Planta (Berl.) 70, 13—25 (1966)

CRYOSTAT TECHNIQUE FOR FRESH PLANT TISSUES AND ITS APPLICATION IN ENZYME HISTOCHEMISTRY

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Received March 21, 1966

Summary. A rapid cryostat technique for sectioning fresh plant tissues is described. The plant tissues are embedded in unfixed brain, frozen with CO_2 and sectioned in a cryostat at temperatures ranging from -12^0 to -18^0 C for different plant materials. Embedding in brain prevents disruption of plant tissues during freezing and sectioning. The sections are allowed to dry in the cryostat and must be warmed slowly to room temperature before starting histochemical experiments to avoid thawing artefacts.

For histological evaluations rapid fixation and staining procedures were elaborated.

The reliability of the technique is shown for different tissues of higher plants.

Cytochemical localization of some enzymes in meristematic cells is given as an example for the application of the cryostat technique in histochemistry. From the dehydrogenases, NAD-diaphorase is found in the cytoplasm, lactic dehydrogenase seems to be bound to the mitochondria and distributed in the cytoplasm, especially near the nuclear membrane; succinic and isocitric dehydrogenases are localized within the mitochondria. The site of aliesterase activity is close to the plasm membrane. No enzyme activity was found in the nuclei.

Introduction

In recent years histochemical investigations on plant tissues have gained more and more interest (Jensen, 1962). Exact preparation and sectioning techniques are essential. The customary method of embedding fixed and dehydrated tissue in paraffin and sectioning the block with a microtome is inadequate for histochemical evaluations of different substances and reactions such as the localization of watersoluble substances, the transport of electrolytes and the determination of enzymes. The investigations mentioned above are possible only if fresh tissue is frozen immediately after excision. Frozen plant tissue cannot be sectioned satisfactorily with the freezing microtome for histochemical examinations. However, after freeze-drying a paraffin-infiltration under vacuum and sectioning with the paraffin microtome is possible (Jensen, 1962). This procedure has many disadvantages because of the complicated equipment needed (Branton and Jacobson, 1961). Many inadequacies of the freezing microtome were avoided by the introduction of the cryostat in the medical-histological technique (Coons et al., 1951).

The use of the cryostat for plant tissues was limited till now. Chayen et al. (1960a, b) and Gahan and Rajan (1965) succeeded in producing

cryostat sections after treating the plant tissue with polyvinyl alcohol for stabilization of cell and tissue structures. Embedding in gelatin was also successful (FRIED and FRANKLIN, 1961). Cryostat sectioning of untreated plant material however was only successful for woody tissue (WEAVER and LAYNE, 1965).

Therefore, it was necessary to find an embedding medium for the plant tissue, the application of which prevents disruption during freezing and sectioning and limits the change of substances in the tissue during embedding to a minimum, so that the tissue retains its original state. Analogous to the possibility to support sensitive animal material with another animal tissue, e.g. retina with liver slices (SUTTER and MEIER-RUGE, 1965), it was tried to embed plant material in a homogeneous plant tissue. Such experiments, e.g. with fresh elder pith, did not show satisfactory results. However certain animal organs, above all brain and liver, yield an excellent embedding material.

There are several reasons to prefer the brain in comparison with the liver. The brain yields a homogeneous embedding medium for the plant tissue. It contains fewer vessels than the liver, an advantage for sectioning. A contamination of the plant tissue would occur more likely with the liver, because this is rich in enzymes and has also a high mineral content. The content of calcium is higher in the liver than in the brain (SCHMIDT and GREENBERG, 1935). Moreover, the liver can accumulate iron (FAZIO and BUFFA, 1959) and copper (WOLFF, 1960).

A preliminary report on this work has been given recently (LÄUCHLI, 1966).

Embedding and Sectioning

Embedding of unfixed plant tissue in brain is shown schematically in Fig. 1. Fresh brain tissue from laboratory animals, generally from rats, was always used. The brain must be cut in such a manner, that the plant tissue can be entirely embedded. The block is affixed to the tissue holder of the microtome of a Dittes-Duspiva cryostat with some distilled water, frozen with CO_2 to about -80^0 C in a few seconds and placed in the cryostat. After about 1 hr the tissue has reached the temperature of the cryostat and can be sectioned.

