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Respiratory Activity during Germination of Seeds of *Cicer arietinum* L. II. Effect of some Metabolic Inhibitors and Mitochondrial Activities

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Changes in respiration rate during the early period of germination of *Cicer arietinum* L. seeds were studied. Three phases were distinguished. During phases I and II the RQ was higher than unity. Sodium fluoride showed no effect on gaseous exchange during phase I while in phases II and III a progressive increase in the inhibition of the respiratory activity was obtained. Iodoacetate inhibited oxygen uptake and carbon dioxide evolution from the beginning of germination. The activities of mitochondrial enzymes increased during the germination period, especially after the first 24 hours. The respiratory control and ADP/O ratio of the isolated mitochondria increased gradually during germination, reaching maximum values at 48 hours, lower, however than the expected theoretical ones.

In a previous paper (2) we found during germination of chick pea, changes in the activities of some enzymes of the glycolytic and fermentative pathways, which suggested a shift from anaerobic to aerobic respiration. In the present work this suggestion has been confirmed. Measurements of the respiratory activity, the effect of some metabolic inhibitors, the oxidative and phosphorylative capacity of isolated mitochondria, as well as the activity of some tricarboxylic acid cycle and electron transport pathway enzymes have been carried out in chick pea seeds during germination.

Materials and Methods

Chick pea (*Cicer arietinum* L.) seeds were soaked in 1 % sodium hypochlorite for 5 min and then washed thoroughly with sterile distilled water. The seeds were then germinated in a dark room at 25° C and 70 % relative humidity on moistened filter paper. Growing embryos were separated from the cotyledons after removing the seed coat at the required time. All subsequent isolation steps were carried out at 0° to 4° C and all glassware and buffers were prechilled.

Isolation of mitochondria. Mitochondria were isolated as described in a previous work (3).

Measurement of respiratory rate. Oxygen uptake and carbon dioxide output by the seeds was determined manometrically in a Warburg apparatus at 25° C. The seeds were kept in a moist atmosphere, that is, water was added into the sidearm of the flask but not into the main chamber containing the seeds.

Oxygen uptake of the mitochondrial fraction was measured polarographycally with a conventional Clark oxygen electrode (Yellow Springs Instruments Co.). All measurements were carried out at 25° C in a stirred chamber. All rates of oxygen consumption are expressed as nmoles O_2/min/mg mitochondrial protein. The total volume of the reaction mixture was 3.5 ml and the standard reaction medium had the following composition: 0.5 M mannitol; 0.2 % BSA; 15 mM potassium phosphate; 4 mM $MgCl_2$; 75 mM potassium phosphate buffer pH 7.2. The substrates, succinate, NADH or malate, were added to the reaction medium after 3 min of thermal equilibrium. When malate was used as respiratory substrate, glutamate was also added to the reaction medium (6). The respiratory rates were calculated from a recorder trace on the basis of 240 μM oxygen in aerated medium. The respiratory control (RC) and ADP/O ratio were determined from the oxygen electrode trace obtained upon addition of ADP, according to the method described by ESTABROOK (10).

Preparation of enzyme extracts. The suspension of mitochondria obtained as previously described was sonicated at 20 kcycles/s for 5 times of 45 s each, with an interval of 2 min between each sonication. The suspension was externally cooled with an ice-salt bath during the procedure. This fraction was designated as sonicated fraction. The sonicated fraction was centrifuged at 41.500 *g* for 2 h and the supernatant was designated as centrifuged sonicated fraction. The two sonicated fractions and the soluble fraction were used as a source of the enzymes without further purification.

Enzyme assays. Citrate synthetase (EC 4. 1. 3. 7) was assayed by the method of BOGIN and WALLACE (1). Isocitrate dehydrogenase (EC 1. 1. 2. 42.) was determined by the method of COX (4). Malate dehydrogenase (EC 1.1.1.37.) was determined by the method of DAVIES (5). Succinate dehydrogenase (EC 1. 3. 99. 1.) was assayed by the method of SINGER *et al.* (18) with a slight modification: the buffer used was Tris-HCl instead of HEPES. Succinate : cytocrome c oxido-reductase (EC 1. 9. 3. 1.) was determined by the method of DOUCE *et al.* (8).

All the protein determinations were made by the method of LOWRY *et al.* (15). Each value reported in the results was the average of at least three separate experiments.

Results

Respiratory activity of the seeds. Figure 1 shows the respiratory changes during the first 72 h of germination. The rate of O_2 uptake and CO_2 output, increases during the first 6 h, followed by a phase of stabilization and lastly by a rapid increase until 24 h, when a slower increase is observed. The respiratory quotient (RQ) is higher than unity in the first 18 h, falls to a value near unity and remains constant. The sharp respiratory increase and the shift in the RQ coincides with the protrusion of the radicle. If the testa is removed a great increase in oxygen uptake is obtained, especially during the first 18 h.

