RESPIRATION RATE AND MITOCHONDRIAL ACTIVITY IN THE COTYLEDONS OF *PISUM SATIVUM* L. DURING GERMINATION

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SUMMARY

From parallel observations on changes in respiration rate *in vivo* and mitochondrial activity *in vitro* it is concluded that the respiration of pea cotyledons during germination is mediated by enzymes of the citric acid cycle and of the electron transfer chain. Its course may be divided into four phases. The sharp rise in respiration rate in the first phase is due to activation by hydration of the enzymes of the two systems during swelling. The fairly constant respiration level in the subsequent phase is only to a minor extent due to a limited oxygen supply but primarily to the full hydration of the enzymes of the electron transfer chain present in the air-dry cotyledons with a certain potential activity. The increase in respiration rate in the third phase is attributed to a rise in activity of the mitochondrial enzymes, whereas the decrease in respiration rate in the fourth phase seems to be due to a disorganization of the subcellular structure of the cotyledons.

1. INTRODUCTION

The germination of seeds starts with the uptake of water. PERNER (1965), ÖPIK (1965), BAIN & MERCER (1966) observed that this uptake is accompanied by the development of a complex subcellular organization in the cotyledons and axis tissue of the seeds. The concomitant rise in metabolic activity is probably partly due to the activation by hydration of enzymes already present in the dry seeds (ÖPIK & SIMON 1963).

The continuing rise in oxygen uptake during germination has been attributed to several mechanisms. MAPSON & MOUSTAFA (1956) and ÖPIK (1965) suppose that soluble oxidases account for this rise. YOUNG *et al.* (1960) and CHERRY (1963) observed an increase in mitochondrial activity, which suggests an important role of the enzymes of the electron transfer chain in this respiration process. Already PFEFFER (1885) and more recently DAVISON (1949), GOKSÖYR *et al.* (1953) and SPRAGG & YEMM (1959) stressed the importance of anaerobiosis in the germination process of seeds.

The object of the present paper is, by comparing the *in vivo* respiration rate measurements with *in vitro* experiments, to assess the role of the citric acid cycle and of the electron transfer chain in the respiration process of pea cotyledons, and also to find indications about the factors controlling the changes in respiration rate.

2. MATERIALS AND METHODS

2.1 Seeds and germination

Whole pea seeds, var. Rondo, selected on equal air-dry weight and equal colour,

were soaked under aeration for 20–22 hrs in tap water and next germinated for the appropriate period on moist filter paper in Petri dishes. Swelling and germination occurred in the dark at 23° C. The period of germination was measured from the time the seeds first came in contact with water to the removal of the cotyledons for experiment. In some experiments prior to soaking the seed coat or both seed coat and axis tissue were carefully dissected from the cotyledons of the air-dry seeds. This will be referred to as naked germinated seeds or excised cotyledons.

2.2 Degree of swelling

In accordance with KÜHNE & KAUSCH (1965) the degree of swelling is defined as the water content expressed in per cents of the dry weight of the cotyledons. Consequently, the degree of swelling was calculated according to the formula [(fresh weight minus dry weight)/dry weight] × 100. The dry weight of the cotyledons is the weight after drying at 105° C for 20 hrs. The dry weight of a sample of cotyledons can be calculated from the fresh weight by means of the swelling time curve.

2.3 Isolation of the mitochondrial fraction from swollen cotyledons

All apparatus and solutions were cooled to 0–5° C before use, and the extracts obtained kept at this temperature until the start of the experiments. The prechilled cotyledons (10 g fresh weight) were ground in a mortar with an equal weight of sand, adding 1 ml of grinding medium containing 0.4 M sucrose and 0.05 M phosphate buffer (pH 7.2) per gram of cotyledons. The resulting slurry was filtered through 8 layers of aseptic gauze, diluted to 35 ml with the grinding medium, and the filtrate centrifuged at 1500 g for 10 min. Next the supernatant fraction was centrifuged at 20,000 g for 15 min. The pellet thus obtained will be referred to as the unwashed mitochondrial fraction. The pellet was resuspended in a medium containing 0.2 M sucrose and 0.05 M phosphate buffer (pH 7.6), to give the equivalent of particles from 1 gram fresh weight of cotyledons per ml of suspension (1g/ml). The pellet was resuspended in 20 ml grinding medium and again centrifuged at 20,000 g for 15 min, in order to obtain a washed mitochondrial fraction. The washed pellet was resuspended in a medium containing 0.2 M sucrose and 0.05 M phosphate buffer (pH 7.2), to give the equivalent of particles from 2 grams of fresh weight of cotyledons per ml suspension (2 g/ml).

