THE HISTOCHEMICAL LOCALIZATION OF DEHYDROGENASES IN THE COTYLEDONS OF PISUM SATIVUM L. DURING GERMINATION

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SUMMARY

Succinate dehydrogenase activity, NAD-linked malate, isocitrate and alcohol dehydrogenase activity and NADH:nitro-blue tetrazolium reductase activity were demonstrated in sections of fresh or frozen pea cotyledons, using nitro-blue tetrazolium as indicator. The localization of the various enzymes shows much resemblance. Initially, high enzyme activity is present in the cells of the epidermis and hypodermis. This activity decreases during germination. In the storage parenchyma, the enzyme activity in almost all cells increases during the first three days of the germination, and decreases subsequently, especially in the cells of the outer abaxial zone. The tracheary elements do not show any dehydrogenase activity. They appear during germination. The activity of the other cells of the vascular system increases during the first three days of germination but hardly changes in the subsequent seven days.

1. INTRODUCTION

The activity of several mitochondrial enzymes and that of alcohol dehydrogenase changes in the cotyledons of peas during the first days of the germination (KOLLÖFFEL 1967, 1968a). These results were obtained by biochemical assays. It was attempted now to determine the localization and the activity of several enzymes histochemically, because comparitively little is known on enzyme localization in pea cotyledons. FREY (1954) ascertained the localization of acid phosphatases. Recently, FLINN & SMITH (1967) investigated the distribution of, mainly, hydrolytic enzymes in the cotyledons of *Pisum arvense*. In agreement with the results of these authors it was found that the enzyme activity is not uniformly distributed throughout the cotyledon.

The biochemical assays of the enzyme activity were performed with extracts from pea cotyledons. During the extraction enzymes could be inactivated to various degrees. Since this would lead to a wrong idea of the development of enzyme activity in the cotyledon, the activity of the extracts was compared with the histochemically determined activity in the sections.

The course of the activity of the succinate dehydrogenase, NAD-linked malate, and isocitrate dehydrogenase agrees with the course of the activity of several mitochondrial enzymes (KOLLÖFFEL 1967). However, there was a discrepancy between the histochemically determined alcohol dehydrogenase activity and the biochemically determined activity (KOLLÖFFEL 1968a).

2. MATERIALS AND METHODS

2.1. Germination conditions

Air-dry seeds of *Pisum sativum* cv. "*Rondo*", selected on equal weight and colour, were soaked in tap water under aeration with ordinary air for 20–22 hr. Next they were washed and transferred to moist filter paper in "germination boxes", set up as follows:

A small Petri dish (9 cm diameter) was placed in a large one (18 cm diameter) and covered with a glass plate (17 cm diameter) wrapped in filter paper, flaps of which continously were immersed in water poured in the larger dish. The water was changed daily. This procedure allowed the seeds to germinate under conditions of constant water supply. The boxes were kept in darkness at 23 °C. After the appropriate germination period, both seed coat and axis tissue were carefully dissected from the cotyledons.

The period between the beginning of soaking and the moment the cotyledons were used for the experiments, will be referred to as the "germination time". The moment the seeds were put in water will be called the "onset of germination". It must be stressed that the radicle emerged about 20 hrs after this time.

2.2. Preparation of the sections and conditions of incubation

Most of the experiments were performed with freehand cut sections of fresh cotyledons. However, for demonstrating succinate dehydrogenase activity, sections were used of cotyledons which had been frozen and subsequently thawed. To this end, whole peas were put in water in tubes which were cooled for 15 min in an ethanol-solid carbon dioxide mixture. In some experiments, in addition parts of cotyledons were used for demonstrating enzyme activity.

The sections were washed immediately three times, each time in approximately 5 ml phosphate buffer (0.05 M, pH 6.4). These "preincubations' lasted altogether about 15 min.

The incubations were performed in small vessels (internal diameter 1.7 cm) at 25° C. A number of sections were put in a vessel. After incubation, the sections were thorougly rinsed with tap water and fixed in a neutralized 10 per cent solution (v/v) of formaldehyde in water. Unfixed sections were used for microscopic examination.

2.3. Incubation media

The following abbreviations will be used: Nitro-BT (nitro-blue tetrazolium chloride, 3,3'-(3,3'-dimethoxy-4,4'-biphenylene)-bis-2-(p-nitrophenyl)-5-phenyl-2H-tetrazolium chloride, The British Drug Houses Ltd.); PMS (phenazine methosulphate, Aldrich Chemical Co., Inc.); NAD(P) and NAD(P)H (Boehringer und Söhne).

