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Histochemical Studies of an Inclusion Body and P-Protein in Phloem of *Xylosma congestum*[1]

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With 2 Figures

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Summary

Histochemical studies revealed that both the P-protein and a spheroidal inclusion body in sieve tubes of *Xylosma congestum* are acidic proteins. This protein may be conjugated to phosphate moieties. Trypsin, pepsin, and trypsin followed by pepsin extractions did not completely break down the P-protein and there was only a partial effect on the inclusion body. This body is considered not to be an "extrusion nucleolus". Tests for DNA, RNA, and lipids were negative for mature sieve elements. Immature sieve elements contained a basic protein fraction.

1. Introduction

Spheroidal inclusions having a dense appearance and in size comparable to nuclei of phloem parenchyma cells have been observed in mature sieve elements of many species. It has been assumed generally that they are nucleoli persisting after the nucleus has disintegrated in the process of sieve tube maturation (*cf.*, ESAU 1969). DESHPANDE and EVERT (1970) have refuted this assumption on the basis of results obtained in light and electron-microscopic studies of five broad-leaved woody species.

Interest in these inclusions stems from observations made during enzyme-histochemical studies of the phloem of *Xylosma congestum* (LOUR.) MERR., an evergreen shrub. The spheroids occur singly in all mature elements and are visible even without staining. The present study started with the hypothesis that they indeed represent persisting nucleoli or remains of the degenerated nucleus. Therefore nucleochemical methods were applied to determine whether they contained any nucleic acid or histones. Results indicate these inclusions

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to be proteinaceous, with staining properties almost identical to those of the P-protein. No visible amount of DNA, RNA, or nucleohistone was detectable. The protein seems to be acidic.

2. Materials and Methods

Bark from *Xylosma* plants was routinely taken at about 1 m below the shoot apex. For comparison, material having immature morphological characteristics was usually included in the study, but the main investigation was carried out on mature tissue far removed from the apex. The tissue was frozen at — 150 °C in an isopentane bath, freeze-dried (JENSEN 1962), embedded in paraplast and sectioned at 10 µm. For some of the methods, slides with affixed freeze-dried sections were immersed in 10% neutral formaldehyde or in formaldehyde : acetic acid : ethanol 5 : 5 : 90. In a few instances, fresh material was sectioned on a sliding microtome at 25 and 40 µm, and used fresh or fixed. The staining schedules are summarized in Table 1. Observations were made on a Leitz Ortholux microscope. Photomicrographs were recorded on Agfachrome CT 18 using an automatic camera. The fluorescence lamp was a Philips mercury burner CS-150, and the exitation filter was either UG 1 for UV or BG 12 for blue light.

