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Alcohol Dehydrogenase and an Inactivator from Rice Seedlings[1]

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ABSTRACT

Alcohol dehydrogenase (ADH) was measured in the various organs of rice seedlings (*Oryza sativa*) growing in air. In extracts from ungerminated seeds, the ADH is stable, but in extracts from seedlings more than 2 days old the enzyme initially present loses activity in a time- and temperature-dependent fashion, due to the presence of an inactivating component which increases with age in roots and shoots. The inactivation can be prevented completely by dithiothreitol, and when this is included in the extraction medium the apparent loss of total ADH in roots and shoots with age is not observed. In seedlings grown in N_2, ADH levels in coleoptile extracts are higher than those in air, the enzyme is stable, and no inactivator can be detected. When seedlings grown for 5 days in air were transferred to N_2 for 3 days, ADH levels increased and there was a decline in inactivator activity. Transfer back to air after 1 day in N_2 led to loss of the accumulated ADH and increase in inactivator. These reciprocal changes and the fact that the inactivator is absent from coleoptiles of seedlings grown in N_2 appear to suggest a regulatory role for the inactivator *in vivo*. However, it is clear that high levels of inactivator and ADH can exist in cells of seedlings grown in air for long periods without loss of enzyme activity, and it is argued that they must normally be separately compartmented.

The developmental pattern of ADH[3] during germination has been examined in a number of seeds (5). It is commonly found that ADH activity shows an early increase followed by a decline as development proceeds and this has been correlated with a phase of fermentative metabolism before the aerobic system is fully developed. It has been demonstrated in maize (2–4) and pea (6) that an inhibitor or inactivator of ADH develops as the measured ADH declines and that this may account for regulation of ADH levels.

Among higher plants, rice is unusual in that it can produce a growing coleoptile under strictly anaerobic conditions (7). Thus, this material allows the examination of the behavior of ADH and the possible inactivator under contrasting conditions of O_2 supply.

MATERIALS AND METHODS

Seed Germination. Hulled seeds of rice (*Oryza sativa* cv S-6) were sterilized by exposure to 95% ethanol for 1 min followed by 25 min in 2% NaOCl after vacuum infiltration for 2 min. Fifty washed seeds were germinated in a sterilized culture dish (8 × 4 cm) or in a sterilized filter flask (250 ml) containing four sheets of filter paper and 7 to 10 ml deionized H_2O. The containers were placed in darkness at room temperature (25 ± 2°C). For anaerobic experiments, N_2 gas (99.998% N_2) was continuously passed through the filter flask.

Preparation of Extracts. Thirty-five seedlings or particular excised tissues from 35 seedlings were homogenized in a chilled mortar in 10 ml of 10 mM Tris-HCl buffer (pH 7.5). In some experiments, the buffer contained 5 mM DTT. Homogenates were centrifuged at 27,000*g* for 10 min at 4°C, and the supernatant solution was used for enzyme analysis after filtration.

Assay of ADH. ADH activity was measured by recording NADH production (1). The reaction mixture (1 ml) contained 0.1 M sodium glycine buffer (pH 9.0), 0.2 mM NAD, 0.1 M ethanol, and 10 to 50 μl of extract. One unit of ADH is that which produces 1 μmol NADH/min under the assay conditions.

Assay of Inactivator. As shown in "Results," seedlings of rice contain a component which induces a loss of ADH activity in extracts. Scandalios and his colleagues (2–4) have investigated a similar response in maize seedlings and refer to the active component as in inhibitor. Since in both systems the response is time dependent, it seems more appropriate to use the term inactivator. The assay that we have used is similar to that of Ho and Scandalios (2), and is based on ADH measurements in extracts of dry seeds which do not contain the inactivator, in the presence and absence of extracts to be assayed. The substrate ADH (dry seed extract) was mixed with an equal volume of extract to be assayed (a). For the control value, substrate ADH (b) and the extract to be assayed (c) were each diluted separately by adding an equal volume of the extraction buffer. After incubation at 30°C for 40 min, the three samples were assayed for ADH. The residual ADH activity in (a) was compared to the sum of the ADH activities in (b) and (c). One unit of inactivator is defined as that giving 50% of the relative residual activity in a total volume of 0.4 ml assay solution (Fig. 4).

Protein determinations were made using a Bio-Rad protein assay kit with γ-globulin as standard.

