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DISTRIBUTION OF ACID PHOSPHATASE AND PHOSPHORYLASE IN RELATION TO STARCH PRODUCTION

BY

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INTRODUCTION

In stems of higher plants acid phosphatase activity is found to be greatest in meristematic tissues, phloem tissue, and the sheath around vascular bundles (YIN, 1945). As a result sugar phosphates can not enter the phloem, and in fact sieve tubes contain sucrose only (WANNER, 1952). A similar mechanism has been found at the surface of yeast cells, where phosphatases break down sugar phosphates, the liberated glucose becoming available to the cell (ROTHSTEIN, 1954). Studies of acid phosphatase activity in endosperms (KUGLER & BENNETT, 1947; WILSON & CUTTER, 1955) confirmed localization of the enzyme in young tissues, in general in differentiating cells, where protoplasmic synthesis takes place.

A relation to carbohydrate metabolism is less clear, but according to YIN (1945) there is such a relationship, while MCGREGOR and STREET (1953) even suggested a connection between starch content and phosphatase in tomato roots.

On the other hand ROTHSTEIN (1954) reports that in the case of yeast, inhibition of the enzyme does not prevent the cells from utilizing available glucose, so that phosphatases do not appear to play an essential or direct rôle in the carbohydrate metabolism of this organism.

It is interesting to note from the above-mentioned papers that, as endosperm cells age and start to synthesize reserve materials, phosphatase activity disappears. The effect would be that sugar phosphates would then become available for starch synthesis, should the latter take place with the aid of phosphorylase. If this is so, then the presence of phosphatase would prevent starch synthesis from taking place, and therefore there would be a relation to carbohydrate metabolism. The fact that phosphorylase may be present in the plastids and mitochondria of endosperm cells long before the latter show any starch production, and in cells which, under natural conditions, never form any starch at all, (DUVICK, 1953), might partially be explained by such a relationship. Other factors, however, could interfere, for example the presence of amylases and other inhibitors, and lack of substrate.

It is also possible, that phosphorylase is not involved in the synthesis of starch, but only in its phosphorolysis (EWART, SIMINOVITCH & BRIGGS, 1954). More work on the determination of the ratio inorganic phosphate glucose-1-phosphate has to be done in various tissues and organisms, before it can be accepted that a mechanism other than that by phosphorylase exists for starch synthesis (e.g. by means of an amylosucrase, as suggested by EWART et al., 1954).

Because of the existing uncertainty, the localization of acid phophatase with a view to its possible relationship with starch synthesis in different storage tissues during their development, was studied.

MATERIALS AND METHODS

Seeds from *Scilla ovatifolia* Bak. were germinated in Petri dishes between filter paper, or grown in pots in the greenhouse. Bulbs of the same species in different stages of development were also investigated. Germination is hypogeal, as is the rule in section *Ledebouria.*

Fruits of waxy corn were obtained from the F_2-progeny of a single seed, kindly supplied by Dr M. M. MACMASTERS. Their development was investigated at several stages after pollination. The plants grew up under unfavourable conditions; many of them only produced a cob long after they had tasseled. For these reasons we could not be too sure about the time of pollination, and development of the fruits might also have been retarded. Some of the plants were grown in a greenhouse, others in a cold frame. There were no other maize plants growing in the neighbourhood, so that no special precautions against cross-pollination were needed.

Phosphatase localization. Sections made with the freezing microtome, or free-hand sections, gave much better results than the paraffin embedding method. The fresh material was frozen in a 3 % solution of gum arabic in water, and cut at 25–35 μ, washed rapidly in water, and transferred to 70 % ethanol for a short period. Free-hand sections were cut straight into 70 % alcohol.

The sections were then floated in a drop of 80 % alcohol, dehydrated, covered with collodion, and further treated following in principle GLICK's modification of GOMORI's histochemical method for acid phosphatase (1949) (incubation with glycerophosphate and lead nitrate, and conversion of the precipitated lead phosphate to PbS). The substrate solution was prepared according to the prescription given by GOMORI (1950). Residual phosphate was removed by treating the sections with 0.1 M citrate buffer at pH 4.8. After this treatment the black colour of cell walls became reduced to a faint brown or yellow colour.

The length of time of incubation in the substrate medium required to obtain a positive reaction, gives some indication of enzyme activity. Seeds, embryos, and young seedlings, required an incubation period of 1—$1^1/_2$ hours only, while older seedlings might require as much as 18 hours to show a distinct reaction. Mature bulbs required a minimum period of 12 hours.

