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THE HISTOCHEMICAL DEMONSTRATION OF ACID PHOSPHATASE IN NECTARIES

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INTRODUCTION

Two opinions may be advanced as to the nature of the activity of the nectaries in the secretion of nectar. Either we can argue that the nectaries have to be considered as tissues with a real glandular function which actively secrete the nectar from substances applied by the phloem or we can consider the nectaries as a kind of passive hydathodes which only conduct the substances delivered by the translocation stream of the vascular bundle. The latter opinion has recently been defended by FREY–WYSSLING (1933) and his collaborators (AGHTE, 1951; ZIMMERMANN, 1953 and FREI, 1955). These authors observed that the nectaries always possess an immediate anatomical connection with the adjacent vascular bundles. If the nectaries secrete a sugar-rich nectar this connection is made up mainly of sieve tubes while in nectaries with a more watery secretion xylem elements predominate. It was experimentally shown that excised flowers or even nectaries placed on a sugar solution secreted the sugar from the solution in the nectar. According to Frey–Wyssling the primary cause of the nectar secretion is the lack of a balance between the supply and the consumption of carbohydrates. In young, still growing parts of plants the supply and the consumption should be well balanced so that nectar secretion does not take place. In adult parts, however, this equilibrium should be disturbed in proportion as the supply of assimilates exceeds their consumption. The remainder is secreted as nectar.

Though the observations of Frey–Wyssling and his collaborators show convincingly that the nectar secretion is connected with the translocation in the vascular bundle we still do not know whether the nectaries merely conduct the substances from the translocation stream or secrete the nectar from the substances in the translocation stream by a process of active secretion. If the nectaries merely conduct the above substances there is no reason to expect that the metabolic activity of the nectaries will be different from that of the adjacent tissues. If, however, the nectaries have a glandular function we may expect that in the nectaries specific metabolic reactions take place which finally lead to the formation of the nectar. In the latter case it is suggested that an eventual difference in metabolic activity between

nectaries and the adjacent tissues could be histochemically demonstrated. Such an investigation should be directed towards enzyme systems taking part in the production of nectar. At present, however, we do not exactly know which enzyme systems are concerned in these reactions; moreover we have the disadvantage that only a very few enzyme systems can be determined histochemically. Therefore we have been limited in our choice to the phosphatases, as these enzymes can be convincingly demonstrated in a histochemical way and as they take part in certain reactions of the metabolism of the carbohydrates.

Several phosphatases exist with different specific substrates and differing pH optima. In plant tissue mainly acid phosphatases occur. We investigated the action of the acid phosphatases which acts on glycerophosphate and have an optimal pH of about 4. The histochemical demonstration of this acid phosphatase was performed by using a method developed for animal tissues by GOMORI (1952). This method is based on the specific property of phosphatase to hydrolize phosphoric esters. The phosphoric acid released by the hydrolysis can be demonstrated by precipitating the phosphate with ions of heavy metals such as lead and cobalt.

METHOD

The histochemical determination starts with a pretreatment of the plant tissue consisting of a series of manipulations finally leading to the sectioning and mounting of the sections. Two methods can be followed. The tissue fixed can be sectioned with the freezing microtome. This procedure has two advantages: the pretreatment of the plant material takes only a very short time and radical chemical manipulations such as fixation and dehydration are avoided. Therefore with this method the inactivation of the enzymes is reduced to a minimum. However, the results of the different experiments were not those expected. The microscopic aspect was very unequal. Perhaps the freezing of the tissue by itself is insufficient to allow the substances from the outside access to the enzymes or perhaps the enzymes are washed out afterwards.

Consequently we are necessarily committed to the paraffin method. Fixation and dehydration precede an embedding in paraffin. The results obtained with this pretreatment proved to be very satisfactory and reproducible. In spite of the theoretical objections which can be raised against the paraffin method, this method proved to be the fittest at the moment.

According to Gomori the recommended pretreatment as described below should give as slight an inactivation as possible in animal tissue.

Slices of tissue, at most 3 mm thick, are chilled for half an hour and afterwards fixed in a previously chilled mixture of equal volumes of acetone and absolute alcohol at 0° C. The fixation takes 24 hours. After fixation the tissue is dehydrated (a process which already starts in the fixation liquid) in absolute alcohol at icebox temperature, again for 24 hours. The alcohol is changed once and finally removed

by transferring the pieces to chloroform, remaining there for half an hour. The manipulation was repeated once. Then the tissue is impregnated with paraffin under reduced pressure for 1–1½ hours at 55° C. After this the tissue is embedded in paraffin. Sections, at most 5 μ, are mounted with albumin-glycerol, dried, dewaxed and coated with a thin layer of collodion. This is done by flooding the dewaxed slides with 0.5 per cent solution of collodion in alcohol-ether, shaking off the excess and hardening the membrane for a minute in 80 per cent alcohol. This collodion membrane protects the tissue against loss of enzyme.

