ACTIVITY OF ALCOHOL DEHYDROGENASE IN THE COTYLEDONS OF PEAS GERMINATED UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

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SUMMARY

Extracts prepared from cotyledons of air-dry pea seeds showed a sizeable alcohol dehydrogenase activity. The activity decreased when the seeds germinated under aerobic conditions, but increased when they were kept under anaerobic conditions. A relatively high alcohol dehydrogenase activity was also found in the cotyledons from seeds which germinated in solutions of one per cent ethanol or acetaldehyde but under otherwise aerobic conditions. These results suggest that the alcohol dehydrogenase activity of the cotyledons during germination is closely related with the endogenous ethanol or acetaldehyde concentration in the seeds.

The alcohol dehydrogenase activity is discussed in relation to previous data on the respiration in the cotyledons during germination.

1. INTRODUCTION

In a previous paper (KOLLÖFFEL 1967), experiments were described on the oxygen consumption by cotyledons of germinating pea seeds and on the determination of the activities of several mitochondrial enzymes, extracted from these cotyledons. Evidence was obtained that in the cotyledons a fermentative system, probably containing alcohol dehydrogenase, was especially active during the early stages of germination. Reports on the alcohol dehydrogenase (ADH) activity in pea seedlings (VIRTANEN *c.s.* 1944; DAVISON 1949; GOKSÖYR *c.s.* 1953; COSSINS & TURNER 1962; SUZUKI 1966) agree only partly and do not give information about the development of the ADH activity in the cotyledons during germination.

In the course of the present study it appeared that during seed germination the oxygen supply probably was a decisive factor in the development of the ADH activity. The effect of anaerobic conditions on the ADH activity, therefore, was investigated.

It was supposed that the observed high ADH activity under these conditions could be the result of an accumulation of substrates derived from the carbohydrate catabolism. To investigate this, pea seeds were allowed to germinate in ethanol or acetaldehyde solutions, and the influence of these substrates on the ADH activity of the cotyledons was determined. An influence of ethanol or acetaldehyde on the ADH activity of actively growing plant organs has been found by several authors (APP & MEISS 1958; HAGEMAN & FLESHER 1960), but has not been investigated for cotyledons.

2. MATERIALS AND METHODS

2.1 Germination and seedling growth

Whole pea seeds, var. Rondo, selected on equal air-dry weight and equal colour, were soaked for 20–22 hrs in tap water. Next they were washed and transferred to moist filter paper.

A small Petri dish (9 cm diameter) was placed in a large one (18 cm diameter) and covered with a glass plate (17 cm diameter) wrapped in filter paper, flaps of which continuously drooped in water poured in the large dish. The water was changed daily. This procedure allowed the seeds to germinate under conditions of high relative humidity and constant water supply. They were kept in darkness at 23°C.

This procedure was varied as indicated under the sections 2.2, 2.3 and 2.4 in order to obtain the different environmental conditions mentioned.

The term "germination time", mentioned in this paper, covers the period between the beginning of soaking until the moment the cotyledons were used for the experiments.

2.2 Aerobic conditions

The seeds were soaked in tap water under aeration with ordinary air. Next they were allowed to germinate on moist filter paper as described.

2.3 Anaerobic conditions

The seeds were placed under tap water in wash bottles and during 10 min nitrogen was passed through.

Next they were transferred to the Petri dishes, which were now placed in large desiccators. Here, the anaerobic conditions were obtained by reducing the internal pressure with a water pump (to 20–30 mm of mercury) and then refilling the desiccator with nitrogen gas from a cylinder (the gas in the cylinder contained at least 99% nitrogen). This process was repeated three times. A beaker with a 10 per cent solution of KOH was placed in the desiccator to remove CO_2.

2.4 Aerobic conditions with ethanol or acetaldehyde

The pea seeds were placed in the solutions of ethanol or acetaldehyde in a large Erlenmeyer flask (1 l). This flask was connected by means of a pump and tubing with a second flask (2 l), which contained the solutions of ethanol or acetaldehyde only. The gas phase consisted of air which was equilibrated with the ethanol or acetaldehyde solutions, and containing sufficient oxygen to allow aerobic germination. The air circulated continuously through the whole system. This procedure limited the volatilization of ethanol and acetaldehyde and did allow aeration of the peas.

After a period of 20–22 hrs the seeds were washed and transferred to the Petri dishes. An ethanol or acetaldehyde solution was poured both at the bottom of the box and at the bottom of the desiccator. The solutions were changed daily.