Clearing in oil of thyme was found to be unnecessary. To avoid treatment with xylol, sections were mounted in Euparal after dehydration in absolute alcohol. Modified this way, the method gave a clear picture of the distribution of acid phosphatase.

Phosphorylase localization. Hand sections were incubated for 24–48 hours at room temperature in a 2 % solution of glucose-l-phosphate, after its pH had been adjusted to 6.0 with citric acid crystals. The slides were then stained with a solution of 0.3 g iodine + 1.5 g KI in 200 ml water. Slides could be made semi-permanent by sealing the edge of the coverslips with "Cutex".

STRUCTURE AND DEVELOPMENT OF SEED AND SEEDLING OF *SCILLA OVATIFOLIA* BAK.

The seed has a rough coat, a large endosperm, and a small tri-lobed, undifferentiated embryo (Fig. 1). The endosperm does not contain

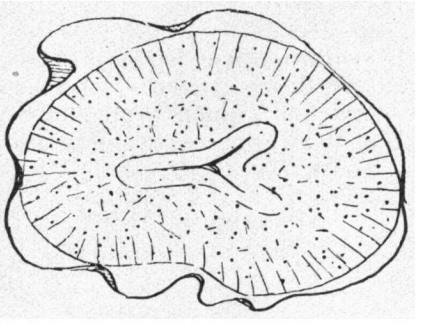

Fig. 1. L.S. of a seed of *Scilla ovatifolia*, showing a tri-lobed, undifferentiated embryo.

starch, the reserve material being stored as hemicellulose in the thick walls of the cells, which show prominent pit canals (Fig. 2).

After the seeds have been soaked in water for a few days, the embryo starts to develop (compare ARBER, 1925; BOYD, 1932; ESAU, 1953).

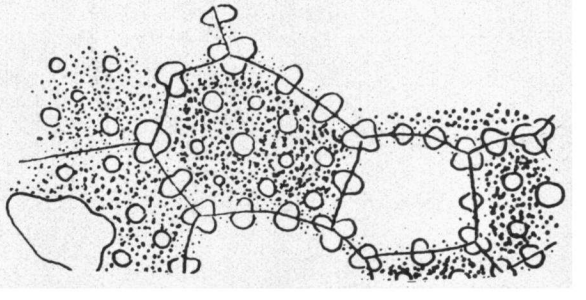

Fig. 2. Endosperm cells of *Scilla* seed.

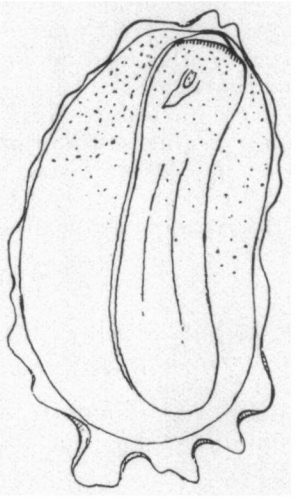

Fig. 3. L.S. of *Scilla* seed, soaked in water for 6 days, before rupture of seed coat. Embryo has formed a cylindrical cotyledon. A first indication of apical meristems is visible.

First an elongated, cylindrical cotyledon is seen (Fig. 3), but later two growing points are differentiated (first near each other) in the lower part of the cotyledon, which protrudes from the seed at this stage (Fig. 4 C, a and b); a root cap region is also present (d). At the

