AN ULTRASTRUCTURAL STUDY OF ACID PHOSPHATASE LOCALIZATION IN PHASEOLUS VULGARIS XYLEM BY THE USE OF AN AZO-DYE METHOD

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SUMMARY

The localization of acid phosphatase during xylem development has been examined in the bean, Phaseolus vulgaris. The azo dye, the final reaction product, is initially prominent in the dictyosomes, vesicles apparently participating in secondary wall formation, and in the middle lamella of the young vessel element. Final reaction particles are also present in mitochondria, chloroplasts, and certain vacuoles and are sparsely scattered in the cytoplasm. At a later stage of vessel differentiation, the azo dye is concentrated in the disintegrating cytoplasm and along the fibrils of the partially hydrolysed primary wall and middle lamella. In the mature vessel element, the azo dye is still present along the disintegrated primary wall at the side of the vessel and covers the secondary wall. In the parenchyma cell adjacent to the vessel element, acid phosphatase localization is found in the dictyosomes, endoplasmic reticulum, mitochondria, small vacuoles, and the middle lamella. The controls from all stages of vessel element development were free of azo dye particles. The concentration of acid phosphatase along the secondary walls of the mature vessels and in the middle lamella between other cells indicates that this enzyme has other functions besides autolysis of the cytoplasm and primary cell wall. Acid phosphatase may participate in the formation of the secondary wall and may also have a role in the secretion and transport of sugars.

INTRODUCTION

During the differentiation of xylem vessel elements, a lignified secondary wall is produced. Then the protoplast is autolyzed, the primary cell wall is partly hydrolysed, and the end walls are removed. Hence, series of mature vessel elements form a hollow tube which functions in water transport (Esau, 1965a, b; Torrey, Fosket & Hepler, 1971).

Although several studies of xylem vessel development have been made by the use of light (cf. Esau, 1965a, b; O'Brien, 1972) and electron microscopy (Cronshaw, 1967; Cronshaw & Bouck, 1965; Esau, Cheadle & Gill, 1966a, b; O'Brien, 1970; O'Brien & Thimann, 1967b; Yata, Itoh & Kishima, 1970) the developmental sequence needs further clarification. We wanted to explore, in the developing xylem, the degradation process which accompanies cell maturation (Matile, 1969) by using acid phosphatase as a marker enzyme.

Gahan & Maple (1966) examined acid phosphatase localization during xylem
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development in Acer with the light microscope and found the enzyme localized in particles in young xylem but in a dispersed state in older tissue. A high acid phosphatase activity was recorded by Hebant (1973) with the light microscope in developing water-conducting tissue of bryophytes. Using the electron microscope, Wodzicki & Brown (1973) found evidence of acid phosphatase activity (Gomori test) at the surface of membranes exposed to the vacuole in differentiating pine tracheids.

We have investigated the development of vessel elements in the xylem of Phaseolus vulgaris using electron microscopy and histochemistry. A part of this study, the results of acid phosphatase localization by means of an azo-dye method (Bowen, 1971), are described in the present paper.

MATERIALS AND METHODS

The pulvini from petioles of Phaseolus vulgaris bean (Tendercrop, Burpee Company), were first fixed in 3 % glutaraldehyde made with 0.1 M cacodylate buffer, pH 7. Then the pulvini were cut with a razor blade into small pieces in the 3 % glutaraldehyde and fixed for 2 h at room temperature. After fixation was completed, the pieces were sectioned into 50-μm sections with a Sorvall TC-2 tissue chopper. The 50-μm sections were washed in the cacodylate buffer for 36 h in order to remove the glutaraldehyde which inhibits acid phosphatase reactions. During the 36-h period the buffer solution was changed several times.

The azo dye enzyme localization was the same as the one used by Bowen (1971). Five milligrams of the substrate, naphthol AS-BI phosphate, were dissolved in 0.25 ml of dimethyl formamide, and 25 ml of distilled water were added. Then 25 ml of 0.2 M acetate buffer, pH 5.2, were mixed in, and were followed by 30 mg p-nitrobenzene diazonium tetrafluoroborate (Kodak), the coupler. After all the components were dissolved, 0.1 ml of 10 % solution of MnCl₂ was added as an activator.

