OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA FROM GERMINATING PEAS

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SUMMARY

1. The P/O ratio, with succinate, of mitochondria from the cotyledons of peas was 1.0–1.5 during the first 2 days of germination; from the third day on it decreased till zero at the fifth day.
2. The uncoupling was caused by a mixture of long-chain fatty acids, which are formed during the homogenization of the tissue.
3. The uncoupling, therefore, is not due to changes in the mitochondria during germination.

1. INTRODUCTION

The activities of mitochondria from germinating seeds have been reported to alter with the age of the seedling. STANLEY (1957) found that the capacity of mitochondria from the endosperm of sugar pine seeds to oxidize succinate, decreased after six days of germination, whereas the capacity to oxidize citrate increased till the tenth day. The capacity of a particulate fraction from the endosperm of germinating castor beans to oxidize succinate was shown to decline after the fourth day (BEEVERS & WALKER 1956). AKAZAWA & BEEVERS (1957) found that these particles from the castor bean showed a maximum of α-oxoglutarate oxidation on the fifth day, whereas the P/O ratio declined from the fifth day, reaching zero at the seventh day of germination.

CHERRY (1963) reported that the oxidative and phosphorylative activities of isolated mitochondria from peanut seeds showed a maximum after eight days of germination, followed by a decline. In the present study the cause of the changes in mitochondrial activity during germination was investigated.

2. MATERIALS AND METHODS

2.1. Germination

Pea seeds (var. Alaska) were soaked in water for ca. 8 hrs. They were then placed in moist, sterilized vermiculite, and kept at 20–25° in the dark.

2.2 Mitochondria

All operations were carried out at 0–4°. The cotyledons were cut from the seedlings and cooled in ice for ca. 2 hr. They were homogenized in a mortar with sand, in a solution containing 0,5 M-sucrose and 0,03 M-KH_2PO_4 adjusted to pH 7.4 with NaOH, (4 ml/g tissue). The pH of the homogenate was 6.9 to 7.2 After filtration through double cheese-cloth, the homogenate was centrifu-

ged at 1500 *g* for 7 min., and the supernatant at 12000 *g* for 20 min. By means of a hand-driven Potter-Elvehjem homogenizer, the 12000 *g* pellet was suspended in fresh buffered sucrose solution (2 ml/g tissue) and recentrifuged. The pellet was taken up in buffered sucrose solution (0.5 ml/g tissue). The intensely green mitochondrial suspension was used immediately in the experiments.

2.3. Respiration

Oxygen uptake was measured in the Warburg manometer in air at 30°. No correction was made for the, relatively low, endogenous respiration. The flasks contained in the main compartment 1 ml of 0.2 M-sucrose, 0.3 M-glucose, 0.02 M-NaF, 0.012 M-$MgCl_2$, 0.0015 M-sodium-ADP and 1 mg/ml hexokinase. The side arm contained 1 ml of 0.5 M-sucrose and 0.06 M-sodium succinate. Both solutions were adjusted to pH 7.4 with NaOH. To the main compartment 1 ml of the mitochondrial suspension, containing 5–9 mg. of protein (from 2 g of tissue), was added. After equilibration for 10 min. the substrate was tipped in. After 30 min. the reaction was stopped by adding 1 ml 20% trichloroacetic acid. In the deproteinized solution inorganic phosphate was determined by the method of HURST & BECKING (1963). In some experiments the glucose-6-phosphate produced was measured according to SLATER (1953). Protein was determined by the method of LOWRY *c.s.* (1951).

2.4. Extraction of supernatant

Protein was removed by precipitation with 4 volumes of cold acetone. After centrifugation, the acetone was removed by distillation under nitrogen gas at diminished pressure. The residual aqueous solution was extracted three times with ether. The extract was dried on $CaCl_2$ and after removal of the ether with a stream of nitrogen, the remaining green oil was taken up in absolute ethanol or light petroleum (b.p. 60–80°)

2.5. Chromatography

For thin-layer chromatography, plates of Kieselgel HF (E. Merck, Darmstadt, Germany) were prepared. The plates were developed with light petroleum (b.p. 40–80°) – ether – acetic acid (90:10:1, by vol.) The spots were made visible by spraying with a 1% iodine solution in methanol.

For gas-liquid chromatography, the methyl esters of the fatty acids were prepared as described by DE BOER & BACKER (1954). Diethyleneglycolsuccinate was the stationary phase and helium gas the mobile phase.

2.6. Free fatty acids

Cotyledons and homogenates were extracted as described by DOLE (1956). The fatty acids could not be titrated because the extract had a yellow-green

colour. They were separated from the other components by thin-layer chromatography, as above, After elution from the gel with chloroform the fatty acids were determined by means of the copper method of DUNCOMBE (1963); oleic acid was used as a standard.

