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Localization of Acid Phosphatase in the Differentiating Root Meristem

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This paper is dedicated to Professor BOHUMIL NĚMEC *on the occasion of his 90th birthday*

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Abstract. The localization of acid phosphatase was studied by Gomori's newer technique and by azo-coupling methods (α-naphthyl phosphate + fast red ITR or fast garnet GBC; AS or AS D phosphate + fast blue B or fast red violet LB) in the root tips of *Vicia faba* L. on paraffin sections (fixation with Wolman's acidified ethanol) and on frozen sections (fixation with Baker's calcium formol). Analogous results were obtained on the material treated in various ways and using different methods. In the broad bean, the reaction is most intense in the cap. In the meristematic zone, the primary core is more intensely stained than the ground parenchyma of the central cylinder. The phloem and xylem poles are usually strongly positive. Using both types of methods on Wolman fixed paraffin embedded material, essentially the same localization of acid phosphatase was found in the root tips of *Ricinus communis* L., *Lupinus luteus* L., *Sinapis alba* L., *Allium cepa* L. as in the broad bean. In *Zea mays* L. the rhizodermis and the hypodermic layers of the primary core were found to be most active. On sections of Wolman fixed paraffin embedded broad bean the most intense reaction was observed at pH 4·2—4·8. In the same material, both the azo-coupling and the Gomori reaction is inhibited by 10^{-2} M NaF, but 10^{-2} M tartaric acid only inhibits the Gomori reaction.

Classical plant anatomy distinguished different tissues either by the shape, proportions, size and the mutual set-up of cells or by the mode of thickening of their cell membranes. These aspects were sufficient for the description of mature tissues. Physiological anatomy began to distinguish tissues by their function. Following partly the conception of Protoplasmatische Pflanzenanatomie, developmental anatomy today is attempting to establish new criteria for the differential characterization of the zones of the meristems. In previous work (BENEŠ, LOJDA, HOŘAVKA 1961, BENEŠ 1962a, 1962b, 1962c) we have already dealt with the question of the extent to which histochemical methods are suitable for the study of tissue differentiation, i.e. if it is possible in a morpho-

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logically more or less uniform meristem to distinguish parts which are metabolically, i.e. chemically different. The present paper reports further studies along the same lines. From the methodical point of view, we wish to compare the material treated by azo-coupling methods with that treated by the Gomori technique.

Material and Methods

The seeds of *Vicia faba* L., var. Chlumecký, *Ricinus communis* L., *Lupinus luteus* L., *Sinapis alba* L., and *Zea mays* L., var. Český bílý koňský zub, were left in water overnight and then germinated on moist filter paper in Petri dishes. The adventitous roots of *Allium cepa* L. were obtained in the usual way; the bulb was placed in the neck of the Erlenmeyer flask so that the basal part was partly immersed in water. The root tips of all objects were then fixed with modified Wolman's mixture and vacuum embedded in paraffin. In addition, the broad bean root tips were fixed with BAKER's calcium-formol and embedded in glycerin-gelatine. Paraffin-embedded objects were further treated in the usual ways, including the use of egg white containing adhesive and the xylene-ethanol series for deparaffinization. The objects embedded in glycerin-gelatine were sectioned by the freezing microtome (BENEŠ et al. 1961, BENEŠ 1962c). In the present work, only transverse sections were studied, as they are anatomically easier to examine.

Three types of methods can be used to determine acid phosphatase in situ: a) indoxyl methods, b) Gomori's methods, c) azo-dye methods.

ad a) The indoxyl methods have only rarely been used so far (HOLT 1954, see DEANE et al. 1960).

