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Effect of Anoxia on Energy Charge and Protein Synthesis in Rice Embryo

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ABSTRACT

Energy charge, adenine nucleotide levels, and protein synthesis were studied during the transfer of rice seedlings from air to anoxia. Within minutes, the energy charge value dropped from 0.90 in air to 0.50 in the seed and 0.60 in the coleoptile after the transfer to a nitrogen atmosphere, and then increased to a value of 0.80 during the subsequent hours. The sum of nucleotides also dropped to 60% of the value in air in the seeds and to 30% in the coleoptiles. However, during the anaerobic growth of coleoptiles, a considerable increase in the nucleotide pool occurred.

The incorporation of amino acids into proteins was measured at different stages in anoxic treatment. In rice embryo, we observed a considerable protein synthesis correlated with a high value of energy charge under anoxia. The analysis of labeled proteins by two-dimensional polyacrylamide gel electrophoresis showed a modified pattern of polypeptides synthesized during anoxic treatment. Some of these proteins were intensively labeled and appeared to be induced by anaerobic treatment.

Our data indicate that high metabolic activity occurs in rice embryo under anoxia, which can be correlated with a high energy charge value. These phenomena may be part of the mechanisms which permit the adaptation of rice embryos to anaerobiosis.

In nonchlorophyllous plant tissues and in animals, it is generally assumed that ATP synthesis is drastically reduced when oxidative phosphorylation is inhibited by a lack of O_2. The knowledge of the balance between ATP regeneration and utilization for metabolic work should permit a better understanding of the mechanisms used by these organisms to survive under anoxic conditions.

In animal tissues, there is a rapid inhibition of protein synthesis after a few min of anoxia (10, 12). In plant tissues, various conditions are found. There is a sharp decrease in protein synthesis in *Arachis* cells (28) and in squash cotyledons (22), even under hypoxic conditions which do not completely limit oxidative phosphorylation. This also occurs in the moss, *Tortula ruralis,* in darkness under anoxia (5). In soybean roots (14), polysomes are destroyed under anoxia resulting in a cessation of protein synthesis, whereas, in maize roots, Sachs and Freeling (24) and Ferl *et al.* (9) were able to demonstrate a low incorporation of labeled amino acids into a limited number of proteins under anaerobiosis. In some cases (5, 10, 22, 28), *in vivo* protein synthesis under hypoxia or anoxia was correlated with ATP level or energy charge. The very different capabilities of protein synthesis may be due to wide differences in the ability of the different tissues or cells to synthesize ATP.

When ATP regeneration is limited in lettuce seeds (23) and in maize root tips (27), energy charge is a parameter correlated with the level of residual metabolic activity. Such a correlation was also demonstrated in blood platelets (1).

Preliminary data obtained in our laboratory indicate that rice embryos or coleoptiles germinated in anoxia or transferred from air to nitrogen have a high energy charge (20, 21) and an active RNA (2) and DNA synthesis (16).

The purpose of this paper is to study the effect of anoxia on adenine nucleotide levels, energy charge, and incorporation of labeled amino acids into proteins in rice embryos. The correlation between energy charge and protein synthesis in anoxia is discussed, and anoxic protein patterns sustain the hypothesis of an adaptation of rice embryo to anaerobic conditions.

MATERIALS AND METHODS

Plant Materials. Rice seeds (*Oryza sativa* L., var. Cigalon) cultivated by the Station d'Amélioration des Plantes (INRA, Montpellier, France) were mechanically husked and sterilized with commercial (NaOCl (150 g Cl/l), as described previously (16).

Germination of Rice Seeds. Seeds were placed in glass flasks with water and shaken at 26 C in darkness. They remained under aerobic conditions for 40 to 48 h until coleoptiles reached 4 to 5 mm.

For anoxia, samples were placed in a nitrogen flow (100 ml/min), as previously described (16). Oxygen content was checked with an O_2 analyzer (WOM, Mécanalyse, France) or by gas chromatography. pO_2 in N_2 was lower than 0.01 KPa. Water, glassware, and other materials were autoclaved. Gases were sterilized by passage through membrane filters (pore size, 0.2 μm).

Bacteriological controls were carried out with an aliquot of incubation medium at the end of each experiment, as already described (16).

Adenine Nucleotide Measurement. For nucleotide determinations, rice samples were placed on wet filter papers in stoppered beakers and treated as described by Raymond and Pradet (23).