2.4 Isolation of the mitochondrial fraction from air-dry cotyledons

Excised cotyledons from air-dry seeds were pulverized with a Braun "multimix". An equal weight of tap water was added to the powder, that was allowed to imbibe for 2 hrs at 0–5° C. From this moment the imbibed powder was treated in the same way as the fresh cotyledons.

2.5 Fragmentation of the mitochondria

A suspension of unwashed mitochondria (1 g/ml) in 0.05 M phosphate buffer

(pH 7.6) was usually fragmented by freezing (ethanol-solid CO_2 mixture) and thawing (0–5° C) three times. In some experiments the suspension was exposed to sonic treatment of 800 KHz in a Lehfeldt sonifier for 3 min at 200–250 Watt. The supernatant obtained after centrifuging the frozen and thawed suspension or the sonicated suspension at 20,000 g for 15 min was used in the standard assays for fumarate hydratase and aconitate hydratase. The sediment was resuspended in a volume equal to the original unwashed mitochondrial suspension.

2.6 General remarks on enzyme activities

All enzyme assays were performed at 25° C, with the exception of NADH- cyt. c oxidoreductase which was performed at 29–31°C. The rate of reaction was always proportional to the amount of enzyme added. Enzyme activities were calculated from a linear portion of the reaction rate time curve. The enzyme activities – calculated for dry weight base – were not corrected for the decrease (about 15%) of the dry weight of the cotyledons taking place during the germination process. Their activities are expressed in units as indicated in the figures.

2.7 Respiration of whole cotyledons

The respiration of whole cotyledons was measured by the Warburg manometric technique. Five cotyledons were placed in the main compartment of the Warburg flask with 0.2 ml 10% KOH in the centre well. During the measurements the cotyledons were kept in moist atmosphere, 0.4 ml water being present in the side-arm.

2.8 Succinate oxidase system

Oxygen uptake by the isolated mitochondria was measured in a conventional Warburg apparatus. The main compartment of the vessel contained, in a total volume of 2.4 ml with a final pH of 7.2: 0.02 M sodium succinate; 0.01 M tris-HCL buffer; 0.0125 M phosphate buffer; 0.2 M sucrose; 0.01 M NaF; 5mM $MgCl_2$; 0.3 mM NAD; 0.3 mM NADP; 0.01 mM cytochrome c; 1 mM ADP and 0.6 ml of a washed suspension of mitochondria (2 g/ml).

This reaction mixture was intended originally for the oxidation of both succinate and other intermediates of the citric acid cycle.

2.9 Succinate dehydrogenase (succinate : (acceptor) oxidoreductase, EC 1.3.99.1.)

Succinate dehydrogenase was measured spectrophotometrically by the reduction of 2,6-dichlorophenol-indophenol in the presence of phenazine methosulphate (PIERPONT 1963). The reaction mixture contained in a final concentration: 0.04 M sodium succinate; 0.05 M phosphate buffer; 0.2 M sucrose; 0.01 M KCN; phenazine methosulphate (0.3 mg/ml); 0.03 mM 2,6 dichlorophenol-indophenol and 0.1 ml unwashed suspension of mitochondria (1 g/ml) in a final

volume of 3 ml with a final pH of 7.6 The dichlorophenol-indophenol was omitted from the optical blank. The rate of reduction was always corrected for the partly non-enzymic reduction that occurred without added succinate. Enzyme activity was calculated from the observed decrease in E_{600nm} by using a molecular extinction coefficient of $20.5 \times 10^3 M^{-1}cm^{-1}$. Enzyme activity was not corrected for infinite dye concentrations (ARRIGONI & SINGER 1962).