The incubation medium for demonstrating NADH: Nitro-BT reductase activity contained: phosphate buffer (0.05 M, pH 6.4), NADH (0.6 mM) and Nitro-BT (0.25 mg/ml). Sections of fresh cotyledons were incubated in 1 ml of this medium for 15 min.

The reaction media for demonstrating the activity of NAD-linked enzyme complexes contained: phosphate buffer (0.05 M, pH 6.4), NAD (1.35 mM), Nitro-BT (0.25 mg/ml) and substrate. Substrates employed were: ethanol (0.4 M), DL-isocitrate (0.05 M) and L-malate (0.05 M). Sections of fresh cotyledons were incubated in 2 ml medium for 30 min.
The reaction medium for demonstrating succinate dehydrogenase activity contained: phosphate buffer (0.05 M, pH 6.4), PMS (0.1 mg/ml), Nitro-BT (0.5 mg/ml) and succinate (0.05 M). Sections of cotyledons which had been frozen and thawed were incubated in 2 ml of this medium for 30 min.

2.4. Control reactions

The unspecific reduction of Nitro-BT by endogenous substrates, catalyzed by unknown dehydrogenases, was followed by incubating sections in a medium without added substrates. The non-enzymatic reduction of Nitro-BT was followed by inactivating the enzymes in the sections by immersing them for 5 min in boiling water. The effect of an inhibitor on the enzyme activity was determined by washing the sections once in 5 ml phosphate buffer (0.05 M, pH 6.4) and subsequently twice in buffer with the inhibitor for altogether 15 min. Besides, the inhibitor was added to the incubation medium.

3. PLANT MATERIAL

In the present study, changes in the distribution and the activity of several dehydrogenases were followed during the first ten days of germination. Because the conditions during the germination of the seeds differed from those reported by other authors (BAIN & MERCER 1966b, SMITH & FLINN 1967), the morphological and anatomical changes occurring during the period of ten days are briefly described below, as a basis of reference with the data from the literature. A description of the anatomy was already given by HAYWARD (1938), BAIN & MERCER (1966 a, b) and SMITH & FLINN (1967). Their results differ, however, in certain minor respects with the present results.

3.1. Morphology

The green air-dry cotyledons are enclosed in an intact seed coat, which remains tightly closed around the cotyledons, even after it has been perforated by the radicle and the epicotyl of the seedling. The radicle penetrated the seed coat about 20 hrs after the seeds had been put in aerated water. The subsequent growth of the root proceeds linearly with time until the roots were approximately 8 cm long after four days. The roots were about 18 cm long after ten days. Secondary roots emerged at the fifth day after the onset of germination.

The hooked epicotyl emerged two to three days after the onset of germination. The shoot was approximately 3 cm long after four days and approximately 10 cm long after ten days.

The dry weight (105° C for 22 hr) of the air-dry cotyledons was approximately 100 mg and dropped about 15% during the first four days of germination. In the

subsequent six days, it dropped till approximately 60% of the original dry weight.

The water content of the air-dry cotyledons was 18 per cent of their dry weight. It increased rapidly to about 120% during the first 10–15 hrs of germination. From this time, until the second day, the water content showed little further increase. In the later stages of germination the dry weight of the cotyledons decreased, which resulted in an increase in the percentage of water. The water content after four days was 150% and after ten days about 270% of the dry weight.

3.2. Anatomy

The cotyledon is approximately a hemisphere with the flat face corresponding to the adaxial face of the leaf. The epidermis as seen from above consists of

