Radioautographic Analysis of the Distribution of Label From ^{3}H-Indoleacetic Acid Supplied to Isolated Coleus Internodes[1]

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Abstract. **Isolated fifth internodes of *Coleus blumei* Benth. were supplied with ^{3}H-IAA at their apical ends. Microradioautography, using thin sections (0.25–0.5 microns) of Epon embedded tissue and liquid emulsion techniques, revealed extremely specific localization of the acetone-insoluble derivatives of IAA. Changes in the patterns of labeling were followed over periods of 3, 24, and 48 hr at various distances from the source of applied auxin. Progressively basipetal labeling of the cell walls of the youngest xylem elements was evident with time. At the end of 24 hr the label was detected also within the cytoplasm or slime of phloem elements. After 24 to 48 hr, labeling of plastids was evident. The results suggest a continued incorporation of IAA derivatives into cell wall precursors.**

Auxins affect vascular differentiation in many plant systems (*e.g.*, 1, 6, 9, 28). In stems of *Coleus*, IAA at physiological concentrations can completely replace the leaves in their effect on tracheary regeneration in either elongating (7) or thickening internodes (23). The endogenous auxin of *Coleus* is apparently IAA and only IAA, judging by R_F values on paper chromatograms, color tests, fluorescence tests, and bioassays (20).

However, we have little evidence as to the locus of action of IAA in causing tracheary differentiation. Published work on the localization of auxins using radioautography has used mostly ^{14}C or relatively thick sections, with consequently poor resolution (13, 16, 26).

Since no experimental techniques are available for the precise localization of both free and bound fractions of exogenously supplied IAA, we have examined the distribution of only the acetone-insoluble fraction in some detail. Our aim was to increase the precision of radioautography by employing tritium-labeled IAA and thin tissue sections (prepared as for electron microscopy) and to increase the efficiency by using *Coleus* fifth internodes for which quantitative relationships between IAA and vascular regeneration have been worked out (23).

Materials and Methods

The plant material consisted of 10 mm median segments excised from the fifth internodes of 7 to 8 week-old plants of *Coleus blumei* Benth. of the Princeton clone. Each internode segment was wounded following the procedure described by LaMotte and Jacobs (10) and stood vertically with its basal end on a receiver block of 1.5 % agar gel resting on a glass slide. The apical end of the segment was covered with a similar donor block of agar containing tritium-labeled IAA. The slides were arranged within covered glass Petri dishes lined with moistened filter paper and placed in a growth chamber under long-day conditions (16 hr light—800 ft-c) and a constant temperature of 24°. Uptake and translocation of the radioactive IAA by the tissue (duplicate segments per treatment) were allowed to proceed for periods of 3 hr, 24 hr, and 48 hr.

Tritium-labeled IAA (^{3}H-IAA), tagged on the benzene ring at the no. 5 carbon position and having a specific activity of 15.8 c/mmole was obtained from Isotopes, Incorporated, Westwood, New Jersey. According to the manufacturers, the radiochemical purity of the compound was better than 99 % at the time of shipment and it was used immediately. The final concentration of ^{3}H-IAA in the donor blocks was 5×10^{-7} M and each block contained approximately 2.0 μc of the label.

After each incubation period, segments were prepared for fixation. A 1-mm thick transverse section was cut from each internode 1 mm below the apical cut surface, and another from the middle of the segment in the region of the wound. In the case of internodes treated with IAA for 48 hr, a slice was also taken 1 mm above the basal cut surface. The

