Mitochondria in the Endosperm of the Germinating Castor Bean: a Developmental Study

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The fatty endosperm tissue of the germinating castor-bean seedling in the space of a few days progresses from the resting condition in the dry seed, through a phase of increasing metabolic activity and growth, to one of decreasing activity and depletion of its substance as its reserves are transported to the growing seedling attached to it. In 8–9 days the tissue has thus completed a cycle of activation, growth and ageing which in many other plant tissues occupies a period of several weeks or months. It was therefore excellent material in which to study the changes that occur in the mitochondria of a developing and ageing tissue. To this end preparations have been made at daily intervals for the determination of such quantities as: mitochondrial content, microsomal content, the intrinsic oxidative activity of the mitochondria under optimum conditions, the efficiency of oxidative phosphorylation and sensitivity to the uncoupling agent dinitrophenol. A preliminary account of this work has appeared elsewhere (Akazawa & Beevers, 1956).

MATERIALS AND METHODS

Most of these have been described in the previous paper (Akazawa & Beevers, 1957). All of the mitochondrial experiments were carried out with the crude unfractionated suspensions which, as before, were prepared so that a known number of beans and weight of endosperm tissue was represented by the volume (1 ml.) of suspension used in each instance.

In experiments in which oxidative phosphorylation was measured, hexokinase (Sigma, Chemical Co., St Louis, Mo., U.S.A.; 1 mg./2 ml. of reaction mixture), glucose (0·05 M) and sodium fluoride (0·02 M) were present in addition to the full complement of cofactors described by Beevers & Walker (1956). An oxidation period of 30 min. was allowed and the reaction was terminated by transferring a 1 ml. volume to 5 ml. of cold M-$HClO_4$. Phosphate was determined according to Fiske & Subbarow (1925).

Adenosine triphosphatase activity determinations. Mitochondrial suspension or supernatant (1 ml.) was added to 5 mg. of adenosine triphosphate (sodium salt, Pabst Brewing Co., Milwaukee, U.S.A.; ATP) and incubated at 25° for 30 min. The liberation of inorganic phosphate was used as the measure of activity.

RESULTS

Changes in mitochondrial content with age of seedling

During the first 5 days of germination there is a progressive increase in the bulk of mitochondria, as judged by the size of the pellet obtained after centrifuging. The data in Fig. 1 show that there is, in fact, a striking and regular increase in mitochondrial N during this time. After reaching a level about four times that at the 2 days stage, there is a fall in the amount of mitochondrial N/bean.

These changes made it of interest to find whether similar ones occurred in the microsomal particles. The microsomal N remained almost unchanged during the first 5 days (Fig. 2); after 5 days there is a decline in microsomal N which occurs concomitantly with that in mitochondrial N. The results in

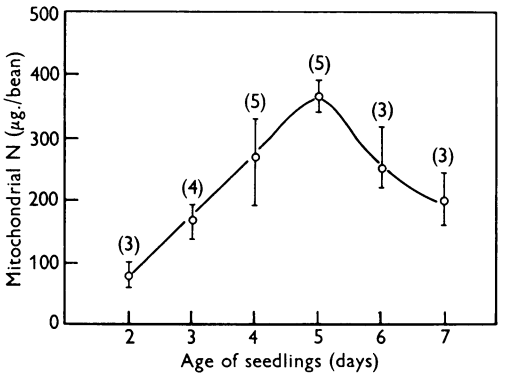

Fig. 1. Changes in the amount of endosperm mitochondrial N with age of seedling. (In Figs. 1, 3 and 4 the open points are the average values obtained in the stated number of experiments, and the vertical bar shows the extent of the variation encountered.)

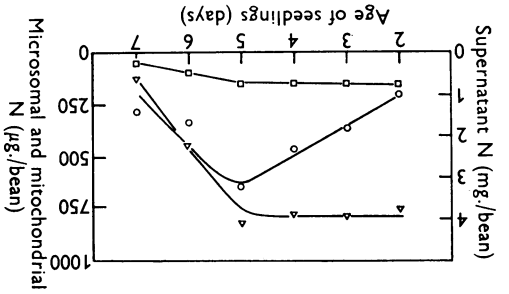

Fig. 2. Effect of age of the seedling on the amounts of total N contained in the supernatant (△), mitochondrial (○) and microsomal (□) fractions.

Fig. 2 also include values for the nitrogen content of the supernatant from the first centrifuging. In this series of experiments, determinations of the N content of mitochondria were made on unwashed sediments. It will be seen that even at its maximum, the percentage of N included in the two particulate fractions is less than 15 % of the total of the homogenate.