No-substrate controls were made by omitting the naphthol AS-BI phosphate from the reaction mixture. Controls involving inhibition of the enzyme were carried out by adding 0.01 NaF to the medium described above.

The 50-μm sections were placed in the enzyme incubation medium or one of the control media for 1.5 or 3 h at room temperature. The sections were rinsed in a 0.1 M cacodylate buffer, pH 7.0, and postfixed overnight at 4 °C in 2 % osmium tetroxide made with the cacodylate buffer. Then the sections were dehydrated through an acetone series and 100 % propylene oxide and embedded in Epon. Monitoring sections for light microscopy and thin sections for electron microscopy were cut on the Porter-Blum (Sorvall) ultramicrotome. A diamond knife was used to cut thin sections; these were placed on 150- or 200-mesh copper grids and examined either unstained or stained with uranyl acetate and lead citrate. The sections were examined with the Siemens 101 electron microscope at 60 or 80 kV.

All the illustrations in this paper are from unstained longitudinal sections of the tissue, and all the electron micrographs showing enzyme localization and controls are from sections incubated for 3 h.

RESULTS

During the differentiation of secondary walls xylem elements possess complete protoplasts. Parts of this kind of element are shown in Figs. 1 and 2. The small black particles in the various organelles and the cell wall are the reaction product, the azo dye, of the enzyme localization. Most of the particles are extremely fine, but since these sections (as well as all the others shown in this paper) were not counterstained with uranyl acetate and lead citrate, the enzyme localization is not obscured by heavy
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metal deposits. The reaction product is conspicuous in the mitochondria and dictyosomes, but is most concentrated in the middle lamella of the wall. Significantly, it also occurs in vesicles which apparently participate in secondary wall formation (see in Fig. 1). The dye is diffusely present in some vacuoles, endoplasmic reticulum, and throughout the cytoplasm. In Fig. 2, in which parts of 2 adjoining vessel elements are shown, the reaction product is present not only in the middle lamella but also in the primary wall to the left of the middle lamella. In the parenchyma cell to the left, above, in Fig. 1, the reaction product is seen in the mitochondrion and in the cytoplasm.

We have not obtained much information on enzyme localization in the vessel end wall. Sassen (1965) has suggested that vesicles incorporated into the end wall while the latter increases in thickness contain enzymes which initiate the disintegration of this wall. In the part of a transverse end wall between 2 developing vessel members shown in Fig. 3 the reaction product is present here and there in the peripheral region of the wall and as a few particles in the middle lamella. In some of the stained and unstained material used in other parts of our xylem study, vesicles occurred in the peripheral region of the end wall. In Fig. 3, however, an association of the enzyme localization near the edge of the end wall with vesicles is not discernible. The end wall in Fig. 3 may not have been in the appropriate stage of development to show the vesicles.

Sometimes the reaction product inside vacuoles of developing vessel elements is somewhat more conspicuous than in Fig. 1. In Fig. 4, for example, numerous exceedingly small particles are associated with the membranes inside the vacuole. In this view, the reaction product is also sparsely present in the cytoplasm. In Fig. 5, enzyme activity is seen concentrated in 2 kinds of organelle, mitochondria and plastids, a feature which was generally recorded by us in developing vessel elements. Again, in this view, some particles of the reaction product are present in the cytoplasm.

As was stated under Materials and methods, 2 types of control were used with the enzyme localization. A control based on inhibition of acid phosphatase reaction by sodium fluoride is shown in Fig. 6, in which a developing vessel member is on the right and a parenchyma cell on the left. No enzyme localization is discernible in the middle lamella, secondary wall, plastid, mitochondrion, dictyosomes, endoplasmic reticulum or the cytoplasm. The non-substrate control of the same stage of development was also free of reaction product.