After this pretreatment the slides are placed in distilled water for the enzyme demonstration. Now the slides are placed for 24 hours in a substrate mixture (27° C.) which contains the substrate for the enzyme. This mixture is composed as follows:

In 500 ml. of a 0.05 M acetate buffer of pH 4.3 0.6 g. of lead nitrate is dissolved and 50 ml. of a 3 % solution of Na-glycerophosphate is added. This mixture is kept in an incubator at 27° C. for 24 hours and is filtered. 30 ml. of distilled water is added to the filtrate to prevent precipitation on evaporation.

In the case of a positive phosphatase reaction a white precipitate of lead phosphate is formed on the sites of activity; moreover, lead may also be bound to proteins. After removal of the protein-bound lead which is not due to an enzymatic activity, the slides are rinsed in water for 2 min. and transferred to a 2 % solution of acetic acid. After 2 min. the slides are rinsed again in water for 2 min. Then the lead phosphate is converted into the black, more stable lead sulfide by immersing the slides in a diluted solution of ammonium sulfide. The collodion membrane is removed with acetone and finally the slides are embedded in a mixture of gelatin and glycerin (following Johansen's method).

There is a possibility that the precipitation of lead phosphate is not due to the action of a phosphatase; it may be caused by phosphate already present in the cells. Besides, lead sulfide can be formed from lead ions bound to substances not removed in the acetic acid. Therefore a control test is performed simultaneously so that any phosphatase present is inactivated by an inhibitor (0.005 M Na fluoride) added to the substrate mixture.

If the slides without inhibitor show a greater amount of the black precipitate we may confirm the presence of acid phosphatase from the results obtained.

In order to prove the reliability of Gomori's method for the demonstration of the presence of acid phosphatases in plant tissue it was first tested on potato tissue, which is known to contain the acid phosphatase (SUMMER and MYRBÄCK, 1951). Using the method above described sections of potato tissue show a brown or black colour according to our expectations.

Microscopic examination shows that there is no well-defined localisation of the lead sulfide in the protoplasma. The precipitation is spread out through the whole cell. This is either due to a scattered

distribution of the acid phosphatases in the protoplasma or to a diffusion of the phosphate occurring during the histochemical determination. In animal tissue this latter phenomenon is observed too and it is probably due to the imperfection of the method. This histochemical method can be used for qualitative purposes only (for the demonstration of the presence and of the distribution of the enzyme in the tissue), because a considerable part of the enzyme activity is lost during the pretreatment.

Results

By means of the method above described the nectaries of *Trifolium repens* L., *Echeveria gibbiflora* DC. and *Euphorbia splendens* Boyer have been tested for the presence of acid phosphatases. Slides of the nectaries were cut at 5 μ and treated in the way described. Fortunately the black precipitate of lead sulfide was hardly present at all in the control tests.

a. Trifolium repens L.

The inner wall of the stamen tube is built up at its base by the nectary which consists of some layers of small cells and is bounded on the outside by the parenchyma and vascular bundles of the stamen tube and the corolla which are connated at their bases. The nectary gives a strong positive phosphatase reaction. The tissues of the stamen tube and the corolla adjacent to the nectary produce a similar very distinct

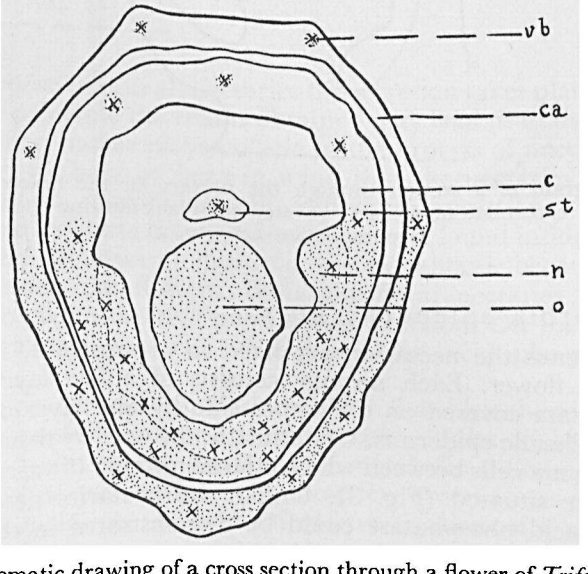

Fig. 1. Schematic drawing of a cross section through a flower of *Trifolium repens* L. c. corolla; ca. calyx; n. nectary; o. ovary; st. stamen; vb. vascular bundle. Black dotting shows the sites of phosphatase activity.

black precipitate. The other parts of the corolla — the base of the wings and of the vexillum — do not contain any phosphatase with the exception of the vascular bundles. The base of the sepal tube also shows at the side of the nectary a black precipitate like that in the receptacle. In Fig. 1 the distribution of the acid phosphatases is indicated by a black dotting in a cross section just above the place where the free stamen is connected to the stamen tube.