Changes in the q_{O_2} (μl. of O_2 absorbed/mg. of N/hr.) values with age of seedlings from which the mitochondria were extracted are shown in Fig. 3. The intrinsic activity of the mitochondria rises to a sharp maximum at 5 days and declines equally sharply thereafter.

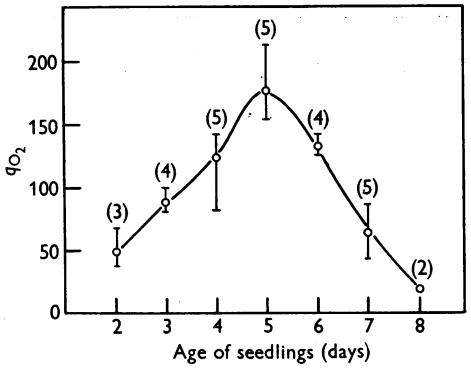

Fig. 3. Changes, with age of seedling, in the q_{O_2} values of the extracted mitochondria. Substrate was α-oxoglutarate.

Oxidative phosphorylation

Oxidative phosphorylation with a reasonably high efficiency occurs in mitochondria from 5 days seedlings which have, in addition to the usual complement of cofactors, the conventional hexokinase–glucose trapping system and fluoride (Table 1). The omission of either the trapping system or fluoride, or the addition of 2:4-dinitrophenol (DNP), leads to reductions in P/O (micromoles of phosphate esterified/microatoms of O_2 uptake) values. The addition of either hexokinase and glucose or DNP stimulates the O_2 uptake of the mitochondria. In subsequent experiments on oxidative phosphorylation the trapping system and fluoride were always added.

Table 2 shows the results of P/O determinations with a variety of substrates. It will be seen that in each case the oxidation is closely coupled to phosphorylation and satisfactorily high P/O values are obtained.

The changes which occur in phosphorylating efficiency as the seedlings age are shown in Fig. 4. The youngest seedlings provide mitochondria in which oxidation is tightly coupled with phosphorylation, for P/O values above 3 have been observed. Values considerably greater than 2 are obtained until the fifth day, after which there is an abrupt fall.

The effect of concentration of DNP on the O_2 uptake of mitochondria from 5-day-old beans is shown in Fig. 5. The greatest stimulation was observed at DNP concentrations of $1–2 \times 10^{-4}$ M. By using these concentrations, the changes in sensi-

Table 1. *Oxidative phosphorylation by 5 days mitochondria under different conditions*

The complete system contained 0·01 M-α-oxoglutarate, 0·05 M-glucose, 0·02 M-NaF, 1 mg. of adenosine triphosphate, 1 mg. of DPN, 0·5 mg. of cocarboxylase, 0·1 mg. of CoA, 1 mg. of hexokinase and 1 ml. of mitochondrial suspension in a volume of 2 ml.

	O_2 uptake (μatoms/30 min.)	Phosphate uptake (μmoles/30 min.)	P/O
Complete system	11·2	24·5	2·19
Without NaF	13·7	21·6	1·58
Without NaF and hexokinase	6·9	5·1	0·74
With DNP (2×10^{-4} M)	10·0	5·9	0·59
Without NaF and hexokinase; with DNP	17·4	0	0

Table 2. *Oxidative phosphorylation in the presence of different substrates*

Each system contained the complete additions shown in Table 1.

Substrate	Age of beans (days)	O_2 uptake (μatoms/30 min.)	Phosphate uptake (μmoles/30 min.)	P/O
α-Oxoglutaric acid	5	9·5	19·4	2·04
Malic acid	5	8·5	16·8	1·98
Citric acid	5	10·4	22·4	2·15
α-Oxoglutaric acid	3	5·9	12·3	2·1
Succinic acid	3	3·5	5·5	1·57
α-Oxoglutaric acid	5	10·5	20·8	1·98
Malic acid	5	11·0	22·0	2·0
Glutamic acid	5	12·8	25·6	2·0

tivity to DNP, which can be taken as a measure of the tightness of coupling, were examined, with the results shown in Fig. 6. The O_2 uptake of mitochondria from all seedlings younger than 5 days is greatly stimulated by DNP, but after this the response is smaller and has disappeared in 7-day-old seedlings.