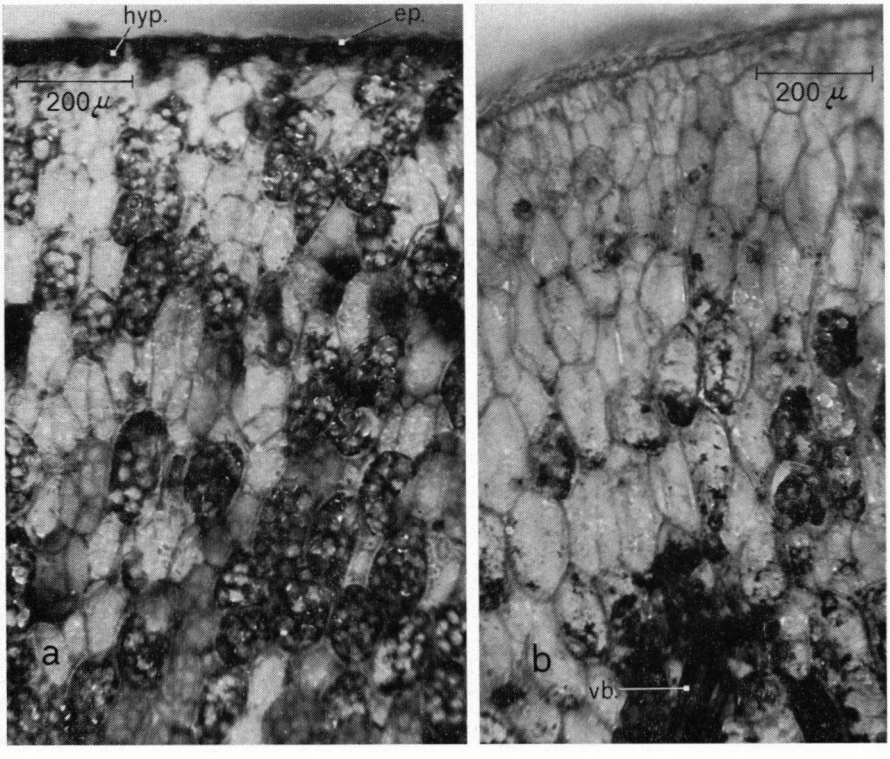

Fig. 1. a. Surface view of a cross-section of the abaxial face of a pea cotyledon after one day of germination showing a strong alcohol dehydrogenase activity in the epidermis (ep.) and hypodermis (hyp.) and a much weaker activity in the storage parenchyma cells.
b. The similar view of a pea cotyledon after ten days of germination. The alcohol dehydrogenase activity has disappeared from epidermis, hypodermis and most of the storage parenchyma cells. Note the heavy diformazan deposits in the vascular bundle (vb.) and the absence of starch in the storage parenchyma cells.

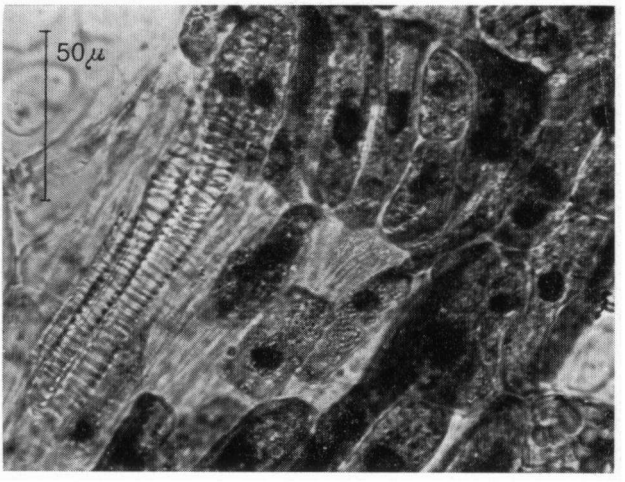

Fig. 2. Freehand cut section of a cotyledon stained with aceto-orcein, showing a detail of a vascular bundle with some primary tracheary elements and a great number of the small uninucleate cells.

small cells (about 8 μ diameter and 90 μ long), which are arranged parallel to each other in small groups. The orientation of these groups is not constant. Stomata are absent.

Below the epidermis is a single row of cells comprising the hypodermis *(fig. 1)*. The cells are more or less cylindrical and are about 25 μ in diameter and about 40 μ high.

The storage parenchyma makes up the bulk of the cotyledon and appears to consist of two zones. The cells of the inner zone, below the flat surface, are about isodiametric with a diameter of about 100 μ. The cells of the outer zone, below the convex surface, are about 90 μ in diameter and 150 μ long with their longest axis perpendicular to the surface *(fig. 1)*.