RESULTS

Stability of ADH in Total Extracts from Seedlings. The ADH activity in extracts from 5-d-old seedlings declines by 50% in 2 h at 0°C and approaches zero at 6 h (Fig. 1). This decline in activity was not prevented by 50 mM ethanol, 0.1 mM NAD, the proteinase inhibitors phenylmethylsulfonyl fluoride (1 mM), pepstatin (1 mM), or leupeptin (0.1 mM), or by 1 mM EDTA. High concentrations of glycerol (30%) gave some protection, but, as shown, the addition of the SH compounds mercaptoethanol and particularly DTT not only completely prevented the decline, but gave considerable stimulation over the zero time values recorded 40 min after beginning the extraction. However, the addition of the SH compounds after 72% of the ADH activity had been lost at 3.5 h led to only a small reactivation.

ADH in extracts prepared from seedlings of different ages grown in air shows an initial increase but declines markedly after 2 d (Fig. 2), a pattern seen in other seedlings by previous workers (5). However, when DTT was included in the grinding mixture, a very different developmental pattern was seen, with ADH increasing throughout the 8-d period. The total ADH activity in anaer-

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[3] Abbreviation: ADH, alcohol dehydrogenase.

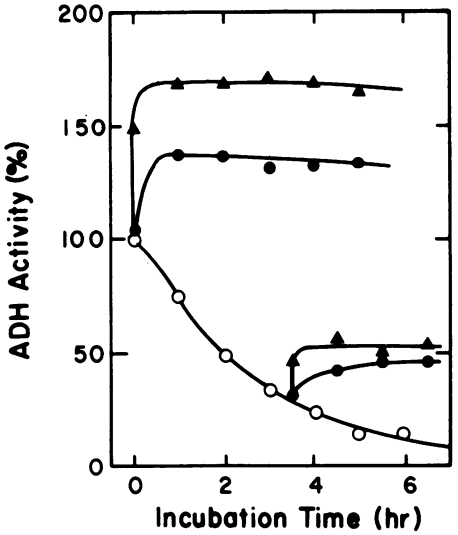

FIG. 1. Effect of SH compounds on stability of ADH in the crude extract from aerobic 5-d-old rice seedlings. The extract was stored at 0°C (○). At the indicated times, 50 mM mercaptoethanol (●) or 5 mM DTT (▲) was added.

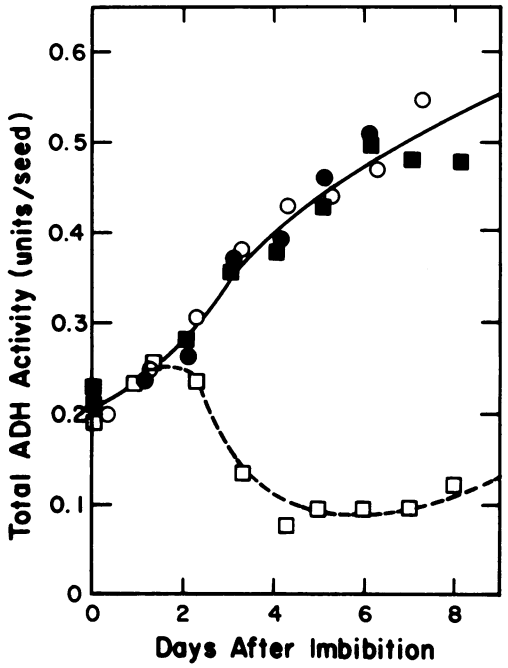

FIG. 2. Developmental patterns of ADH activity during germination in N_2 (○, ●) and in air (□, ■). (●, ■) values obtained from extracts made in the presence of 5 mM DTT; (○, □), those from extracts made in the absence of DTT. The activities were measured 60 min after beginning the extractions.

obic seedlings increased at a similar rate and was completely unaffected by DTT, and ADH activity in extracts from anaerobic seedlings is stable at 0°C in contrast to that from aerobic seedlings (Fig. 1).

It thus appears that in aerobic seedlings greater than 2 d old there is a progressively greater inactivation of ADH during the time prior to assay and that, since DTT prevents this decline, the usual assay for ADH gives misleading results. Apparently, the inactivator is present in increasing quantities in extracts from older aerobic seedlings but is absent from dry seeds and from seedlings grown in the absence of O_2.