ATP, ADP, and AMP were measured using the bioluminescence method according to Pradet (18) and Saglio *et al.* (26).

Labeling, Extraction, and Analysis of Proteins. Rice seedlings aerobically grown for 48 h were used for a 4-h pulse labeling of proteins. A [^{14}C] L-amino acid mixture (New England Nuclear; 185 x 10^7 Bq/Matom carbon) was used after ethanol and traces of O_2 were flushed away by nitrogen. After different times under anoxia, labeled amino acids (185 × 10^4 Bq) in 0.5 ml were added with a syringe to flasks containing 12 rice seedlings in 1 ml of water under nitrogen flow. After 4 h of labeling, the samples were washed with cold distilled H_2O. The aerobic control was labeled in air.

Ten embryos were dissected out and homogenized in 1 ml buffer solution (9.5 M urea, 2% (w/v) NP-40[3], 2% ampholines

[3] Abbreviations: NP-40, Nonidet P-40; IF, isoelectric focusing.

[comprised of 1.6% pH range 5 to 7 and 0.4% pH range 3 to 10], and 5% (v/v) β-mercaptoethanol). The homogenate was centrifuged for 20 min at 40,000*g*. Aliquots of the supernatant (S40) corresponding to 0.75 embryo were analyzed by two-dimensional polyacrylamide gel electrophoresis, according to O'Farrell (17). The gels were treated with dimethylsulfoxide and 2-5 diphenyloxazole for fluorography (6, 13). They were dried under vacuum and exposed to Cronex-4 film (Dupont de Nemours), which was developed by a standard process.

Aliquots of S40 were used for studies of amino acid uptake and incorporation into proteins. For uptake determination, an aliquot (10 μl) was solubilized with NCS (Amersham/Searle) and counted in a toluene scintillation mixture containing acetic acid (3.4 ml/l). Another aliquot (50 μl) was used for acid insoluble determination; 0.5 ml 10% trichloroacetic acid was added, and the cooled precipitate was collected on a glass fiber filter (GF/C Whatman), washed with 5% trichloroacetic acid and 70% ethanol, dried, and counted after NCS treatment.

Protein Determination. Proteins of the trichloroacetic acid precipitate were solubilized in 0.1 N NAOH and estimated by the method of Lowry *et al.* (15) with BSA (Sigma) as standard.

RESULTS

Adenine Nucleotides and Energy Charge under Anoxia. When aerobic rice seedlings were transferred to nitrogen, the energy charge value dropped within 30 min from 0.90 to 0.60 in coleoptiles and to 0.50 in the remaining part of the seed (Fig. 1).

During the following hours, the energy charge increased to 0.80, and this value was maintained for 48 h. The anaerobic treatment induced a 70% drop of the adenine nucleotides pool in coleoptiles and a 40% drop in the remaining part of the seeds. This pool (Σ = ATP + ADP + AMP) increased 10 times during the following 48 h while it remained stable in the other part of the seedling (Fig. 1).

When seedlings (first germinated in air for 48 h and then maintained under anoxia for a subsequent period of 24 h) were again transferred to air, the level of the energy charge increased from 0.80 to 0.90 within 1 min. This new value remained stable for hours. When these seedlings were again transferred to nitrogen after 15 min or 1 h of air treatment, the energy charge level decreased to about 0.75 (Fig. 2). This value is close to that observed after 24 h of anoxia. It is high when compared to the value observed at the beginning of the first anoxic treatment.

Analysis of Proteins under Aerobic and Anaerobic Conditions. When aerobically grown rice seedlings are transferred to nitrogen, the total protein content of embryos remains stable while it increases in aerobic controls (Fig. 3). During the anoxic treatment, only coleoptile growth occurs, and, under these conditions, rice seedlings can survive for several days.

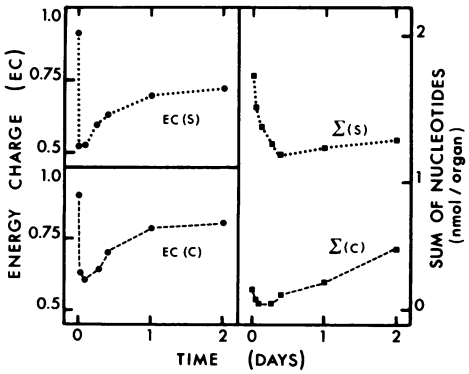

FIG. 1. Changes in energy charge (EC) (●) and in the sum of nucleotides (Σ = ATP + ADP + AMP) (■) in rice coleoptiles (C) and in the remaining part of the seeds (S) during the transfer from air to nitrogen.