DIEZEL (1958) has recommended the Dittes-Duspivia cryostat for rapid sectioning of pathological material and for histochemical determinations of enzymes in animal tissues. This cryostat is also very convenient for sectioning plant tissues. The quality of the sections depends on the temperature of the cryostat and the section thickness. Optimal conditions must be determined empirically for each tissue. The water content of the tissues is very important. Generally one has to section tissues rich in water at higher temperatures than tissues with a low water

content. Because fresh plant material has usually a higher water content than animal tissue, optimal section thichness is greater for plant material. The optimal cryostat temperature for the brain is about -14^0 to -20^0 C. If a block is sectioned at temperatures beyond this range, partial disruption may appear in the embedding medium.

This method was tested for different plant tissues and the optimal cryostat conditions were elaborated. The results are given in the table. They should serve only as terms of reference.

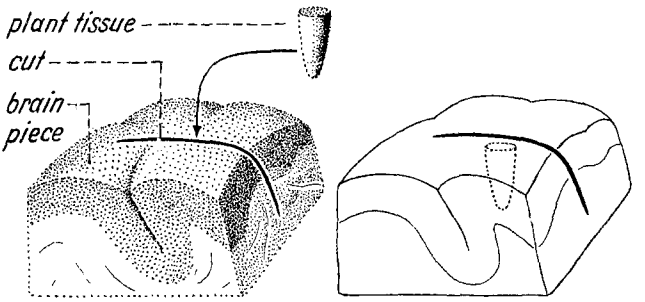

Fig. 1. Embedding of fresh plant tissues in unfixed brain for cryostat sectioning (schematical representation)

Table. *Cryostat Conditions for Sectioning Fresh Plant Tissues Embedded in Brain*

Plant tissue	Optimal sectioning temperature (° C)	Section thickness (μ)	
		For routine work	Minimal
Apical meristems	−16 to −18	12—16	10
Leaves	−14 to −16	20	16
Roots and stems	−11 to −13	20	16

The sections are affixed to cover-slips in the cryostat by cautious thawing and allowed to dry in the cryostat for about 2 hr. Because of the appearance of disruptions and also of damages from condensation water (MEIER-RUGE et al., 1964), the sections must not be warmed immediately to room temperature, but be placed in a box (e.g. made of styropor), containing dry ice and a drying agent (silica gel). After evaporation of the dry ice the sections warm slowly to room temperature with the box. They are now ready for histochemical investigations.

Fixation and Staining

For histochemical experiments it is necessary to have fixed and eventually stained sections for the purpose of comparison. Simple and rapid fixation and staining procedures for crystat sections of plant material are now described.

Reagents

FAA: 50% ethyl alcohol — glacial acetic acid — 40% formaldehyde 90:5:5.
Formalin pH 7,0: 100 ml 40% formaldehyde
900 ml distilled water
4 g $NaH_2PO_4 \cdot 2\ H_2O$
8 g $Na_2HPO_4 \cdot 2\ H_2O$.
Haemalum: 2 g haematoxylin
0,2 g $NaJO_3$
50 g $KAl(SO_4)_2 \cdot 12\ H_2O$
20 g chloralhydrate
1 g citric acid
1000 ml distilled water
(allow the reagent to stand for 1 week before use).
HCl — alcohol: 10 ml 25% HCl
1000 ml 50% ethyl alcohol.

Apical meristems

1. Fix sections in FAA for 30 min.
2. Wash with 50% alcohol and then with distilled water for 5 min each.
3. Stain in haemalum for 1 hr.
4. Wash with running water for 5 min.
5. Destain in HCl — alcohol for a few sec (this should be controlled by observing sections under the microscope).
6. Wash with running water for 10 min.
7. Dehydrate by passing through 30%, 50%, 70%, 90%, 96% and 2 changes of absolute alcohol for 5 min each.
8. Place in a mixture of equal parts of absolute alcohol and xylene and then place in pure xylene twice for 5 min each.
9. Mount with Eukitt.

Nuclei and chromosomes stain blue, cytoplasm light — gray and primary cell walls appear colourless.

Roots and stems

1. Fix sections in FAA for 30 min.
2. Wash with 50% alcohol and then with running water for 5 min each.
3. Mount with glycerin — gelatin.

Observe sections with polarized light.

If stained sections are preferred, use the safranin — fast green staining (JENSEN, 1962; p. 90).

