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Correlation between Oxygen Availability, Energy Charge, and Protein Synthesis in Squash Cotyledons Isolated from Germinating Seeds

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ABSTRACT

The influence of O_2 availability on the rate of protein synthesis, the levels of RNA and of adenylates, and the value of the energy charge in squash (*Cucurbita maxima*) cotyledons isolated from seeds germinated for 15 or 28 hours at different O_2 concentration (3% or 20% O_2) has been investigated.

The rate of protein synthesis is five times lower in cotyledons maintained in 3% O_2 than in those maintained in 20% O_2. Also net RNA synthesis is almost blocked in 3% O_2, while in 20% O_2 it proceeds almost linearly for 48 hours.

The different RNA contents cannot explain the different rates of protein synthesis.

The results of shift experiments (cotyledons shifted from 20% to 3% O_2 or vice versa) show that the rate of protein synthesis is strictly correlated with actual O_2 availability and is largely independent of the one in the previous period. O_2 controls the development of the adenylate pool and particularly the increase of ATP level. Thus, both the adenylate pool and the values of the energy charge ratio are lower in cotyledons grown in 3% than in 20% O_2.

The shifts of O_2 availability induce rapid changes of ATP, ADP, and AMP levels and thus of the values of the energy charge, which are about 0.7 at 3% O_2 and higher than 0.8 at 20% O_2, independent of previous O_2 availability.

The rate of protein synthesis appears to be largely independent of the levels of the single nucleotides and better correlated to the energy charge values.

Resumption of metabolic activities in the early phases of germination is controlled by both endogenous and external factors. Among environmental conditions, water and O_2 availability play a main role. The mechanism through which water and O_2 control seed germination is not satisfactorily understood.

A suitable material for the study of the effect of O_2 on seed germination is supplied by squash seeds (*Cucurbita maxima*). In this material, O_2 availability at the onset of germination is physiologically limited by the presence (under the teguments) of the inner membrane (residual of nucellus and of endosperm) which strongly limits O_2 exchange (4, 23). O_2 availability of intact seeds in the early phases of germination is nearly equal to that of seeds deprived of the inner membrane and incubated in the presence of 3% O_2. Such an O_2 concentration appears to be strictly limiting: in fact, increasing pO_2 (up to 20% O_2) strongly stimulates QO_2 and the development of many enzymic activities involved in the resumption of metabolic activities at the onset of seed germination (5, 23).

It seems that the effect of O_2 on seed germination involves an effect on the systems of transcription and translation of genetic information. The control exerted by O_2 on these systems should imply, in its turn, the regulation of other metabolic parameters as, for example: (*a*) an effect on the level of ATP, as promptly utilizable energy made available by respiration; (*b*) an effect on the value of the energy charge (1) as an important factor regulating both the production of ATP from respiration and its utilization for metabolic work.

In this investigation, we have studied the effect of O_2 availability on the rate of protein synthesis, the levels of RNA and of adenylates, and the value of the E.C.[1] in squash cotyledons incubated in high (20%) or low (3%) O_2 concentration.

MATERIALS AND METHODS

Squash seeds (*Cucurbita maxima*) sterilized with 1% w/v NaClO for 10 min were decoated and deprived of the inner membrane after 1 hr of imbibition and then germinated at 30 C in the dark in open Petri dishes on wet filter paper. The dishes were put in desiccators through which humidified gas mixtures were flown continuously. Cotyledons were isolated after the desired times of germination.

Determination of the various parameters was performed immediately after isolation or after different periods of incubation (from 7 to 90 min) in distilled H_2O in Erlenmeyer flasks. Gas mixtures were bubbled continuously into the medium during incubation through a rubber plug provided with two holes.

Gas Mixtures. Mixtures of O_2 and N_2 were used during the experiments. The concentrations of O_2 in the gas mixture expressed in units of volume per volume per cent at 1 atm pressure, were 3% and 20%. The gas flow was held constant by different pressure regulators (ASA, Sesto S. Giovanni, Italy).

Rate of Protein Synthesis. The rate of protein synthesis was determined by means of L-[^{14}C]leucine (obtained from Amersham) in the presence of 0.1 M leucine. In our material the amount of radioactivity incorporated in the proteins is independent of the uptake rate only at leucine concentrations higher than 50 mM (data not reported). Trichloroacetic acid-soluble and -insoluble fractions were prepared as described (16).

Levels of RNA. The RNA content of the cotyledons was measured according to Cocucci and Sturani (11).