One of the striking features of mitochondria from older endosperm is their high adenosine triphosphatase activity. The relationship between the development of this activity and the age of the seedlings is shown in Fig. 7. Adenosine triphosphatase activity is initially fairly low in the mitochondria, but a sharp increase begins after 5 days of germination; the activity of the supernatant, on the other hand, remains at a low level throughout.

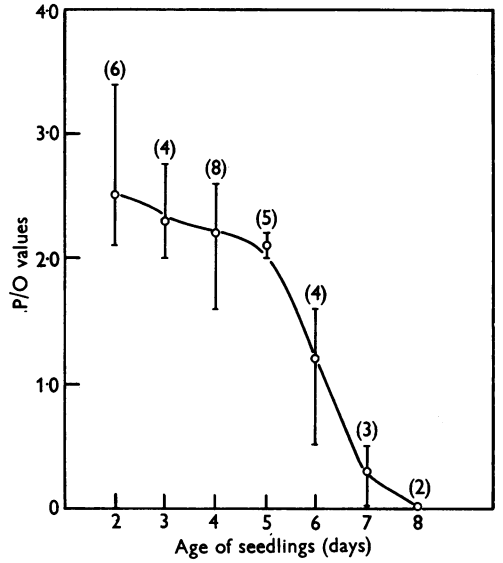

Fig. 4. Effect of age of the seedling on the efficiency of oxidative phosphorylation of the extracted mitochondria. Substrate was α-oxoglutarate.

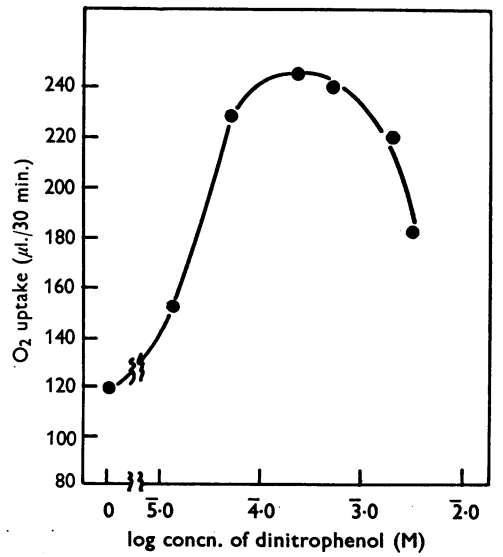

Fig. 5. Effect of a range of DNP concentrations on the O_2 uptake of mitochondria from 5-day-old beans. Substrate was α-oxoglutarate.

DISCUSSION

The results show clearly that there are striking differences in the amounts of mitochondrial nitrogen extractable by the present methods from beans of different ages. Further, in two important attributes, namely their intrinsic oxidative activity and their phosphorylating efficiency, the mitochondria show equally remarkable changes with age. If, as seems likely, these changes in activity reflect the properties of the native mitochondria *in vivo*, they are clearly of importance and must be considered, along with the differences in mitochondrial content, in attempts to gain an insight into the metabolism of the tissue itself.

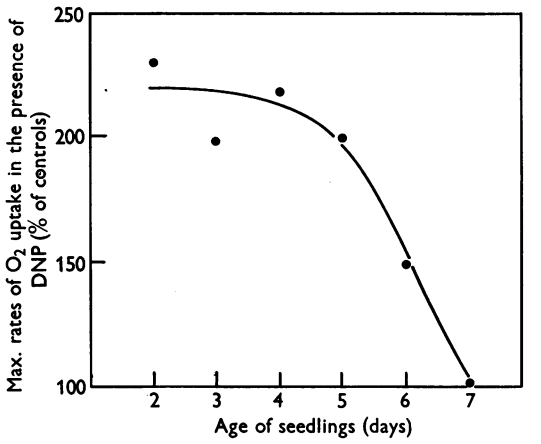

Fig. 6. Changes in the mitochondrial response to DNP during ageing of the seedling. (DNP, $1\text{–}2 \times 10^{-4}$ M.)

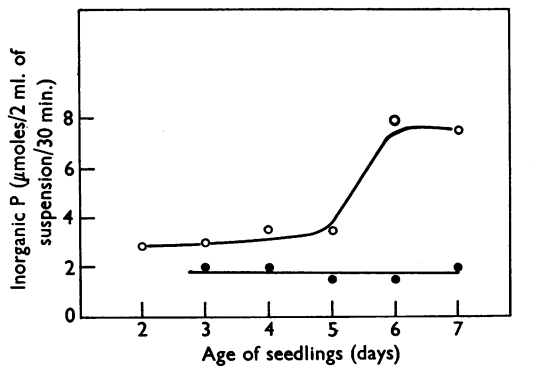

Fig. 7. Effect of age of the seedling on the adenosine phosphatase activities of mitochondria (○) and supernatant (●).