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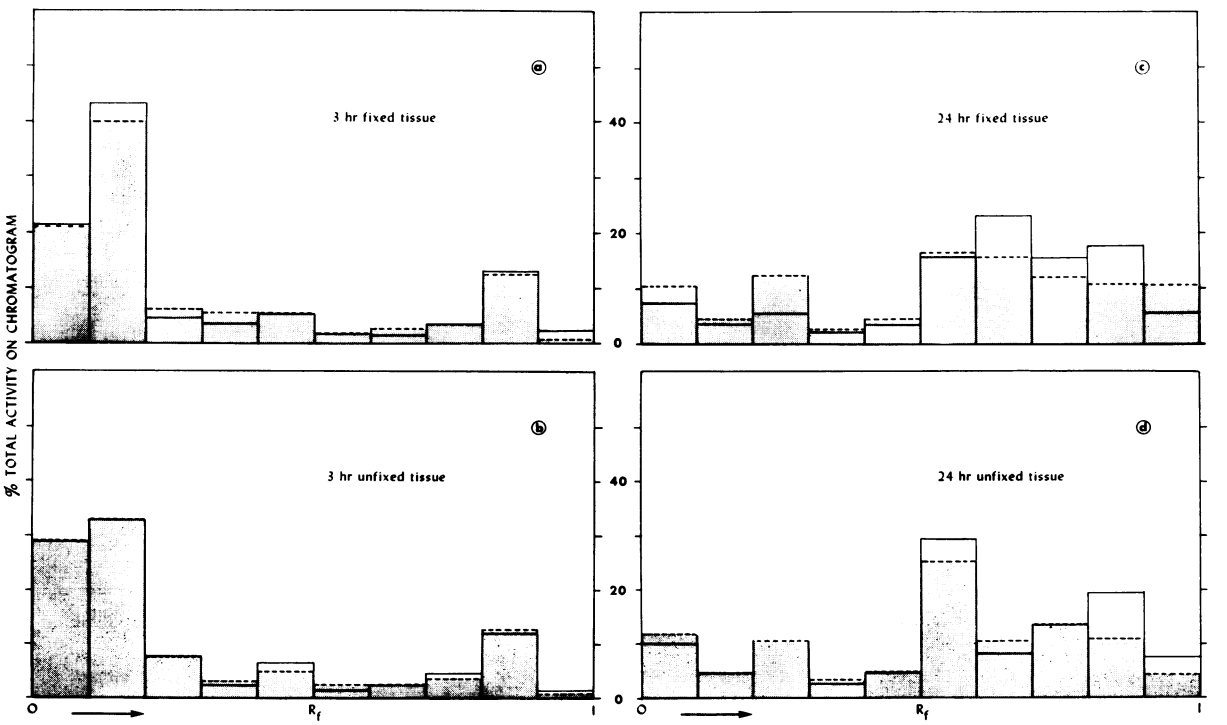
tissue was fixed in cold 4 % (v/v) glutaraldehyde + 1 % (w/v) paraformaldehyde in 0.1 M phosphate buffer at pH 7.2. The first 30 min of fixation took place over ice under vacuum, followed by 2 and one-half hr in the refrigerator. After fixation, the slices were rinsed in three 20-min washes of cold phosphate buffer and subdivided into smaller cubes of tissue. Secondary fixation was in cold 1 % osmic acid in the same vehicle for 1 hr. The fixed tissue was rinsed in 4 changes of buffer and dehydrated in a graded acetone series. The material was allowed to remain in 100 % acetone overnight at 2°, followed the next day by 2 more changes of acetone preceding gradual infiltration by Epon 812. Thin sections (0.25–0.5 microns) of the Epon blocks were cut with a diamond knife on a Sorvall MT-1 ultramicrotome. Sections selected for uniformity of interference colors under reflected light were affixed to non-subbed slides. Approximately 20 slides, each with 3 transverse sections, were prepared from every block sectioned.