ad b) Two years after the development of the alkaline phosphatase technique, GOMORI (1941, see PEARSE 1960) introduced his first method for acid phosphatase. It appeared that the precise localization and reproducibility of the results depended on the ratio of the concentrations of individual components of the medium in addition to the substrate and pH. On the basis of this knowledge GOMORI (1950, see PEARSE 1960) introduced a more reliable modification, the advantage of which was confirmed by HOLT (1959). GLICK and FISCHER (1945) modified the older Gomori technique for plant material. In the present paper the Gomori technique of 1950 was used as recommended by LOJDA (1961). Stock solution was prepared as follows: In 1,000 ml. acetate/acetic acid buffer pH 5·2 (5·4 g. Na acetate . 3 H_2O was dissolved in H_2O, to which 0·6 ml. glacial acetic acid had been pipetted and H_2O added to give 1,000 ml.) 1·2 g. $Pb(NO_3)_2$ was dissolved. Prior to application, 0·3 g. glycerophosphate (Lachema) dissolved in 10 ml. H_2O was added to 100 ml. stock solution and pH adjusted to 5·2 with 1N HCl, the solution was mixed several times and then filtered after 30 min. standing. The paraffin sections were incubated for 3 hours, the frozen sections for 5—30 min., both at 37° C. The controls were incubated in medium minus substrate. After incubation the sections were rinsed twice for 30 seconds in H_2O exposed for 30 min. to a fresh solution of yellow ammonium sulphide (10% commercially available solution was diluted 1 : 10 with H_2O). Then the sections were washed thoroughly in water. Both with the Gomori technique and the azo-coupling methods an aqueous medium "Gama" (BENEŠ 1962a) was used for mounting the preparations. Since the controls were negative, rinsing with dilute acetic acid after incubation was not considered necessary.

ad c) YIN (1945, see DEANE et al. 1960) first used the azo-coupling methods at acid pH level. The azo-coupling methods were thus used for demonstration of acid phosphatase in plants four years before they were applied to study animal acid phosphatase in situ. Since then only SURREY (1955, see van FLEET 1962) and WALEK-CZERNECKA (1962), as far as we know, used these methods on plant material. However, in animal objects azo-coupling methods for the demonstration of acid phosphatase were developed by many authors (see DEANE et al. 1960, PEARSE 1960). At the present time, simultaneous methods are mostly used, with α-naphthyl phosphate or phosphates of the AS naphthol series as substrate. PEARSE (1960) reports a standard method with α-naphthyl phosphate. The ratio of the substrate to diazonium salt (10—20 mg. substrate : 20 mg. salt) is not suitable according to some authors (LOJDA 1958, GÖSSNER 1958). In the present work the following medium was used: 10 mg. α-naphthyl phosphate (provided by Mr. MÜLLER, 1st Institute of Pathology, Faculty of Medicine, Charles University, Prague) is dissolved in 50 ml. 0·05—0·5M acetate/acetic acid buffer pH 5·2, to which 50 mg. of Echtrosalz ITR (Rohner) or Echtgranatsalz GBC (Rohner) is added and then the solution is filtered. No substantially improved localization was observed at the anatomical level using hexazotized pararosaniline. The paraffin sections are incubated for 1—2 hours, the frozen sections for about 30 minutes,

both at 37° C. For demonstration of acid phosphatase with AS and AS D naphthyl phosphate (synthetised by Dr. VEREŠ, Isotope Laboratory, Institute of Biology, Czechoslovak Academy of Sciences, Prague, according to CHYTIL and MÜLLER 1961) the formulae for ASBI phosphate as given by BURSTONE (1958, see PEARSE 1960) were used: 0·5 ml. stock solution (2% solution of AS or AS D naphthyl phosphate in dimethylphormamide kept in the refrigerator; no decomposition of substrate was noted within 2 weeks) was pipetted drop by drop into 50 ml. 0·05 – 0·5M acetate/acetic acid buffer pH 5·2, then 70 mg. Fast blue B (Schwarze) or fast red violet LB (Verona) was added and the solution filtered. The paraffin sections were incubated for 2 – 3 hours, the frozen sections for 30 – 60 minutes, both at 37° C. The incubation in medium minus substrate served as the control.

The advantages and the drawbacks of the methods for demonstration of hydrolases in plant material were discussed elsewhere (BENEŠ 1962c). The azo-dye methods are suitable for the observation of the pH optimum and for a continuous control of staining intensity during incubation. The Gomori technique is adequate for the study of substrate specificity.