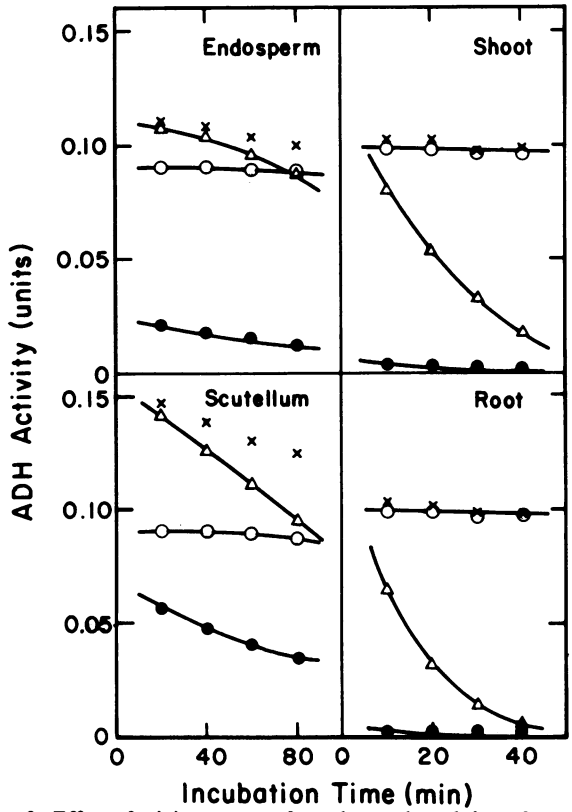

FIG. 3. Effect of mixing extracts from dry seeds and those from tissues of aerobically germinated seedlings on the stability of ADH at 30°C. (△), ADH activity of the combined solution of dry seed extract with an equal volume of tissue extract; (○), ADH activity of the dry seed extract diluted with an equal volume of grinding buffer; (●), ADH activity of the tissue extract diluted with an equal volume of grinding buffer; (×), the sum of the two ADH activities from the dry seed extract and from the tissue extract.

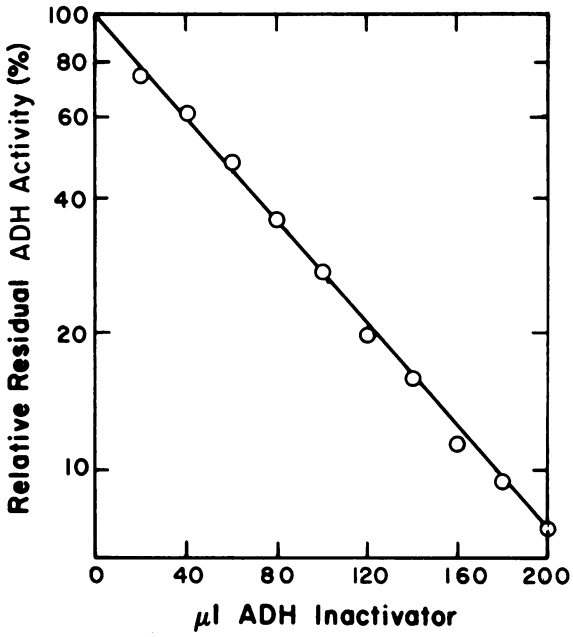

FIG. 4. Relation between the relative residual ADH activity and the amount of inactivator. The inactivator solution was prepared from roots of aerobic 7-d-old seedlings. The substrate ADH was prepared from dry seeds. The ADH-inactivator mixture was incubated at 30°C for 40 min, and the relative residual activity was measured as described in "Materials and Methods."