Leaves

1. Fix sections in formalin pH 7.0 for 30 min.
2. Wash with running water for 5 min.
3. Mount with glycerin — gelatin.

Observe sections with visible light or phase contrast illumination.

Results and Discussion

Fig. 2 shows a cross section through a root tip of maize after embedding in brain. The thin cell walls and the first intercellular spaces of the periblem are well preserved, also the first vessels. Nuclei in mitosis could repeatedly be observed with higher magnification.

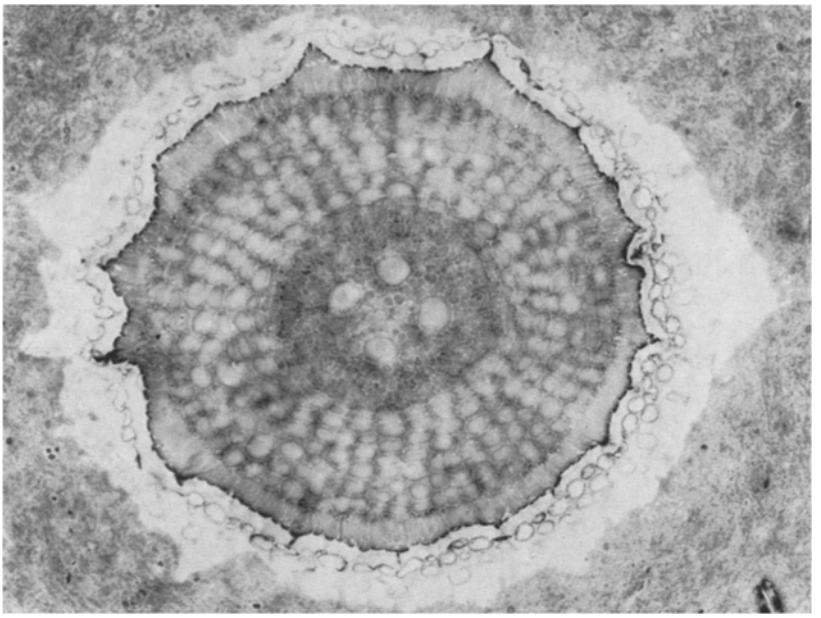

Fig. 2. Cross section through a root tip of maize. Haemalum-staining. ×120

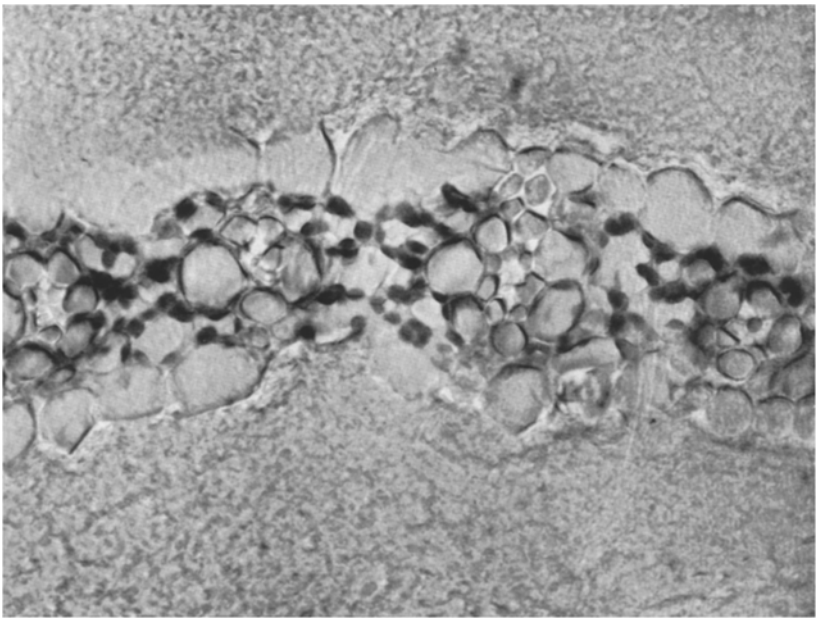

Fig. 3. Cross section through a maize leaf. Visible light. ×500

A part of a cross section through a maize leaf is represented in Fig. 3. The epidermis remains intact only with optimal embedding in brain. At higher magnification the chloroplasts show grana. Nuclei are seen with phase contrast illumination.