Effect of inhibitors on respiratory activity. Table I shows the effect of 10 mM NaF and 5 mM iodoacetate on respiratory

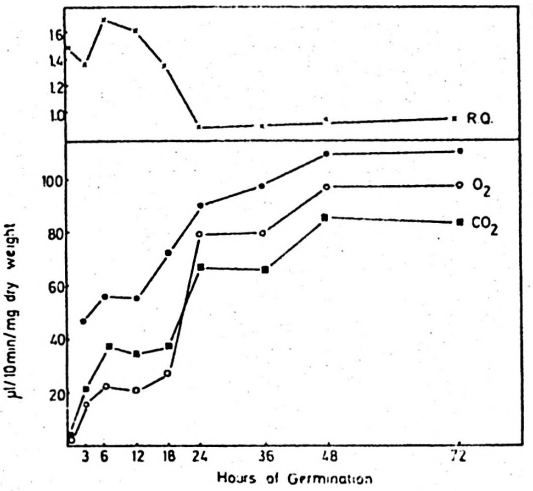

Fig. 1. *Respiratory activity of the complete seeds :oxygen uptake (○); carbon dioxide output (■); respiratory quotients (×); oxygen uptake without seed coat (●).*

activity. NaF shows no effect on gaseous exchange during the first 8 h of germination while it shows a progressive increase in the inhibition of the respiratory activity from 8 h onwards. The effect of iodoacetate was quite different since O_2 uptake and CO_2 evolution were considerably inhibited from the beginning of the germination time. This inhibition increases progressively reaching nearly total inhibition at 24 h.

Change in the enzymatic activities. Each of the assayed enzymes was present at low but significant levels in the ungerminated seeds. All the mitochondrial enzymes with the exception of malate dehydrogenase show a similar pattern of changes during germination (figs. 2 and 3). The activity increases during the first 12 h, remaining constant until 24 h. At this time a sharp rise in activity is observed reaching a maximum between 72 and 96 h. Changes undergone by the soluble fraction enzymes (isocitrate and malate dehydrogenases) show a similar pattern to that of mitochondrial malate dehydrogenase, reaching a maximum at 24 h followed by a very slight increase. Between 12 and 34 h the activity of the soluble isocitrate dehydrogenase is about twice the level of the mitochondrial one, thereafter the levels are very similar. The activity of the mitochondrial malate dehydrogenase is 10 to 15 times higher than that detected in the soluble fraction.

Respiratory activity of isolated mitochondria. Changes produced during the germination time in the oxidative and phosphorylative capacity of the isolated mitochondria are shown in table II. Isolated mitochondria were able to oxidize NADH, succinate and malate, NADH being the best oxidized substrate. The oxidative ability of the mitochondria follows a similar pattern of changes during germination with the three substrates. The respiratory rate in state 3 and 4 increases during the first 12 h, remains constant between 12 and 24 h and then a new and sharp rise in oxygen consumption is observed, reaching the maximum

Table I. *Effects of inhibitors on respiratory activity of germinating seeds of* Cicer arietinum L.

Hours of germination	Treatment	O_2 uptake µl/10 min/g d. w.*	Inhibition %	CO_2 output µl/10 min/g d. w.	Inhibition %
4	Control	15.4		20.3	
	NaF	15.2	1.3	20.2	—
	IA**	9.8	36.3	9.4	53.7
8	Control	24.2		36.4	
	NaF	24.1	—	36.3	—
	IA	14.0	42.3	14.0	61.6
16	Control	27.1		36.9	
	NaF	16.8	38.1	22.7	38.4
	IA	11.3	58.2	12.3	66.4
24	Control	81.5		72.9	
	NaF	24.9	69.5	40.9	43.9
	IA	8.0	90.2	8.1	88.9

* Dry weight; ** iodoacetate.