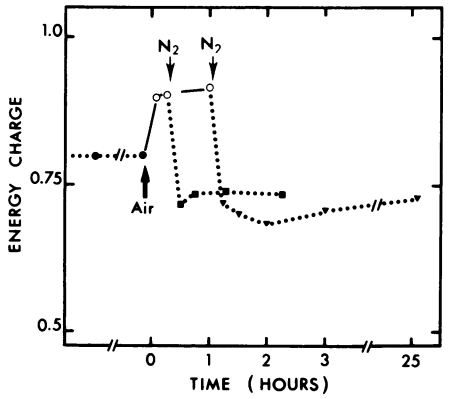

FIG. 2. Energy charge of aerobically germinated rice seedlings submitted to anoxia for 24 h (●) and then transferred to air for 15 min or 1 h (○) before another anoxic treatment (■, ▼).

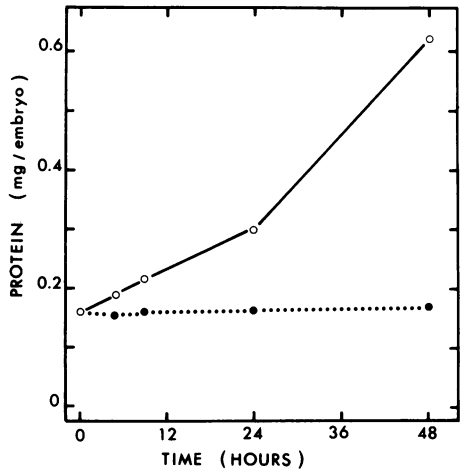

FIG. 3. Total protein content of rice embryos from seedlings aerobically grown for 2 days and then submitted to air (○) or nitrogen (●).

Table I. *[^{14}C] Amino Acid Mixture Uptake and Incorporation into the Acid-Insoluble Fraction of Rice Embryos for 4 h under Aerobic or Anaerobic Conditions and Rate of Protein Synthesis*

Treatment before the 4-h Labeling[b]	Uptake	Incorporation	Incorporation/Uptake	EC[a]
	cpm × 10^{-6}/embryo			
Air[c]	4.7	0.56	0.12	0.90
N_2 (1 h)	1.7	0.10	0.06	0.60–0.70
N_2 (24 h)	2.5	0.22	0.09	0.80
N_2 (48 h)	2.9	0.22	0.08	0.80

[a] EC, Energy charge values during the labeling period.

[b] Treatment applied to rice seedlings grown in air for 2 days.

[c] Aerobic control was labeled during 4 h in air just after 2 days of aerobic germination.

Proteins of rice seedlings grown in air for 48 h were labeled for 4 h with a [^{14}C] amino acid mixture under different conditions (in air and after 1 h, 24 h, and 48 h of anoxia). Precursor uptake and incorporation into proteins are shown in Table I.

After 1 h of anoxia, the level of incorporation represented 20% of that observed in air. It rose to 40% after 24 h and remained stable up to 48 h. During this anoxic treatment, precursor uptake was also reduced. If we assume that there is no important modification of the intracellular pool of amino acids or degradation of

the precursor during the treatment, the ratio between incorporation and uptake of amino acids allows a comparison of the rate of protein synthesis in air and in anoxia. In these conditions, the rate of protein synthesis was 50% of that in air after 1 h of anoxia and reached 70% after 24 h (Table I). The higher rate of protein synthesis in rice embryos under anoxia is found when energy charge has reached its highest value (Fig. 1; Table I).

The separation of labeled proteins is shown in Figure 4. A number of proteins were labeled under anoxia, and the distribution of radioactive spots was different in aerobic or anaerobic samples. For example, some polypeptides, intensively labeled in air, became less labeled or disappeared as anoxic treatment continued (spots 3, 7, and 8). On the other hand, some proteins were labeled to a greater extent under anoxia (spot 9). The longer the treatment, the higher the relative intensity of some spots (spots 5, and 6). Moreover, some polypeptides appeared during the anoxic treatment (for example, spots 1, 2, and 4). These proteins either were not labeled in air or were synthesized at such a low rate that their labeling was below the limit of detection.