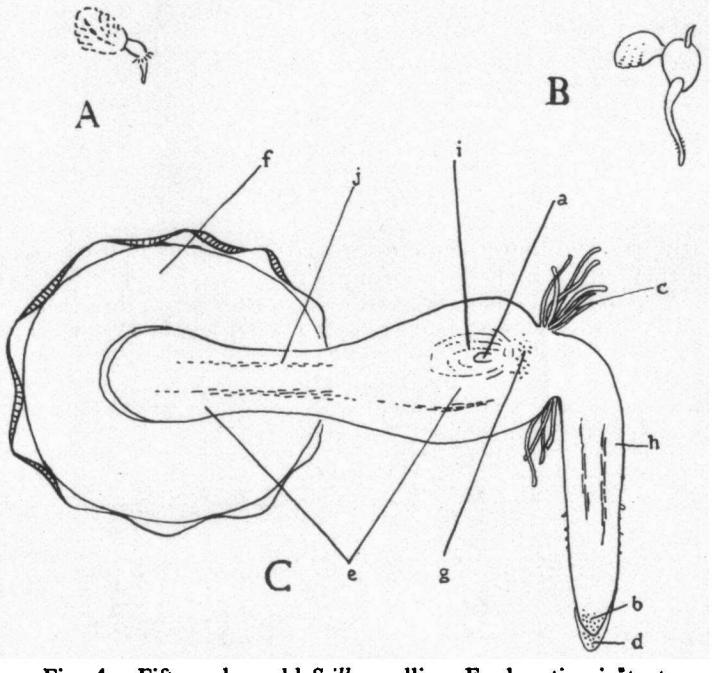

Fig. 4. Fifteen days old *Scilla* seedling. Explanation in text.

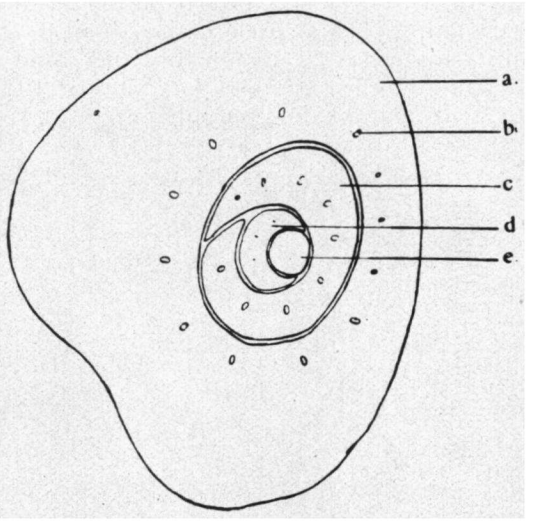

Fig. 5. T.S. of a very young bulb of *Scilla ovatifolia*. Explanation in text.

same time procambium strands develop in the cotyledon (j), and a ring of unicellular hairs (resembling root hairs in structure and function) appear at its base (c). These hairs shrivel as soon as the radicle elongates and typical root hairs are produced.

The portion of the cotyledon (e) outside the seed, and surrounding the plumule, swells, while the rest remains in the endosperm (f) and acts as an absorptive organ. Vascular bundles now develop in the cotyledons and root (h).

During following stages the root elongates further and the cotyledon swells considerably. This bulb-like structure should not be confused with the true bulb, which is finally built up by swollen leaf bases. The cotyledon acts for a long time as a protective sheath, and is pierced by the first leaf to appear (Figs. 4B and 5); it shrivels eventually. After the emergence of the second leaf, adventitious roots appear at the base of the young bulb, around the primary root.

RESULTS

A. Scilla seedlings

Distribution of acid phosphatase. Prior to germination seeds (Fig. 1) showed some phosphatase activity, which was evenly distributed throughout the tissue (2 hours incubation). As the embryo developed (Fig. 3) the activity increased considerably, so that the incubation time had to be reduced. Greatest activity was found in the meristematic regions (Fig. 4C, a and b). In all cases the enzyme appeared concentrated in the nucleus, and particularly in the nucleolus (Fig. 6). Not

much importance can be attached to distribution on the intracellular level, however, as diffusion and preferential absorption can produce erroneous results (NOVIKOFF, 1951). On the other hand a relationship has been demonstrated by VITAGLIANO and NICOLA (1948) and others between nucleic acids and alkaline phosphatases.