3. RESULTS

3.1. Uncoupling

Table 1 shows that the oxidative capacity of mitochondria from cotyledons diminishes with the period of germination, and that the phosphorylating capaci-

Table 1. Oxidative phosphorylation by mitochondria from cotyledons of germinating peas.

Germination time (days)	Phosphate esterified (μmoles/30 min.)	O_2 uptake (μatoms/30 min.)	P/O
1	8.4	6.9	1.22
2	6.9	5.2	1.34
3	6.3	7.6	0.83
4	1.6	8.8	0.18
5	0.4	6.4	0.06
6	-0.3	5.7	0
7	-0.1	3.7	0
8	-0.3	4.2	0
10	0.0	2.2	0

ty falls rapidly after about two days. As shown in *table 2*, the mitochondria from peas germinated for two days become uncoupled by adding the supernatant from peas germinated for five days. On the other hand, the mitochondria from 5-days peas are recoupled by adding the supernatant from 2-days peas, or by washing with this supernatant. These observations are most readily explained by assuming the presence of a supernatant factor which restores coupling during the first days of germination and, later on, a supernatant factor with uncoupling activity. Restoration of the phosphorylating capacity was also obtained by adding bovine serum albumin *(tables 3* and *4)*. Washing the mitochondrial pellet with a 1% solution of serum albumin restored the phosphorylation up to the seventh day of germination, and by including extra albumin in the Warburg flasks, even mitochondria from 8-days peas were capable of phosphorylation. From the requirement for ever larger amounts of serum albumin it is clear that the amount of uncoupling factor present on the mitochondria increases during germination.

3.2. Identification of the uncoupling factor

From the supernatant fraction of 5-days peas, which has a strong uncoupling

Table 2. Effect of supernatant on oxidative phosphorylation.

Exp. no.	glucose-6-phosphate produced (μmoles/30 min.)	O_2 uptake (μatoms/ 30 min.)
1 2-day mitochondria	17,8	13,7
2-day mitochondria + 1 ml. 5-day supernatant	1,6	20,7
no mitochondria; 1 ml. 5-day supernatant	0,4	3,6
2 5-day mitochondria	3,2	15,3
5-day mitochondria + 1 ml. 2-day supernatant	25,9	30,0
no mitochondria; 1 ml. 2-day supernatant	4,9	1,1
3 5-day mitochondria	6,2	23,7
5-day mitochondria, washed in 2-day supernatant	34,1	27,2
5-day mitochondria + protein from 1,3 ml 2-day supernatant	22,9	30,3

[1] Protein was precipitated by trichloroacetic acid; the sediment was washed twice with buffered sucrose solution.

Table 3. Effect of bovine serum albumin on 5-day mitochondria.

mg bovine serum albumin added to the flasks	glucose-6-phosphate produced (μmoles/30 min.)	O_2 uptake (μatoms/30 min.)
none	2,3	14,4
10	11,2	28,0
30	37,4	29,1
90	42,4	31,7
180	39,8	30,2

effect, the active principle can be extracted with ether *(table 5)*. The extract of the supernatant from 7-day peas was separated by thin-layer chromatography into 5 components. These were eluted from the gel with methanol. When added, in ethanolic solution, to mitochondria from 1-day peas, only one component showed uncoupling activity. It was identified as a mixture of long-chain fatty acids in the following way: stearic and oleic acids co-chromatographed with the active component, and gas-chromatographic analysis showed it to be a mixture of about 70% $C_{18:2}$ and about 10% C_{16} and $C_{18:1}$ each. Very small quantities of C_{18} and of an unknown substance were present. We thus may conclude that the uncoupling of mitochondria is caused by long-chain fatty acids. The uncoupling capacity of fatty acids is demonstrated in *table 6* with

Table 4. Restoration of oxidative phosphorylation by bovine serum albumin (BSA) during germination

Mitochondria were washed in buffered sucrose solution enriched with 1% BSA (w/v). They were given a second wash in the normal buffered sucrose solution (A). The effect of including extra BSA is shown under B. The mitochondria were of the same batches used in Table 1.
ΔP, μmoles phosphate esterified in 30 min;
ΔO, μatoms oxygen taken up in 30 min.