DISCUSSION

The data presented in this paper show some biochemical aspects of the adaptation of rice embryos to anoxic conditions. The energy charge values of normoxic tissues are always higher than 0.80 (3, 19, 23). When animal or nonchlorophyllous plant tissues are transferred from air to nitrogen, the energy charge drops quickly and stabilizes at a lower value ranging from 0.60 to 0.20, according to the tissue. When these tissues are kept under nitrogen for hours or days, this value remains stable in some while it decreases slowly

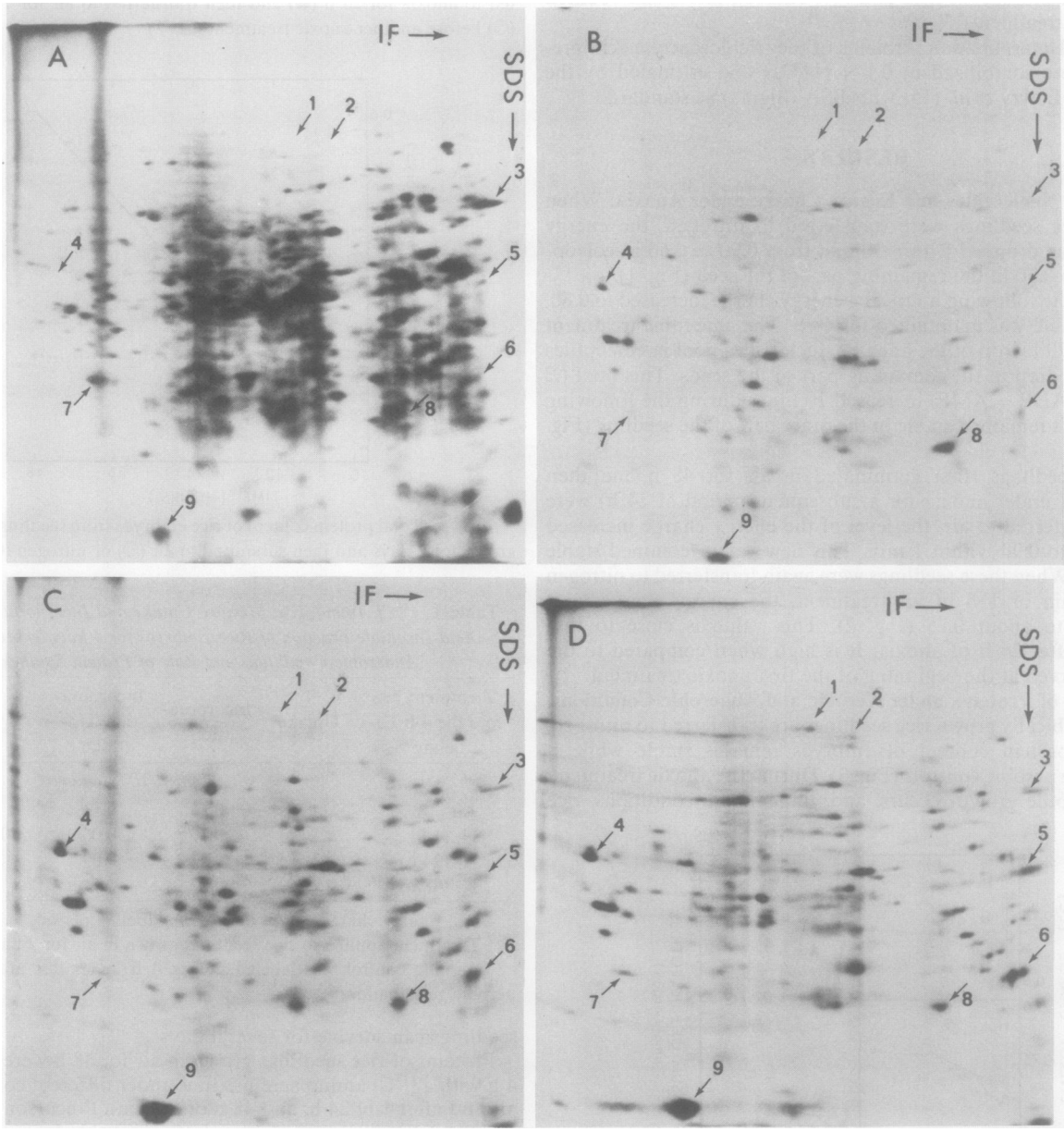

FIG. 4. Distribution of rice embryo proteins as a function of molecular weight and relative isoelectric point. Proteins of seedlings aerobically grown for 2 days were labeled with [^{14}C] amino acids for 4 h under different conditions (in air [A] and after 1 h [B], 24 h [C], and 48 h [D] in nitrogen) and were then subjected to equilibrium in the first dimension and on SDS-15% polyacrylamide gel electrophoresis in the second dimension. Samples corresponding to 0.75 embryo (110–120 μg of proteins) were loaded on the gels. The autoradiograms were exposed for 6 days. Some protein spots are marked with numbers for comparison between aerobic and anaerobic treatments.

in others. For a review, see Raymond and Pradet (23).