Enzyme localization is prominent during the late stages of secondary wall formation. This kind of stage is depicted in a strongly biased longitudinal section of a vessel element (below) and a parenchyma cell (above) in Fig. 7. In the vessel member, the enzyme localization is concentrated in the remnants of cytoplasm located along the margins of the primary wall and is also present along the margin of the secondary wall and in the middle lamella. Except for the degenerating cytoplasm along the walls, the rest of the contents of the differentiating vessel member had disintegrated. In the parenchyma cell, the reaction product is visible in the dictyosome and mitochondrion and here and there in the cytoplasm.

The view in Fig. 8, a control for Fig. 7, likewise shows a vessel element in a late
stage of differentiation of the secondary wall as well as parts of 2 parenchyma cells (above). In this control the acid phosphatase reaction was inhibited by sodium fluoride. No reaction product is present in the middle lamella, the primary or secondary wall, or in the disintegrating protoplast. Differentiation of the vessel member in Fig. 8 is somewhat less advanced than that of the vessel member in Fig. 7, for more degenerating cytoplasm is present in the cell lumen in Fig. 8. The dark material observed in the autolysing cytoplasm in this figure does not indicate enzyme localization since similar material was present in tissue fixed without reference to enzyme localization. Moreover, the dark material in Fig. 8 does not have the particulate structure observed in the enzyme localization shown in Fig. 7 and in the other pertinent electron micrographs. The no-substrate controls for this enzyme localization test were also free of the azo dye.

Parts of vessel elements at later stages of differentiation than that in Fig. 7 are shown in Fig. 9. The vessel element on the left is completely mature, the one on the right has some distintegrating cytoplasm. The stain is most evident along the fibrous material which has remained after the partial hydrolysis of primary wall and middle lamella. The reaction product is present also in the disorganized cytoplasm on the right side. A similar combination of parts of vessel elements is shown at a low magnification in Fig. 10. The vessel element on the right is completely mature; the one on the left has degenerating cytoplasm along the secondary thickenings and in the lumen of the cell. An enlargement from the same section in Fig. 11 shows some reaction product in vesicles and free in the disorganized cytoplasm.

The reaction product continues to be discernible after maturation of a vessel element. Part of a mature vessel element and adjoining parenchyma cell are depicted in Fig. 12. The reaction product is concentrated along the partially hydrolysed primary wall on the side of the vessel and covers the secondary wall (cf. Fig. 13). In the parenchyma cell the acid phosphatase localization is evident in the dictyosomes, endoplasmic reticulum, mitochondria, a small vacuole, and the middle lamella (left). No localization is observed in the larger vacuoles.

Parts of another mature vessel (centre) and associated parenchyma cells (right and left) are illustrated in Fig. 13. As in Fig 12, the reaction product of acid phosphatase is concentrated along the partially hydrolysed primary wall on the side of the vessel element and extends over the secondary wall thickenings. In contrast to the situation in Fig. 10, the lumen of the vessel element in Fig. 13 is free of protoplast, for the latter was completely disintegrated. The enzyme localization is evident in the middle lamella between 2 parenchyma cells (left). At the low magnification in this figure, the reaction product is barely discernible in some organelles in the parenchyma cells.

The last electron micrograph (Fig. 14) is a no-substrate control of parts of 2 vessel elements (right) and a parenchyma cell (left). The central element is more mature than the one to the right, which still contains some disorganized cytoplasm in the lumen. No reaction product is found in the partly hydrolysed primary walls, in the disintegrating cytoplasm at the far right, in the material covering the secondary wall thickenings, or in the parenchyma cell.
DISCUSSION

It has been well documented that young tracheary xylem cells contain all the usual plant cell constituents including nuclei, mitochondria, endoplasmic reticulum, plastids and dictyosomes. During maturation of the future conducting xylem elements, degenerative changes occur in the cytoplasmic components (Cronshaw & Bouck, 1965; Esau et al. 1966a, b; O'Brien & Thimann, 1967a; Yata et al. 1970; and the present study).