A cross section through a maize root is shown in Fig. 4. At the sectioning temperature of -12^0 C the brain embedding has partially

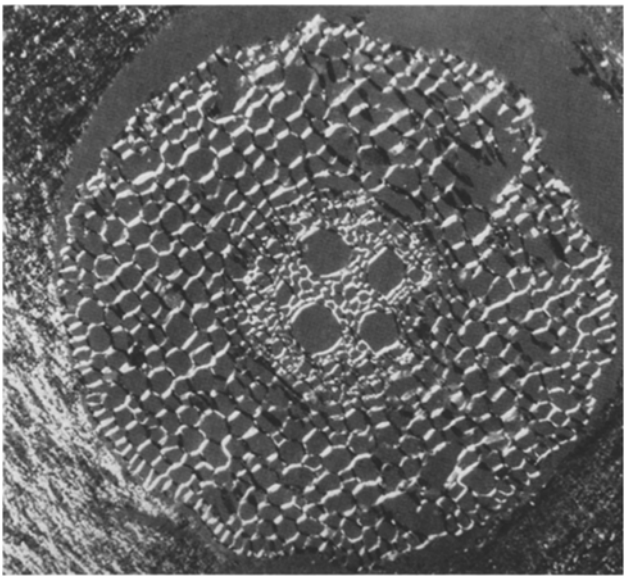

Fig. 4. Cross section through a maize root. Polarised ultraviolet light. ×95

broken away from the root. The epidermis and the cortex show slight damage. The cell walls are strongly birefringent.

Problems of mineral transport through plants (LÄUCHLI, 1965) and the localization of enzymes in tissues and cells are investigated with this technique. The initial results of the enzyme histochemical studies are now described.

Methods of Enzyme Determination

The enzyme studies were done with cross sections (16 μ) through root tips of maize.

The following enzymes were determined:

1. Dehydrogenases. NAD-diaphorase was determined according to NACHLAS et al. (1958), succinic dehydrogenase (SDH) according to NACHLAS et al. (1957); and the method of HESS et al. (1958) was used for lactic dehydrogenase (LDH) and isocitric dehydrogenase (ICDH). The tetrazolium salt (TNBT) (MEIER-RUGE, 1965a) was applied for all determinations. Sections were incubated for 1 hr. The mounting medium was hydramount.

2. Esterase. α-Naphthyl acetate esterase (aliesterase) was determined according to the method of PEARSE (1960, p. 886) with the diazonium salt Blue B. Sections