3. Observations

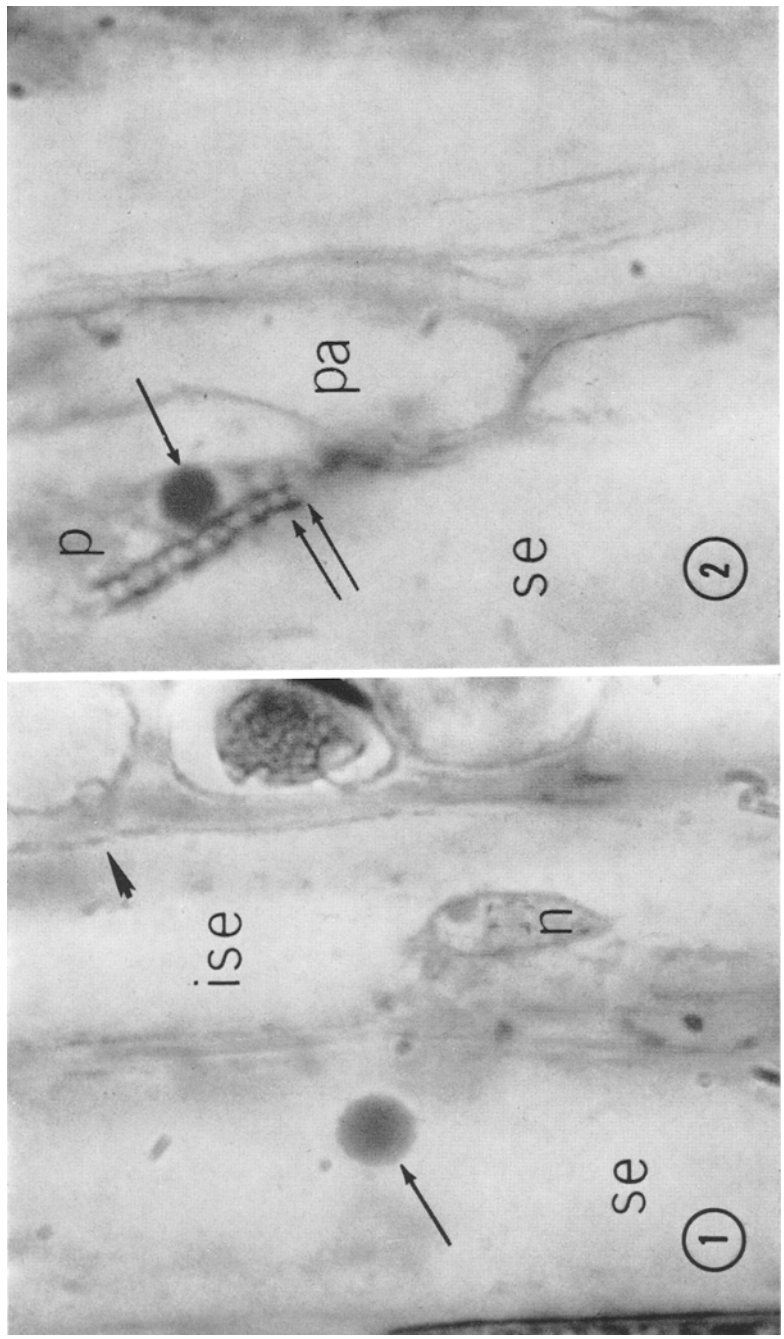
3.1. Total Protein

There was good agreement between aniline blue black and tetra-azotized dianisidine staining. Furthermore, these dyes complement each other: with aniline blue black much cellular detail is obtained, whereas tetra-azotized dianisidine is a quantitative stain, forming covalent bonds with the protein side chains (DANIELLI 1953). Both methods are specific for proteins. With aniline blue black, proteins are revealed in the P-protein, the storage granules, the thin layer of cytoplasm, the plasmalemma, and in the spheroidal inclusion which stains most intensely. Tetra-azotized dianisidine does not give such a clear picture of cellular organization, but again the spheroidal inclusion stains most intensely.

After trypsin and pepsin extractions up to 24 hours, and trypsin followed by pepsin (PEARSE 1960), the cells assumed a very disorganized appearance. After trypsin, proteins from the nucleus and nucleolus disappeared. The P-protein and the spheroidal inclusions shrank, especially with pepsin, but the breakdown was far from complete.

3.2. Acidic Proteins

Unmasking the proteins with trichloracetic acid (TCA) and staining with a basic dye such as toluidine blue at an acidic pH allows for further classification of the proteins (CHAYEN *et al.* 1969). At pH 4 the P-protein and the spheroidal inclusion stained positively with toluidine blue. At pH 1 this test was negative, which indicates that these proteins are acidic, and that they are not conjugated to sulphates. They still may be conjugated to phosphates, since organic phosphates stain with difficulty below pH 4. EBEL's test for



Figs. 1 and 2. Freeze-dried phloem of *Xyloma congestum* stained for total protein with aniline blue black. The globoidal inclusion (single arrows), the nucleus (*n*), the P-protein (p), the P-protein strands through the sieve pores (double arrows), and the plasmalemma (arrow-head) contain the stain. Sieve element = *se*; phloem parenchyma = *pa*; immature sieve element = *ise*. ×5,800

polyphosphates (CHAYEN *et al.* 1969) at pH 4 and below was definitely positive. A test for carbohydrates (PAS reaction, CHAYEN *et al.* 1969) was negative.

3.3. Basic Protein

The method of ALFERT and GESCHWIND (1953) was used to further characterize the sieve element proteins. The spheroidal inclusions remained unstained. But there was a gradient of decreasing color intensity between the cytoplasm of the most immature and the fully differentiated sieve element. Immature sieve elements appeared to possess basic proteins not only in their nuclei, but also in the precipitated cytoplasm. This color diminished as the cells developed and was absent in the fully mature elements. Trypsin extraction before staining eliminated the color. To obtain coloring it was necessary to treat the cytoplasmic basic protein the same as the nuclear histone, *i.e.*, staining was obtained only after fixation and 5% TCA extraction of the freeze-dried material.

3.4. Nucleic Acids

In the Feulgen reaction, nuclei stained pink with purplish-red nucleoli. The spheroidal inclusion always remained unstained. Azure B at pH 4 stained nuclei bluish green, imparting a fine granular appearance. Nucleoli were dark blue. The spheroidal inclusion and the P-protein stained turquois; extraction for RNA did not diminish this color. Furthermore, bovine serum albumin, treated exactly the same as the tissue sections, stained similarly to P-protein.

