Flooding-mediated induction of alcohol dehydrogenase in rice seedlings: Involvment of new synthesis of enzyme molecules in the induction.

Wu-Fu Tong(1) Jieh-Juen Yu(1) and Shau-Wen Lin(2)

(1) To whom reprint requests should be addressed Department of Biology, National Taiwan Normal University.

(2) Graduate Institute of Science Education, National Taiwan Normal University

Abstract

Alcohol dehydrogenase (ADH) activity was induced in roots and shoots of four day old seedlings through flooding. ADH activity in shoots increased rapidly and in three days reached a level about twenty fold that of untreated shoots. Reducing the oxygen content in the flood water by nitrogen gas saturation caused further an increase in enzyme activity. ADH inactivator existed only in air-grown seedlings and accumulated with development. Cycloheximide effectively inhibited the increase of ADH activity, while only partially retarding the decay of enzyme activity after the induced seedlings are transferred to air-grown conditions. A twelve-hour incubation with 70% deuterium oxide

flooding resulted in a density increase of 0.01kg/l of the enzyme population with no change in the band width of the enzyme profile. The results suggest that rice ADH is an enzyme with rapid turnover and the induction under conditions of oxygen deficiency will enhance the de novo synthesis of enzyme molecules.

Key words: Alcohol dehydrogenase, Rice induction Density labeling.

Introduction:

Higher plants generally obligate aerobes, which can tolerate anaerobic conditions for various lengths of time. Under anaerobic conditions, plants will respond with metabolic change by increasing the ADH activity in order to meet energy requirements (Davies, 1980). Despite the regulation of enzyme activity by inactivator, de novo synthesis of ADH has been demonstrated in maize scutella (Lai and Scandalios, 1977) and in maize roots (Sachs and Freeling, 1978).

Rice is commonly recognized as the most flooding tolerant plant of all the cerals (Tsuji, H., 1973). ADH induction and the ADH inactivator accumulation in rice seedlings has been investigated and the conclusion drawn that ADH and ADH activator might be located in separate cell compartments (Shimomura and Beevers, 1983). Little information is available concerning the mechanism of ADH induction in rice by anaerobiosis, in particular the lack of the evidence of activation or de novo synthesis of enzyme in vivo in response to the increase of ADH activity. Recently Ricard et al., (1986) demonstrated that an increase in the translatable level of mRNA for two ADH peptides in rice embryos, is induced by anoxic treatment.

In this paper we will analyze the kinetics of ADH induction in developing rice seedlings under condition of oxygen deficiency. We intend to show that the increase of ADH activity depends on cellular protein synthesis and the rapid turnover of ADH molecules reflects the deuterium

incorporation and density shift of enzyme populations.

Materials

Rice seeds of *Oryza sative cv.* Tainung 67 were donated by the Institute of Botany, Academia Sinica, Taipei. Glycine, Tris, ethylenediamine tetraacetic acid, NAD^+, polyvinyl pyrrolidone, N,N-methylene-bis-acrrylamide, 2-nitrophenyl-B-D-galactopyranoside, deuterium oxide and cycloheximide were purchased from Sigma, St. Louis, USA; Acrylamide, zinc chloride and sodium dodecyl sulfate from Merck, Darmstadt, WestGermany; Dithiothreitol, cesium chloride, β-galactosidase, yeast ADH from Boehringer Mannheim GmbH, West Germany.

Methods

Treatment of seedlings:

Seeds were sterilized and germinated as described previously (Tong and Lin, 1988). Similarly sized four day old seedlings were selected for treatment. One set of seedlings was transfered to the flask flooded with de-ionized water. The others were subjected to both flooding and nitrogen gas bubbling (10 mins.) The flasks were then sealed with an air-tight rubber stopper. Cycloheximide treatment was carried out at a concentration of 20 ug/ml. For both the enzyme induction and decay experiments, cycloheximide was used instead of deionized water for the flooding and saturation of filter paper. For the density labeling experiments, the seedlings. were treated with 70% deuterium oxide solution. All the treatments proceeded at 25°C, in the dark.

Extraction and assay of enzyme and ADH inactivator:

Thirty roots or shoots were homogenated in a prechilled mortar with 1.0 ml of 10 mM Tris-HCl buffer solution (pH7.6), containing 0.5mM zinc chloride, 10 mM 2-mercaptoethanol, 0.5 g insoluble polyvinylpyrrolidone and 0.5 g quartz sand. The homogenate was centrifuged at 5,600 xg for 20 mins., at 4 °C, and the supernatant served as a crude extract for assay of both ADH and inactivator activities.

ADH activity was determined by modifying the method of Bonnichsen and Brink (1955). The assay mixture contained 0.1 M glycine-NaOH buffer (pH 9.0), 75 mM ethanol and 0.26 mM NDA^+. After the addition of enzyme solution to the mixture, the initial rate of NAD^+ reduction was measured at 340 nm with a Gilford 250 spectrophotometer. One unit of ADH activity was defined as the amount which catalyzed 1.0 μmole of NAD^+ per min.