Some studies on xylem element differentiation indicate that vacuolar digestion of cytoplasmic components is involved in the protoplast degradation. Wodzicki & Humphreys (1972, 1973), who examined cytodifferentiation of maturing pine tracheids using scanning and transmission electron microscopy, stated that autolysis of the protoplast began with the formation of cytoplasmic spherules which contained cell organelles. The organelles were subsequently reported to be released into the central vacuole where they were digested. Wodzicki & Humphreys favour the hypothesis that digestion of separate cytoplasmic components occurs in the vacuolar sap containing hydrolases, and they find evidence that such digestion of organelles occurs before the parietal layer of cytoplasm breaks down. Matile & Winkenbach (1971) had earlier reported the occurrence of a similar mechanism in protoplast degradation during senescence of the morning glory corolla. Their electron micrographs provide evidence of intravacuolar digestion of cytoplasmic components before the final lysis of the cytoplasm.

Gahan & Maple (1966), whose work was based on light microscopy, envisioned a localization of acid phosphatase in particulate structures and subsequent release and diffusion of the enzyme throughout the differentiating xylem cell. Matile (1969), however, suggested that Gahan & Maple were actually observing the development of a vacuolar lysosomal apparatus instead of a release of enzymes from lysosomes.

We did not observe the exact stage of xylem element differentiation that was considered by Wodzicki & Humphreys, and enzyme localization in vacuoles was not a prominent feature in our material. One possibility is that the azo dye localization is specific for an acid phosphatase isozyme which is concentrated not in the secondary lysosomes but rather in the dictyosomes and cell walls.

In our material, however, the young xylem cells do contain a few vacuoles (Fig. 4) which have the necessary characteristics of secondary lysosomes, that is, they are delimited by a single membrane, contain at least one hydrolytic enzyme, and enclose material which is apparently breaking down (cf De Duve & Wattiaux, 1966; Gahan, 1973; Matile, 1969). Among the investigators who have studied xylem development using electron microscopy only O'Brien & Thimann (1967a, b) mentioned the presence of lysosomes. Without performing biochemical or enzyme localization tests these authors suggested that the crystal-containing bodies in oat coleoptiles might be lysosomes serving as the source of hydrolases. Morphologically, the crystal-containing bodies observed by O'Brien & Thimann are similar to microbodies (Tolbert, 1971) and not to lysosomes (De Duve & Wattiaux, 1966). In their ultrastructural study of acid phosphatase localization (Gomori method) in pine xylem, Wodzicki & Brown
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(1973) observed bodies resembling lysosomes in parenchyma cells but not in differentiating tracheids. Hence, the role of lysosomes in xylem formation is still unresolved.

The indication that acid phosphatase is localized in the dictyosomes of young xylem elements in the bean (Figs. 1, 2) supports the view that acid phosphatase is packaged by the dictyosomes and perhaps transported to other locations by dictyosome vesicles. The large vesicles associated with the dictyosomes in bean xylem rarely show dark particles (Figs. 1, 2), but some of the particles located in the cytoplasm might be enclosed in small dictyosome vesicles. Without counterstains such vesicles are difficult to discern. Enzyme localization, however, is detectable in the vesicles that are apparently participating in secondary wall formation (Fig. 1, ve, and top of Fig. 2). Some of the reaction product also seems to be associated with the microtubules adjacent to the secondary wall (Fig. 1, mt). What a role acid phosphatase might have in wall formation is unknown, but recent studies have shown that hydrolytic enzymes have an important function during wall formation in fungi (Bartnicki-Garcia, 1973). The close association between the microtubules and vesicles (Fig. 1) may indicate that dictyosome vesicles, which presumably contain cell-wall precursors and/or cell-wall-synthesizing enzymes, are directed by microtubules that are oriented parallel to the microfibrils during secondary wall formation (Hepler & Palevitz, 1974; Robards, 1968). O’Brien (1972), however, has appropriately cautioned about the difficulties of obtaining evidence concerning the direction of movement of vesicles from static pictures.

Disintegration of unlignified cell walls occurs during the late stages of vessel element maturation. The disintegration of the primary wall stops at the middle lamella if a parenchyma cell is adjacent to the vessel element with a disintegrating protoplast. According to O’Brien (1970), the parenchyma cell apparently forms a protective layer which may prevent the digestion of its primary wall. Although some investigators have reported a deposition of a special wall layer on the side of the parenchyma cell next to a mature vessel, the protective function of this layer is being questioned (Chafe, 1974; Czaninski, 1973).