Since in *Trifolium* the phosphatase content of the nectary is not distinguished from the adjacent tissues the investigation of these nectaries cannot provide any insight into the participation of the phosphatases in the nectar secretion.

b. Echeveria gibbiflora DC.

In *Echeveria* flowers the nectaries are, as white cushions, found upon the receptacle between the corolla and the stamens. These nectaries which also consist of small cells are at their base founded on large parenchyma cells. The phosphatase distribution shows a very distinct difference between the nectary cells on the one hand which give a positive phosphatase reaction and the adjacent parenchyma cells on the other hand which react entirely negatively (Fig. 2).

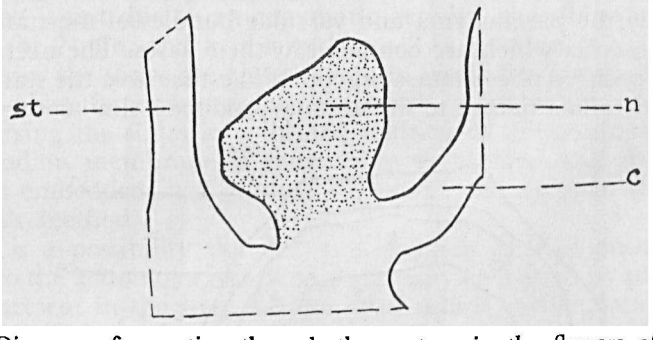

Fig. 2. Diagram of a section through the nectary in the flowers of *Echeveria gibbiflora* DC. c. corolla; n. nectary; st. stamen. Black dotting shows the sites of phosphatase activity.

c. Euphorbia splendens Boyer

In this genus the nectaries, arranged in groups of 4, are situated outside the flower. Each nectary consists of a few layers of small cells which are covered on the upper side by one layer of columnar cells, the palissade epidermis. Underneath, the nectary tissue is founded on parenchyma cells between which a large number of vascular bundle elements are situated (Fig. 3). Unlike the nectaries described previously no acid phosphatase could be demonstrated in the nectaries of *Euphorbia*. The palissade epidermis reacts negatively too. The parenchyma and the vascular bundles, on the contrary, give a positive reaction. It must be mentioned that the reaction of the parenchyma

is possibly an artefact caused by a diffusion of phosphate from the vascular bundles which are present in this tissue in a great number. This idea is favoured by the observations that the vascular bundles of *Trifolium* contain active phosphatases and that the phosphate produced by the enzyme action diffuses in the neighbouring parenchyma.

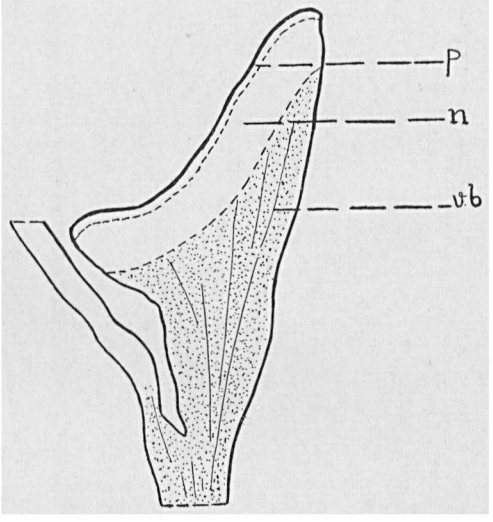

Fig. 3. Diagram of a section through the nectary of *Euphorbia splendens* Boyer. n. nectary; p. palissade epidermis; vb. vascular bundle. Black dotting shows the sites of phosphatase activity.

Discussion

If we suppose that in all nectaries the secretion takes place according to the same principle the results obtained may lead us to the conclusion that acid phosphatase plays no role in the process of nectar secretion.

However, we have to bear in mind that the nectaries which were used belong to morphologically different types. The nectaries of *Trifolium* and *Echeveria* are intrafloral: they are found inside the flowers where as the nectaries of *Euphorbia* are extrafloral, because they are situated outside the flowers. Both intrafloral nectaries investigated appeared to give a positive phosphatase reaction. On the other hand in the extrafloral nectaries of *Euphorbia* no acid phosphatase was present.

The experiments described above show that histochemical methods may be successfully applied to enzymatic studies in higher plants. The results point to the existance of metabolic differences between both types of nectaries. However, these few observations do not allow for further conclusions; more detailed investigations of a great number of nectaries belonging to various plant species are necessary. In the absence of the required data generalizations about the nature and the activity of nectaries are premature.

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