Activity of the ADH inactivator was measured according to the method of Lai and Scandalios (1977). Yeast ADH served as a substrate for the inactivator. The activity of yeast ADH was adjusted to 2 units before assay. Three sets of mixture were then prepared as follows: a. 0.5 ml of yeast ADH mixed with 0.5 ml of buffer (10 mM Tris-HCl pH 7.5 containing 10mM 2-mercaptoethanol); b. 0.5 ml of sample mixed with 0.5 ml buffer, c. 0.5 ml of yeast ADH mixed with 0.5 ml of sample. After incubation at 37°C for one hour, the mixtures were assayed for ADH activity. The activity of β-galactosidase was determined using the method described by Hestrin et al. (1955).

Equilibrium centrifugation:

Isopynic density gradient centrifugation was conducted as described by Acton and Schopfer (1975). 2.1 g of cesium chloride was dissolved in 10 mM Tris buffer (pH 7.4) containing 10 mM 2-mercaptoethanol, to a final volume of 3.5 ml. One and a half milliliters of enzyme extract containing about 2 mg of protein with 1 to 3 units of ADH activity, and 10 μl of β-galactosidase (15 U) from E. coli as a density marker, were then added. The final mixture of cesium chloride solution with a density of 1.2978 kg/l was subjected to density gradient centrifuging in a fixed angle rotor of an MSE Prepspin 65 centrifuge at 40,000 rpm for 40 hours. Unlabeled controls were always done in parallel experiments. Density values were calculated from refractive index measurements and corrected according to the position of the marker.

Results

Air-grown etiolated seedlings exhibited very low ADH activity in both roots and shoots. ADH activity in the seedlings was induced by flooding (Fig. 1). During the treatment, root growth was retarded and it's ADH activity increased about ten fold in 24 hours, while shoot growth was promoted and it's ADH activity in shoot was induced much higher than that in root tissues. The differnce in the levels of ADH activity induced may reflect the metabolic and morphological diversity of tissues in their response to anaerobiosis. When the flood water was deprived of oxygen by nitrogen saturation, further increase of ADH activity was induced in both roots and shoots. This indicates that the magnitude of ADH induction in these tissues corresponds to the diminished oxygen content in the environment.

It has been reported that ADH inactivator exists in plant tissues (Suzuki and kyuwa, 1972; Ho and Scandalios, 1975; Shimomura and Beevers, 1983). In air-grown rice seedlings the activity of ADH inactivator was detected in five day old seedlings, and increased rapidly with growth, while in the extract from flooded seedlings, no inactivator activity could be measured (Fig. 2).

Results suggest that the production of ADH inactivator in both roots and shoots is retarded by oxygen deficiency.

ADH inactivators extracted from eight day old air grown shoots or roots inhibited ADH activity to 50% in one hour and completely stopped it within three hours of incubation. When the extract was first boiled at 100 °C for 3 mins. and then subjected to the inactivator assay, the inhibitory effect of the inactivator was not detected (Fig. 3). Apparently, the ADH inactivator was denatured by the heat treatment.

In order to test if the increase of ADH activity depends on newly synthesized enzyme molecules, the seedlings were treated with cycloheximide

(20 μg/ml) at the zero, 12 and 24 hour points of flooding (Fig. 4). Cycloheximide was found to completely inhibit the increase of ADH activity, and the level was not maintained after treatment. Twenty four hours after induction, when the ADH activity was high, cycloheximide treatment resulted in a decline of the activity level, while treatment at the very beginning of the induction, when ADH activity was low, resulted in no observable reduction. Thus it seems that the decline in ADH activity depends on the level of activity at the time of treatment. Increasing the cycloheximide concentration to 100 μg/ml produced no significantly different results (data not shown). It appears that cycloheximide affects on the turnover of the ADH molecules, particularly the synthesis of new enzymes. Since inactivator activity was not detected during the inductive period, it's interference in the developing pattern of ADH activity seems unlikely.

When seedlings from ihductive state were transferred to air grown conditions, ADH activity decreased very rapidly and ceased within 24 hours (Fig. 5).

During the ADH decay period, ADH activity was not stopped, but was slowed down by cycloheximide treatment. Cycloheximide may cause some alteration in the inactivator accumulation or enzyme degradation.

Fig. 6 shows the change in density of ADH molecules after 12 hours of labeling with 70% deuterium oxide during induction. The density increase of ADH molecules was 0.01 kg/l. In addition to a density increase, a transient band width change of the enzyme profile in the density gradient occurred midway towards the saturation level of labeling (Tong and Schopfer, 1976). The band width of ADH enzyme profiles in labeled and nonlabeled control samples showed not obvious difference. Thus, it is believed that after 12 hours of labeling of the enzyme population, deuterium

incorporation is close to the steady state levels. From this, one may deduce that ADH induction by oxygen deficiency most likely results from a rapid synthesis of new enzyme molecules.