In the bean xylem, a strong acid phosphatase activity is indicated in vessel elements in the last stages of differentiation (Figs. 7–14). The phenomenon is apparently correlated with autolysis of the protoplast (Figs. 7, 8) but it is notable that the mature vessel element continues to show enzyme localization along the walls (Fig. 13). Perhaps acid phosphatase serves other functions besides autolysis. Hall & Sexton (1972) identified peroxidase localization in maturing xylem elements of pea and suggested involvement of the enzyme in lignification of the secondary wall. The occurrence of enzymes in vessels in the nearly mature and mature states requires further study, and the phenomenon must be evaluated in relation to the evidence that high acid phosphatase activity in parenchyma cells associated with the vessels seems to be part of a mechanism bringing about the periodic release of sugar into vessels (Sauter, Iten & Zimmermann, 1973).

High concentrations of acid phosphatase have also been reported in the conducting tissues of bryophytes. Hébant (1973), who made light-microscope studies of the localization of β-glycerophosphatase (Gomori technique) and naphthol AS-MS
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phosphatase (Burstone technique) in different species of bryophytes, observed a peak of strong acid phosphatase activity in water-conducting elements toward the end of their development. The author suggested that this intense activity was correlated with autolysis of the protoplasts. Moreover, he proposed that the occurrence of acid phosphatase activity in parenchyma cells associated with the conducting elements in the highly developed gametophytes of the Polytrichiales supports the hypotheses that phosphatases may participate in the storage and mobilization of carbohydrates and possibly in transport phenomena in general.

The most outstanding feature of acid phosphatase localization in the bean xylem tissue is the consistency with which the enzyme is identifiable in the cell walls. In the vessel element specifically, the enzyme appears to be associated with the partial hydrolysis of the primary wall characteristic of this cell in its late stages of differentiation (O'Brien, 1970). But acid phosphatase is localized in walls of parenchyma cells as well, particularly in the middle lamella region. The presence of hydrolytic enzymes in plant cell walls has been identified by biochemical (Stephens & Wood, 1974; Suzuki & Sato, 1973) and histochemical (Hall & Sexton, 1974; Poux, 1970) methods. Acid phosphatase specifically has been associated with isolated plant cell walls (Kivilaan, Beaman & Bandurski, 1961; Lampert & Northcote, 1960; Stephens & Wood, 1974; Suzuki & Sato, 1973; Yamaoka, Hayashi & Sato, 1969).

Several studies on enzyme localizations at the electron-microscope level suggest that presence of acid phosphatase in cell walls of higher plants is a common phenomenon (Hall & Sexton, 1974; Halperin, 1969; Poux, 1970). These ultrastructural localizations of enzyme, however, were carried out by means of lead-coupling methods that have been shown in some instances to result in artifacts because of non-specific lead staining (Shnitka & Seligman, 1971; Weber, Franke & Kartenbeck, 1974). The advantage of the azo-dye method is that the primary reaction product is insoluble and apparently does not produce non-specific staining as the lead-coupling technique does. All our controls were completely free of azo-dye particles.

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REFERENCES


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Fig. 1. Longitudinal section through parts of developing xylem vessel element (right) and parenchyma cell (left, above). Enzyme localization by the use of the substrate naphthol AS-BI phosphate and simultaneous coupling with p-nitrobenzene diazonium-tetrafluoroborate. Enzyme localization is present in middle lamella (ml), vesicles (ve) in secondary wall (sw), dictyosomes (d), endoplasmic reticulum (er), mitochondria (m), possibly in microtubules (mt), vacuole (v), and scattered in the cytoplasm. x 36000.

Fig. 2. Parts of 2 adjoining developing vessel elements in longitudinal section. Enzyme localization as in Fig. 1. Reaction product is present in middle lamella (ml) and primary wall to the left of it; at the margin of the secondary wall (sw) thickening; in dictyosomes (d) and the cytoplasm. x 36000.
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Fig. 3. Part of transverse end wall between 2 developing vessel members. Enzyme localization by the use of the substrate naphthol AS-BI phosphate and simultaneous coupling with p-nitrobenzene diazonium-tetrafluoroborate. Sparse enzyme localization along periphery of end wall and in middle lamella (ml). × 36,000.