The cotyledon has a complex reticulate vascular system. It consists of some tracheary elements with lignified spiral thickenings (*fig. 2*) which appear one to two days after the onset of germination. Sometimes a few phloem elements were observed. These tissues are capped with a great number of small cells (about 10μ in diameter and 60μ long) which are rich in cytoplasm. Starch was never observed in these cells. They hardly show any visible anatomical changes during germination. When fresh sections were stained in 0.1% orcein in 45% acetic acid (JENSEN 1962) nuclei were observed in these cells even after ten days from the onset of germination *(fig. 2)*.

3.3. Mobilization of starch

Starch was indicated by putting whole cotyledons, parts of cotyledons, or sections in a solution of iodine in potassium iodide. It appeared that big starch

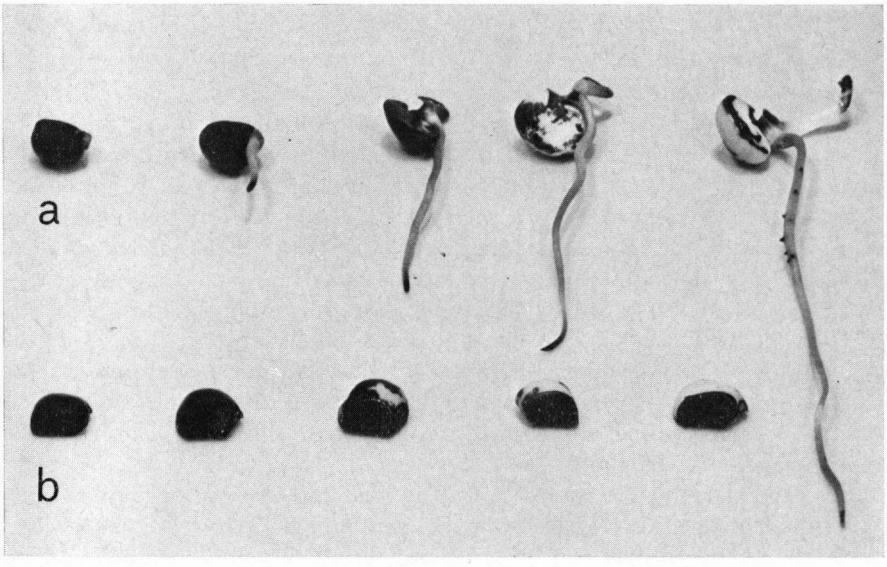

Fig. 3. a. The photograph shows the disappearance of starch from the hypodermis of whole cotyledons during the first five days of germination. Microscopic examination revealed only starch of the hypodermis stained by iodine. The epidermis never contained starch.
b. Parts of cotyledons stained with iodine show that the starch had not yet disappeared from the storage cells.

grains were present in large quantities in all cells of the storage parenchyma until about ten days from the onset of germination. In the subsequent days, the cells of the outer storage parenchyma, below the convex face of the cotyledon, became depleted of their starch. The cells of the hypodermis *(fig. 3)* initially contained a few small starch grains. Those of the convex face first lost their starch reserves. After four to seven days, all starch had disappeared from the hypodermis. The cells of the vascular bundle sheath contained several little starch grains. There were still some left after about ten days of germination. Starch never was observed in the other cells of the vascular bundle and in the epidermis.

These results show that the degradation of starch started at the convex face of the cotyledon and subsequently proceeded in a wave inwards. In this respect the results are in agreement with those of RYWOSCH (1909) and SMITH & FLINN (1967). However, whereas the degradation of starch in the cotyledons of *Pisum arvense* (SMITH & FLINN 1967) is already completed in twelve days, it was found in the present study that after a fortnight the cells of the inner storage parenchyma of *Pisum sativum* still contained numerous starch grains.

4. HISTOCHEMICAL RESULTS

4.1. Extracellular and unspecific diformazan production

Dehydrogenase activity was indicated by following the enzyme catalyzed reduction of Nitro-BT by several substrates. The diformazan, produced during the reaction, stained the sections bluish purple. Cells of these sections then contained distinct, stained particles. In addition, sometimes a diffuse deposit was observed.

In the incubation media of the sections, hardly any diformazan production occurred, indicating that neither the enzymes responsible for the reduction of Nitro-BT nor the reduced tetrazolium salt readily diffused into the media (see also section 4.3). However, if unwashed sections were transferred directly into the incubation media, diformazan production also occurred there. In the assay of the NADH: Nitro-BT reductase activity, the diformazan deposit in the sections was frequently less than that in the washed sections, suggesting that the NADH became limiting. The enzymes responsible for the staining in the media likely escaped from damaged cells.