Nuclei stained with acridine orange fluoresce yellow and cytoplasmic RNA fluoresce red (CHAYEN *et al.* 1969). Such results were obtained for *Xylosma* cells. The spheroidal inclusion of sieve elements fluoresced orange-brown and this color remained unchanged after RNAse treatment or 5% TCA extraction.

3.5. Lipids

Sudan IV, nile blue (JENSEN 1962) and pyrenin B at pH 11.3 (STRUGGER 1949) were used. The results for lipids were negative with all methods. Results of staining tests are summarized in Table 1.

4. Discussion

The P-protein of *Xylosma* appears to have properties of an acidic protein. It is not conjugated to sulfates, polysaccharides, or lipids, but may be conjugated to phosphates. This is suggested because EBEL's test for polyphosphates was positive. YAPA and SPANNER (1973) also came to the conclusion that the P-protein of *Tetragonia* and *Lycopersicum* is acidic in nature. They used various protein-extracting enzymes and found pepsin to be effective

Table 1. *Staining Schedule for the P-Protein and Globular Inclusion Body of Xylosma congestum*

Chemical component	Stain	Result	Control	Reference
Total proteins	Aniline blue black	+	Trypsin, pepsin	FISHER 1968
	Tetra-azotized dianisidine	+	Trypsin, pepsin	CHAYEN *et al.* 1969
Acidic proteins	5% TCA, then toluidine blue pH 4	+	Pepsin	CHAYEN *et al.* 1969
Simple vs. conjugated proteins	5% TCA, then toluidine blue pH 1	—	none	CHAYEN *et al.* 1969
Phosphates, polyphosphates	Ebel's test	+	none	CHAYEN *et al.* 1969
Carbohydrates	PAS	—	Acetylation	JENSEN 1962
Lipids	Nile blue, Sudan IV	—	none	JENSEN 1962
	Pyronine B pH 11.3	—	none	STRUGGER 1949
Nucleic acids	Feulgen	—	DNAse	JENSEN 1962
	Azure B	—	DNAse, RNAse	JENSEN 1962
	Acridin orange	—	DNAse	CHAYEN *et al.* 1969
Basic proteins	Fast green, pH 8.1	—	Trypsin	ALFERT and GESCHWIND 1953

both for fresh and formaldehyde-fixed tissue. They discussed the fact that pepsin attacks peptide bonds in the vicinity of tyrosine, phenylalanine, and possibly glutamate and aspartate, and that the sap from yet another plant, *Heracleum,* has relatively large amounts of the latter two amino acids. In the present studies, pepsin removed P-protein far from completely, but this could result from a difference in technique rather than in the nature of P-protein. For example, fresh tissue was not employed, and sections always were 10 μm thick, whereas YAPA and SPANNER's were never more than 1 μm.

Seemingly the most puzzling observation in this study is that the protein of immature sieve elements appears to contain an alkaline component. It would be surprising to find that the P-protein filaments change in amino acid composition during the ontogeny of a sieve element. However, immature sieve elements are known to have a large amount of ribosomes, which of course are unresolvable entities in a light-microscopic study. Since staining occurred only after TCA extraction, *i.e.,* after nucleic acid removal and unmasking of the reactive sites, it could be that these basic proteins are structural components of the ribosomes. The globoidal inclusion of *Xylosma* may be similar to inclusions described in other species. Judging from results of the cytochemical tests employed, this structure is considered not to be an extruded nucleolus. Rather it seems to be a proteinaceous body with chemical proper-

ties closely resembling those of P-protein, but distinct from those of nucleoli. This is especially well demonstrated with toluidine blue at pH 4: the globular inclusion and P-protein stain metachromatically with a color change towards the shorter wavelenth (blue to turquois); nucleoli stain orthochromatically. Furthermore, nucleic acid extractions do not alter the staining properties of the inclusion body.

The inclusion is much larger than even the most prominent nucleolus. Nucleoli appear quite inconspicuous after tetra-azotized dianisidine treatment, indicating a quantitative difference between nucleolar protein content and that of the inclusion. In living, unstained sections of *Xylosma*, nuclei and nucleoli are translucent, whereas the globoidal inclusion is quite dense. An actual extrusion of the nucleolus from a degenerating nucleus was never observed. While the nucleus enlarged and lost its chromaticity, the nucleolus retained its normal size. Nuclei and globoids never were observed together in the same sieve element; this was not the case in other species, as reported by DESHPANDE and EVERT (1970). In *Xylosma*, immature sieve elements never contained the globoid; fully mature elements always did. Therefore the development and the function of this inclusion remain obscure, but it is possible that it represents yet another conformation of P-protein (ESAU and CRONSHAW 1967).

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