Discussion

When the growing rice seedlings were flooded, the root growth was retarded, while shoot growth was promoted. This phenomena could help the seedlings in nature to pass through the surrending water and get oxygen from the air as quickly as possible. In higher plants, Davies (1980) has concluded that rice is one of the few cases which contains the mechanism to maintain a higher energy charge and is allowed limited growth. Enhancement of the fermentative metabolism in seedlings induced by anaerobiosis, responds to the increase of ADH activity from which more energy is produced to supply the tissues for life and growth. Under the condition of oxygen deficiency the growing shoots were found to have increased their ADH activity more rapidly than that of the retarded roots (Fig. 1). The degree of ADH induction was further enhanced in shoots as well as in roots, when the flood water was saturated with nitrogen gas. This indicated that the increase in ADH activity depended on the decrease of oxygen concentration. Similar results have been reported on the induction of anaerobic metabolism in the roots of rice and wheat (Bertani and Brambilla, 1982).

ADH inactivator appeared after four days in seedlings grown in air, and it accumulated with seedling development, while in flooded seedlings, no ADH inactivator was detected. When the flooded seedlings were transferred to air-grown conditions, ADH inactivator did appear, and ADH activity decreased. This decrease was very rapid. The time required for 50% loss off activity (t1/2) was estimated to be 10 hours, which was faster than the rate of inactivator accumulation. Thus, the existence of inactivator seems

not to be a major factor in the rapid decay of enzyme activity. It has been found that most inactivators are bonded to membranes. The ADH is cytosolic, and suggests that inactivator and ADH are separately compartmentalized within the cells (Shimomura and beevers, 1983). Recently Kang et al., (1986) have shown that the inactivation of ADH is closely related to fatty acid oxidation. Therefore, the accumulation of ADH inactivator in air-grown seedlings seems to be a consequence of metabolic requirements, rather than to regulate the ADH activity, involved directly in the fermentative metabolism of cells.

Cycloheximide effectively inhibited the increase of ADH activity during induction, suggesting that the activity increase was a translation dependent process. Treatment with 70% deuterium oxide in a 12 hour inductive period resulted in a density increase of ADH molecules of 0.01 kg/l with no change in the band width (Fig. 6). The result was similar to the induction of phenylalanine ammonia-lyase in which the 0.01 kg/l density shift and unchanging band width indicated that a saturated incorporation of deuterium was nearly reached (Tong and shopfer, 1976). According to the estimation, a 0.01 density unit increase may correspond to the incorporation of one deuterium per amino acid residue (Austine et al., 1970). Thus, it is suggested that ADH is a rapid turnover molecule and that induction by oxygen deficiency will accelerate the de novo synthesis of this enzyme. The result supports the finding by Richard et al. (1986), in which the increase of translatable levels of mRNA for ADH peptids is induced by anoxic treatment.

In contrast to the maize system, little information concerning the genetic study of rice ADH is available. Rice ADH isozymes have been analyzed from both embryos (Ricard et al., 1986) and seedlings (Tong and Lin, 1988). Similar isozyme patterns were observed in both tissues under oxygen

deficiency: one isozyme band existed in uninduced tissues and two additional weak bands existed after induction. A comparison of the ADH activities induced showed there was a ten fold increase in shoot growth with 24 hours anoxic treatment, while only a two fold increase in embryo growth. This suggests that the growing seedlings represent a better subject for study on the fermentative response induced by anoxia.

Acknowledgement. We wish to thank Prof. Dr. J. T. Shih for his critical reading of this manuscript and Mr. S. I. Yuan for his technical assistance.

Literature Cited

Acton, G. J. and P. Schopfr. 1975. Control over activation or synthesis of phenylalanine ammonia-lyase by phytochrome in mustard (Sinapis alba)? Biochim. Biophys. Acta. 404: 231-242.

Anstine, W., J. V. Jacobsen, J. G. Scandalios and J. E. Varner. 1970. Deuterium oxide as a density label of peroxidases in germinating barley embryos. Plant Physiol. 45: 148-152.

Bertani, A. and I. Brambilla. 1982. Effect of decreasing oxygen concentration on some aspects of protein and amino acid metabolism in rice roots. Z. Pflanzenphysiol. 107: 193-200.

Bertani, A. and I. Brambilla. 1982. Effect of decreasing oxygen concentration on wheat roots: Growth and induction of anaerobic metabolism. Z. Pflanzenphysiol. 108: 283-288.

Bonnichsen, R. K. and N. G. Brink. 1955. Liver alcohol dehydrogenase. Methods Enzymology. 1: 495-500.

Davies, D. D. 1980. Anaerobic metabolism and the production of organic acids. In Davies, D. D.(ed.) the biochemistry of plants: A comprehensive treatise. Vol. 2, Acad. Press Inc. N.Y. 581-611.

Hestrin, S., D. S. Feingold and M. Schramm. 1955. Hexoside hydrolases. Methods Enzymology. 1 : 241.

Ho, D. T. H. and J. G. Scandalios. 1975. Regulation of alochol dehydrogenases in maize scutellum during germination.

Plant Physiol. 56: 56-59.

Lai, Y. K. and J. G. Scandalios. 1977. Differential expression of alcohol dehydrogenase and it regulation by an endogenous alcohol dehydrogenase-specific inhibitor during maize development. Differentiation 9:111-118.