	A				B		
germination time (days)	ΔP	ΔO	P/O	mg BSA added to flasks	ΔP	ΔO	P/O
1	8.8	6.2	1.42	30	8.0	6.2	1.29
2	6.1	5.5	1.12	30	7.4	5.6	1.31
3	7.3	7.8	0.94	30	7.2	7.4	0.97
4	9.3	9.3	1.00	30	8.3	7.8	1.05
5	8.3	9.5	0.88	30	9.3	8.8	1.05
6	9.2	11.7	0.80	30	12.0	10.5	1.14
7	1.1	8.9	0.12	30	7.5	10.3	0.73
8	0.9	8.5	0.10	90	5.6	8.9	0.63
10[1]	1.1	5.3	0.21	90	0.5	6.7	0.08

[1] incubation time was 60 min.

Table 5. Effect of extracts from supernatants on oxidative phosphorylation by 2-day mitochondria.

0.1 ml of extract in absolute ethanol corresponds to 1.3 ml of supernatant.

addition to the flask	glucose-6-phosphate produced (μmoles/30 min.)	O_2 uptake (μatoms/30 min.)
0.1 ml. ethanol	24.2	16.4
0.1 ml. extract 2-day supernatant	20.0	15.8
0.1 ml. extract 5-day supernatant	5.3	13.9

oleic acid. Bovine serum albumin is shown to reverse the effect of the fatty acid. From the data in *table 6* it can be calculated that 1 mole of serum albumin binds 6 moles of oleic acid. This is in good agreement with the number of 7 moles bound per mole serum albumin which has been found by BJÖRNTORP *c.s.* (1964).

3.3. Source of the fatty acids

The question now arose whether the fatty acids were liberated as a consequence of the homogenization, or were already present in the intact seed. *Fig. 1* shows that the fatty acid content of the seeds remains low during germination. The amount in the homogenate is also low during the first three days, but rises strongly on the fourth day, in parallel with the uncoupling of the mitochondria.

Table 6. Effect of oleic acid and bovine serum albumin (BSA) on 2-day mitochondria. A solution of oleic acid in hexane was pipetted into the flasks, and the solvent removed by a stream of nitrogen.

Oleic acid to the flask	Phoshate esterified (μmoles/30 min.)	O_2 uptake (μatoms/30 min.)	P/O
none	16.1	10.8	1.49
0,3 μmole	12.6	9.9	1.28
0,9 μmole	6.6	10.0	0.65
3,6 μmole	0.0	6.2	0.0
3,6 μmole + 30 mg BSA	11.0	10.8	1.02

It is concluded that the loss of phosphorylating capacity of the mitochondria during germination normally does not occur in vivo, but is an artifact of preparation. (The uncoupling at the tenth day may have another cause, since there was no accumulation of fatty acids in the homogenate of 10-day peas.)

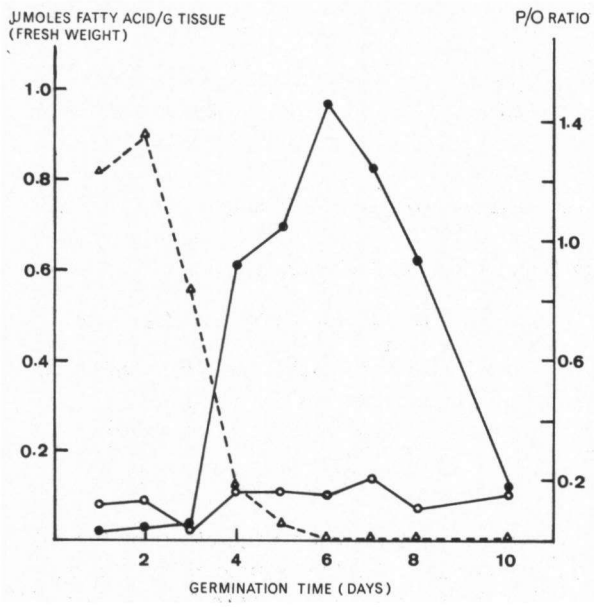

Fig. 1. Fatty acid content of cotyledons and their homogenates during germination. Cotyledons were homogenized in a Waring Blendor for 30 sec. The homogenate was kept in ice for 30 min. and subsequently, was extracted (DOLE 1966). Extraction of the cotyledons themselves was carried out by homogenizing in the presence of Dole's extraction medium. o, content of the cotyledons; •, content of homogenate after 30 min. at 0°; △, P/O ratio (taken from table 1).

4. DISCUSSION

Our results show that the decrease in phosphorylation – and to some extent also in respiration – during germination, is not due to alterations in the mitochondria *themselves*, but to an increased concentration of free fatty acids on the isolated mitochondria. Unlike AKAZAWA & BEEVERS (1957), we therefore believe that the decrease probably is not a physiological phenomenon, but an artifact

of isolation which is due to the changed metabolic state of the tissue after several days of germination. Similar effects have been observed in a number of animal tissues (WOJTCZAK & LEHNINGER 1961; WOJTCZAK & WOJTCZAK 1960; CHEFURKA 1964, 1966).

