Stabilization of Adenine Nucleotide Ratios at Various Values by an Oxygen Limitation of Respiration in Germinating Lettuce (*Lactuca sativa*) Seeds

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The concentrations of adenine nucleotides were determined in germinating lettuce (*Lactuca sativa*) seeds after transitions from air to hypoxic or anoxic atmospheres. The ratio ATP/ADP and the energy charge were rapidly lowered after the transitions and remained stable at low values for hours. The energy charge in anoxia stabilized at a value close to 0.3. After 24 h in anoxia the energy charge rose rapidly to high values (0.9) when N₂ was replaced by air. The metabolic properties of lettuce seeds had then been conversed for hours at low energy charge. In hypoxia the O₂ uptake was decreased and the energy charge was stabilized at values intermediate between that in air and that in anoxia. When the O₂ partial pressures (*p*O₂) were 5 and 2 kPa, the values of O₂ uptake were one-third and one-sixth of that in air, and the energy charges were 0.7 and 0.5. These results show that the energy charge is regulated over a wide range of values. The ratio ATP/ADP and the energy charge are indicators of the limitation of metabolic activity by hypoxia.

ATP/ADP and ATP/AMP ratios (Gevers & Krebs, 1966) as well as the energy charge that integrates them (Atkinson & Walton, 1967) have been proposed as regulation parameters of the cellular metabolic activity. The energy-charge values for many tissues were tabulated (Bomsl & Pradet, 1968), and from these data it was asserted (Pradet, 1969) that the energy charges of most of the actively metabolizing tissues range between 0.8 and 1. This was confirmed by many workers (Chapman *et al.*, 1971; Knowles, 1977; Chapman & Atkinson, 1977). Atkinson (1977) considers that the most likely values for energy charge range between 0.87 and 0.94.

Low energy-charge values have been observed for more or less long periods after modifications of the metabolic balance: metabolic shift-up resulting from additions of carbon substrates (Atkinson, 1977), or shift-down produced by limitation of (a) oxidative phosphorylation in lettuce (*Lactuca sativa*) seeds (Pradet, 1969), *Escherichia coli* (Chapman *et al.*, 1971), rat brain (Ridge, 1972), rat liver (Kinnula & Hassinen, 1978), rice (*Oryza sativa*) embryos (Pradet & Prat, 1976), *Azotobacter vinelandii* (Leung & Schramm, 1978), (b) photophosphorylation in anaerobic wheat (*Triticum vulgare*) leaves (Bomsl & Sellami, 1974; Sellami, 1976) and

chromatium (Miovic & Gibson, 1973) and (c) carbon-substrate availability in various bacteria (see references in Chapman & Atkinson, 1977), yeast (Ball & Atkinson, 1975), ascites-tumour cells (Yushok, 1971; Live & Kaminskis, 1975) and rat lymphocytes (Mendelsohn *et al.*, 1977).

In some cases the decrease in energy charge is followed by an increase towards the initial values (Chapman *et al.*, 1971; Pradet & Part, 1976) or by the death of the organisms (Chapman *et al.*, 1971; Saglio *et al.*, 1979). From such observations, Atkinson (1977) concluded that '...the range of energy charge values that is compatible with steady metabolism and growth is very narrow...'

However, some results have shown a stabilization of energy charge at low values for periods up to several hours (Yushok, 1971; Ridge, 1972; Hutchinson & Hanson, 1974; Live & Kaminskis, 1975; Chulavatnatol & Haesungcharern, 1977; Leung & Schramm, 1978).

We demonstrate in the present paper that the energy charge in germinating lettuce seeds can be stabilized at any value between 0.9 and 0.3 according to the limitation of respiration by O₂ concentration. The hypoxic and anoxic states are stable for, and can be reversed after, a period of 19 h. Under these conditions, the energy-charge level and

consequently the corresponding ATP/ADP or ATP/AMP ratios are directly correlated to the metabolic activity.

Materials and Methods

Lettuce seeds

Seeds of lettuce (*Lactuca sativa*, var. Reine de Mai, pleine terre) were stirred for 30 min in commercial sodium hypochlorite (150 g of Cl/litre), rinsed for 10 min in a Büchner funnel with 4 litres of sterile water, stirred for 5 min in 0.01 M-HCl (Abdul-Baki, 1974) and rinsed again for 15 min with 6 litres of sterile water. After this treatment, there was no bacterial or fungal contamination as checked by plating-out ground seeds on a medium containing (in 1 litre) Bacto yeast extract, 3 g; bacto-peptone, 5 g; agar, 15 g; glucose, 10 g; final pH, 7.3. The rate of germination and the concentrations of nucleotides were unchanged when compared with those in untreated seeds. The seeds were immersed in water and agitated to permit a good aeration after 6 h they were placed on wet filter papers in stoppered beakers and gassed with mixtures of O₂ and N₂ at various partial pressures. The stoppered beakers were fitted with a cylindrical funnel closed by a Teflon stopcock to allow the introduction of cold diethyl ether for killing.