Adenine nucleotide levels. Four different methods of ATP extraction from the cells were tested: incubation in boiling water for 10 min (9), treatment with liquid N_2 and 4.5 N perchloric acid (15), or with liquid N_2 and 0.6 N perchloric acid

[1] Abbreviation: E.C., energy charge.

(13) or with 0.6 N perchloric acid at 0 C. In all cases the cotyledons were homogenized in a mortar in the presence of seasand.

The best results with respect to the adenine nucleotide levels were obtained in this material extracting with 0.6 N perchloric acid. Killing in liquid N_2, as compared to extraction at 0 C gave the same results; therefore extraction of adenylates was always performed in 0.6 N perchloric acid at 0 C. The O_2 concentration was kept constant respectively at 20% or 3% during extraction with perchloric acid. The supernatant obtained by centrifugation for 5 min was filtered, neutralized with K_2CO_3 in the presence of 0.1 M triethanolamine HCl/NaOH buffer at pH 7.8, and centrifuged again. The enzymic assay of ATP (17), ADP, and AMP (14) was performed on the supernatant. Internal standards of ATP, ADP, and AMP added during homogenization were recovered at the end of the treatment to the extent of 90%, 85%, and 84%, respectively. Substrates and enzymes for enzymic assay of adenylates were obtained from Boehringer.

All of the data reported in this paper are the mean of two or more experiments run in triplicate. The values of ATP, ADP, and AMP and E.C. are expressed ± SD.

RESULTS AND DISCUSSION

Effect of Oxygen on Content of RNA and on Rate of Protein Synthesis. The increase of enzymic activities at the beginning of germination largely depends on *de novo* synthesis of enzymes (8, 18, 19). Therefore, the different development of enzymic activities at the two pO_2 considered (3% and 20% O_2) mainly reflects a different activity of the protein-synthesizing machinery. The results reported in Figure 1 show that indeed in cotyledons grown and maintained in low pO_2 the rate of protein synthesis—which reaches a plateau after only 15 hr—is only 20% of that measurable in cotyledons maintained in high pO_2.

The different activity of the protein-synthesizing machinery corresponds to a different level of RNA in the cotyledons grown in the two different pO_2 (Fig. 2). In fact, the level of RNA remains almost constant throughout the first 48 hr of germination in the cotyledons grown in 3% O_2, while it increases progressively in the cotyledons grown in 20% O_2.

However, there is no correlation between RNA levels and the rates of protein synthesis observed in the cotyledons grown in 3% or 20% O_2; in fact, the values of the ratios between the rate of protein synthesis (Fig. 1) and the level of RNA (Fig. 2) are markedly lower for the cotyledons maintained in low pO_2. Therefore, in the cotyledons maintained in low pO_2 there is a great fraction of RNA which appears not to be involved in protein synthesis.

Figure 3 demonstrates that the rate of protein synthesis is controlled by the value of pO_2 during the measurement and does not depend on the conditions of low or high O_2 availability in the previous period (0–15 or 28 hr). In fact, the rate of protein synthesis at low pO_2 appears as low in cotyledons maintained all of the time in low pO_2 as in cotyledons grown in high pO_2 (for 15 or 28 hr) and shifted to low pO_2 when the rate of protein synthesis was measured. Similarly, the rate of protein synthesis is equally high when measured in conditions of high pO_2, independent of the previous treatment in high or low pO_2.

The data in Table I show the changes of the levels of RNA in cotyledons maintained in the same conditions of high or low pO_2 or shifted from high to low or from low to high pO_2 for 45 min. These data demonstrate again the absence of any correlation between the rate of protein synthesis and the amount of total RNA. In fact, even if the shift from low to high pO_2 induces a significant increase of the level of RNA, the amount of total RNA remains—at least for 45 min—less than half of the amount of RNA present in the cotyledons maintained in high pO_2. This of course does not exclude that important, short lived mRNAs may be synthesized in this period of time.

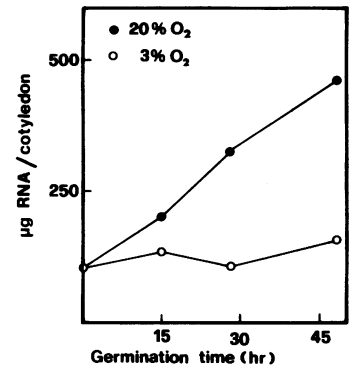

FIG. 1. Effect of O_2 on rate of protein synthesis in isolated cotyledons. Rate of protein synthesis was measured at different times of germination over a 30-min period immediately following isolation of cotyledons and is expressed as nmol of L-[^{14}C]leucine incorporated/cotyledon.