2.5 Extraction of enzymes from swollen cotyledons

Prechilled cotyledons were ground in a mortar with an equal weight of sand. The grinding medium contained 0.4 M sucrose and 0.05 M phosphate buffer (pH 7.2) One ml was added for each gram of cotyledons. The resulting slurry was filtered through 8 layers of aseptic gauze, diluted to 35 ml with the grinding medium, and the filtrate centrifuged at 1500 × g for 10 min. Next the supernatant fraction was centrifuged at 20,000 × g for 15 min. The sediment thus obtained was used to determine the activity of several mitochondrial enzymes (KOLLÖFFEL 1967). The supernatant was used as the source of ADH. It contained the equivalent of 5 g or 10 g cotyledons (fresh weight) per 35 ml medium. Practically all ADH activity was found to be present in this fraction.

2.6 Extraction of enzymes from air-dry cotyledons

Excized cotyledons from air-dry seeds were pulverized with a Braun "multimix". An equal weight of tap water was added to the powder that was allowed to imbibe for 2 hrs at 0–5 °C. From this moment the imbibed powder was treated in the same way as the preparation from fresh cotyledons.

2.7 Alcohol dehydrogenase (alcohol: NAD oxidoreductase EC 1.1.1.1)

The ADH activity was determined spectrophotometrically by measuring the oxidation of NADH in the presence of acetaldehyde. The reaction mixture contained in final concentrations: 0.05 M phosphate buffer; 0.40 M sucrose; 0.01 M acetaldehyde; 0.225 mM NADH and 0.1 ml enzyme preparation in a total volume of 2.0 ml with a final pH of 7.2. The reaction was started with the addition of acetaldehyde. After 15 sec the decrease in absorbance at 340 nm was followed in a Unicam S.P. 800 recording spectrophotometer against a blank containing the enzyme, buffer, sucrose and NADH. The enzyme was essayed at 25 °C. The reaction proceeded linearly with time only for 1 to 3 min. Enzymic activity is expressed as μmoles of NADH oxidized/min/g dry weight. It was calculated from the initial reaction velocities of at least three different dilutions of one extract. A molar extinction coefficient of $6.22 \times 10^3 M^{-1} cm^{-1}$ was used.

Acetaldehyde was distilled before use and diluted with water. A 0.2 M solution was stored at –8 °C for several days.

The addition of sucrose to the reaction mixture was not necessary; it even decreased the reaction velocity.

3. RESULTS

3.1 Germination and seedling growth

The germination of the pea seeds was accompanied by a rapid uptake of water. The course of water uptake falls into two phases. Initially the water content of the cotyledons increased rapidly to about 120 % of their dry weight. From about 10–15 hrs, the amount of water showed little further increase. However, in the

later stages the dry weight of the cotyledons decreased, which resulted in a slow increase in the percentage of water to approximately 150% (about 90 hrs after the seeds have been put in water).

The number of cells remained virtually constant during the life of the cotyledons. There might be a relatively small change in cell number caused by the differentiation of the procambium bundles. Therefore, we think that changes in the enzymic activity of the cotyledons as a whole does represent real physiological changes in their cells. The radicle emerges about 20 hrs after the seeds have been put in aerated water. The subsequent growth of the root proceeded linearly with time.

In a nitrogen atmosphere the appereance of the radicle was postponed about 40 hrs. The elongation of the root was strongly retarded. When transferred to air the growth soon reached the level of that of the controls (in water).

The presence of alcohol in the medium (10 ml/l water) reduced the elongation of the roots to about half that of the roots in water. Seeds put in solutions of acetaldehyde (1 ml/1 water) did not germinate at all.

3.2 The course of the ADH activity during germination

The activity of ADH is calculated on a dry weight basis (105°C for 20 hrs). The average dry weight of the cotyledons drops approximately 15% over a period of 90 hrs from the onset of germination. Consequently, values obtained after 90 hours of germination are about 15% too low.

Neither the seed coat nor the imbibition water showed ADH activity. This suggests that the results were not influenced by microbial contaminations.

In a previous paper (KOLLÖFFEL 1967) evidence was given that the enzymes of the citric acid cycle and the electron transfer chain are present in the air-dry cotyledons. It was thus interesting to investigate whether also ADH is dormant in the air-dry cotyledons or whether it only appears upon soaking and germination. The enzyme was extracted from an imbibed powder of air-dry cotyledons. As it is not likely that during the imbibition of this powder with water (maximally 2 hrs at 0–5°C) enzyme synthesis has occurred the ADH activity of this extract represents the potential ADH activity from the air-dry cotyledons.

The ADH activity remains constant *(fig. 1)* for a period of about 30 hrs from the onset of germination. The activity decreases considerably in the subsequent 35–40 hrs and then it remains constant again at the lower level.