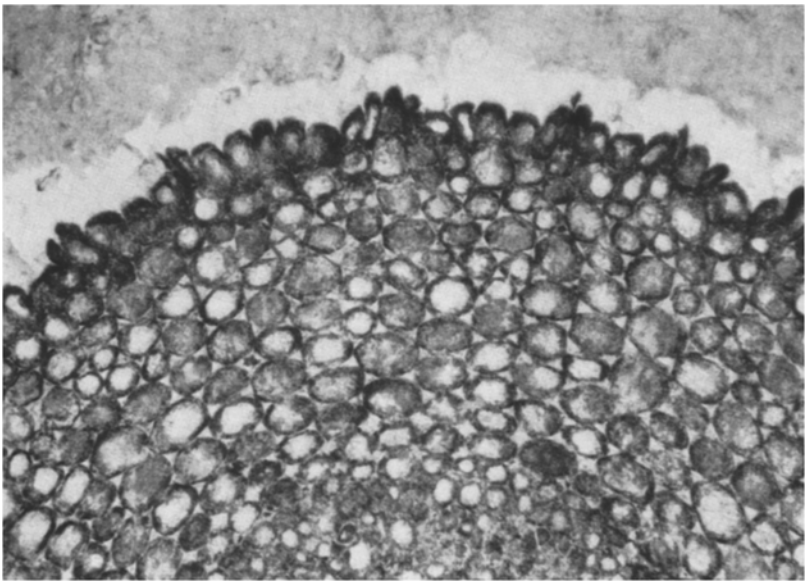

Fig. 5a. High activity of NAD-diaphorase throughout the root section. ×250

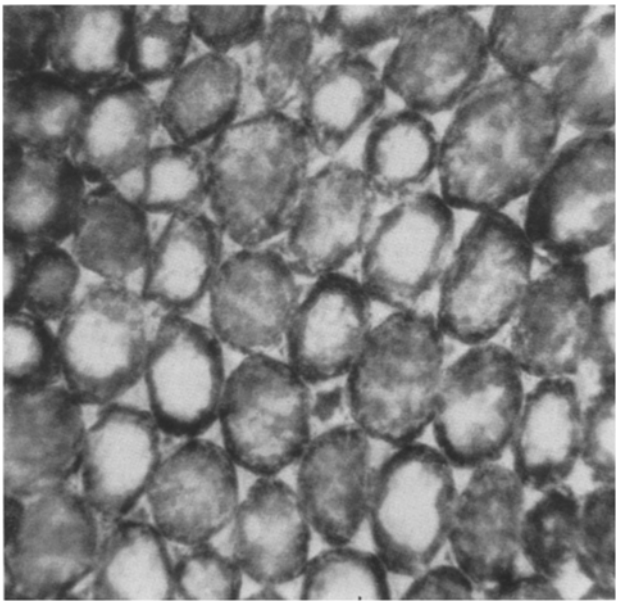

Fig. 5b. Intracellular localization of NAD-diaphorase in the cytoplasm. ×650

Figs. 5—8. Localization of some enzymes in root tips of maize (cross sections)

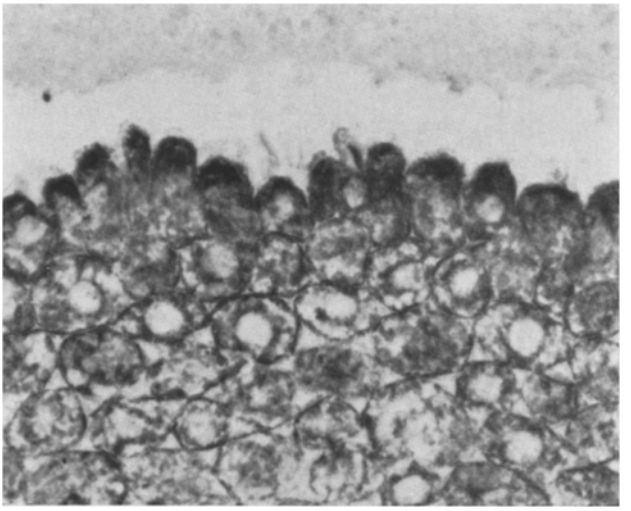

Fig. 6. Demonstration of lactic dehydrogenase showing the enzyme activity in the cytoplasm, especially near the nuclear membrane, and bound to the mitochondria. ×450

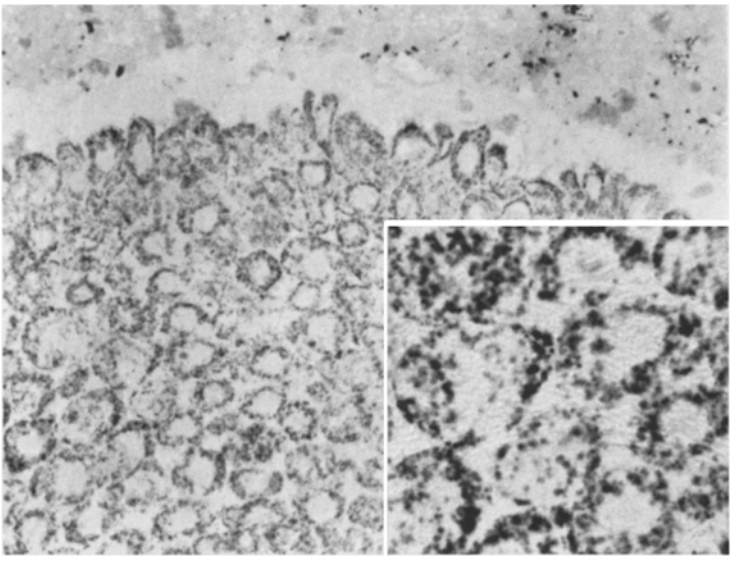

Fig. 7. Localization of succinic dehydrogenase in the mitochondria. ×300, enlarged portion ×650

were incubated for 30 to 60 min. A post-incubation with 10% potash alum for 30 min was applied to inhibit N_2 formation after mounting (MEIER-RUGE and MEIER, 1963). The mounting medium was glycerin-gelatin.