Fig. 4. Part of protoplast of developing vessel element. Enzyme localization as in Fig. 3. Some reaction product of acid phosphatase in vacuole (v) and cytoplasm. × 36,000.

Fig. 5. Part of protoplast of developing vessel element. Enzyme localization as in Fig. 3. Reaction product is conspicuous in mitochondria (m) and plastid (pl). × 36,000.

Fig. 6. Parts of developing vessel member (right) and parenchyma cell (left). Control: inhibition of acid phosphatase reaction by NaF. No reaction product in either protoplast or wall layers. Details are: d, dictyosomes; er, endoplasmic reticulum; m, mitochondrion; ml, middle lamella; pl, plastid; sw, secondary wall. × 36,000.
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Fig. 7. Parts of vessel element (below) in late stage of differentiation and parenchyma cell (above). The section of the xylem wall is strongly biased. Enzyme localization by the use of the substrate naphthol AS-BI phosphate and simultaneous coupling with \( p \)-nitrobenzene diazonium-tetrafluoroborate. Enzyme localization concentrated along the primary wall (ptv) of vessel element. Reaction product is also present at margin of secondary wall (sw), in middle lamella (ml), and in dictyosome (d), mitochondrion (m) and cytoplasm of parenchyma cell. \( \times 30,000 \).

Fig. 8. Parts of vessel element (below) in late stage of differentiation and 2 parenchyma cells (above). Control: inhibition of acid phosphatase reaction by NaF. No reaction product. Details: ml, middle lamella; ptv, primary wall; sw, secondary wall. \( \times 30,000 \).
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Fig. 9. Parts of 2 adjoining vessel elements, the one to the left completely mature, the one to the right with some disorganized cytoplasm. Enzyme localization by means of the substrate naphthol AS-BI phosphate and simultaneous coupling with p-nitrobenzene diazonium-tetrafluoroborate. Incubation time, 3 h. Reaction product most conspicuous along remainder of partially hydrolysed primary walls (ptw) and middle lamella (ml). sv, secondary wall. × 24,000.

Fig. 10. Parts of 2 adjoining vessel elements, the one to the right completely mature, the one to the left with disorganized cytoplasm along the secondary thickenings and in the lumen of the cell. Enzyme localization as in Fig. 9. Sparse reaction product in remnants of cytoplasm (cf. Fig. 11). × 7200.

Fig. 11. An enlarged micrograph from the same section as was used for Fig. 10. It shows some reaction product in vesicles and free in the disorganized cytoplasm. × 12,000.

Fig. 12. Parts of mature vessel element (right) and adjoining parenchyma cell. Enzyme localization as in Fig. 9. Reaction product is concentrated along the partially hydrolysed primary wall on the side of the vessel and also covers the secondary wall (cf. Fig. 13). In the parenchyma cell, enzyme localization is evident in dictyosomes (d), endoplasmic reticulum (er), mitochondria (m) and a small vacuole (v). Reaction product is present in the middle lamella in the wall to the left but not in the one to the right. sv, secondary wall. × 30,000.
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Fig. 13. Parts of mature vessel element (centre) and parenchyma cells (right and left). Enzyme localization by the use of the substrate naphthol AS-BI phosphate and simultaneous coupling with ρ-nitrobenzene diazonium-tetrafluoroborate. Reaction product of acid phosphatase is concentrated along the partly hydrolysed primary wall on the side of the vessel element and extends over the secondary wall (m) thickenings. In the wall between two parenchyma cells to the left, enzyme localization is present in the middle lamella (ml). No enzyme localization in the 2 walls between the vessel element and adjacent parenchyma cells (cf. Fig. 12). Some reaction product is visible in the chloroplasts (ch). × 12000.

Fig. 14. Parts of 2 vessel elements (right) and a parenchyma cell (left). Control: no substrate. No reaction product in partly hydrolysed primary walls, in the material covering the secondary wall (m) thickenings, the disorganized cytoplasm on the extreme right, or in the parenchyma cell. × 12000.
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