Kang, M. Y., S. Shimomura and T. Fukui. 1986. Inactivation of alcohol dehydrogenase in rice seedlings is related to microsomal fatty acid -oxidation system. J. Biochem. 99: 549-559.

Ricard, B., B. Mocquot, A. Fournier, M. Delseny and A. Pradet. 1986. Expression of alcohol dehydrogenase in rice embryos under anoxia. Plant Molec. Biol. 7: 321-329.

Sachs, M. M. and M.. Freeling. 1978. Selective synthesis of alcohol dehydrogenase during anaerobic treatment of maize. Molec. Gen. Genet. 161: 111-115.

Shimomura. S. and H. Beevers. 1983. Alcohol dehydrogenase and an inactivator from rice seedlings. Plant Physiol. 71: 736-741.

Shimomura, S. and H. Beevers. 1983. Alcohol dehydrogenase inactivator from rice seedlings: Properties and intracellular location. Plant Physiol. 71: 742-746.

Suzuki, Y. and K. Kyuwa. 1972. Activation and inactivation of alcohol dehydrogenase in germinating pea cotyledons. Physiol. Plant. 27: 121-125.

Tong. W. F. and P. Schopfer. 1976. Phytochrome-mediated de novo synthesis of phenylalanine ammonia-lyase: An approach using preinduced mustard seedlings. Proc. Natl. Acad. Sci. USA 73:4017-4021.

Tong, W. F. and S. W. Lin. 1988. Purification and biochemical properties of rice alcohol dehydrogenase. Bot. Bull. Academia Sinica 29: 245-253.

Tsuji, H. 1973. Growth and metabolism in plants under anaerobic conditions. Environ. Control Biol. 11: 79-84.

Legends

Fig. 1. Time course of ADH activity in developing seedlings.

Four day old dark grown seedlings were treated either

with flooding or with nitrogen gas saturated floodings. -●-roots and -○- , shoots of air-grown seedlings;-✚- ,roots and - ■ -, shoots of seedlings treated with flooding;-★-, roots and - □ - , shoots of seedlings, treated with nitrogen gas saturated flooding

Fig. 2. Time course of ADH inactivator in developing seedlings. Yeast ADH was used as substrate for the inactivator.-★-roots and-◇- shoots of seedlings treated with flooding;- ✚ - roots and -●- , shoots of air grown seedlings.

Fig. 3. Temperature effect on ADH inactivator.

ADH inactivator was extracted from shoots of eight day old, air-grown seedlings. The extracts were incubated either at 25°C or 100°C for 3 mins. and then the inactivator activity was estimated. Yeast ADH served as substrate for the inactivator. -○- , root and -□- , shoot extracts were assayed with inactivator preincubatd at 25°C. -★- , root and -■- , shoot extracts were assayed with inactivator preincubated at 100°C.

Fig. 4. Effect of cycloheximide on ADH induction.

Four day old, air-grown seedlings were treated with flooding and transferred at 0, 12 and 24 hours to be flooded in the same volume of 20 ug/ml cycloheximide solution. After treatment, shoots were extracted and assayed for ADH activity. -○- , air-grown shoots; -□- , shoots treated with flooding; -●- , shoots submerged in 20 ug/ml cycloheximide solution.

Fig. 5. Effect of cycloheximide on ADH decay.

Four day old, air-grown seedlings were treated with flooding for 24 hours and then transferred to air-grown conditions on filter paper which was wetted either with distilled water or with 20 μg/ml cycloheximide solution. Shoots of seedlings were extracted and assayed for ADH activity. - □ - , shoots from flooded seedlings; - ○ - , shoots from seedlings transferred to air-grown conditions; - ■ - , shoots from sedlings transferred to air-grown conditions and treated with cycloheximide

simultaneously.

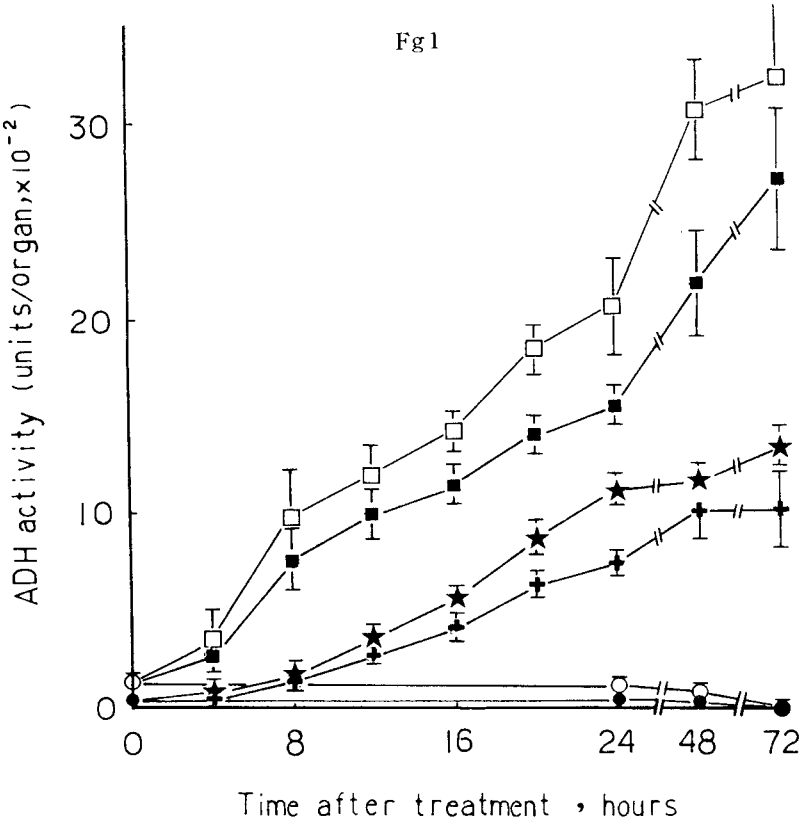
Fig. 6. Activity profiles of isopynically banded alcohol dehydrogenase.