Gases

Gases were sterilized by passage through a 0.22 µm Millipore filter and humidified by bubbling through sterile water in a wash-bottle. The gas flow was about 100 ml/min.

The O₂ content of gas was checked with an O₂ analyser (W.O.M.: Société Mecanalyse, Rueil Malmaison, France) and by gas chromatography (2 m column of a molecular sieve of pore size 0.5 nm, at room temperature, with He as carrier). pO₂ was also controlled at the exit of the beakers. For anaerobiosis the pO₂ in N₂ was lower than 0.01 kPa.

Killing and sampling

Preliminary results have shown that the concentrations of nucleotides change rapidly when the pO₂ is modified (Pradet *et al.*, 1968). Thus if the seeds are in contact with air during sampling, the concentrations of nucleotides will dramatically change, even if this contact lasts only a few seconds.

In order to avoid this error, the seeds were frozen before sampling by pouring cold diethyl ether (−100°C) into the beaker through a funnel. Diethyl ether was removed by decantation and the seeds were stored at −25°C.

Enzyme inactivation and nucleotide extraction

Frozen seeds were homogenized in 0.6 M-tri-

chloroacetic acid (5 ml for 100 mg of seeds) by using a Polytron homogenizer. The homogenate was centrifuged at 20000 g for 10 min, the precipitate was re-extracted twice with 3 ml of 0.1 M-trichloroacetic acid and centrifuged. The extracts were pooled and extracted three times with 3 vol. of diethyl ether to remove trichloroacetic acid. Traces of ether were eliminated from the extracts by bubbling with air and the extracts adjusted to pH 6.8 with KOH (0.2 M). All operations were carried out between 0 and 2°C.

Determination of nucleotides

ATP was assayed by the bioluminescence method (Strehler & Totter, 1952) with a Pico ATP biophotometer (Société Jobin et Yvon, longjumeau, France). ADP and AMP were converted into ATP by the method of Pradet (1967), as modified by Saglio *et al.* (1979).

Results

In germinating lettuce seeds, respiration is limited when the pO₂ becomes lower than 15 kPa (Pradet, 1969). This pO₂ is called the 'critical oxygen pressure'. Hypoxia is the condition in which respiration is limited by oxygen availability, and

Fig. 1. Stabilization of the energy charge in germinating lettuce seeds exposed to hypoxic or anoxic atmospheres

Zero time corresponds to the beginning of gas flow at various oxygen pressures: O, 5 kPa; Δ, 2 kPa; □, <0.01 kPa. At the times indicated after changing the atmosphere, killing was performed as described in the Materials and Methods section; samples for zero time were killed in air. Each point is the mean of three determinations on three samples. Bars indicate S.D. Energy charge was calculated by the formula:

$$\frac{2[\text{ATP}] + [\text{ADP}]}{2\Sigma} \quad \Sigma = [\text{ATP}] + [\text{ADP}] + [\text{AMP}]$$

we call anoxia the condition in which the production of energy-rich bonds by respiration is very low as compared with that in anaerobic pathways (Pradet & Bomsel, 1978).

Transition from air to hypoxia or anoxia

Fig. 1 shows that transferring the lettuce seeds from air to a hypoxic or an anoxic atmosphere produces a steep fall in the energy charge that reaches, in a few minutes, a value close to the one at which it will be stabilized during the entire period of hypoxia or anoxia, that is, 0.7, 0.5 and 0.3 at pO_2 values of 5 kPa, 2 kPa and less than 0.01 kPa respectively.

The size of the pool of adenine nucleotides remains stable within the range of experimental error. The concentrations of ATP, ADP and AMP vary slightly during the 2 h after the transition from air to 2 kPa of O_2 and to N_2 ; this produces a transient increase of the mass-action ratio of the adenylate kinase up to 2, followed by a decrease to values corresponding to an apparent equilibrium (Fig. 2).

O_2 uptake remains stable during the entire period of hypoxia at about one-third and one-sixth of its maximum value when the pO_2 is 5 and 2 kPa respectively (Fig. 3).