The decrease in ADH activity coincided with the perforation of the seed coat by the elongating radicle. The seed coat limited the oxygen diffusion to the cotyledons. In order to investigate whether this decrease was related to the better oxygen diffusion, pea seeds were allowed to germinate under aerobic and anaerobic conditions. *Fig. 2* shows that the ADH under these conditions initially remains almost constant. The activity increases after about 30 hrs until after 90 hrs from the onset of germination it is almost doubled. The transfer from anaerobic to aerobic conditions after 36 hrs or 60 hrs resulted in a lowered ADH activity. Apparently the environmental conditions during germination strongly influence the ADH activity from the cotyledons.

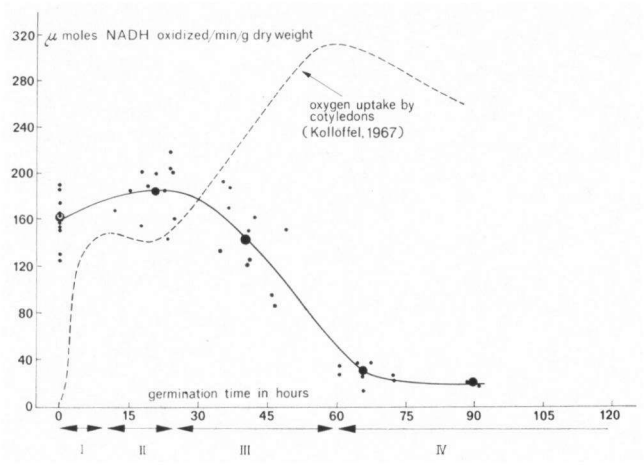

Fig. 1. Alcohol dehydrogenase activity and oxygen uptake (respiration rate) of cotyledons from pea seeds, germinated under aerobic conditions. The course of the respiration rate is divided into four phases (I, II, III and IV). Big dots are the average of a group of values.

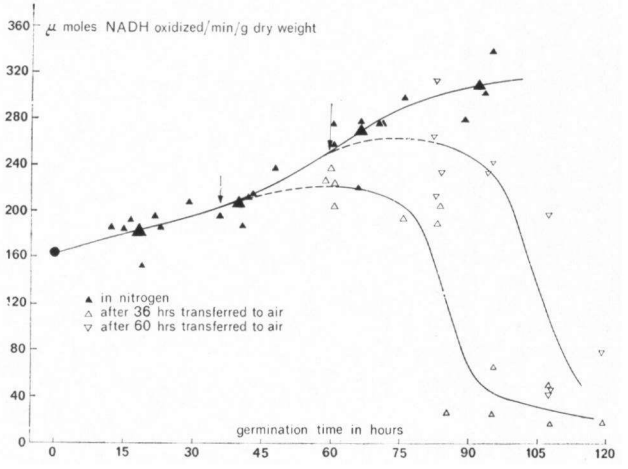

Fig. 2. Alcohol dehydrogenase activity of cotyledons from pea seeds, germinated under anaerobic conditions. The seeds were transferred to aerobic conditions after 36 hrs or after 60 hrs from the onset of germination. Big black triangles are the average of a group of values.

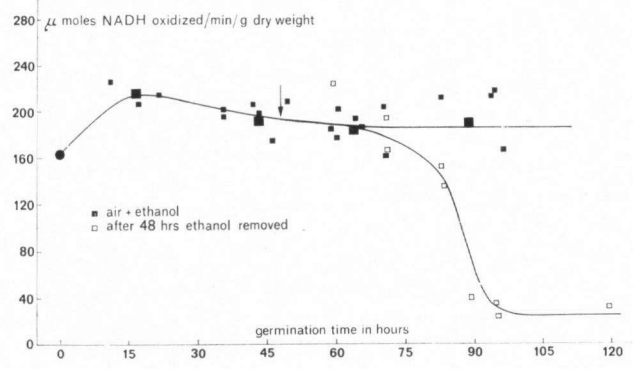

Fig. 3. Alcohol dehydrogenase activity of cotyledons from pea seeds, germinated under aerobic conditions in an ethanol solution (10 ml/l tap water). The seeds were transferred to tap water after 48 hrs from the onset of germination. Big squares are the average a of group of values.

3.3 The influence of acetaldehyde and ethanol on ADH activity

Pea seeds were allowed to germinate under aeration in solutions of acetaldehyde or ethanol. Acetaldehyde prevents the decrease in ADH activity *(table 1)*. When the seeds germinated in a 0.1% solution (v/v) of ethanol, the moment at which the ADH activity started to decrease was postponed for about 10 hrs. It remained at a high and constant level *(fig. 3)* when the seeds germinated in a 1% solution (v/v) of ethanol. The activity was depressed, however, by the transfer from the ethanol solution to tap water. These data suggest that endogenous ethanol and acetaldehyde also bring about the enhanced ADH activity under anaerobic conditions *(fig. 2)*.