In objects with high activity (*Ricinus communis* L., *Zea mays* L.) the localization of acid phosphatase was investigated in relation to different incubation times.

The pH optimum and the inhibition by NaF or tartaric acid were studied on paraffin sections of broad bean root tips. For the investigation of the pH optimum either 200 ml. medium for the Gomori's reaction or 200 ml. medium for the azo-coupling reaction (AS-naphthyl phosphate + Fast red violet LB) was prepared. The medium was divided into 50 ml. aliquots and adjusted with 1N HCl, or 1N NaOH to pH 4·2; 4·8; 5·2; 5·5 as measured by means of indicator papers (Phan Lachema). Random control with the pH meter (Radiometer) showed the accuracy of the adjustment of pH to be within $\pm 0{\cdot}3$ pH unit. The effects of the inhibitors were studied using the preincubation of the slides in 0·05M acetate/acetic acid buffer pH 5·2 containing the inhibitor (10^{-2}M NaF, Lachema, 21 mg./50 ml., or 10^{-2}M tartaric acid, Association for Chemical and Metallurgical Production, 75 mg./50 ml., in both cases for 30 min. at 37° C) followed by the incubation of the slides in complete medium containing the same amount of the inhibitor as stated above. Since the pH decreases to about 4·5 after the addition of tartaric acid, the buffer, in which the inhibition controls were preincubated, was adjusted to the same pH with 1N HCl, whereas the complete medium containing tartaric acid was adjusted to pH 5·2 with 1N NaOH.

Results

On transverse sections of the broad bean root, at the distance of about 1 to 2 mm. from the apex, the reaction is strongest in the cap. Beginning with the rhizodermis the reaction is weaker, nevertheless the primary core is more intense than the ground parenchyma of the central cylinder. In the central cylinder the xylem and phloem poles are rather intense (fig. 1—4), but the relative intensity of the reaction in different parts of the central cylinder varies in individual root tips treated simultaneously. The Gomori technique and the azo-coupling methods give essentially the same results on both paraffin (fig. 1, 2) and frozen sections (fig. 3, 4).

In all the other objects except for *Zea mays* essentially the same localization of acid phosphatase was found as in the broad bean using both the Gomori technique and the azo-coupling methods, on paraffin sections (fig. 5—7). About 0·5—1 mm. from the apex the cap is most intense and the primary core displays a stronger reaction than the central cylinder. In the onion the selective reaction in the pericambium is striking (fig. 5, 6). In *Zea mays* at the distance of about 1 mm. the cap is no longer visible. The most intense reaction is found in the rhizodermis and in the hypodermic layers of the primary core. In all objects tested the relative intensity of the reaction in individual parts of the root changes, depending on the distance from the apex.

Except for one case, at the intracellular level no reaction was observed which was more intense in the nucleus than in the cytoplasm. Only with the

Gomori technique was more intense staining of nuclei noted at the sites of very low activity (fig. 6).

When incubating paraffin sections of broad bean root tips in Gomori's medium of different pH, the most intense reaction was obtained at pH 4·8. At pH 5·2 the reaction is still quite intense, but it is weak at pH 5·5. Only some of the roots revealed an intense reaction at pH 4·2, the others were rather weak at this pH. Quite irregular fluctuation of the intensity of Gomori's reaction was observed in single sections at pH 4·2. Using the azo-coupling method, the reaction was intense at pH 4·2 and 4·8, at pH 5·2 some weakening of the intensity may be seen, at pH 5·5 the reaction was weak. At pH 4·2 the azo-coupling reaction was intense even in objects that have only a slightly visible Gomori reaction at this pH. Localization of acid phosphatase did not change when incubation was made at various pH levels.

In paraffin sections of the broad bean root meristem both the Gomori and the azo-coupling reaction were inhibited by NaF. However, tartaric acid inhibited only the Gomori reaction.