Transition from 2 kPa of O_2 to N_2

After 19 h at 2 kPa of O_2 , seeds were transferred to N_2 . The energy charge decreased to a value of 0.2 in

Fig. 3. Stabilization of oxygen uptake by germinating lettuce seeds in hypoxia

Oxygen uptake was determined in a Warburg apparatus at 23°C in 15 ml vials containing 150 mg (dry wt.) of seeds and 2 ml of water. Respiration in air of each sample was measured every 15 min during 1 h. Vials were then subjected to the hypoxic atmospheres. Measurements were made every 90 min. The atmosphere was changed every 4–5 h. O, 5 kPa of O_2 ; Δ, 2 kPa of O_2 . Each point represents the O_2 uptake as a percentage of that in air, i.e. $430 \pm 7 \mu l$ of O_2/h per g dry wt. Results are given as means $\pm$ s.d. of measurements on three samples.

Fig. 2. Adenine nucleotide concentrations, pool size and adenylate kinase mass-action ratio in germinating lettuce seeds exposed to hypoxic or anoxic atmospheres

These data are from the experiment of Fig. 1. a_1 and a_2 , Transition from air to nitrogen; b_1 and b_2 , transition from air to 2 kPa of O_2 ; c_1 and c_2 , transition from air to 5 kPa of O_2 ; a_1 , b_1 and c_1 : nucleotide concentrations, expressed as a percentage of the pool size. ●, ATP; ▲, ADP; ■, AMP; a_2 , b_2 and c_2 : Δ, pool size ($\Sigma = [ATP] + [ADP] + [AMP]$) in nmol/g dry wt. O, Adenylate kinase mass-action ratio calculated by the formula:

$$K = \frac{[ATP] \times [AMP]}{[ADP]^2}$$

Fig. 4. Modification of the energy charge in germinating lettuce seeds after its stabilization at low levels by changing the O_2 concentration

Germinating lettuce seeds were treated as indicated in Fig. 1. At the end of the period of hypoxia in 2 kPa of O_2 (a) or anoxia (b) the atmosphere was changed for N_2 (a) or air (b) and samples were killed at the times indicated after this change.

10 min (Fig. 4a). This decrease is very similar to that observed after the transition from air to N_2 , showing that no fermentative pathway had been induced during the period of hypoxia (see the Discussion). As the respiration is stable, it may be concluded that, in hypoxia, the stable energy charge correlates with a stable metabolic activity.

Transition from N_2 to air

After the seeds had been exposed to anoxia during 24 h, the N_2 atmosphere was replaced by air. Fig. 4(b) shows that the increase in energy charge is rapid. In 1 min it reaches a value of 0.88. This shows that the dramatic decrease of energy charge and the metabolic modifications induced by anoxia are associated with the conservation of cellular structures, which allows an immediate utilization of O_2 when it becomes available.

Discussion

A lettuce seed is essentially an embryo. The endosperm is small and contains only 4% of the adenine nucleotides of the seed. From the beginning of the imbibition the respiration is active and coupled with phosphorylation (Pradet *et al.*, 1968). The present data concern an organism that is developing actively. Therefore the low energy-charge levels observed in hypoxia and anoxia cannot be explained by the lack of energy-metabolism regulation that could be supposed to occur in a 'futureless' storage tissue (Chapman *et al.*, 1971).

As stressed by Atkinson & Walton (1967), Bomsel & Pradet (1968) and more recently by Kremen (1976), the use of the parameter energy charge *in vivo* is valid only if the equilibrium

between ATP, ADP and AMP is maintained by the adenylate kinase. It can be verified that the equilibrium is maintained *in vivo* by calculating the mass-action ratio of the reaction, which varies *in vitro* between 0.4 and 1.4 according to magnesium concentration (Bowen & Kerwin, 1956; McBlair, 1970). At high energy charge, the concentration of AMP is very low and the relative error in its determination by the bioluminescence method is too high to allow the calculation of the adenylate kinase mass-action ratio. At an energy charge lower than 0.7 it can be calculated with a good accuracy. Fig. 2 shows that after the transition from air to N_2 its value slowly rises during the first 30 min and reaches about 2, then it falls during 2 h and remains stable at about 1. These data are not in contradiction with previous results (Bomsel & Pradet, 1968). They cannot be interpreted as an evidence that the activity of adenylate kinase is insufficient to equilibrate the nucleotide pool, because the mass-action ratio is maintained when the rate of modification of nucleotide concentrations is maximum, i.e. during the first 3 min. On the contrary, the disequilibrium appears during the following half hour, when the nucleotide concentrations are nearly stabilized at their values in anoxia. The rise in the mass-action ratio of the adenylate kinase reaction could be due to the formation of two or more pools of nucleotides with different energy charges. Even if each pool was equilibrated by adenylate kinase, the overall data would show an apparent disequilibrium. As a consequence the lowest values reached by the energy charge do not reflect the actual state of the adenine nucleotide pool in the cytoplasm, and the rise from 0.12 to 0.3 cannot be interpreted as a rise of the metabolic activity as in *E. coli* (Chapman *et al.*, 1971) or rice embryos (Pradet & Prat, 1976).