For demonstrating the several dehydrogenases, it was necessary to add, besides the substrate, also NAD to the incubation media. However, tetrazolium salts are readily reduced in media with NAD only at an alkaline pH (see e.g. PEARSE 1961). As this unspecific diformazan production could be a source of error in the assays of the dehydrogenase activity, it was investigated whether this reaction also occurred in sections of pea cotyledons.

In media with Nitro-BT alone, no diformazan production occurred at pH values from 5 to 7. At slight alkaline pH levels, a weak reaction occurred. If sections were incubated in a medium with both Nitro-BT and NAD, no reaction occurred at a slightly acid pH. However, the diformazan production increased progressively with the pH of the medium. It was strong at a slightly alkaline pH.

These results indicate that in fact the Nitro-BT was reduced unspecifically in a medium with NAD at an alkaline pH. As a consequence a pH of the medium of 6.4 was choosen for demonstrating the several dehydrogenases.

4.2. NADH: Nitro-BT reductase

When sections were incubated in a medium with NADH, a heavy diformazan deposit was already found within 15 min. No reaction occurred in sections of boiled cotyledons or when NADH was absent. Phenyl-mercury acetate (10^{-4}M) inhibited the diformazan production. Potassium thiocyanate (10^{-3}M) and sodium azide (10^{-2}M) were without effect. Evidently, the sections contained a relatively high NADH: Nitro-BT reductase activity.

Both a soluble pyridine nucleotide-quinone reductase (WOSILAIT & NASON 1954) and a mitochondrial NADH: cyt. c reductase (KOLLÖFFEL 1967) have been demonstrated biochemically in germinating pea cotyledons. KALINA (1966) demonstrated both a soluble and a particle-bound NAD(P)H tetrazolium reductase in embryos of *Vicia faba*. Thus, it is possible that the diformazan deposit in the sections of the pea cotyledons must be attributed to the activity of both a soluble and a mitochondrial enzyme.

Sections incubated in a medium containing NADPH (0.6 mM) showed a moderate diformazan deposit in 15 min. Thus, besides NADH: Nitro-BT reductase, also a NADPH: Nitro-BT reductase is probably present in these sections. The possibility exists that, via a transhydrogenase reaction, this NADPH: Nitro-BT reductase may contribute to a diformazan production which erroneously may be held for NADH: Nitro-BT reductase activity.

The following evidence, however, is opposed to this supposition: (1) The dye reduction in a medium with NADH was faster than that found on incubating sections in a medium with NADPH. (2) Addition of NADP to the incubation medium with NADH was without effect on the diformazan production.

During the first eight days of germination, the overall NADH: Nitro-BT reductase activity remained practically at the same level. Although the activity slightly decreased in the subsequent days, a relatively high activity was still left at the tenth day of germination.

4.3. Alcohol dehydrogenase

Sections of cotyledons showed a very weak diformazan production when incubated in a medium with ethanol. NAD promoted this reaction. Control sections with NAD, but without ethanol, showed a very weak diformazan production only.

If, instead of ethanol, propanol (0.4 M) or butanol (0.4 M) were used as substrate also a heavy diformazan deposit was obtained, but the reaction was much weaker in the presence of methanol (0.4 M). Similar results were obtained with extracts from pea cotyledons (COSSINS c.s. 1968).

Sections of boiled cotyledons reduced the Nitro-BT not at all. Phenyl-mercury acetate (10^{-4}M) completely inhibited the diformazan production whereas potassium thiocyanate (10^{-4}M) and sodium azide (10^{-3}M) inhibited the reaction only partially.

These results clearly show that alcohol dehydrogenase activity can be demonstrated in sections of pea cotyledons. The first step in the reaction sequence was the oxidation of ethanol and the concomitant reduction of NAD, catalyzed by alcohol dehydrogenase. The subsequent oxidation of NADH by Nitro-BT was catalyzed by NADH: Nitro-BT reductase. Thus in fact, the activity of the alcohol: Nitro-BT reductase complex was measured and the NADH: Nitro-BT reductase was localized. Alcohol dehydrogenase activity was found in the soluble fraction of an extract of pea cotyledons (KOLLÖFFEL 1968 a). It has been shown (KALINA & GAHAN 1965, 1968) that some soluble dehydrogenases may diffuse from frozen sections of animal and plant tissues during incubation for a histochemical reaction. In the incubation medium, these soluble dehydrogenases catalyzed the transfer of electrons from the substrate to NAD. The NADH, thus formed, diffused back to the sections where it was reoxidized by a tissue-bound tetrazolium reductase, with the concomitant reduction of a tetrazolium salt to its corresponding formazan. According to KALINA & GAHAN this phenomenon may lead to a wrong localization of the enzyme activity. It was necessary to investigate whether or not this phenomenon also may oc-