3. Phosphatases. The method of BARKA and ANDERSON (1962) was tested for the determination of acid phosphatase, but some alterations must be worked out for optimal localization in plant tissues. Glucose-6-phosphatase was determined

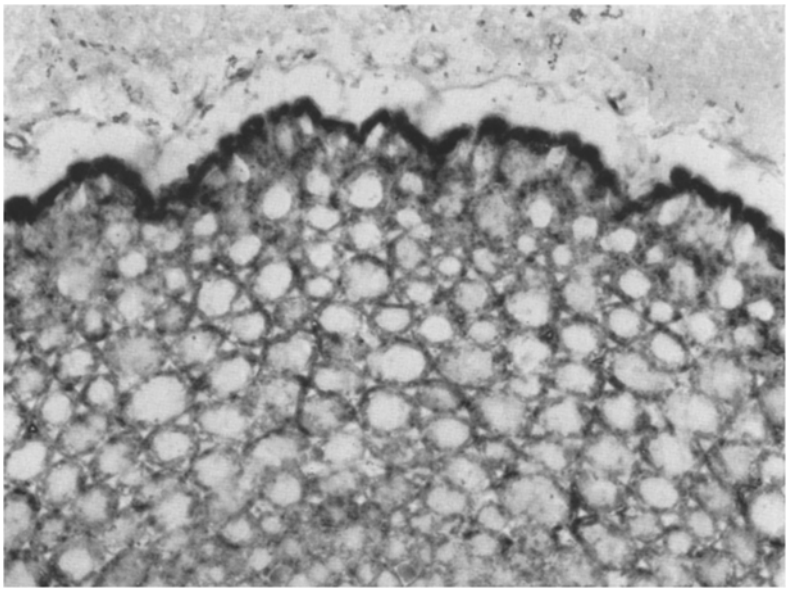

Fig. 8a. Distribution of aliesterase with high activity in the dermatogen near the outer cell walls. ×300

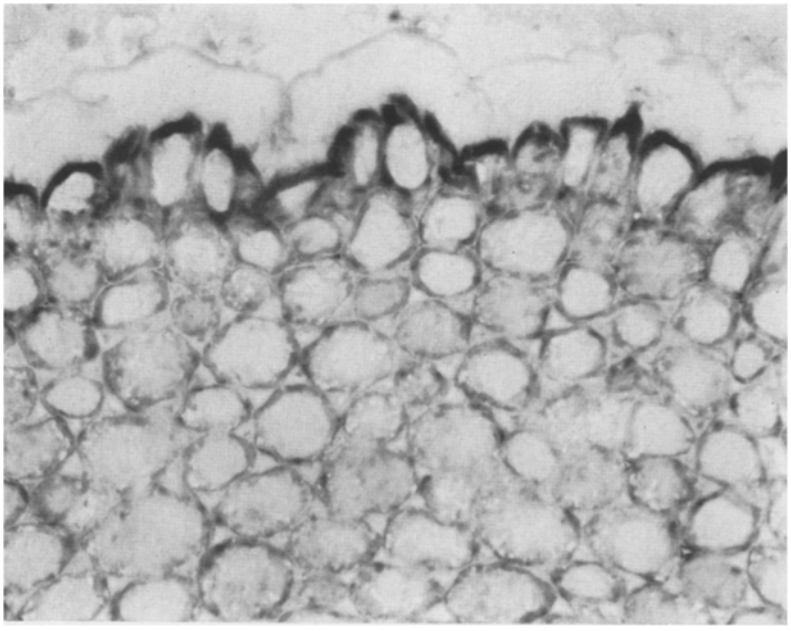

Fig. 8b. Cytochemical localization of aliesterase in the cytoplasm close to the plasm membrane. ×475

according to CHIQUOINE (1953). Sections were incubated for 2 hr. The mounting medium was hydramount.

No counter stains were applied after the enzyme determinations.

Incubation mixtures without the substrate were used as controls; they gave no staining reaction or only a weak one.

Using the cryostat technique, exact localization of enzymes in plant cells, similar to animal materials, is possible.

The reaction with the tetrazolium salts of dehydrogenases involving NAD as a coenzyme is mediated by the NAD-diaphorase. This intermediate enzyme has a high activity throughout the root section, especially in the dermatogen (Fig. 5a). Intracellularly the NAD-diaphorase is found in the cytoplasm; the nuclei do not show any enzyme activity (Fig. 5b).

LDH seems to be bound to the mitochondria and distributed in the cytoplasm. A fairly high activity is localized near the nuclear membrane (Fig. 6). Furthermore, the nuclei are represented exactly by negative staining. In animal cells the LDH is distributed mainly in the cytoplasm (MEIER-RUGE, 1965b).