2.10 Nadh:cytochrome c oxidoreductase (EC 1.6.99.3.)

Enzyme activity was measured spectrophotometrically by a slight modification of the method of NASON & VASINGTON (1963). The reaction mixture contained: 0.05 M phosphate buffer; 0.2 M sucrose; 5 mM KCN, cytochrome c (0.15%); 0.3 mM NADH and 0.1 ml of a suspension of unwashed mitochondria in a final volume of 2 ml and a pH 7.6. At zero time the reduced NAD solution was added to the cuvette, and within 15 sec the increase in E_{5000nm} was measured for 6 min in a Unicam S.P. 800 recording spectrophotometer against a cuvette without NADH as an optical blank. Enzyme activity was calculated by using a molecular extinction coefficient of $18.5 \times 10^3 M^{-1}cm^{-1}$.

2.11 Fumarate hydratase (L-malate hydro-lyase EC 4.2.1.2.) and aconitate hydratase (citrate hydro-lyase EC 4.2.1.3.)

Both enzymes were measured spectrophotometrically by a slight modification of the method of RACKER (1950). In final concentrations the reaction mixture for fumarate hydratase contained: (PIERPOINT 1960) 0.04 M sodium malate; 0.045 M tris-HCl buffer; 0.6 mM cysteine and 0.3 ml of an enzyme preparation in a final volume of 3 ml and a pH of 8.0.

The reaction mixture for aconitate hydratase contained 0.027 M sodium citrate, 0.045 M tris-HCl buffer and 0.3 ml of an enzyme preparation in a final volume of 3 ml and a pH of 8.0.

In both assays use was made of the supernatant fraction of a three times frozen and thawed mitochondrial suspension. Increase in E_{240nm} was measured in a Zeiss M 4 Q III spectrophotometer, with the reaction mixtures without substrate as an optical blank. Enzyme activities were calculated from the observed changes in $E_{240\ nm}$ by means of a molecular extinction coefficient of 2.4×10^3 $M^{-1}cm^{-1}$.

3. RESULTS

3.1 Respiration of whole cotyledons

The course of respiration of the cotyledons (*fig. 1*) can be analysed, for convenience, into four phases as follows:

I. A rapid rise in respiration rate for the first 8-10 hrs.

II Hereupon follows a phase of about 15 hrs during which respiration is maintained at a fairly constant level, or even tends to drop somewhat.

III. A second slower rise extending from about 25 hrs to about 60 hrs from the onset of germination.

IV. A slow drop in respiration rate after about 60 hrs.

Further study of these results is made easier by plotting respiration rate directly against water content (*fig. 4*). It is now seen that in phase I respiration rate increases linearly with swelling up to a degree of about 100%. There exists, however, no linear relation between swelling and respiration in phase II and III.

The extent to which the respiration of the cotyledons during the respiration

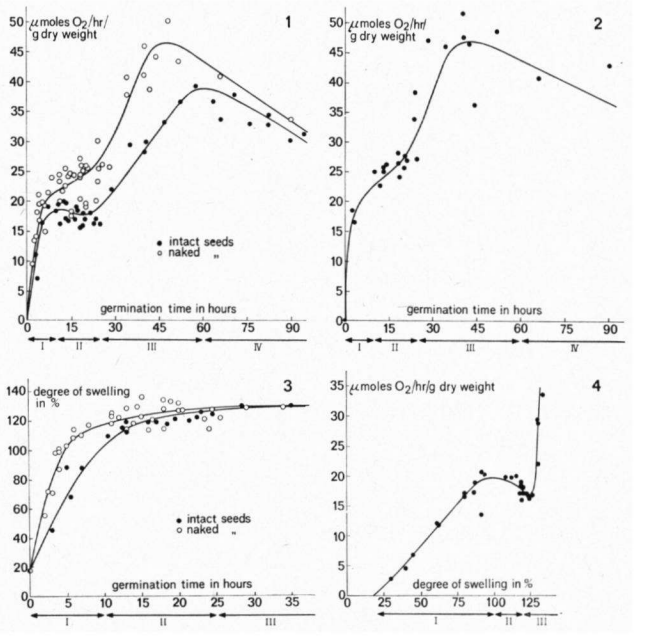

Fig. 1. The course of the respiration rate of cotyledons from intact germinated seeds (●) and of cotyledons from seeds germinated without seed coat (naked cotyledons ○). The course of the respiration rate is divided into four phases (I, II, III and IV).