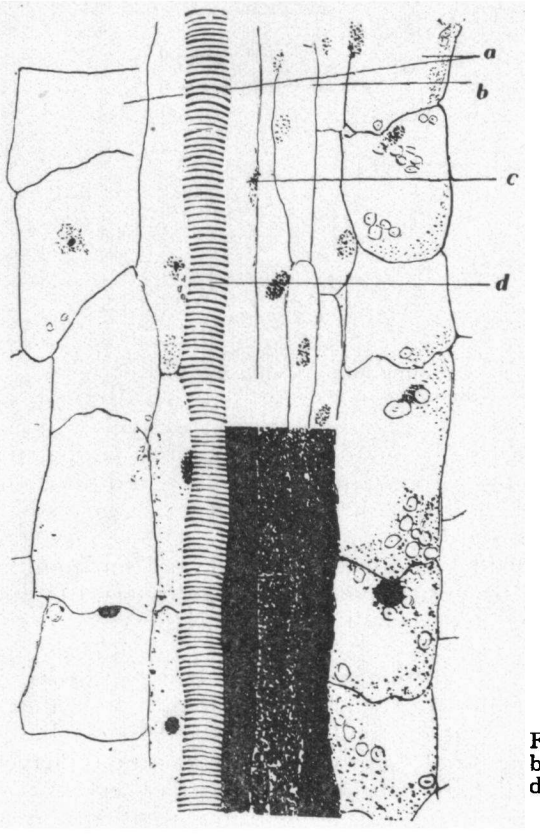

Fig. 6. L.S. of a vascular bundle in the first leaf of a 20 days old bulb of *Scilla ovatifolia*. Explanation in text.

In the young seedling phosphatase activity gradually decreased to nil in endosperm and cotyledon, except in the procambial strands. At the same time vascular bundles were differentiated, highest activity was found in the sieve tubes and phloem parenchyma. The same applied to the developing tissues in the primary root, and shoot.

In a transverse section of a young bulb (Fig. 5) the growing point (e) showed a very strong phosphatase activity, and the second leaf (d) still contained some of the enzyme. In the first leaf (c) and the cotyledonous sheath (a) activity was limited to the phloem elements in the vascular tissues (b). A longitudinal section of a vascular bundle in the first leaf is shown in Fig. 6, the top part before, and the bottom part after the phosphatase test. Note the heavy precipitate in phloem

parenchyma (b), sieve tubes (c), and nuclei; the more dispersed particles of PbS in the large cells of the starch sheath (a); and no reaction at all in the xylem vessels (d).

Distribution of phosphorylase. Slight activity could be detected in the peripheral endosperm cells, the cotyledon and the root cap of germinating seeds. The two youngest leaves of a small bulb showed strong phosphorylase activity in the plastids, although they did not contain starch. It is remarkable that the small starch granules formed in these leaves after incubation with glucose-l-phosphate, stained red with iodine solution, in contrast to the larger starch granules which are naturally present in the scales formed by the older leaves. This contrast could be emphasized by allowing the preparation to become dry, and then adding water. The older leaves showed some slight phosphorylase activity in various cells, some of which became uniformly light to dark blue after incubation.

Distribution of starch. Seeds and very young seedlings did not contain starch; it appeared first after differentiation of root and shoot meristems, and accumulated in the tissue between the two growing points, remaining localized at the base of the plumule, while the root elongated (Fig. 4C, g). Gradually more of these small round starch granules were found in the cotyledons, especially in the cells surrounding the developing vascular strands. Neither root nor root cap contained starch.

As the leaves developed they swelled at the base; above the swelling starch deposits were limited to a sheath around the vascular bundles. In the transitory zone small starch granules became visible in the parenchyma tissue. Below the transitory zone they both increased in number and size, until all cells of the swollen part were filled with large starch granules. Thus older scales (approximately 2 cms long) at the periphery of a bulb were more full of starch granules than were the inner, younger scales.

B. Waxy maize endosperm

Phosphatase distribution. From 14–24 days after pollination highest activity was found in the pericarp, in the remainder of the nucellus, and in the outer layers of the endosperm. Towards the centre of the endosperm nuclei were found to have swelled and collected small starch grains (brown-staining with iodine) around them. These stages have been described by Lampe (1931) (see her plate III, Figs. 5 and 6). Phosphatase activity decreased considerably inside the cambial part of the endosperm, which later forms the aleurone layer (Straus 1954). The inner cells still showed some activity, the nuclei staining diffuse brown with dark nucleoli, as already observed by Kugler and Bennett (1947).

Phosphorylase distribution. The young starch-free endosperm (e.g. 12 days after pollination) showed phosphorylase activity, especially at its base. But also, when brown-staining starch granules appeared (15 days after pollination), phosphorylase could often be demonstrated. In such

cases many cells stained deep blue with iodine after incubation with glucose-l-phosphate, in others the colour was violet. Stained cells were probably damaged and had attained an increased permeability. When sections were left long enough in the substrate medium, often indications of phosphorylase activity could be found in older endosperms. These results corroborate those of PAECH and KRECH (1953). As a rule incubation over night showed phosphorylase activity in starch-free endosperms, but none when brown-staining starch granules had developed.

The enzyme remained for a while in the nucellus, and disappeared about 20 days after pollination. It is noteworthy that the endosperm, after incubation with glucose-l-phosphate, produced blue-staining starch; that produced by the nucellus stained red to violet.