A. ADH extracted from shoots of four day old, air-grown seedlings flooded for 12 hours.

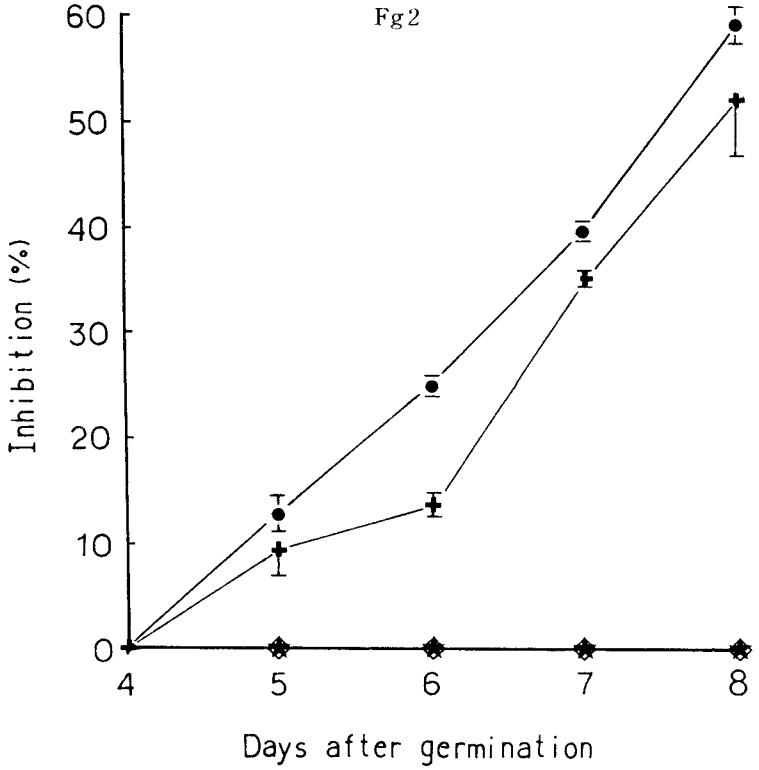
B. ADH extracted from shoots of four day old, air-grown seedlings flooded with 70% deuterium oxide for 12 hours.

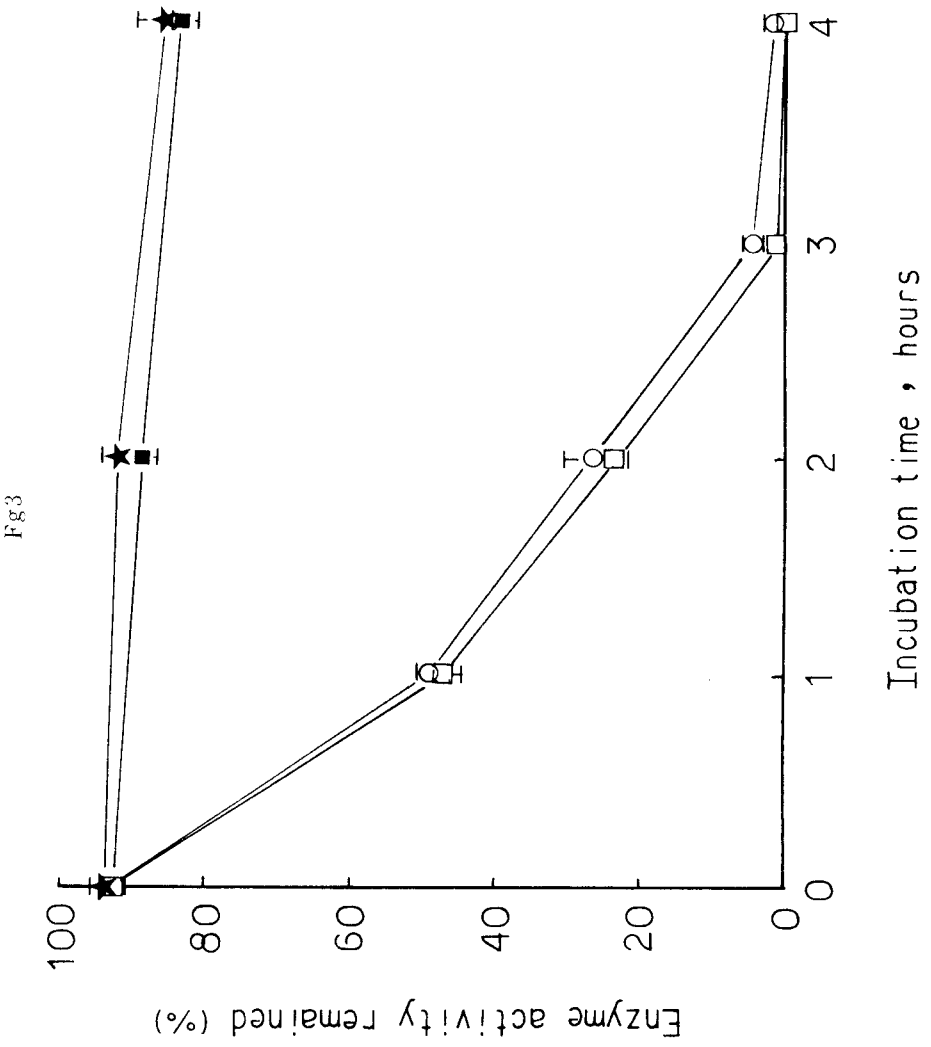
● -, density of CsCl gradient (kg/l); - ○ -, activity of β- galactosidase (Density marker);- ■ -, ADH activity.