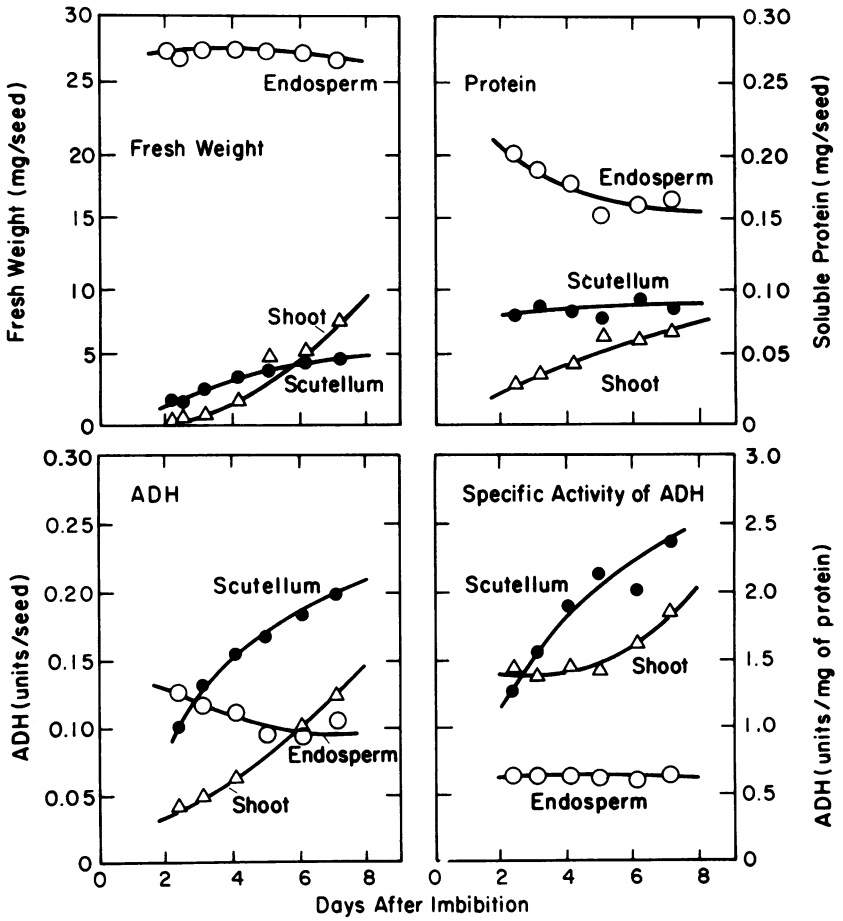

FIG. 5. Developmental patterns of ADH in organs from rice seedlings during germination in N_2.

Assay of ADH Inactivator in Aerobic Seedlings. When extract from dry seeds is incubated at 30°C, the ADH activity remains essentially constant for 80 min (Fig. 3). Extracts from different tissues of 7-d seedlings, prepared in the absence of DTT, have different ADH activities but all decline with time (Fig. 3). When these extracts are added to the extract from dry seeds, the residual ADH activity declines with time and is less than the sum of the two separate activities. That is, the inactivator present in the tissue extracts brings about the loss of the ADH in the dry seed extract. This is particularly marked in the extracts from root and shoot, less so in that from scutellum and small in that from the endosperm.

The loss of ADH activity in combined extracts is dependent on the concentration of inactivator (Fig. 4). When log of percent residual ADH activity in the combined extracts is plotted against the amount of extract containing the inactivator, a straight line is observed; and this is the basis for estimation of inactivator units as described in "Materials and Methods."

ADH and Inactivator Distribution in Tissues of Rice Seedlings during Growth in Air and N_2. The growth characteristics of seeds in N_2 are shown (Fig. 5). No roots are produced under these conditions and, as shown, there is a small loss of fresh weight from the endosperm, and a marked gain in the scutellum. The gain in fresh weight of the shoot (coleoptile) is due entirely to cell enlargement. The protein assays show that the loss of protein from the endosperm is largely accounted for by the gain in that of the shoot.

All measurements of ADH were made on extracts prepared in the presence of DTT. The total activity and specific activity in both scutellum and shoot increase progressively from day 2, with the scutellum contributing most of the activity at the earliest stages before coleoptile elongation begins. Total ADH in the endosperm declines, but the specific activity remains essentially constant. The amounts of ADH inactivator were too low in all tissues to measure accurately.

Figure 6 shows the corresponding measurements in aerobically grown seedlings. The more vigorous growth under these conditions is shown by the much larger fresh weight of the shoots (including leaves), the production of roots, and a correspondingly greater loss in endosperm fresh weight. There are parallel changes in soluble protein in the various tissues.

In the aerobic seedlings, the scutellum at all stages shows the highest ADH activity. The total ADH activity in the shoots remains constant from day 3 and lower than that of the anaerobic shoots. However, the developmental patterns of ADH differ between coleoptile and leaves (Fig. 7). The total ADH activity in the coleoptiles decreases after the onset of germination but that in the leaves continues to increase. A significant increase is also observed in the roots. The specific activities in both roots and shoots show a steady decline until day 5 and then remain essentially constant. The changes in ADH in the endosperm are quite similar to those observed in anaerobiosis (Fig. 5).