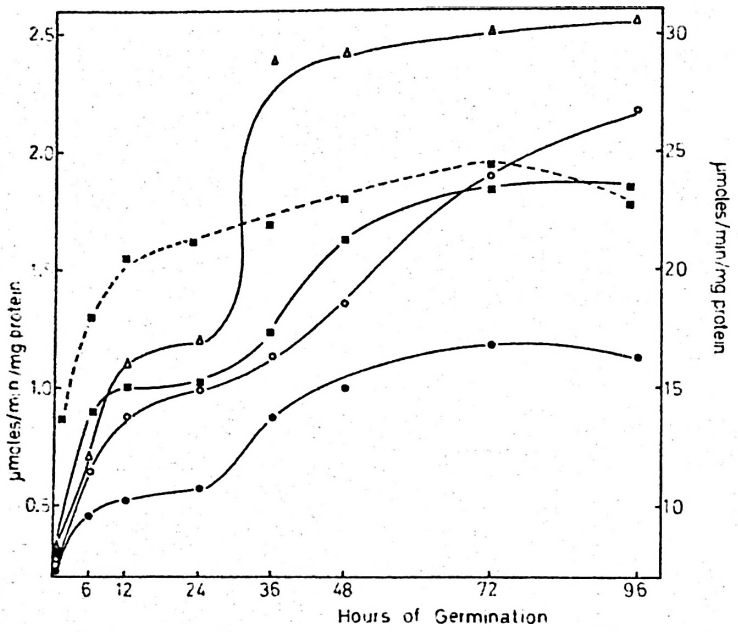

Fig. 2. *Citrate synthetase, succinate dehydrogenase, succinate: cytochrome c oxido-reductase and isocitrate dehydrogenase activities from cotyledons of germination chick pea seeds.*

Citrate synthetase (△) (mitochondrial centrifuged sonicated fraction; scale 10-30 µmoles/min/mg protein); succinate dehydrogenase (○) (mitochondrial sonicated fraction; scale 10-30 µmoles/min/mg protein); succinate: cytochrome c oxido-reductase (●) (mitochondrial sonicated fraction; scale 0.5-2.5 µmoles/min/mg protein); isocitrate dehydrogenase (- - ■ - -) (soluble fraction; scale 0.5-2.5 µmoles/min/mg protein); isocitrate dehydrogenase (—■—) (mitochondrial centrifuged sonicated fraction; scale 0.5-2.5 µmoles/min/mg protein).

at 96 h. The oxidative ability of the mitochondria isolated from dry seeds (0 time) is extremely low, they are uncoupled when NADH or succinate are the substrates used and they are unable to utilize malate until some imbibition time (6 h) has elapsed. The respiratory control and ADP/O ratio increases gradually during germination, reaching the maximum at 48 h, this is followed by a decline in both values. The behaviour of mitochondria isolated at 72 and 96 h of germination, i.e, the increase in oxidative ability but decrease in the respiratory control and ADP/O ratio could be explained by the presence in the chick pea mitochondria of an alternate pathway cyanide-resistant of electron transport which increases with germination time (3).

Discussion

The increase in O_2 uptake during germination, as has been observed in pea (12) and *Phaseolus mungo* (16) consists of several phases. In seeds of *Cicer arietinum* three phases can be distinguished: phase I, an initial rapid increase in the respiratory activity lasting 8 h; phase II, a plateau after swelling has been more or less completed; phase III, a second increase beginning at about 18 h when the seed envelope is broken and free gas exchange without limitation by

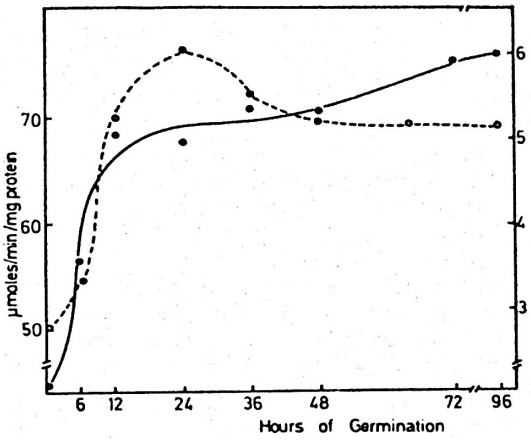

Fig. 3. *Malate dehydrogenase activity from cotyledons of germinating chick pea seeds.* Soluble fraction (○) (scale 0-6 µmoles/min/mg protein) and mitochondrial centrifuged sonicated fraction (●) (scale 0-70 µmoles/min/mg protein).

Table II. *Oxidative and phosphorylative activities of mitochondria isolated from cotyledons of germinating seeds of* Cicer arietinum L.