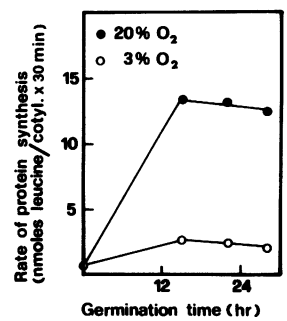

FIG. 2. Effect of O_2 on RNA content of cotyledons isolated at different times of germination.

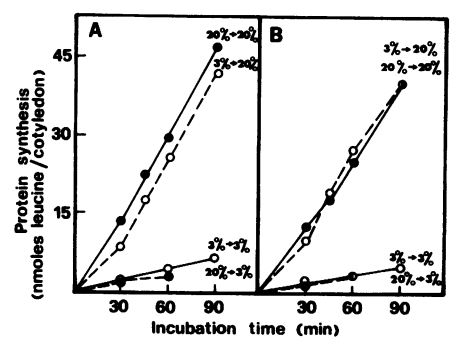

FIG. 3. Protein synthesis in cotyledons isolated after 15 (A) or 28 hr (B) of germination under 3% (open symbols) or 20% (closed symbols) O_2 concentration and maintained during the measurement in the same O_2 concentration (continuous line) or transferred respectively under 20% (open symbols, broken line) or 3% (closed symbols, broken line) O_2 concentration. Protein synthesis is expressed as nmol of L-[^{14}C]leucine incorporated/cotyledon.

Effects of Oxygen on Levels of Adenylates and Value of Energy Charge. The increase of pO_2 from 3% to 20% O_2 brings about a rapid large stimulus of QO_2, which likely means an increase of oxidative phosphorylation (23). This fact and the very quick response of protein synthesis to the shifts of pO_2 suggest that the ATP level and/or the phosphorylation degree of adenylates (expressed by the values of the energy charge as formulated by Atkinson [1]) play a role in the regulation of the rate of protein synthesis by O_2. The involvement of adenylate levels in the control of protein synthesis has been pointed out by Freudenberg and Mager (12) and by Rupniak and Quincey (24) in reticulocytes and ascite tumor cells; in plant materials, a good correlation between ATP levels, E.C. values, and the rate of protein synthesis has been reported by Bewley and Gwóźdź

(2) and by Verma and Marcus (26). The values of ATP, ADP, AMP, and of the E.C. in cotyledons isolated from seeds germinated for 22 hr in 3% or 20% O_2 are reported in Table II. The levels of all of the nucleotides are lower in the cotyledons grown in 3% O_2 than in those grown in 20% O_2. The largest difference is in the level of ATP, which in limiting O_2 is less than half of that in 20% O_2. As a consequence there is a change in the value of the E.C. from very high values (0.85), typical of growing cells, in high pO_2, to a substantially lower value (0.75), in low pO_2 (6).

The behavior of the adenylate system in response to the shift of O_2 availability from 3% to 20% O_2 or vice versa was then studied. The levels of ATP, ADP, AMP, and E.C. of cotyledons isolated after 22 hr of germination in 3% or 20% O_2, then incubated for 30 min in various pO_2 conditions, are reported in Table III. The data show that the shift to high pO_2 of cotyledons coming from low pO_2 brings about in 30 min a strong decrease of the levels of ADP and AMP and a considerable increase of ATP level in comparison with the controls maintained in 3% O_2. Besides, as the increase of ATP is larger than the decrease of ADP and AMP, an increase of the adenylate pool is evident. Due to the increase of ATP and the decrease of ADP and AMP, the E.C. rises to a value also larger than that of cotyledons maintained in 20% O_2 all of the time. Conversely, the shift to 3% O_2 of cotyledons grown in 20% O_2 brings about a partial conversion of ATP to ADP and AMP and a consequent lowering of the E.C. to a value lower than that of controls maintained in 3% O_2. Thus, 30 min after the shift to the two pO_2 under consideration, the values of the E.C. are satisfactorily correlated to the rates of protein synthesis. This situation is already clear 7 min after the pO_2 shift, at least from high to low pO_2 (Table IV); in fact, under these conditions, an increase of ADP and, also larger, of AMP and a severe decrease of ATP are clearly detectable after such a short period. Changes of adenylate levels brought about by a 7-min-long shift from 3% to 20% O_2 are less significant; however, a slight increase of ATP level and decrease of ADP and AMP occur also in this short period. The delay of the response of the adenylate system to the increase of pO_2 might depend on a poor development of the mitochondrial oxidizing enzymes in conditions of low pO_2 (23) and/or on a slow rate of diffusion of O_2 in the tissue. Nevertheless, in this case also the change of the E.C. value is well correlated with the rate of protein synthesis. In fact, while the shift from high to low pO_2 causes the rate of protein synthesis to fall immediately, the highest increase of the rate of protein synthesis brought about by the shift from 3% to 20% O_2 is somewhat delayed.