Assays for ADH inactivator show that neither the endosperm nor the scutellum (in contrast to maize [2, 3]) have significant amounts of inactivator, but that there is a striking increase in both total and specific activities in shoots and roots after day 2 (Fig. 6) as suggested by the data of Figure 2.

Effect of Anaerobiosis on Levels of ADH and the Inactivator. Seedlings grown for 5 d in air were subjected to various treatments, and the effects of ADH and the inactivator were measured over the subsequent 3 d (Fig. 7). In seedlings maintained in air, both fresh weight and soluble protein content in roots and leaves

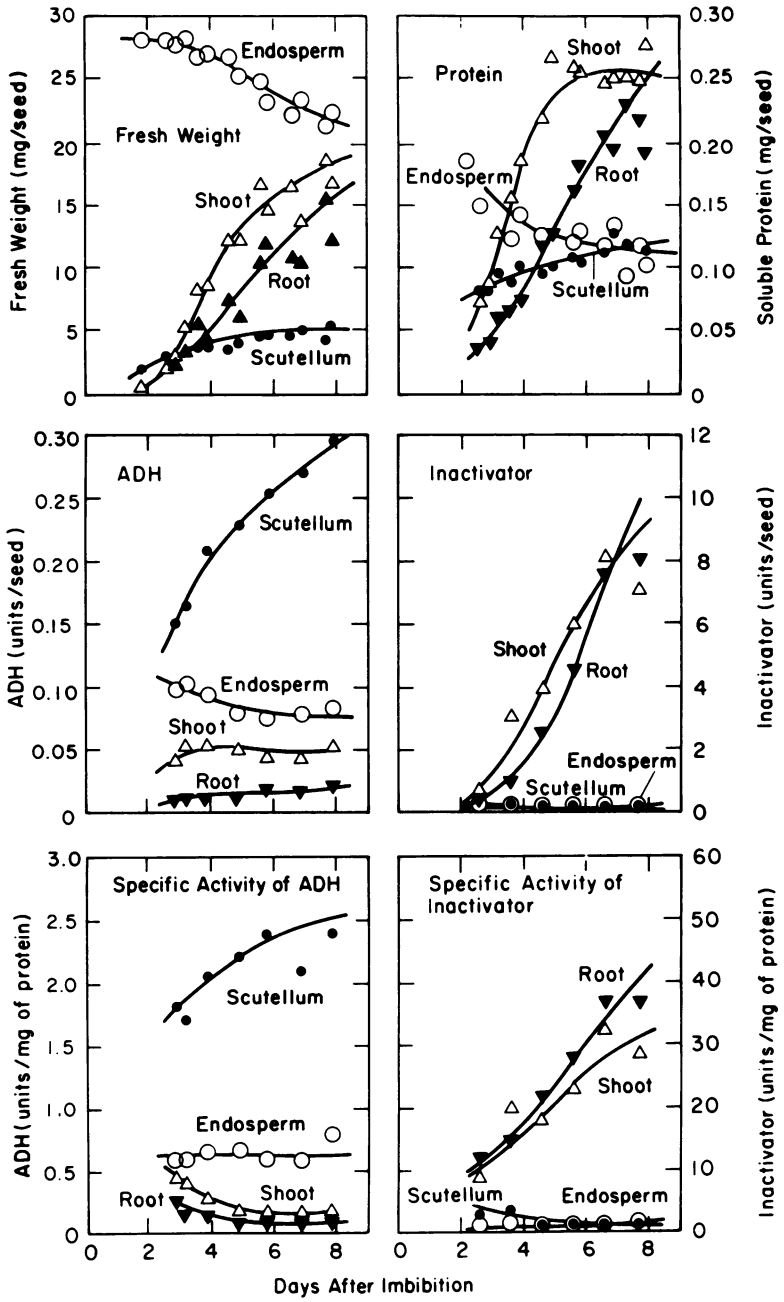

FIG. 6. Developmental patterns of ADH and ADH inactivator in organs from rice seedlings grown in air.

increased over the 3-d period and the soluble protein content in coleoptiles decreased, while the specific activities of ADH in these organs remained essentially constant. Transfer to N_2 prevented the increases in fresh weight and protein content, but induced a 3- to 5-fold increase in both specific and total ADH activities in these tissues. Cycloheximide strongly depressed the increase in ADH observed in N_2 and had no effect on the aerobic levels. Transfer back to air after 1 day in N_2 resulted in sharp losses in ADH activity in all three tissues. These losses in ADH levels were found not only in the specific activity but also in the total activity in roots as well as coleoptiles, while in leaves the total activity did not decline.