Substrate	Hours of germination	Oxygen uptake nmol/min/mg protein		Respiratory control	ADP/O ratio
		State 3	State 4		
Succinate (8 mM)	0	3.2	3.2	—	—
	3	10.3	8.7	1.2	0.6
	6	24.4	15.6	1.5	1.2
	12	36.4	18.0	2.0	1.3
	24	36.5	19.7	1.9	1.3
	36	52.5	25.5	2.0	1.3
	48	72.1	34.9	2.0	1.3
	72	78.7	40.0	1.9	1.2
	96	95.5	46.6	2.0	1.2
NADH (1 mM)	0	3.6	3.6	—	—
	3	13.1	8.9	1.4	0.6
	6	35.4	14.8	2.4	1.1
	12	61.1	22.2	2.7	1.3
	24	59.1	18.7	3.1	1.3
	36	91.4	27.4	3.3	1.3
	48	108.8	32.7	3.3	1.3
	72	128.7	44.3	2.9	1.1
	96	137.0	45.0	3.0	1.2
	3	4.9	4.9	—	—
	6	19.6	10.6	1.8	1.1
	12	17.4	9.1	1.9	1.4
	24	19.9	8.1	2.2	1.6
	36	28.6	13.5	2.2	1.6
	48	30.3	12.2	2.4	1.9
	72	37.9	15.9	2.3	1.7
	96	41.9	18.6	2.2	1.6

the testa becomes possible. These three phases can be observed not only in the complete seeds (fig. 1) but also in the isolated mitochondria (table II) and in the mitochondrial enzymatic activities (figs. 2 and 3).

During phases I and II the RQ was higher than unity, due probably to the fermentative activity observed during this time in chick pea cotyledons (2) as well as in other seeds (7, 14). The seed coat plays an important role in this fermentative activity, limiting oxygen supply to the seed, since its removal before the protrusion promotes an increase in oxygen uptake about three times higher than in the intact seed. Nevertheless, the changes in oxygen uptake in seeds germinated without the seed coat follow a similar pattern to that observed in intact seeds. Whereby, it seems possible that some other mechanisms besides the testa impermeability to oxygen could be involved in the fermentative activity observed during the first hours of germination (2). The behaviour of the metabolic inhibitors in *Cicer arietinum* is different from that observed in *Ph. mungo* (17) in which O_2 uptake was insensitive to iodoacetate and NaF during the early germination. In chick pea, O_2 uptake as well as CO_2 output were sensitive to iodoacetate from the very early germination.

The diminished oxidative ability of mitochondria isolated from dry or recently imbibed seeds could be explained, in agreement with WILSON and BONNER (20) by the fact that these mitochondria could be deficient in cyt. c and lack the tight coupling of phosphorylation to respiration.

The data presented here and those shown in a previous paper (2), indicate that the germination period between 18 and 24 h is a crucial one during the development of the seedling in *C. arietinum*. It is at the moment of the protrusion of the radicle that decline in the RQ and other metabolic changes take place. As shown previously (2), aldolase, triosephosphate deshydrogenase and pyruvate kinase, as well as alcohol dehydrogenase, reach maximal activity during this time, and inmediately, a pronounced decrease in their activity is observed. On the other hand, the activity of the TCA cycle enzymes and the respiratory activity reach a plateau during this period of germination, followed by a sharp rise in both activities.

As mentioned above, the testa plays an important role in this metabolic change, since its rupture by the radicle coincides with the second increase in O_2 uptake, the decrease in glycolytic and fermentative activities and the stabilization and further increase in the activity of the Krebs cycle enzymes and respiratory activity in mitochondria. Nevertheless, the metabolic regulation of the shift from anaerobic to respiration, may result from the fact that the mitochondrial structure is not well developed at this stage of germination, as was indicated by ELDAN and MAYER (9) in lettuce seeds and by SOLOMOS *et al.* (19) in pea cotyledons.

The RC values and ADP/O ratios of mitochondria isolated from chick pea cotyledons were lower than the expected theoretical ones. Similar results have been found in other plant tissues (11, 13). In agreement with LANCE (13) an increased participation of an alternate cyanide-resistant and non phosphorylating electron transport pathway can be adduced as a reason in order to explain this low activity. As a matter of fact we have detected in chick pea cotyledons this alternate pathway (3) which activity increases in parallel with germination time.

Resumen

Se han investigado los cambios respiratorios que se producen durante el período inicial de la germinación de semillas de *Cicer arietinum* L. En este período pueden distinguirse tres fases. El cociente respiratorio es superior a la unidad durante las fases I y II. El fluoruro sódico no afecta el intercambio gaseoso durante la fase I, mientras que en las fases II y III la inhibición de la actividad respiratoria incrementa progresivamente. El iodoacetato inhibe la absorción de oxígeno y la liberación de dióxido de carbono desde el principio de la germinación. La actividad de los enzimas mitocondriales aumenta en el transcurso de la germinación, principalmente a partir de las 24 horas. El control respiratorio y la relación ADP/O de las mitocondrias aisladas aumenta gradualmente durante la germinación de las semillas, alcanzando el máximo valor a las 48 horas. No obstante, los valores obtenidos fueron menores que los esperados teóricamente.

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