An effect of endogenous fatty acids on mitochondria from plant tissue homogenates has been demonstrated by DALGARNO & BIRT (1962). Accumulation of fatty acids in homogenates of cotyledons from castor beans, cotton seeds and peanuts after some days of germination has been observed by ST. ANGELO & ALTSCHUL (1964).

Our results are, however, in complete disagreement with those of YOUNG *c.s.* (1960). With the same variety of peas and the same methods, these authors still found a P/O ratio of 0.4 on the tenth day.

In conclusion, our results illustrate the point made by BENSON (1964) that, "Lipase action is an ever present problem in analysis of plant cell fractions".

ACKNOWLEDGEMENT

The investigation was carried out with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z. W. O.).

REFERENCES

AKAZAWA, T. & H. BEEVERS (1957): Mitochondria in the endosperm of the germinating castor bean: A developmental study. *Biochem. J.* **67**:115–118.

BEEVERS, H. & D. A. WALKER (1956): The oxidative activity of particulate fractions from germinating castor beans. *Biochem. J.* **62**:114–120.

BENSON, A. A. (1964): Plant membrane lipids. *Ann. Rev. Plant Physiol.* **15**:1–13.

BJÖRNTORP, P., H. A. ELLS & R. H. BRADFORD (1964): Albumin antagonism of fatty acid; effects on oxidation and phosphorylation reactions in rat liver mitochondria. *J. Biol. Chem.* **239**:339–344.

BOER, TH. J. DE & H. J. BACKER (1954): A new method for the preparation of diazomethane. *Rec. Trav. Chim. Pays-Bas* **73**:229–234.

CHEFURKA, W. (1964): Dinitrophenol-induced adenosinetriphosphatase activity in relation to in vitro aging of mitochondria. *Abstracts 6th Int. Congress Biochem. (New York)* VIII: 646.

CHEFURKA, W. & T. DUMAS (1966): Oxidative phosphorylation in in vitro aged mitochondria. *Biochemistry* **5**: 3904–3911.

CHERRY, J. H. (1963): Nucleic acid, mitochondria, and enzyme changes in cotyledons of peanut seeds during germination. *Plant Physiol.* **38**:440–446.

DALGARNO, L. & L. M. BIRT (1962): Free fatty acids in carrot-tissue preparations and their effect on isolated carrot mitochondria. *Biochem. J.* **87**:586–596.

DOLE, V. P. & H. MEINERTZ (1960): Microdetermination of longchain fatty acids in plasma and tissues. *J. Biol. Chem.* **235**:2595–2599.

DUNCOMBE, W. G. (1963): The colorimetric microdetermination of long-chain fatty acids. *Biochem. J.* **88**:7–10.

HANSON, J. B., C. M. WILSON, M. J. CHRISPEELS, W. A. KRUEGER & H. R. SWANSON (1965): Ribonuclease and other factors involved in the respiratory senescence of maize scutellum. *J. Exp. Bot.* **16**:282–293.

HURST, R. O. & G. C. BECKING (1963): Hydrolysis of calf thymus DNA by pancreatic DNAse *Canad. J. Biochem. Physiol.* **41**:469–480.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL (1951): Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–276.

SLATER, E. C. (1953): Spectrophotometric determination of fructose-1,6-diphosphate, hexose-monophosphates, ATP and ADP. *Biochem. J.* **53**:157–167.

ST. ANGELO, A. J. & A. M. ALTSCHUL (1964): Lipolysis and the free fatty acid pool in seedlings. *Plant Physiol.* **39**:880–883.

STANLEY, R. G. (1957): Krebs cycle activity of mitochondria from endosperm of sugar pine seed. *Plant Physiol.* **32**:409–412.

WOJTCZAK, L. & A. B. WOJTCZAK (1959): Uncoupling of oxidative phosphorylation and inhibition of ATP-P_i exchange by a substance from insect mitochondria. *Biochim. Biophys. Acta* **39**:277–286.

WOJTCZAK, L. & A. L. LEHNINGER (1961): Formation and disappearance of an endogenous uncoupling factor during swelling and contraction of mitochondria. *Biochim. Biophys. Acta* **51**:442–456.

YOUNG, J. L., R. C. HUANG, S. VANECKO, J. D. MARKS & J. E. VARNER (1960): Conditions affecting enzyme synthesis in cotyledons of germinating seeds. *Plant Physiol.* **35**:288–292.