The levels of inactivator in all three tissues continued to increase, as expected, when the seedlings were maintained in air, but in N_2, on the contrary, there was a considerable decline.

There is a rapid development of the ADH inactivator and a decline in ADH activity when coleoptiles from seedlings grown in N_2 for 9 d are transferred to air (Fig. 8).

DISCUSSION

The present results show that in extracts of rice seedlings growing in air an ADH-inactivating component is present which can strongly affect ADH activity. Similar observations have been made previously on maize (2–4) and pea seedlings (6). Its action can certainly lead to erroneous estimates of ADH activity, the more so as time between extraction and assay is prolonged, and particularly in older tissues where the amounts of inactivator are greater. In rice, inclusion of DTT in the extraction medium prevents ADH inactivation completely and greatly modifies the apparent decline of ADH with age. DTT has no effect on the ADH activity in extracts from seedlings less than 2 d old or from anaerobically germinated seedlings which were shown not to contain the inactivator. DTT has only a small restorative effect when added after inactivation *in vitro* at 0°C was extensive (Fig. 1). However, when it was added 40 min after grinding was begun

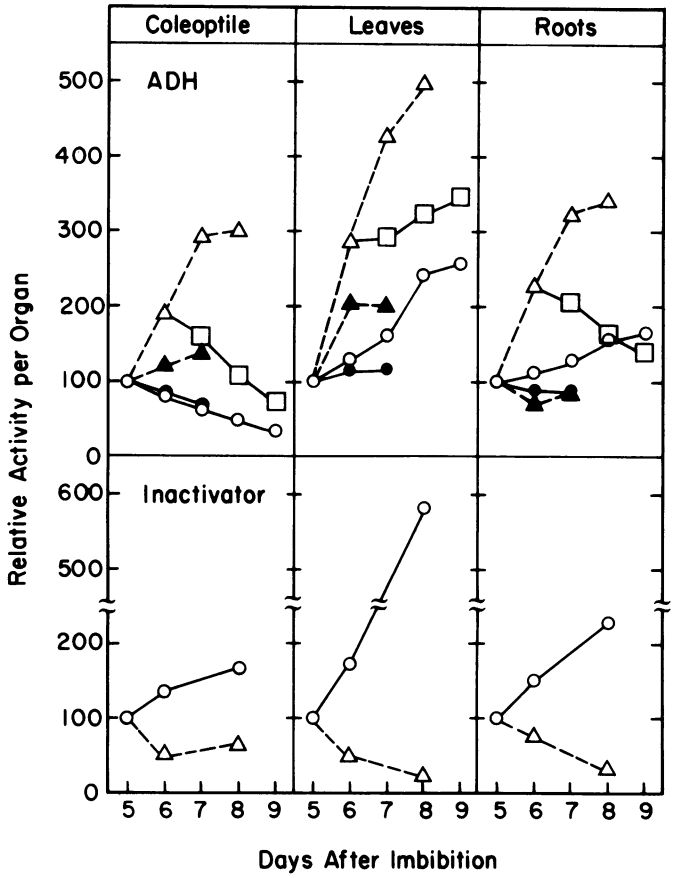

FIG. 7. Effect of temporal anaerobiosis on the activities of ADH and its inactivator in rice seedlings. Five-d-old rice seedlings grown in air were the starting material. (○), Activities of the seedlings maintained in air; (●), under aerobic conditions in the presence of 0.2 mg/ml cycloheximide; (△), under anaerobic conditions; (▲), under anaerobic conditions in the presence of 0.2 mg/ml cycloheximide; (□), 1-d anaerobiosis and then transferred back to air.