Fg1


ADH activity (units/organ,×10⁻²)
30
20
10
0
0
8
16
24
48
72
Time after treatment , hours

Fg2


Inhibition (%)
60
50
40
30
20
10
0
4
5
6
7
8
Days after germination


Enzyme activity remained (%)
100
80
60
40
20
0
0
1
2
3
4
Incubation time , hours

Fg3

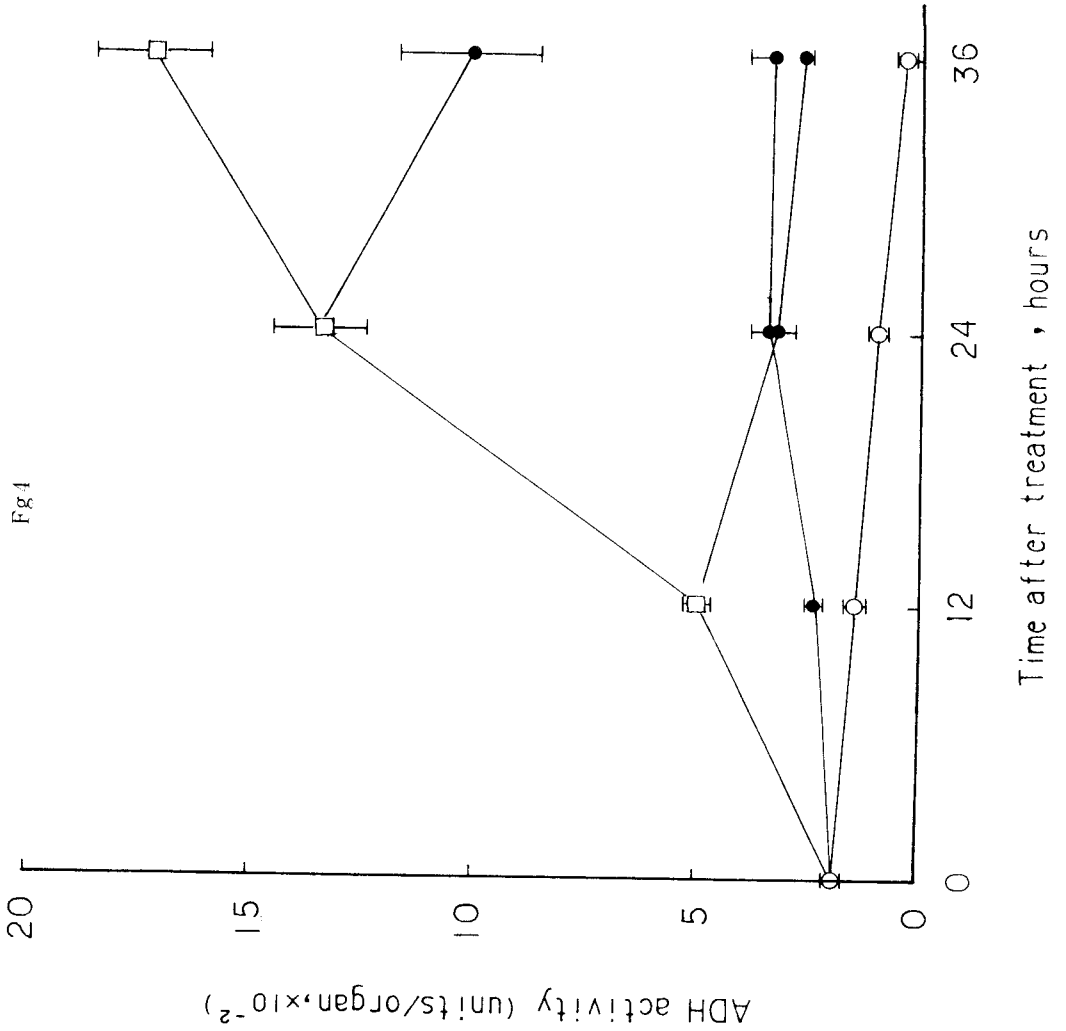
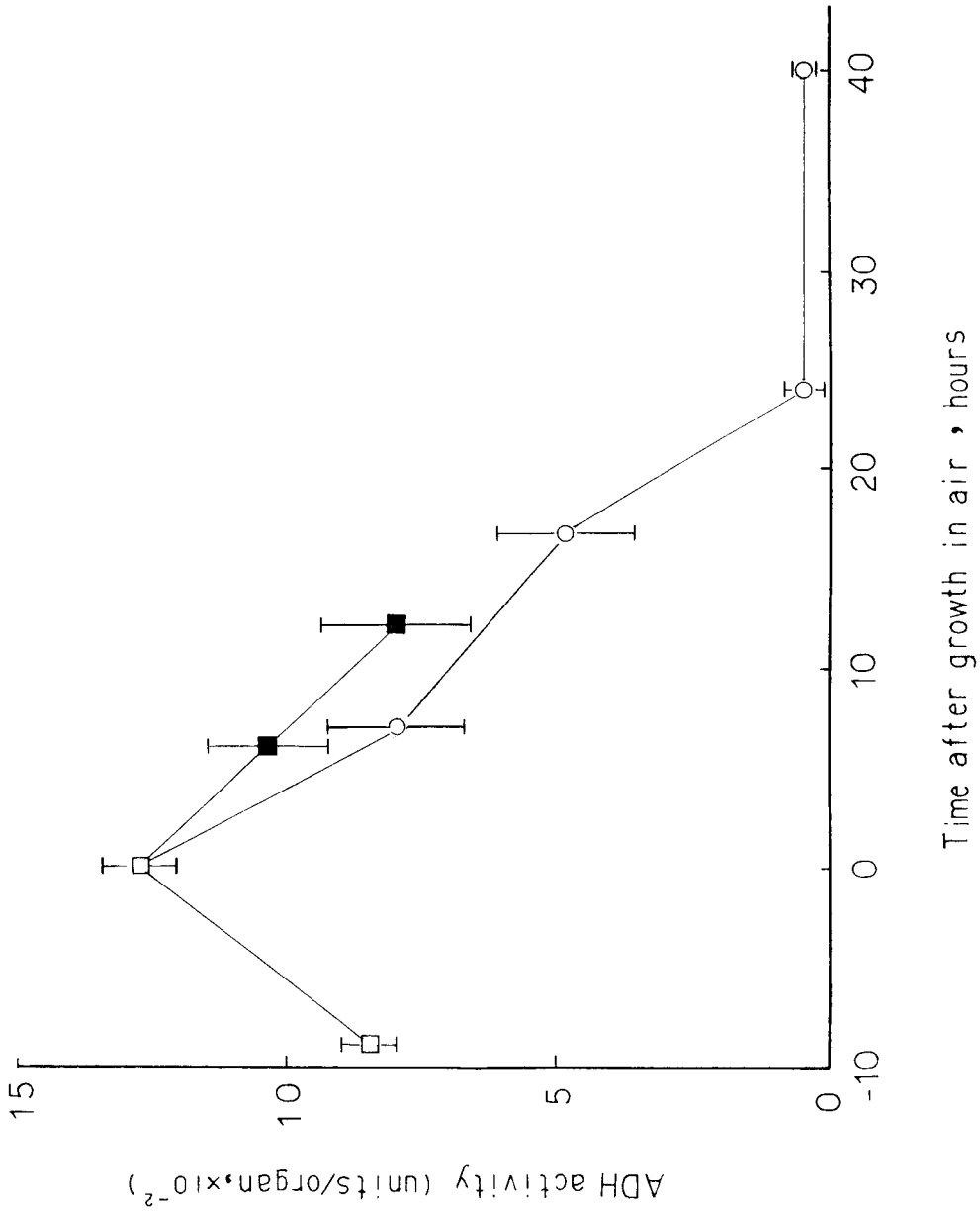
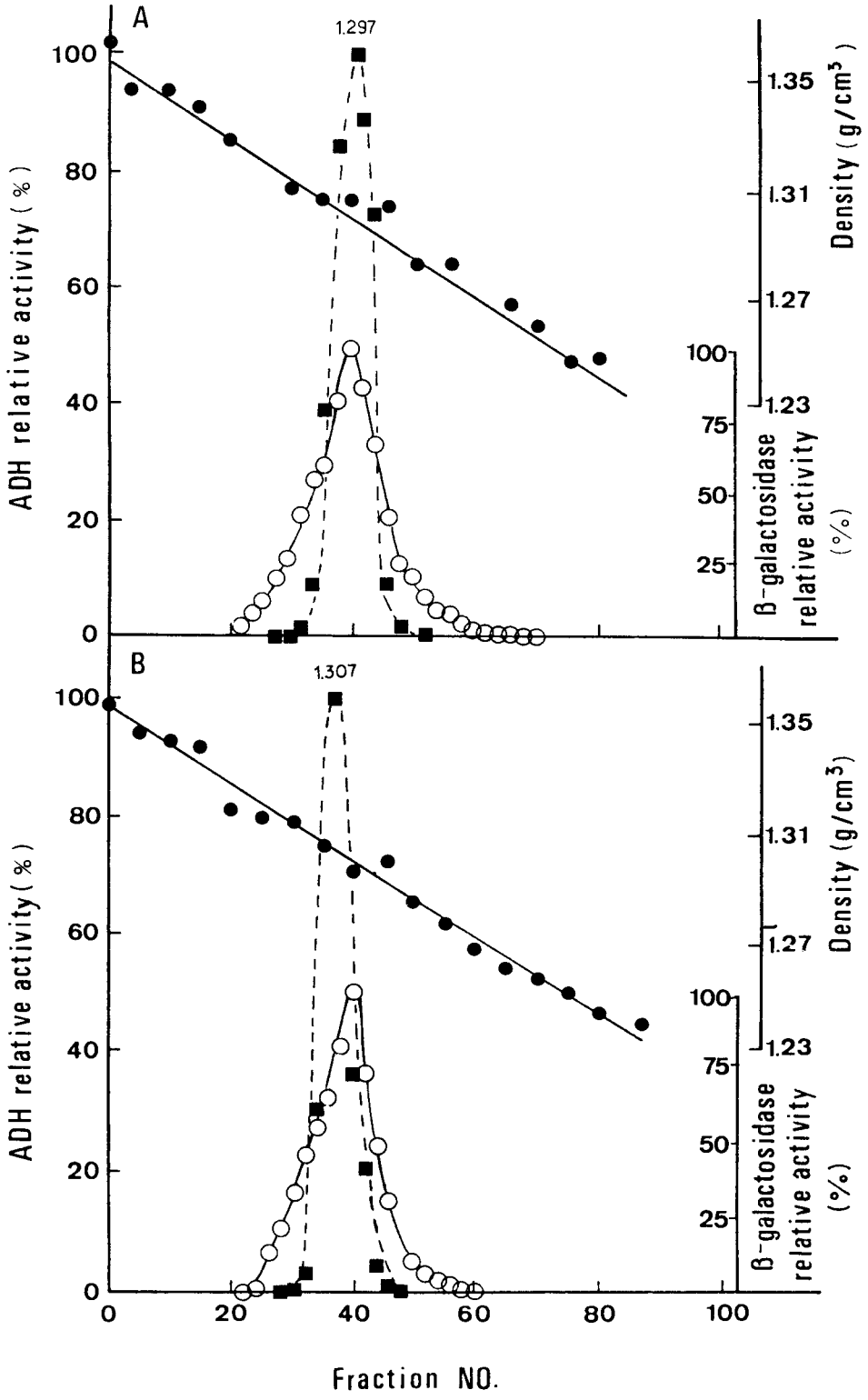