cur during the determination of the alcohol dehydrogenase activity, because alcohol dehydrogenase is a soluble enzyme and NADH:Nitro-BT reductase was retained in the sections during incubation (section 4.1). Sections of cotyledons germinated for about seven days showed a low alcohol:Nitro-BT reductase activity. They were incubated together with sections of cotyledons germinated for one day, which were known to have a high activity. If alcohol dehydrogenase was lost in the medium it was anticipated that – in accordance with the experiments of KALINA & GAHAN – both sections would show a heavy diformazan deposit. However, the diformazan deposit in the sections of the cotyledons germinated for one day remained high and that of the sections germinated for seven days remained low. When sections were washed three times as usually and next transferred to phosphate buffer (0.05 M, pH 6.4) samples of this medium contained hardly any alcohol dehydrogenase activity, showing that no enzyme diffused into the buffer. The alcohol dehydrogenase activity was determined spectrophotometrically by measuring the oxidation of NADH in the presence of acetaldehyde (KOLLÖFFEL 1968a). It can be assumed that alcohol dehydrogenase was retained likewise in the sections during their incubation in the complete medium.

The overall alcohol:Nitro-BT reductase activity of the sections increased slightly during the first three days of germination. In the subsequent days, the activity decreased. After about ten days, the sections showed only a slight reaction.

Some results of this investigation have been reported already in a preliminary paper (KOLLÖFFEL 1968b).

4.4. Malate dehydrogenase and isocitrate dehydrogenase

Sections incubated in a medium with malate but without NAD showed hardly any diformazan production. The reaction was stimulated when NAD was added to the incubation medium. Now, it was significantly higher than that found in sections incubated in a medium without malate but with NAD. The diformazan deposit was absent in sections of boiled cotyledons. Similar results were obtained if, instead of malate, isocitrate was used as substrate. Thus, it is clear that both NAD-linked malate dehydrogenase and NAD-linked isocitrate dehydrogenase activity can be demonstrated in fresh sections of pea cotyledons. Both malate and isocitrate dehydrogenase activity were inhibited by phenylmercury acetate (10^{-4}M).

The activity of both enzymes in sections of cotyledons of newly germinated peas was low in comparison with the rest of the enzymes investigated. Their overall activity only slightly increased during the first three days of germination but decreased in the subsequent days.

In addition to these NAD-linked dehydrogenases, also a NADP-linked malate and isocitrate dehydrogenase activity was demonstrated. The activity of these NADP-linked enzymes was less than that of the NAD-linked enzymes.

4.5. Succinate dehydrogenase

When sections of fresh cotyledons were incubated in a medium with the following compounds, they stained: (1) very weakly with succinate; (2) intensely with PMS; (3) intensely with succinate plus PMS. Thus, owing to the high unspecific dye reduction in the presence of PMS, it was impossible to demonstrate succinate dehydrogenase activity in these sections. When sections of frozen cotyledons were incubated in these media, they stained: (1) very weakly with succinate; (2) moderately with PMS; (3) strongly with succinate plus PMS. Now, the diformazan production in the medium with succinate and PMS was higher than that in the control medium without succinate.

The staining pattern of the frozen sections was identical to that of the fresh sections. The amount of reduced dye, however, was less. Freezing especially minimized the unspecific dye reduction. Similar results were obtained by KOENIGS (1966). The fresh sections only contained bluish purple diformazan deposits. Areas of the frozen sections sometimes contained in addition a small amount of a light red deposit.

Sections of boiled cotyledons showed only these light red deposits. The diformazan production in the frozen sections incubated in the complete medium was strongly inhibited by malonate (0.05 M) and by oxaloacetate (0.05 M).