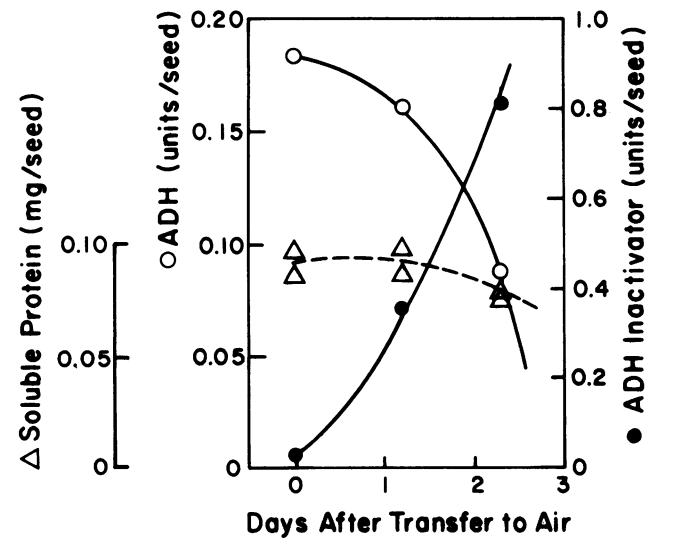

FIG. 8. Change of the activities of ADH and its inactivator in coleoptiles of anaerobic 9-d-old rice seedlings after transfer to air.

(time 0 in Fig. 1), it produced immediated reactivation and the ADH level was in fact somewhat higher than that which could be estimated by extrapolation to be present when grinding was begun. Thus, DTT can reactivate ADH in the early phase of inactivation. The estimation of initial ADH activity in the absence of DTT by extrapolation is hazardous, since local concentration of inactivator during grinding is likely to be higher than that in the final extract and the temperature during preparation was probably higher than 0°C. Nevertheless, it cannot be ruled out that a small fraction of the ADH activity measured when DTT was included in the grinding medium was due to reactivation of ADH already existing in a partially inactivated form and, thus, that values obtained in the presence of this protectant are slightly overestimated.

There is no net decrease in total ADH activity during periods when the inactivator is increasing during growth under aerobic conditions (Fig. 6). The decline in specific activity of ADH in leaves and roots is due to the increasing protein content of these organs. The decline in total ADH of the coleoptiles parallels the loss in protein and thus is not due to a specific loss of ADH. The only instances in which a rapid decline in total ADH activity, conceivably due to the ADH inactivator, was seen, are those in Figure 7 where, after 1 d of anaerobiosis, during which ADH levels increased, coleoptiles and roots were returned to air, and the ADH levels returned to the previous aerobic levels in the subsequent 3 d (see also Fig. 8).

There are several other observations which suggest that the inactivator may be responsible for the control of endogenous levels of ADH. First, when the seedlings are grown under anaerobic conditions, ADH levels in the coleoptile show strong increases and no inactivator can be detected (Fig. 5). Second, when aerobically grown seedlings are transferred to N_2, ADH levels rise and the inactivator declines. The opposite reciprocal relations are shown when anaerobic coleoptiles are returned to air (Fig. 7 and 8).

Nevertheless, there are compelling arguments against the notion that the inactivator is actually operating *in vivo* at all times and in favor of the view that it becomes fully effective only on disrupting the tissue. Chief among these is that the amount of inactivator present for example in 7-d shoots and roots is capable of completely inactivating the ADH in the extracts in 1 h at 30°C, yet fully active ADH can be obtained from the tissue simply by preventing the action of the inactivator with DTT. Clearly, the inactivator and ADH can coexist in the same cells for days without loss of ADH activity.

Where protein synthesis was stopped by cycloheximide (Fig. 7), the level of ADH remained steady in the presence of inactivator. Thus, it does not seem possible that ADH levels are maintained by active resynthesis of enzyme replacing that lost due to the inactivator. Again, it is clear that in air the ADH levels remain constant in spite of the fact that inactivator levels were increasing by 2- to 4-fold over a 3-d period (Figs. 6 and 7).

Thus, we are faced with a dilemma. It seems that, to account for the coexistence of ADH and its inactivator in cells, some compartmentation must be involved. If indeed the inactivator has some regulatory function *in vivo*, the additional postulate must be made that there is temporal regulation of the degree to which the two entities come into contact. ADH is a cytosolic enzyme and in work with the maize scutellum. Lai and Scandalios (4) concluded that the inactivator was a specific proteinase also present in the cytosol. In a subsequent paper, we show that the rice inactivator is different from that in maize and we examine its intracellular localization.

In relation to previous results from corn, the situation in the scutellum is of interest. The corn scutellum was shown to be a rich source of inactivator (3), while in rice the levels are very low, and ADH levels in the scutellum increase strongly both in seedlings in air and in N_2.

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