Discussion

According to PRESNOV (1954), the phosphatases were discovered in 1905 by IVANOV in broad bean seedlings. Phosphatases are usually defined as hydrolytic enzymes that split off the phosphoric acid residue from ester (C—O—P), anhydride (P—O—P, and also C—O—P) or the amide bond (N—P). Recent findings in vitro showed that some phosphatases were identical with transphosphorylases or transphosphatases (see reviews by AXELROD 1958, SCHWARZE 1958). Phosphatases have usually been classified on the basis of pH optimum and substrate specificity (ROCHE 1950, ROCHE and NGUYEN-VAN THOAI 1950). In contrast with animal tissues, non-specific phosphomonoesterases of the second type by the Roche classification, i.e. phosphatases with the optimum in the acid range, are most usual in plants. However, even in plant material a series of specific phosphatases was found, some of them with the optimum at alkaline pH. Enzymologically, the question of plant phosphatases is so unclear that the author of the article on phosphatases (SCHWARZE 1958) in the most comprehensive compendium of plant physiology available today does not attempt to deal with them in detail.

Localization of acid phosphatase in plants was mostly considered in association with carbohydrate metabolism (FREY 1954). In animal material, VORBRODT (1958) investigated the association between the localization of acid phosphatase in situ and proteosynthesis and PRZELECKA et al. (1962) the relation between alkaline phosphatase and lipid dynamics. The findings of marked activity particularly of alkaline phosphatases in the nuclei or the chromosomes (they seem to be erroneous on the basis of present knowledge) suggested that phosphatases may participate in the metabolism of nucleic acids. All these aspects would certainly be of interest in considering the localization of acid phosphatase in the meristems. However, so long as the role of phosphatases in metabolism is not sufficiently clear biochemically, it will be difficult to consider its physiological function.

When evaluating the localization of acid phosphatase in detail, we attempt

in fact to make a quantitative evaluation. The question remains of the extent to which the reactions for demonstration of hydrolases are quantitative, i.e. what is the correlation between the intensity of staining and the activity of the enzyme. Even the intensity of staining alone is difficult to consider because the thickness of the absorbing layer (see BENEŠ 1962c) changes due to vacuolization of the cytoplasm and the shade of the final reaction product also varies.

It is certainly inaccurate to make a static description of localization. Individual parts of the root may give different results at different sites, e.g. the proliferating part of the root cap and its dying part, primary core of the meristematic and prolongation zone etc.

At the present time, there are two reviews on the histochemical demonstration of enzymes in plants (VAN FLEET 1952, 1962). HILBE and MARRON (1941, see VAN FLEET 1962) were the first to make a histochemical demonstration of phosphatase in plants. In addition to the papers reported in the review of VAN FLEET (1962) alkaline phosphatase in plants was also demonstrated histochemically by ROSS and ELY (1951), PFEIFFER (1956), SHARMA (1953), SHARMA and CHATTERJEE (1961). The following papers on acid phosphatase were not included in the review by VAN FLEET (1962): MANINGAULT and JUSSIER (1951), MCGREGOR and STREET (1953), FREY (1954), WILSON and CUTTER (1955), SHARMA (1956), HORÁK (1957), AVERS and GRIMM (1959b), SLEPTCHENKO (1959), AVERS and KING (1960), FREY-WISSLING and HÄUSERMANN (1960), SCHLÖSSER (1960), AVERS (1961), PODDUBNAYA-ARNOLDI et al. (1961), SHARMA and CHATTERJEE (1961).

Even though the literature on the histochemical demonstration of phosphatase in plants is far from being so extensive as that related to animal tissues, it is impossible to summarize all the results on the localization of acid phosphatase in plant tissues, because important methodical details would be omitted. It was therefore decided to deal in detail with the localization of acid phosphatases in the root tips.