ADH activity (units/organ,×10⁻²)
20
15
10
5
0
0
12
24
36
Time after treatment, hours

Fig. 4


ADH activity (units/organ,×10⁻²)
15
10
5
0
-10
0
10
20
30
40
Time after growth in air, hours

Fg5

Fg 6


A
1.297
B
1.307
ADH relative activity (%)
Density (g/cm³)
β-galactosidase relative activity (%)
Fraction NO.

淹水誘導水稻幼苗的乙醇去氫 ： 誘導機制中具有酶分子的新合成

童 武 夫[1] 余 玠 諄[1] 林 曉 雯[2]

(1)國立臺灣師範大學生物學系

(2)國立臺灣師範大學科學教育研究所

淹水處理萌芽第四天的水稻幼苗，能誘導乙醇去氫酶活性的增高。處理的幼莖中乙醇去氫酶活性急劇增高，到第三天時，其酶活性高達正常幼莖酶活性的 20 倍。若再於水中加灌氮氣，降低其溶氧量，則更加强酶活性的誘導。酶抑制物祇見於正常生長的幼苗，並隨著幼苗的生長而增加。

Cycloheximide 能有效抑制酶活性的誘導 。當淹水幼苗移回正常生長後，酶活性會急速消失，此時 Cycloheximide 的處理祇能稍為緩和，但不能阻止酶活性的消失。若在酶誘導期間以重水標示，則在 12 小時後，可使酶分子密度增高 0.01 Kg/l ，但酶帶的寬度則無明顯差異。此結果顯示水稻幼芽所誘導的乙酶去氫酶，其換新速度相當快，且此因缺氧所誘導的酶活性增高，顯然是促進酶分子新合成所致。

關鍵詞 :Alcohol dehydrogenase, Rice induction, Density labeling.