Fig. 2. The course of the respiration rate of excised cotyledons.

Fig. 3. The swelling of cotyledons from intact seeds (●) and of cotyledons from seeds germinated without seed coat (○).

Fig. 4. The relation between the degree of swelling and the respiration rate of cotyledons from intact germinated seeds.

rate measurements may have been limited by the supply of oxygen was studied by investigating the effect of high oxygen tension on respiration rate.

As appears from *table 1* high oxygen tension during the measurements caused only a slight, fairly constant, rise of the respiration rate of cotyledons from intact and naked germinated seeds. This rise, being independent of the germination time of the cotyledons, does not account for the differences in the respiration rate of the cotyledons during the course of the germination.

Table 1. The effect of high oxygen tension on the rate of respiration of cotyledons from intact seeds and from seeds germinated without seed coat at different phases of germination. Oxygen uptake was measured manometrically first in air, and next in 100% oxygen. Oxygen uptake expressed as μ moles 0_2/hr/g dry weight.

hours germination	cotyledons from intact germinated seeds		cotyledons from seeds germinated without seed coat	
	in air	in oxygen	in air	in oxygen
11	16.2	19.1	20.0	22.6
14	16.8	20.8	20.1	24.9
18	15.4	18.8	19.2	23.3
19	18.0	21.0	15.6	23.2
24	18.1	23.8	23.3	29.0
40	30.0	34.0	41.2	46.2

However, the respiration rate may have been influenced by the oxygen supply during swelling and germination. This was indicated by the observation that the rise in respiration rate at the transition from phase II to phase III corresponded closely in time to the perforation of the seed coat by the elongating radicle of the seedling and consequently with better oxygen supply. The influence of the seed coat on respiration rate was therefore investigated. From the data of *table 2* it may be concluded that the intact seed coat limited the gaseous diffusion.

Table 2. The effect of peeling the seed coat from whole seeds on respiration rate. Oxygen uptake of whole seeds was measured manometrically, next the seed coats were peeled off – but left in the flasks – and measurement of oxygen uptake was resumed. Oxygen uptake expressed as μ moles 0_2/hr/g dry weight.

hours germination	whole seeds	seed coat peeled off
12	10.9	22.0
20	13.5	21.2

Also after having been perforated by the radicle of the seedling the seed coat may limit the oxygen diffusion because even then it remains tightly closed around the cotyledons. However, the seed coat can not have acted as a barrier to diffusion of oxygen during the respiration rate measurements of whole cotyledons, because it was then always stripped off artificially from the cotyledons.

How far the respiration rate is influenced by the limited oxygen supply during swelling and germination was studied by comparing the oxygen uptake of cotyledons from intact germinated seeds with that of cotyledons from seeds germinated without seed coat. As appears from *fig. 1*, there is only a slight difference between both respiration rate time curves. The sharper rise of the respiration rate in phase I is probably partly due to the more rapid swelling of the naked cotyledons (*fig. 3*). The higher respiration rate in phase II and III

seems to be induced by the better oxygen supply. In spite of the removal of the seed coat prior to soaking their remains a phase (II) in which the rise in respiration rate is much lower than in the preceeding (I) and following (III) phase.

The transition from phase II to phase III corresponds with the emergence of the seedling radicle and with a faster growth of the axis tissue. In order to investigate whether this growth is related to the respiration of the cotyledons, oxygen uptake of excised cotyledons was compared with that of cotyledons from naked germinated seeds. As appears from *fig. 2* there is no difference between the respiration of naked cotyledons and excised cotyledons and there is probably no influence of the axis tissue on respiration rate.