In rice seedlings, a fast initial decrease of energy charge was observed, but it subsequently was followed by a slow increase to higher values. However, the original aerobic value (0.90) was not reached. In facultative anaerobic microorganisms, high values are also observed in anoxia. For example, in yeast, Ball and Atkinson (4) observed a transient decrease to 0.75, followed in a few minutes by a rise to the initial value (0.85).

As shown by the rapid rise of energy charge in anaerobic embryos when returned to air, the oxidative capabilities are conserved for long anoxic periods. Costes and Vartapetian (7) already showed that mitochondria from anoxic rice coleoptiles exhibit tightly coupled oxidative phosphorylation and respiratory control when transferred to air. But, in these experiments, mitochondria were prepared in a medium containing O_2, and some O_2-dependent induction could arise during extraction. Our *in vivo* results ruled out this last assumption, and all data stressed the point that oxidative phosphorylation is preserved during anoxic treatment. This behavior is an indication of an adaptation to anoxia because it shows that this tissue is immediately able to recover an efficient energy metabolism as soon as O_2 is provided again.

Moreover, the ability of rice seedlings to maintain high values of energy charge after some hours of anoxia was retained when the anoxic treatment was interrupted by transient aeration. This result suggests that aerobic embryos adapt to anoxic conditions after the transfer to this environment.

This is confirmed by the study of the pattern of proteins synthesized in rice embryo. During the first hours under anoxia, only a small number of polypeptides are intensively labeled, but, after 24 or 48 h, many polypeptides are synthesized. Anoxic treatment of rice seedlings for longer times (up to 9 days) still allows protein synthesis in the embryo. There is a decrease in the incorporation of amino acids, but some polypeptides remain highly labeled (B. Mocquot, unpublished results). Our data confirm the recent demonstration that protein synthesis is possible in plants under anoxia (9, 24). However, our results are very different from those recently found in maize root by Sachs *et al.* (25). In this plant material, aerobic protein synthesis is halted, and a set of 22 new polypeptides is synthesized after several h of anaerobiosis.

As there is a stabilization of protein level in rice embryo during the anoxic treatment, the labeling of many polypeptides indicates an important turnover of proteins during the aerobic-anaerobic transition. Moreover, the modifications observed in the distribution and intensity of the polypeptide spots indicate a change in the expression of the genome following anoxic treatment, and some new proteins which appear during the treatment may be peculiar to anaerobic metabolism.

The rise in energy charge during the first hours of anoxia may be due to the synthesis of new proteins which could allow an increase of energy production. Sachs and Freeling (24) and Ferl *et al.* (9) demonstrated that two isoenzymes of alcohol dehydrogenase (ADH_1 and ADH_2) are among the few polypeptides synthesized under anaerobiosis. These data agree with the fact that, in most plant tissues, ethanol production is the main pathway which permits ATP production and proton elimination under anoxia. Some other pathways are at work in anaerobiosis (8). The proteins synthesized at the beginning of anoxic treatment may permit other metabolic pathways to take place. These, in turn, allow a more efficient anoxic metabolism for days in rice seedlings similar to that observed in subaquatic animals (11).

The value of energy charge has been shown to be related to metabolic activity when ATP regeneration is limited (1, 23, 27). High energy charge values correspond to a high metabolic activity while low energy charge values correspond to a low metabolic activity.

In rice embryo, we have found a considerable protein synthesis correlated with a high value of energy charge (0.80) after some hours in anoxia. The value of 0.80 is a nearly normal value at which most cellular processes may take place normally (3). During the first hours after the transfer from air to nitrogen, however, protein synthesis and energy charge values are lower. Likewise, in maize roots under anaerobiosis, the low protein synthesis observed by Sachs *et al.* (25) may be correlated with low values of energy charge (27).

Thus our results are consistent with the view that there is a metabolic adaptation of rice embryos to anaerobiosis which allows a survival of the rice seedlings for several days in the absence of O_2.

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