The production of new mitochondrial material during the early stages of germination increases the potential oxidative activity of the endosperm, and this production coincides with the increases in oxygen uptake actually observed in the intact tissue (Beevers & Walker, 1956). During this period of increasing mitochondrial nitrogen there is no decrease in the level of microsomal nitrogen. There is no evidence then in the castor-bean endosperm for the reciprocal changes in these constituents which have been described recently by Lund, Hanson & Kahn (1956) in growing corn roots.

During the first 5 days, as germination proceeds, not only is there an increase in the mitochondrial content, but the mitochondria themselves have higher intrinsic activity. The results of analyses for various components (Akazawa & Beevers, 1957) give no clue as to what might be responsible for the increasing q_{O_2} values of mitochondria, since the relative amounts of the components did not alter noticeably. Whatever the nature of this 'activation' it is clearly not accomplished at the expense of phosphorylating efficiency, because the P/O values are quite high.

This phase is succeeded by one of decreasing mitochondrial nitrogen and lowered q_{O_2} values as the endosperm ages, softens and loses its substance to the seedling proper. However, it appears that the potential usefulness of the mitochondria to the metabolism of the endosperm during this period is not limited solely by their decreasing amount and reduced q_{O_2} values. From the fifth day onwards there is a very sharp decline in the phosphorylating efficiency, as shown by the fall in the P/O value; by 7 days this ratio is very low indeed.

These changes in phosphorylating ability are reflected in the degree of stimulation of oxygen uptake by DNP. With mitochondria from the younger seedlings, the oxygen uptake is more than double the control rates, but subsequently the response decreases and none is observed in 7 days mitochondria. This seems to be the first report of stimulation of oxygen uptake of plant mitochondria by DNP.

It will be recalled (Akazawa & Beevers, 1957) that whereas the protein, phospholipid and RNA contents of the mitochondria declined together after the 5 days stage, that of the acid-soluble phosphate ('nucleotide') fraction showed a much steeper decline, which began somewhat earlier. It is now suggested that this change might be the underlying cause of the subsequent decline in oxidative and phosphorylative ability, and that the decrease in acid-soluble phosphate presages the early demise of the mitochondria. It is, of course, quite conceivable that at the 4 days stage some physical change occurs in the mitochondria which renders them 'leaky' and so instigates the progressive loss in acid-soluble phosphate. A further possibility, which might be difficult to separate from this, is that there is a breakdown of the components of the acid-soluble-phosphate fraction within the mitochondria themselves. It is significant in this regard that the adenosine triphosphatase of the mitochondria, although initially low, shows a striking rise after 5 days; but again, while this must certainly be of significance in limiting the oxidative, and more particularly the phosphorylative, abilities of the older native mitochondria, it may conceivably merely reflect their deteriorating physical condition. In this connexion it should be pointed out that it is not the increase in adenosine triphosphatase *per se* which is responsible for the lower P/O values at the later stages, since the enzyme was shown to be inhibited at the fluoride levels used during the P/O determinations.

The conclusions are that whereas in the first 5 days of germination the increasing metabolic activity of the endosperm is paralleled by changes in the content of mitochondria, which have a high respiration and phosphorylating ability, the subsequent declines in mitochondrial content and, more particularly, in q_{O_2} and P/O values, are such as to lower progressively any useful contribution of the mitochondria to the metabolism of the endosperm, which is, of course, approaching the end of its existence.

SUMMARY

1. Mitochondria prepared daily from the endosperms of germinating castor beans have been used to study changes in: (*a*) the amount of mitochondrial nitrogen/bean; (*b*) the q_{O_2} with α-oxoglutarate as substrate; (*c*) the phosphorylating efficiency (P/O ratios with α-oxoglutarate as substrate); (*d*) the sensitivity to 2:4-dinitrophenol; (*e*) the adenosine triphosphatase activity.

2. During the first 5 days there are striking increases in (*a*) and (*b*), the P/O values are considerably greater than 2, and the addition of dinitrophenol more than doubles the rate of oxygen uptake.

3. After 3 days there are marked decreases in (*a*), (*b*), (*c*) and (*d*) and an increase in (*e*).

4. The bearing of these changes on the metabolism of the endosperm tissue is discussed.

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