As appears from these results it was possible to demonstrate succinate dehydrogenase activity by incubating frozen sections in a medium with PMS and succinate. PMS is known to accept electrons directly from succinate dehydrogenase and is capable of transferring these electrons non-enzymatically to Nitro-BT (see *e.g.* GAHAN & KALINA 1968). Thus, succinate dehydrogenase activity is measured directly without the intervention of components of the electron transfer chain.

The overall succinate dehydrogenase activity slightly increased during the first three days of the germination and next decreased in the subsequent days. After about ten days very little activity was left.

4.6. Localization of the various dehydrogenases

For tracing the localization and the activity of the various dehydrogenases, either whole cotyledons, parts of cotyledons or sections were incubated in the various incubation media. Sites of dehydrogenase activity were indicated by a bluish purple diformazan deposit.

The activity of the various tissues of the cotyledon as observed at sections or at parts of cotyledons differed considerably. The changes of the localization and the activity of the various enzymes closely parallelled each other during germination.

When cotyledons of peas, germinated for one to three days, were incubated in the various incubation media, numerous cells of the epidermis and the hypodermis contained diformazan deposits *(fig. 3)*. These deposits were absent in cotyledons of peas germinated for about ten days, showing that the enzyme activity in the cells of the epidermis and the hypodermis was high initially but decreased

during germination. These observations were confirmed by experiments with sections of such cotyledons.

The majority of the cells of the outer abaxial zone of the storage parenchyma and all cells of the inner storage parenchyma initially showed a lower enzyme activity than those of the epidermis and the hypodermis. The activity increased during the first three days of germination but decreased in the subsequent days, especially in the cells of the outer abaxial zone of the storage parenchyma. After about ten days only a few cells, scattered in the outer storage parenchyma, showed a slight dehydrogenase activity, whereas those of the inner storage parenchyma all still showed a slight activity. Thus, although the cells of the storage parenchyma appear morphologically similar, they differ physiologically. When parts of cotyledons were incubated in the various media, only the cells right below the cut surface stained.

The dehydrogenase activity of the majority of the cells of the vascular strands increased during the first three days of germination, but hardly changed in the subsequent days. This was especially evident for succinate dehydrogenase. The tracheary elements showed no dehydrogenase activity.

These observations revealed that the dehydrogenase activity was not uniformly distributed throughout the cotyledon. During germination, the enzyme activity decreased, especially in the peripheral, abaxial zone of the cotyledon. The diformazan deposits were always uniformly distributed in the various cells.

The overall NADH: Nitro-BT reductase activity of a section was relatively high. The activities of alcohol dehydrogenase and succinate dehydrogenase were lower whereas the NAD-linked malate and isocitrate dehydrogenase showed only a weak activity. So, the overall enzyme activities of the various enzymes, as determined by the previously mentioned methods, differed considerably.

5. DISCUSSION

During the germination of pea seeds, the storage materials of the cotyledons are broken down, mainly by the action of hydrolytic enzymes. The products move into the growing parts of the seedling, after being converted to transportable forms. Thus, cotyledons of germinating pea seeds are characterized by catabolic processes. Anabolic processes are involved in the development of a subcellular organization (BAIN & MERCER 1966b), the synthesis of an enzymic equipment and the differentiation of the vascular tissue. During the development of the seedling, the cotyledons deteriorate and become a senescing tissue. It is obvious that dehydrogenases are involved somehow in almost all of these processes.

Malate, isocitrate and alcohol dehydrogenase activity and NADH: Nitro-BT reductase activity were demonstrated in sections of fresh cotyledons. For demonstrating succinate dehydrogenase activity, it was necessary to use sections of frozen cotyledons. Dehydrogenase activity was determined by following the reduction of Nitro-BT by electrons from the several substrates. The reduction of Nitro-BT by NADH was brought about by its direct interaction with NADH:

Nitro-BT reductase. Also in the reduction of Nitro-BT by succinate probably only one enzyme was involved. Therefore, the amount of diformazan was directly related with these enzyme activities. The enzyme catalyzed reduction of Nitro-BT by ethanol, malate and isocitrate, however, depends upon the combined effect of two enzymes working in sequence, respectively the dehydrogenase and the NADH:Nitro-BT reductase. The oxidation of exogenous NADH always occurred readily. Assuming that the oxidation of endogenous generated NADH proceeds at the same rate, it is improbable that this latter reaction is the rate limiting step in the overall reaction for the NAD-linked dehydrogenases. In the assays of these enzymes the NADH:Nitro-BT reductase activity was localized in fact. Since neither diformazan nor enzyme diffused into the incubation medium and control sections hardly showed any reaction, a diformazan deposit yet marked specific dehydrogenase activity.