Table 1. The ADH activity in extracts of cotyledons from pea seeds, kept under aerobic conditions in solutions of acetaldehyde (1 ml/l tap water). ADH activity expressed as μmoles of NADH oxidized/min/g dry weight. Each value represents the average of at least four different extracts.

hours germination	ADH activity
0	162
18	172
43	193
65	182
85	184

4. DISCUSSION

A comparison between the ADH activity of the powder from air-dry cotyledons and that of cotyledons from seeds soaked for about 20 hrs in water reveals only little difference. The ADH activity in vivo in this period is thus probably determined primarily by the degree of swelling of the cotyledons in water. Initially this activity remained constant but it decreased as germination proceeded until after 75 hrs very little activity was left. These results agree well with the results of DAVISON (1949) and COSSINS & TURNER (1962). In contrast, VIRTANEN *c.s.* (1944) and GOKSÖYR *c.s.* (1953) have found that the activity of this enzyme in whole pea seedlings and in pea cotyledons respectively, rose to a maximum in the first two days and then rapidly declined. Although the various experiments were not always made under sufficiently similar conditions to permit good comparisons, there is a general tendency that the ADH activity decreased with germination time.

The production of ethanol by germinating peas has often been demonstrated (see e.g. GOKSÖYR *c.s.* 1953). SPRAGG & YEMM (1959) have shown that the RQ of pea seeds during germination in air, fell from a value of around three to a value around one. The data of these authors as well as the present data strongly suggest that the observed changes in ADH activity indeed represent real changes in the alcoholic fermentation in vivo during germination.

During the germination time of 90 hours four phases of different respiration rate could be distinguished *(fig. 1* and KOLLÖFFEL 1967). The sharp rise in respiration rate in the first 8–10 hrs (phase I) has been attributed to the activation of enzymes of the citric acid cycle and of the electron transfer chain, upon hydration. A further increase in the respiration did not occur for the next 10–15 hrs (phase II) probably because the activity of several enzymes of the electron transfer chain remained almost constant during this time. The increase in respiration rate in the subsequent 30–35 hrs (phase III) coincided with an increase of the activity of enzymes of the electron transfer chain. This fact strongly supports the explanation proposed for phase II.

In situ, the cotyledons are enveloped by the seed coat. The intact seed coat limits the diffusion of gases (SPRAGG & YEMM 1959; KOLLÖFFEL 1967) and consequently "natural" anaerobic conditions exist inside the seed coat. The oxygen consumption of isolated cotyledons was considerably higher than in situ. The oxygen uptake as shown in *fig. 1* however, presents a reasonable impression of the respiration course in situ.

Fig. 1 further shows clearly that as germination proceeds, the ADH activity decreases whereas simultaneously the respiration rate increases. These rapid changes coincided with the rupture of the seed coat by the emerging radicle and thus with an improved gas exchange. It was anticipated therefore that experimental anaerobic conditions would increase the ADH activity. A comparison of *fig. 1* with *fig. 2* reveals that there is no difference in ADH activity during the first 30 hrs from the onset of germination, but that the ADH activity of the cotyledons kept under anaerobic conditions is nearly doubled after 90 hrs *(fig. 2)*, whereas the activity of the cotyledons kept under aerobic conditions was strongly reduced. Similar results were obtained with rice coleoptiles (APP & MEISS 1958), roots and shoots of corn seedlings (HAGEMAN & FLESHER 1960) and with the roots of a number of aquatic and marsh plants (CRAWFORD 1967).

The prevention of the decrease of the ADH activity in the presence of ethanol or acetaldehyde *(fig. 3)* suggests that these substances also enhance the ADH activity under natural and experimental anaerobic conditions. COSSINS & TURNER (1962, 1963) and COSSINS & BEEVERS (1963) reported numerous data which showed that both the endogenous ethanol which accumulated when pea seeds imbibed water prior to germination and exogenous ethanol were oxidatively metabolized. LIU *c.s.* (1965) described the purification of an acetaldehyde dehydrogenase from germinating pea cotyledons. These data suggested that the release from the anaerobic conditions was followed by a decrease of the ethanol concentration in the cotyledons and by a decrease of the ADH activity. APP & MEISS (1958) gave evidence that ethanol is the inducer of rice shoot ADH, whereas HAGEMAN & FLESHER (1960) supposed that in young corn seedlings ADH was induced by acetaldehyde. The latter authors failed to demonstrate any effect of ethanol on ADH activity under either aerobic or anaerobic conditions.

Further experiments are now in progress to elucidate the mechanism of the ADH activation in the pea cotyledons.

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ERRATUM

In the article "Activity of alcohol dehydrogenase in the cotyledons of peas germinated under different environmental conditions" by C. KOLLÖFFEL (Acta Bot. Neerl. **17** (1), 1968), in the last sentence of the third paragraph page 76 "a number of aquatic and marsh plants" should read "a number of *non*-aquatic and marsh plants".