3.2 Mitochondrial activity

3.2.1 Distribution of the mitochondrial enzymes after fragmentation of the mitochondria

From preliminary experiments it emerged that it was difficult to obtain high fumarate hydratase and aconitate hydratase activities with mitochondrial suspensions. Because of the possible inaccessibility of the mitochondria to the substrates it seemed necessary to disrupt them by freezing and thawing or by sonication.

Table 3. Distribution of the activity of several mitochondrial enzymes among the supernatant and sedimental fraction obtained after freezing and thawing or sonication a mitochondrial suspension. The reaction mixtures were composed as described under the section materials and methods. They always contained 0,2 M sucrose when mitochondrial suspensions were tested, whereas the sucrose was omitted when supernatant or sedimental fractions were tested. Mitochondria obtained from cotyledons at different phases of germination showed the same distribution. The enzyme activities are expressed in units as indicated in the figs. 6, 7 and 8.

	succinate dehydrogenase	NADH-cyt.c oxidoreductase	fumarate hydratase	aconitate hydratase
original mitochondria	0.21	1.14	0.16	0.00
supernatant freezing/thawing	0.00	0.03	0.34	0.32
sediment freezing/thawing	0.14	0.96	0.08	0.08
supernatant sonication	0.05	0.31	0.42	0.31
sediment sonication	0.11	0.63	0.03	0.06

After the mitochondria had been disrupted the highest fumarate and aconitate hydratase activity was detected in the supernatants (*table 3*) and hardly any in the sediments. Both enzymes are likely to be readily solubilized. Because of these results freezing and thawing, being a milder method and giving more reproducible results, was used as a routine method for obtaining enzyme preparations with high fumarate- and aconitate hydratase activities.

The highest succinate dehydrogenase and NADH-cyt. c oxidoreductase activity was found in the suspensions with intact mitochondria. After sonica-

tion little activity was recovered in the supernatant, and after freezing and thawing scarcely anything. Most of the activity was recovered, however, in the sediments. Both enzymes are likely to be firmly bound to the mitochondrial membrane. In the determination of the activity of succinate dehydrogenase and NADH-cyt. c oxidoreductase the original unwashed mitochondrial suspension was applied as usual.

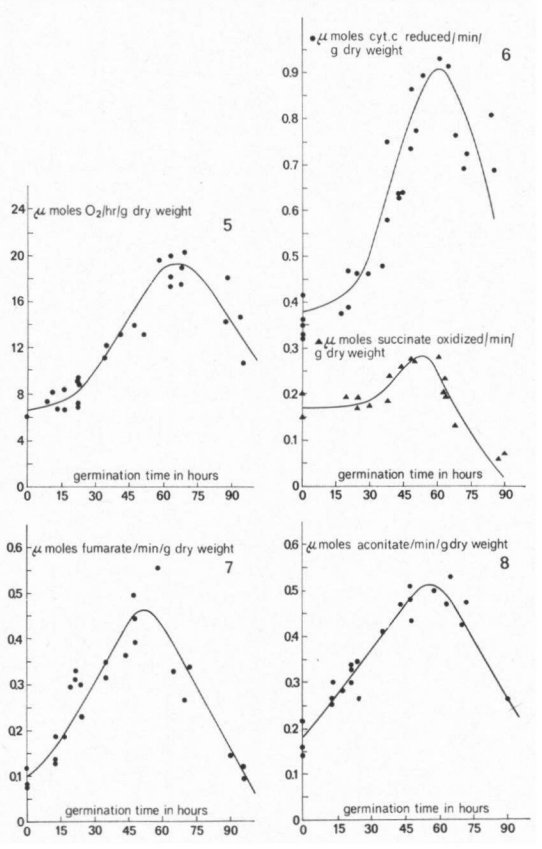

Fig. 5, 6, 7 and 8. The course of the activity of the succinate oxidase system (fig. 5.); succinate dehydrogenase (fig. 6.); NADH cyt. c oxidoreductase (fig. 6); fumarate hydratase (fig. 7.) and aconitate hydratase (fig. 8.).