The localization of the various dehydrogenases showed much resemblance to that of the acid phosphatase in the cotyledons of *Pisum sativum* (FREY 1954) and to the localization of succinic dehydrogenase, cytochrome oxidase and a number of hydrolytic enzymes in the cotyledons of *Pisum arvense* (FLINN & SMITH 1967).

Initially, a relatively high enzyme activity was observed in the cells of the epidermis and the hypodermis. The mobilization of reserve materials also began at the periphery of the cotyledon. Although the dehydrogenases are not directly involved in the degradation of reserve materials, it is obvious that their activity in the peripheral cells initially also must be high, because the general metabolic activity of these cells will be high. FLINN & SMITH, investigating the localization and the activity of succinic and cytochrome oxidase in the cotyledons of *Pisum arvense*, obtained similar results.

The vascular system, except the mature tracheary elements, shows a relatively high enzyme activity. Their tracheary elements only develop during germination. The vascular system is concerned most probably with the transport of reserves from the cotyledons to the axis tissues and hence, the general metabolic activity will be relatively high.

The majority of the cells of the outer storage parenchyma showed initially dehydrogenase activity, whereas the minor part showed no reaction at all. BAIN & MERCER (1966b) observed that the cells of the storage parenchyma were not alike at the ultrastructural level. Some cells became disorganized to some extent during the development of the cotyledons. They were recognizable again in the cotyledons during seedling development. According to BAIN & MERCER (1966b), these cells may have served as channels for the mass movement of substances through the cotyledon. An alternative possibility, based on the fact that certain cells of the storage parenchyma had a higher cytoplasmic RNA content and also a higher level of enzyme activity than the other cells of the storage parenchyma, is that these scattered cells are specialized as sites of enzyme synthesis (FLINN & SMITH).

The breaking of the dormancy and the recovery of the metabolic activity started when the seeds were put in aerated water. The water content of the coty-

ledon was already very high after one day of germination. Almost all cells of the cotyledon, except some cells of the outer storage parenchyma, then showed dehydrogenase activity. The activity of the various cells differed considerably. After about ten days almost all cells of the outer abaxial zone of the storage parenchyma and those of the epidermis and hypodermis had lost their dehydrogenase activity and their starch reserves. So, at any moment during the germination of the seeds, the cotyledon consists of cells, differing considerably in function and in metabolic activity. Changes in the enzymatic activity of the cotyledons as a whole, as demonstrated by biochemical assays does represent only changes in the enzymatic activity of the storage parenchyma cells, constituting the bulk of the cotyledonary cells. However, even this tissue consists of cells of different metabolic state. The number of cells of the cotyledon remains virtually constant during germination. There might be a relatively small change in cell number caused by the development of the tracheary elements.

Succinate dehydrogenase, NAD-linked malate and isocitrate dehydrogenase are components of the mitochondrial citric acid cycle. Their activity increased in the majority of the cells of the storage parenchyma during the first three days of germination but slightly decreased thereafter. After ten days, there was still a minor part of the initial activity left. These values are semi-quantitative because the colour intensities of the diformazan deposits of the several sections were compared macroscopically. They agree with the results of a previous investigation on changes in the activity of some mitochondrial enzymes (KOLLÖFFEL 1967), which were obtained by biochemical assays. The alcohol dehydrogenase activity also increased slightly during the first three days of the germination but decreased in the subsequent days. In contrast, the alcohol dehydrogenase activity of extracts prepared from cotyledons three days after the onset of germination was considerably lower than that of extracts from cotyledons germinated for one day (KOLLÖFFEL 1968a). This discrepancy was probably the result of some inhibitor of alcohol dehydrogenase which could be extracted from cotyledons of peas germinated for several days, but not from cotyledons germinated for one day.

The development of a complex subcellular organization in the storage cells of the cotyledons during germination was correlated with the absorption of water by the cotyledons (BAIN & MERCER 1966b). In the present investigation, it was found that the water content of the cotyledons was already very high after one day of germination. Therefore it is improbable that the increase in dehydrogenase activity during the first three days of germination must be attributed exclusively to the development of a subcellular structure.

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