stage only the enzymes existing in the dry seed are impelled with imbibition. Subsequently the protein synthesis starts,

and differences between aerobic and anaerobic conditions appear. In both species prolonged soaking, especially in highly anaerobic conditions (water bubbled with nitrogen), causes a substantial increase of the aerobic pathway dehydrogenases (ADH and LDH), while MDH slightly decreases (Schramm and Mazurowa 1975).

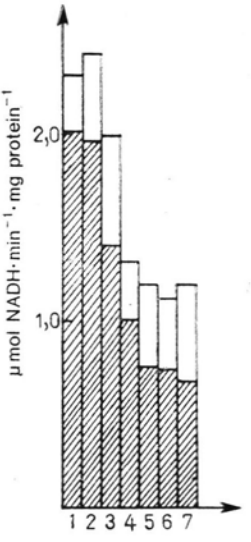

Fig. 5. Comparison of activity of aerobic pathway dehydrogenase (MDH, hatched) and anaerobic pathway dehydrogenases (ADH + LDH, blank) of seven species of Leguminous plant seeds (see Fig. 1)

Significant differences occur in enzyme activities in both species under study. The total activity of three dehydrogenases is twice as high in beans as in peas. MDH activity particularly is about three times higher in beans, whereas ADH and LDH activities are twice as high in peas. In beans changes in enzyme activities are less distinct, and have the same character in three dehydrogenases, i.e. slow decrease occurs after a maximum in 6 (nitrogenated) or 12 (aerated) hours. In peas, however, visible differences in the activity of individual dehydrogenases during 48 hours soaking become evident, especially in MDH and ADH. MDH, being the most active enzyme in dry seeds, appears sensitive to lack of oxygen, and in anaerobic conditions after 6 hours of soaking decreases, falling in 48 hours below the activity of ADH. However, in aerated water this activity rises continuously. ADH, on the other hand, rises very quickly in peas during the imbibition period, especially in aerated water, becoming in 12 hours the most active enzyme; afterwards this activity decreases. In nitrogenated water the increase of ADH activity is very temporary and stabilizes already in 12 hours.

The results suggest, that similarly as in case of experimental flooding of roots (McManmon and Crawford 1971), peas (and probably also field pea and horse bean) are highly intolerant to anaerobic soaking conditions (taking the ADH activity as a measure). Beans apper distinctly

less sensitive to the lack of oxygen, although the problem of "soaking injury" has been closely investigated thoroughly on beans (Orphanos and Heydecker 1968). However, although only limited amounts of pyruvate seem to be metabolized in soaking Leguminous seeds by way of TCAC (Wager 1959, 1961; Sherwin and Simon 1969), differences in enzyme activity and metabolic pathway between individual species can be considerable.

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Aktywność dehydrogenaz cytoplazmatycznych NAD⁺ -zależnych w liścieniach nasion roślin motylkowych

Streszczenie

Badano aktywność dehydrogenez cytoplazmatycznych NAD+-zależnych torów: tlenowego (dehydrogenaza jabłczanowa — MDH) i beztlenowego (dehydrogenazy: alkoholowa — ADH i mleczanowa — LDH) w liścieniach nasion 7 gatunków roślin motylkowych pęczniejących przez 24 godziny w normalnych warunkach. Stwierdzono znaczne różnice indywidualne zarówno w sumarycznej aktywności enzymów, jak zwłaszcza w stosunku aktywności MDH do dehydrogenaz towarów beztlenowych (ADH + LDH). Aktywność LDH była zawsze o 3 rzędy wielkości niższa niż aktywność MDH i ADH. Szczegółowo przebadano aktywność dehydrogenaz w nasionach grochu (*Pisum sativum* L.), jako bardzo wrażliwego, oraz fasoli (*Phaseolus vulgaris* L.) jako stosunkowo mało wrażliwej na niedobór tlenu, pęczniejących przez 48 godzin w wodzie przewietrzanej powietrzem oraz przewietrzanej azotem. Stwierdzono z jednej strony duże różnice indywidualne, w szczególności odnośnie do aktywności MDH i ADH oraz z drugiej strony ogólną prawidłowość większej aktywności wszystkich dehydrogenaz w nasionach pęczniejących w wodzie przewietrzanej powietrzem niż azotem. Nasiona grochu okazały się bardziej wrażliwe na brak tlenu niż nasiona fasoli.