3.2.2 Succinate oxidase system

Mitochondrial suspensions obtained from cotyledons of newly germinated seeds (phase I and II) showed an oxygen uptake which was linear with time for at least 60 minutes. The succinate oxidase activity from older cotyledons (phase IV) declined with time and therefore the activity was calculated from the linear portion of the curve (15-30 min). The mean values of triplicate flasks after correcting for the endogenous respiration have been plotted in *fig. 5.* The

succinate oxidase activity increases a little between 0 and 20 hrs, but after that it rises much more till it reaches a peak at about 60 hrs after the beginning of the germination.

During germination the endogenous respiration of the mitochondrial suspensions showed a slight constant increase from about 0.6 μ moles O_2/hr/g dry weight to about 2.4 μ moles O_2/hr/g dry weight.

3.2.3 Succinate dehydrogenase and nadh-cyt. c oxidoreductase

When in the NADH-cyt. c oxidoreductase assay much diluted mitochondrial suspensions were used, the activity was not proportional to added enzym. It was estimated therefore with undiluted or slightly diluted mitochondrial suspensions. The reaction proceeded then linearly with time for 1 to 2 min. The activity has been plotted, together with that of succinate dehydrogenase, in *fig. 6*. The activity of both enzymes remains fairly constant for about 20 hrs, after that it increases till it reaches a peak about 60 hrs after the beginning of the germination.

3.2.4 Fumarate hydratase and aconitate hydratase

A solubilized enzyme preparation was used in the assays of both enzymes. Their activity increases during germination till it reaches a peak about 60 hrs after the onset of the germination (*fig. 7* and *fig. 8*).

4. DISCUSSION

When the course of the respiration rate during germination of whole cotyledons (*fig. 1*) is compared with the course of the activity of several mitochondrial enzymes, such as the succinate oxidase system (*fig. 5*), succinate dehydrogenase (*fig. 6*), NADH-cyt. c oxidoreductase (*fig. 6*) or fumarate hydratase (*fig. 7*) and aconitate hydratase (*fig. 8*), it will appear that changes in the respiration rate run parallel with changes in activity of these enzymes. Only during the first 10 hrs of germination is there a difference between the course of the activity of the *in vivo* and of the *in vitro* system. The respiration rate in this phase increases sharply, whereas the activity of the isolated enzymes remains practically at the same level. This difference is due to the artificially accelerated hydration of the mitochondrial enzymes during their isolation, which brings about their immediate activation.

The succinate oxidase system (the complexes I, III and IV of an electron transfer particle), succinate dehydrogenase (complex I) and NADH-cyt. c oxidoreductase (complex II) represent almost fully, the enzymes of the electron transfer chain. The fumarate hydratase and aconitate hydratase represent the enzymes which catalyse the citric acid cycle. Assuming that the investigated enzymes are a reliable index for most of the enzymic activities within the mitochondrion and that their changes in activity *in vitro* represent real changes in activity *in vivo*, it may be concluded from these experiments that the respiration

of the pea cotyledons during germination is mediated by enzymes of the citric acid cycle and the electron transfer chain.

The enzymes of the electron transfer chain were recovered after freezing and thawing or sonicating a mitochondrial suspension predominantly in the sedimental fraction, whereas the enzymes of the citric acid cycle were found in the supernatant fraction. These results are in accordance with those obtained predominantly with mammalian tissues (BACHMANN *et al*, 1966). They indicate that also in mitochondria from plant tissues the enzymes of the electron transfer chain are localized on the inner membrane of the mitochondrion, whereas the citric acid cycle enzymes are localized on the outer membrane.

The respiration rate of pea cotyledons changes with germination time (*fig. 1*) and for convenience four phases have been distinguished.