Distribution of starch. Five days after pollination the nucellus contained many blue-staining starch granules, the largest in the centre of the tissue measuring about 6 μ. These granules gradually disappeared; from 12–15 days after pollination only a sprinkling of small granules were left, and after 20 days the nucellus had none left.

The pericarp contained many starch granules throughout the period investigated (up till 24 days after pollination), but the outer half contained many more, and larger ones, than the inner half.

DISCUSSION

The high activity of acid phosphatase found in meristematic tissues and phloem elements confirms the results of other investigators. The enzyme may be present in tissues that never produce any starch at all (the afore-mentioned; endosperm of *Scilla*); it may disappear when starch is formed (bulb scales and cotyledon of *Scilla*, endosperm of maize); but it may also be present in starch containing cells (starch sheath of vascular bundles, developing pericarp of maize).

These results are not indicative of a simple relation between phosphatase and starch synthesis. The only general line that can be detected is that young, growing cells contain acid phosphatase, and that the activity decreases when the cell matures and its metabolic activity becomes less.

It is known that sugar phosphates permeate only with much difficulty into the cytoplasm of living cells, and may even have toxic effects (PAECH & KRECH, 1953; SHAW, 1954). One function of the phosphatase therefore may simply be to make free sugars available for transport. Meristematic cells need a constant supply of sugars, which have to travel from cell to cell. In the phloem only sucrose is found, the phosphatase in the phloem and the vascular sheath forming not so much a barrier against the entrance of sugar phosphates, as making free sugars available for sucrose synthesis, the mechanism of which is unknown. Could this be an indication that transport in the phloem does not take place by mass flow, (in which case sugar phos-

phates could be transported just as well,) but rather that diffusion processes are involved?

The sucrose later could become hydrolyzed, and the liberated glucose phosphorylated; conversion of glucose-6-phosphate into Cori-ester would give the basis for starch synthesis by means of phosphorylase. It is also possible that sucrose is directly converted to starch with the aid of amylosucrase, as suggested by EWART et al. (1954). Amylosucrase was found to synthesize glycogen in *Neisseria perflava* (HEHRE, 1949), showing that starch or glycogen may not always be produced from glucose-l-phosphate. So far a similar enzyme has not been demonstrated in higher plants, although there appears to be a very close connection between the presence of sucrose and starch synthesis (see also PORTER, 1953; PORTER & MAY, 1955). In this connection the following observations are of interest: Tomato roots utilize sucrose at a much greater rate than they do glucose, only the glucose moiety is taken up, fructose accumulating in the medium (ROTHSTEIN, 1954); in developing endosperm cells of waxy maize sucrose concentration appears to reach a maximum 15 days after pollination (LAMPE, 1931). However, PORTER's (1953) experiments with tabacco leaves speak against the amylosucrase mechanism.

Explanations of starch granule composition and formation on the basis of a phosphorylase system appear to be very difficult (BADENHUIZEN, 1954). The present study shows again that phosphorylase may be present in tissues which do not produce starch, as e.g. the endosperm, the youngest leaves, and the root cap of *Scilla* seedlings; the test may further be negative or positive in starch containing tissues (compare also DUVICK (1953) and YIN (1945).

It is interesting that starch produced by incubation of waxy maize endosperm tissue with glucose-l-phosphate stained blue with iodine. In contrast the youngest leaves of *Scilla* produced red-staining starch under the same conditions. Normally starch produced by waxy maize endosperm stains brown with iodine, that produced by *Scilla* stains blue. This indicates the artificiality of the test, the result of which is also dependent on cells becoming more or less damaged. It is therefore very difficult to obtain a true picture of phosphorylase distribution. Moreover it becomes clear that the enzymic mechanism of the test is different from that operating under natural conditions. Further discrepancies will be discussed in another paper.

The above-mentioned arguments are not in favour of starch synthesis taking place through the phosphorylase system, although they do not disprove it.

SUMMARY

The distribution of acid phosphatase and of phosphorylase was studied in developing seedlings of *Scilla ovatifolia*, and in young stages of endosperm development in waxy maize. No definite relation to starch synthesis could be found. It is likely that phosphatase is involved in the transport of sugars. The artificiality of the phosphorylase test is pointed out, in that its mechanism appears to be different from that responsible for starch synthesis under natural conditions.

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