Pradet (1969) has shown that, in germinating lettuce seeds 20 min after transition from air to a hypoxic atmosphere, the ATP/ADP and ATP/AMP ratios had values between that found in air and that found in N_2 .

The stabilization of the energy charge and respiration in hypoxia shows that, by controlling the respiratory activity of lettuce seeds by the oxygen concentration, it is possible to induce and maintain metabolic steady states corresponding to energy-charge values between those obtained above the critical O_2 pressure and anoxia. Some results obtained on other higher-plant tissues (Raymond *et al.*, 1978) and the results obtained in rat brain tissue (Ridge, 1972) and liver (Kinnula & Hassinen, 1978) show that this may be a general phenomenon. This observation contradicts Atkinson's (1968, 1977) proposition about the narrow range of energy-charge values in living cells in steady metabolism.

Moreover it shows that, in germinating lettuce seeds under hypoxia, there is a relation between the

Fig. 5. Relation between metabolic activity and energy charge in germinating lettuce seeds in hypoxia or anoxia

The rate of synthesis of energy-rich bonds in hypoxia was calculated from the O_2 uptake, measured as indicated in Fig. 3 at 21, 10, 5, 2 and 1 kPa of O_2 , assuming that uptake of one O_2 atom allows the formation of three energy-rich bonds. In anoxia, metabolic activity was calculated from the rate of ethanol accumulation during the first 6 h in anoxia (the net production of energy-rich bonds was assumed to be one per mol of ethanol. Ethanol was measured enzymically as described by Bernt & Gutman (1972). Lactate and malate were determined during the same period as described by Gutman & Wahlefeld (1972). No accumulation of these two compounds was observed. Metabolic activity: ●, in hypoxia; ○, in anoxia.

level of energy charge and the metabolic activity (Fig. 5). The metabolic activity can be calculated from the oxygen uptake, because phosphorylation at the substrate level is comparatively low; this is to be expected here, as this activity seems low in anoxia (Fig. 5).

Such a relation seems to exist in other organisms or cells when the limitation of metabolic activity results from a limitation of ATP-regenerating pathways (Bomssel, 1973; Mendelsohn *et al.*, 1977; Rasi-Caldogno & De Michelis, 1978), not when it results from other limitations (Pradet, 1969; Cocucci & Marre, 1973; Live & Kaminskas, 1975).

It is not established whether the level of energy charge in anoxia is related to the metabolic activity allowed by fermentative processes: this would be in agreement with Krebs' (1973) concept of an indirect control of rate through equilibrium involving phosphate potential and redox state of pyridine nucleotides; it seems to be supported by various observations.

The value of energy charge in anoxia varies over a

wide range. Where low values (0.3 or below) were reported, they corresponded to low metabolic activities (Yushok, 1971; Leung & Schramm, 1978; Hutchinson & Hanson, 1974). Organisms able to grow or maintain a high activity under anoxia or without respiration have a high energy charge (Chapman *et al.*, 1971; Yushok, 1971; Ball & Atkinson, 1975; Knowles, 1977; Saglio *et al.*, 1979; Pradet & Prat, 1976).

The low level of the energy charge of lettuce seeds in anoxia can be correlated with their surprising aptitude to survive in this condition for a long time (up to 3 weeks; results not shown), and to preserve their cellular structures. The protective effect of low energy charge may be correlated with the lack of the energy that appears to be required for protein degradation in various organisms (Olden & Goldberg, 1978). It is possible that 'intermediate' values of energy charge (between 0.4 and 0.6) are lethal, whereas low values (below about 0.3) permit survival.

The methods for nucleotide determination currently used permit the calculation of energy charge values with a good accuracy. This makes this parameter an invaluable tool in energy-metabolism studies, if used as a marker for the metabolic states where the energy supply is limiting. Since the critical oxygen pressure varies among organisms and the metabolic activity in anoxia or hypoxia depends on the activity of fermentative pathways and the availability of nutrients, the quantitative relation between the energy charge and the limited metabolic activity has to be established for each biological material under given conditions.

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