YIN (1945) found phosphatase activity to be highest in the whole root meristem of *Lamium album* and *Iris germanica*, but in the more developed zone the activity was weaker in the primary core than in the procambial tissues, the cap being inactive. In wheat, GLICK and FISCHER (1946) found an intense reaction in the cap; the nuclei and nucleoli in the root meristem were also very active. DYAR (1950) compared the localization of phosphorylase and phosphatase in pea root tips and found strong acid phosphatase activity in the cap. MCGREGOR and STREET (1953) observed on paraffin sections of tomato roots that the whole meristem showed a high activity, particularly the root-cap and the differentiating xylem. The nuclei were more intensely stained than the cytoplasm. FREY (1954) found the periblem to be more positive than the plerome in the root-tips of various seedlings, however, later the reaction was most intense in vascular tissues. JENSEN (1956) noted that the cells of the root-cap and the cells of the developing vascular tissues were most active in the onion, broad bean and pea. HORÁK (1957) obtained a positive reaction in the meristem and the vascular tissues in adventitious roots of the cuttings of *Vitis vinifera*. Avers and her colleagues reported acid phosphatase in root tips in a number of works performed originally at the University of

Miami, Coral Gables, Fla. and later at the State University, New Brunswick, N. J. In her earliest studies AVERS (1958), AVERS and GRIMM (1959a) revealed non-specific phosphatase by Gomori's technique with glycerophosphate. In the paper of AVERS and GRIMM (1959b) the demonstrated enzyme was taken for glucoso-6-phosphatase. Nevertheless, the assumption of specific glucoso--6-phosphatase (compare with e.g. ALLEN (1961) in animal tissues) should be supported by further evidence. In recent work, AVERS and KING (1960) studied the intracellular localization of some enzymes including acid phosphatase. In a more recent paper AVERS (1961) was concerned with ATPase, 5-nucleotidase, glucoso-6-phosphatase and acid phosphatase. However, the differences in the intracellular localization may be due to factors other than different substrates. It would be useful to compare the results in objects fixed and treated in different ways, because e.g. the different permeability of individual components of the medium can play a role in living roots. SHARMA and CHATTERJEE (1961) investigated the intracellular localization of acid and alkaline phosphatase in smear preparations of the root tip of *Vicia faba*. It would be of interest to confront their results on alkaline phosphatase in the nucleus and the chromosomes with the findings in animal tissues (NOVIKOFF. 1952 in DEANE et al. 1960, AMENTA 1961). In root tips, they obtained a positive reaction even after rather drastic treatment of objects and just the reaction for alkaline phosphatase was strongly positive, whereas the reaction for acid phosphatase was slightly visible. In our previous work (BENEŠ et al., 1961) we failed to demonstrate alkaline phosphatase in broad bean root tips.

It can be concluded from the literature on the localization of acid phosphatase in apical root meristems that the findings as made independently by various authors are quite in agreement at the anatomical level. The root tips mostly display an intense reaction in the meristematic zone, the most intense staining in the root cap and in the procambium. In the present paper essentially the same localization of acid phosphatase was found.

At the intracellular level, many authors observed an intense reaction in the nucleus and the nucleolus. In our preparations we obtained comparable results exceptionally: only with the Gomori reaction at sites of very weak activity. In animal material, the majority of the authors consider the intense reaction in the nucleus to be an artefact. A detailed study of intracellular localization should be supported by further investigations.

The reaction for acid phosphatase does not seem suitable for the histochemical study of the differentiation of the root meristems, because its localization is not selective enough even though the activity is not equal in all parts. It would, however, be of interest to study the localization of acid phosphatase in the endodermis and the adjacent layers of the primary core in *Allium cepa* roots from the aspect of WILLIAMS' views (BENEŠ 1962c).

ROSS and ELY (1951) did not obtain a positive reaction at pH 9·4 with glycerophosphate, α-naphthyl phosphate (they detected the liberated phosphate) and a series of other substrates. However, some substrates gave a positive reaction even at this pH level. MCGREGOR and STREET (1953) studied a wide pH range; they also failed to obtain positive results using the Gomori technique with glycerophosphate above pH 6. Neither did FREY (1954) find the positive reaction at pH below 3 and above 8 using the Gomori technique

with glycerophosphate; the optimum pH was in the zone immediately above pH 5. In the present paper we studied the intensity of the reaction in media of various pH on paraffin sections of broad bean root tips. The optimum was at pH 4·2 to 4·8 with the azo-coupling reaction and at pH 4·8 with the GOMORI's method; at pH 4.2 this technique was not reliable enough. For routine work we used a somewhat more alkaline medium to obtain a more contrasting picture.