PHASE I

The sharp rise in respiration rate of whole cotyledons during the first 10 hrs of germination is attributed to the activation by hydration of enzymes of the electron transfer chain and citric acid cycle already present in the air-dry cotyledons. This conclusion is based on the following evidence: (1) The respiration of the whole cotyledons increases linearly with the degree of swelling (*fig. 4*). This fits in with the results of ÖPIK & SIMON (1963) for bean cotyledons. (2) It was possible to isolate active enzymes from an imbibed powder of air-dry cotyledons. It is not likely that during the imbibition of this powder with water, lasting maximal 2 hrs at 0–5°C, enzyme synthesis has occurred. This agrees with the results of RIGGIO BEVILAQUA (1964), who ascertained $NADH_2$– and $NADPH_2$ oxidoreductase activities in acetone precipitates of dry and germinating pea seeds.

PERNER (1965) concluded from electron microscopic investigations of embryonic root cells of air-dried seeds that during dormancy cell organells and cytoplasmatic structures were preserved. In spite of considerable shrinkage there is no mechanical damage.

BAIN & MERCER (1966) observed that increasing water content in the cotyledons of germinating pea seeds was associated with increasing development of the mitochondrial structure. These authors suppose that the mitochondria could have arisen from "ghosts" of mitochondria which had lost their enzymic machinery as cells became dormant (pro-mitochondria); or they may have arisen *de novo* as the cotyledons became hydrated. The biochemical data of the present study indicate, however, that an origin from pre-existent mitochondria seems the most likely.

PHASE II

In this period, lasting between about 10 hrs and about 25 hrs from the onset of germination, the hydration of the cotyledonary tissue is completed and the reorganization of the ultrastructure is continued (BAIN & MERCER 1966). Probably all pre-existent enzymes have been activated now by hydration and

thus both respiration and enzymic activity remain at a fairly constant level. This lag phase in the respiration rate of whole cotyledons is attributed to a minor extent to the limited oxygen supply, contrary to the opinion of SPRAGG & YEMM (1959). The evidence for this is: (1) Neither an increase of the oxygen tension during respiration (2) nor the removal of the seed coat from the cotyledons before swelling did result in a raise of this lag phase. The testa, acting as a barrier to oxygen, reduces the respiration rate of the cotyledons *in situ*.

PHASE III

The increasing respiration rate of whole cotyledons in this phase is associated with increasing mitochondrial activity. It was not yet investigated whether this increase in activity may be the result of an actual increase in the number of mitochondria (BREIDENBACH *et al.*, 1966), or whether it may be the result of both an increase in the mitochondrial content and a higher intrinsic mitochondrial activity (AKAZAWA & BEEVERS 1957). It is very unlikely that this rise in mitochondrial activity and respiration rate is due to a further hydration of the cell content, as was the case in phase I.

PHASE IV

Respiration rate and mitochondrial activity drop after about 60 hrs from the onset of germination. CHERRY (1963), ÖPIK (1965) and BAIN & MERCER (1966) observed that the mitochondrial ultrastructure in peanut, bean and pea cotyledons, respectively, desintegrates after several days of germination. An indication in the present study supporting these observations is, that during the succinate oxidase assays the oxygen uptake by mitochondria from newly germinated seeds remains constant whereas that of older cotyledons decreases with time.

Histochemical observations were made in order to know more about the significance of the observed changes in mitochondrial activity. In experiments with vital redox indicators, such as Janus Green and blue tetrazolium chloride, particle bound succinate dehydrogenase activity was found mainly in the procambium and less in the epidermal and subepidermal layers. Succinate dehydrogenase activity was also localized in certain cells of the storage parenchyma. During the differentiation of the procambium, taking place within 3 to 4 days, the activity disappeared from the potential xylem, but remained in the adjacent cells. These procambium bundles are the only tissue which undergoes a differentiation during germination. Investigations into the correlation between this differentiation and the changes in mitochondrial activity are in progress.

MAPSON & MOUSTAFA (1956) and ÖPIK (1965), in comparing *in vitro* experiments with the *in vivo* respiration of pea and bean cotyledons, respectively, attributed the rise in respiration partly to the increase in soluble oxidases. The role of these soluble oxidases together with the role of anaerobiosis will be investigated in relation to the present results.

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