It is interesting that the rate of protein synthesis is largely independent of the levels of the three nucleotides separately considered. Therefore it is difficult to define a threshold of ATP, ADP, or AMP levels under or over which protein synthesis is inhibited.

Table I. Effect of the shift of oxygen availability on RNA content

RNA content (expressed in µg/cotyledon) in cotyledons isolated from seeds germinated for 28 hr under 3% or 20% O_2 measured immediately after isolation (a); changes in RNA content after 45 min of further incubation in 3% or 20% O_2 (b).

germination for 28 hr	µg RNA/cotyledon (a)	treated for 45min	changes in RNA content (µg/cotyledon) in 45min (b)
3% O_2	110	3% O_2	-2
		20% O_2	+34
20% O_2	315	3% O_2	-8
		20% O_2	+1

Table II. Adenine nucleotide levels and energy charge in cotyledons after 22 hr of germination at different pO_2

	ATP	ADP	AMP	adenylates	E.C.[1]
	nmol/cotyledon[2]				
20% O_2	29.0±1.0	7.4±0.5	1.9±0.2	38.3	0.85±0.02
3% O_2	11.5±0.6	5.1±0.5	1.8±0.1	18.4	0.76±0.02

[1] $\frac{ATP + 1/2\ ADP}{ATP+ADP+AMP}$

[2] Fresh weight per cotyledon=90 mg at 3% O_2 and 100 mg at 20% O_2

CONCLUSIONS

The results reported in this paper show that in squash cotyledons O_2 availability affects the rate of protein synthesis, the accumulation of RNAs and of the adenylate pool, and the values of the E.C. Oxygen-dependent changes of the rate of protein synthesis—which is certainly a limiting factor for the development of metabolic activities in the early phase of germination—are largely independent of changes of total RNA content but are positively correlated to the phosphorylation degree of the adenylates independent of the levels of the single nucleotides.

The fact that the rate of protein synthesis appears correlated to the values of the E.C. seems physiologically meaningful because, in this manner, the rate of protein synthesis can be promptly controlled as pO_2 changes, independent of the size of the adenylate pool (3, 25).

The correlation between the values of the E.C. and the rate of protein synthesis reported in this paper suggests a general mechanism through which the ratios between ATP, ADP, and AMP and hence the E.C. may have a regulatory function in germinating seeds, as has been shown (7, 9, 10, 20-22), and emphasizes the use of this parameter to investigate the control of metabolic processes by energy availability in eucaryotes (2, 12).

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Table III. Adenine nucleotide levels and energy charge after 30 minutes from the shift to different pO_2

Levels of adenylates measured after 30 min of incubation of isolated cotyledons in condition of pO_2 equal or different from those of germination (22 hr).

germination for 22 hr	treatment for 30 min	ATP	ADP	AMP	adenylates	E.C.
		nmol/cotyledon				
3% O_2	3% O_2	9.3±1.1	4.5±0.6	1.5±0.2	15.3	0.75±0.02
	20% O_2	16.7±1.2	2.9±0.5	1.0±0.2	20.6	0.88±0.02
20% O_2	3% O_2	13.7±1.4	10.4±0.8	4.9±0.2	29.0	0.65±0.02
	20% O_2	24.1±0.5	6.7±0.6	2.1±0.2	32.9	0.83±0.01

Table IV. Adenine nucleotide levels and energy charge after 7 min from the shift to different pO_2

Levels of adenylate measured after 7 min of incubation of isolated cotyledons in conditions of pO_2 equal or different from those of germination.

germination for 22 hr	treatment for 7 min	ATP	ADP	AMP	adenylates	E.C.
		nmol/cotyledons				
3% O_2	3% O_2	11.6±0.6	4.8±0.4	2.1±0.1	18.5	0.76±0.02
	20% O_2	13.0±0.8	4.0±0.3	1.8±0.2	18.8	0.81±0.02
20% O_2	3% O_2	19.1±0.9	8.7±1.5	4.2±0.9	32.0	0.73±0.03
	20% O_2	27.5±1.8	6.6±0.8	1.5±0.2	35.6	0.86±0.02

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