10^{-2}M NaF inhibited both the Gomori's and the azo-coupling reaction in paraffin sections of the broad bean. With this concentration of NaF no greater precipitation of the medium took place. 10^{-2}M tartaric acid blocked the Gomori reaction only. The cause of this is not known. When using NaF or tartaric acid inhibition, it proved suitable to expose the tissue to the inhibitor prior to incubation in the medium with the inhibitor, as recommended by LOJDA (1961).

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K. Beneš, J. Opatrná, Ústav experimentální botaniky ČSAV, Praha: **Lokalisace kyselé fosfatázy v diferencujícím se kořenovém meristému.** — Biol. Plant. 6 : 8—16, 1964.

U kořenových špiček bobu *Vicia faba* L. jsme sledovali novější metodou Gomoriho s glycerofosfátem a azokopulačními metodami (α-naftylfosfát + fast red ITR nebo fast garnet GBC; AS nebo ASD fosfát + fast blue B nebo fast red violet LB) v parafinových řezech (fixace Wolmanovou směsí) a v řezech zmrazených (fixace Bakerovým kalciumformolem) lokalizaci kyselé fosfatázy. Stejné výsledky jsme dostali u různě zpracovaného materiálu i při aplikaci různých metod. U bobu je nejintenzjevnější reakce v čepičce, primární kůra jeví v meristematické zóně silnější zbarvení než střední válec. V středním válci bývají silně pozitivní lýkové a dřevní póly. U většiny ostatních testovaných objektů (kořenové špičky skočce obecného *Ricinus communis* L., vlčího bobu žlutého *Lupinus luteus* L., hořčice bílé *Sinapis alba* L., cibule kuchyňské *Allium cepa* L.) byla zjištěna metodami obou typů v podstatě stejná lokalizace kyselé fosfatázy jako u bobu. U kukuřice *Zea mays* L. byla nejaktivnější rhizodermis a hypodermální vrstva primární kůry. Na parafinových řezech bobu jsme zjistili nejintenzivnější reakci při pH 4,2 až 4,8; azokopulační i Gomoriho reakce je na tomto materiálu inhibována 10^{-2}M NaF, 10^{-2}M kyselina vinná inhibuje jen Gomoriho reakci.

К. Бенеш, Я. Опатрна, Институт экспериментальной ботаники ЧСАН, Прага: **Локализация кислой фосфатазы в дифференцирующейся корневой меристеме.** —Biol. Plant. 6 : 8—16, 1964.

Мы изучали у корневых верхушек боба *Vicia faba* L. при помощи нового метода Гомори с глицерофосфатом и при помощи азокопуляционных методов (α-нафтилфосфат + фаст ред ITR или фаст гарнет GBC; AS или ASD-фосфат + фаст блю B или фаст ред виолет LB) в парафиновых срезах (фиксация смесью Вольмана) и в замороженных срезах (фиксация кальцийформолом Бейкра) локализацию кислой фосфатазы. Одинаковые результаты мы получили в по-разному обработанном материале и в случае применения разных методов. У конского боба наиболее интенсивна реакция в калиптре, первичная кора является в меристематической зоне более окрашенной по сравнению с центральным стержнем. В центральном стержне находятся иногда интенсивно положительные лубяные и древесные полосы. У большинства изученных объектов (корневые верхушки клещевины *Ricinus communis* L., желтого люпина *Lupinus luteus* L., горчицы белой *Sinapis alba* L., лука *Allium cepa* L.) методами обоих типов установлена в сущности такая же локализация кислой фосфатазы, какая имеет место и у конского боба. У кукурузы *Zea mays* L. самой активной являлась ризодерма и гиподермальный слой первичной коры. На парафиновых срезах конского боба мы установили самую интенсивную реакцию при pH 4,2—4,8; азокопуляционная реакция и реакция Гомори ингибирована на этом материале 10^{-2} M NaF, 10^{-2} M виноградная кислота тормозит только реакцию Гомори.