This enzyme, characterizing the anaerobe glycolysis, was found rarely in higher plants (DAVIES et al., 1964). It could be shown in isolated mitochondria from pea epicotyls by DAVIES (1956), but the cytochemical demonstration of the LDH was not clearly successful (KOENIGS, 1966). Using this cryostat technique, it is now possible to study the distribution of LDH in the different plant tissues.

In Figure 7 the distribution of SDH, the limiting enzyme of the citric acid cycle, is shown. Similar to the results of KOENIGS (1966), the enzyme activity is only weak. With higher magnification, an exact mitochondrial localization of the SDH can be seen. AVERS and KING (1960) used intact roots for the determination of the SDH; they could show the localization to be in the mitochondria by direct microscopic observation of the dermatogen. This finding was confirmed by the electron microscopic demonstration of diformazan particles in mitochondria after the test for SDH (AVERS and TKAL, 1963).

The distribution of the ICDH corresponds with that of the SDH.

The aliesterase, an enzyme of great importance for the metabolism of proteins and lipids in animal cells (MEIER-RUGE, 1965b), shows a very high activity in the dermatogen near the outer cell walls (Fig. 8a). Cytochemically, the aliesterase is localized in the part of the cytoplasm towards the cell wall, therefore close to the plasm membrane (Fig. 8b). This is contrary to the findings in animal cells, where the enzyme is distributed generally throughout the whole cell except the nucleus.

Glucose-6-phosphatase has only a very weak activity in the examined sections. The experiments on the acid phosphatase are not yet finished.

This cryostat technique is superior to earlier used methods for cytochemical studies of enzymes (AVERS, 1958; AVERS and KING, 1960; VANDEN BORN, 1963; KOENIGS, 1966). All these mentioned methods do not consider the possibility, that soluble enzymes in cytoplasm may diffuse during the preparation. Actually, a diffusion of enzymes must have occured in the earlier investigations, because no enzyme localizations in plant cytoplasm were reported. Yet the occurence of enzymes in various parts of the plant cell must be expected according to findings on animal material.

The described cytochemical localizations of enzymes give evidence, that the cellular structures are mainly preserved in cryostat sections of meristematic tissue. Likewise the diffusion of enzymes is prevented, since only definite parts of the cells revealed an enzyme reaction in all examined sections, and no enzymes were found in the nuclei and intercellular spaces.

Zusammenfassung

Es wird eine einfache Kryostatschnitt-Technik für natives Pflanzengewebe beschrieben. Die Gewebe werden in unfixiertes Gehirn eingebettet, mit CO_2 eingefroren und in einem Kryostat bei Temperaturen geschnitten, die je nach Pflanzenmaterial zwischen -12^0 und -18^0 C liegen. Die Gehirneinbettung verhindert ein Zerreißen der Planzengewebe während des Einfrierens und Schneidens. Man läßt die Schnitte im Kryostaten trocknen und erwärmt sie langsam auf Zimmertemperatur, um Auftauartefakte zu vermeiden.

Einfache Fixierungs- und Färbeverfahren werden für histologische Untersuchungen angegeben.

Die Zuverlässigkeit der Kryostatmethode wird an Hand von Schnitten durch verschiedene Pflanzengewebe gezeigt.

Als Beispiel für die Anwendung der Kryostat-Technik in der Histochemie dient die cytochemische Lokalisierung einiger Enzyme in meristematischen Zellen. Von den untersuchten Dehydrogenasen ist die NAD-Diaphorase im Cytoplasma nachweisbar, die Milchsäuredehydrogenase scheint sowohl gebunden in den Mitochondrien als auch im Cytoplasma, vor allem in der Umgebung der Kernmembran vorzukommen. Bernsteinsäure- und Isocitronensäuredehydrogenase sind in den Mitochondrien lokalisiert. Die Aktivität der Aliesterase befindet sich nahe der Plasmamembran. Im Zellkern konnte keines der untersuchten Enzyme nachgewiesen werden.

Acknowledgments. This investigation was carried out partially in the pathological institute of the university of Basle. I wish to thank Prof. Dr. A. WERTHEMANN for the permission to work in his institute, P.D. Dr. W. MEIER-RUGE for valuable discussions and Miss V. UNTERRICKER for technical assistance.

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