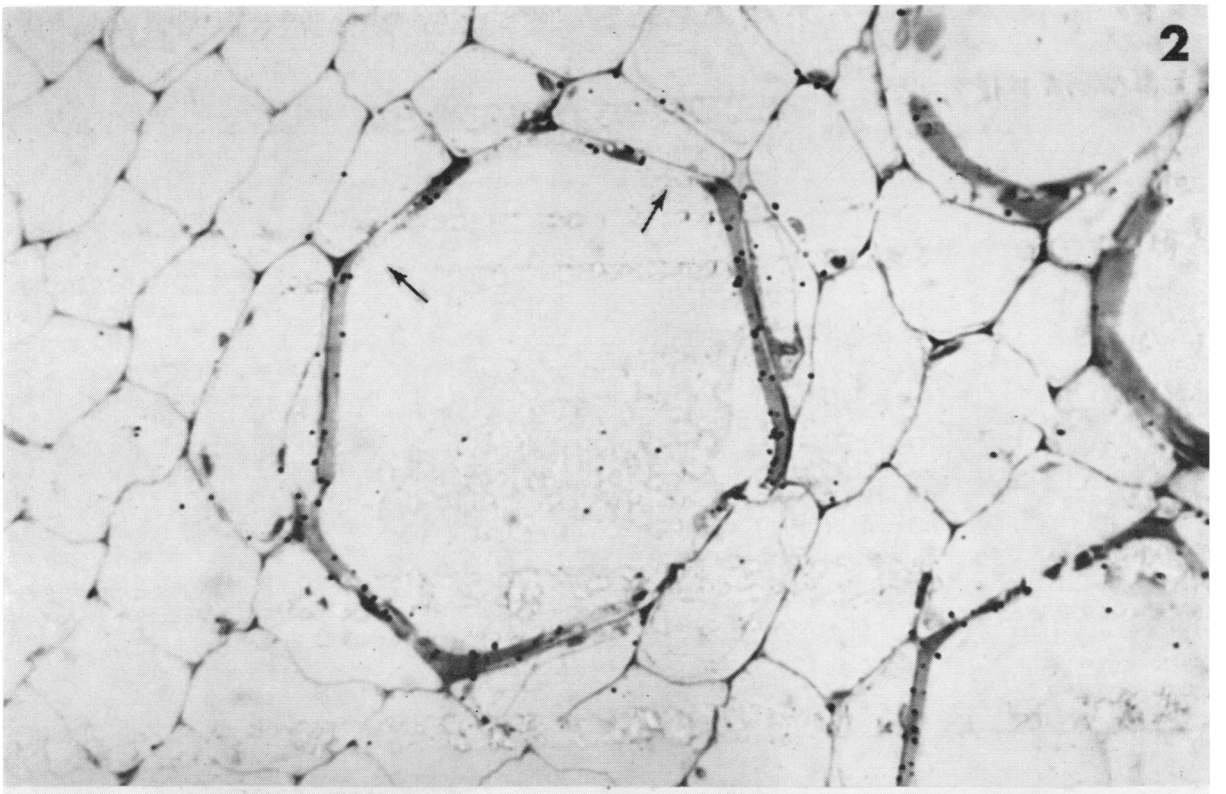
Radioautography was carried out employing methods adopted from Caro (2), Prescott (15), and Stevens (22). Kodak NTB-2 liquid nuclear emulsion was diluted 1:3 with water and the slides coated using the dipping technique. The coated sections were exposed for 2 weeks at room temperature. The radioautograms were developed using the following procedure: 6 min in Kodak Microdol-X (diluted 1:3), 15 sec in 1 % acetic acid, 15 sec in distilled water, 5 min in Kodak Rapid Fixer, 5 to 7 min rinse in running water.

Sections were stained with two 20-sec treatments with Toluidine Blue O at 60°. The final slides were photographed through a Leitz Ortholux microscope using Kodak High Contrast Copy film and a Kodak Wratten 15G yellow filter.

Determination of the Distribution of Radioactivity. A duplicate experiment, covering the 3 hr and 24 hr periods, was performed in order to assess the fraction of absorbed label rendered acetone-insoluble within the tissue, and any possible degradative effects of the fixatives on the solvent-extractable fraction. At the end of each experimental period, half the number of internode segments was dropped directly into 100 % acetone. The others went through the fixation procedure and after the post-osmic rinse were also extracted with acetone. Donor and receiver blocks were dropped into scintillation fluid containing 25 % ethanol in Toluene-PPO-POPOP (14). All samples were allowed to extract for a minimum period of 48 hr in the refrigerator. Half the volume of each acetone extract was transferred to a scintillation vial, dried down under an infra red lamp, and the residue taken up in scintillation fluid for counting. Samples from the acetone extracts of duplicate fixed and unfixed internode segments were spotted and run on Eastman thin-layer Chromatogram sheets. The resolving solvent was isopropanol:28 % ammonia:H_2O (8:1:1) and



FIGS. 1a-1d. Histograms representing thin layer chromatography of acetone extracts of fixed and unfixed *Coleus* internodes treated with ^{3}H-IAA for periods of 3 hr and 24 hr. Broken and unbroken lines on each figure represent duplicate samples. No degradative effect of the fixation procedure is apparent.



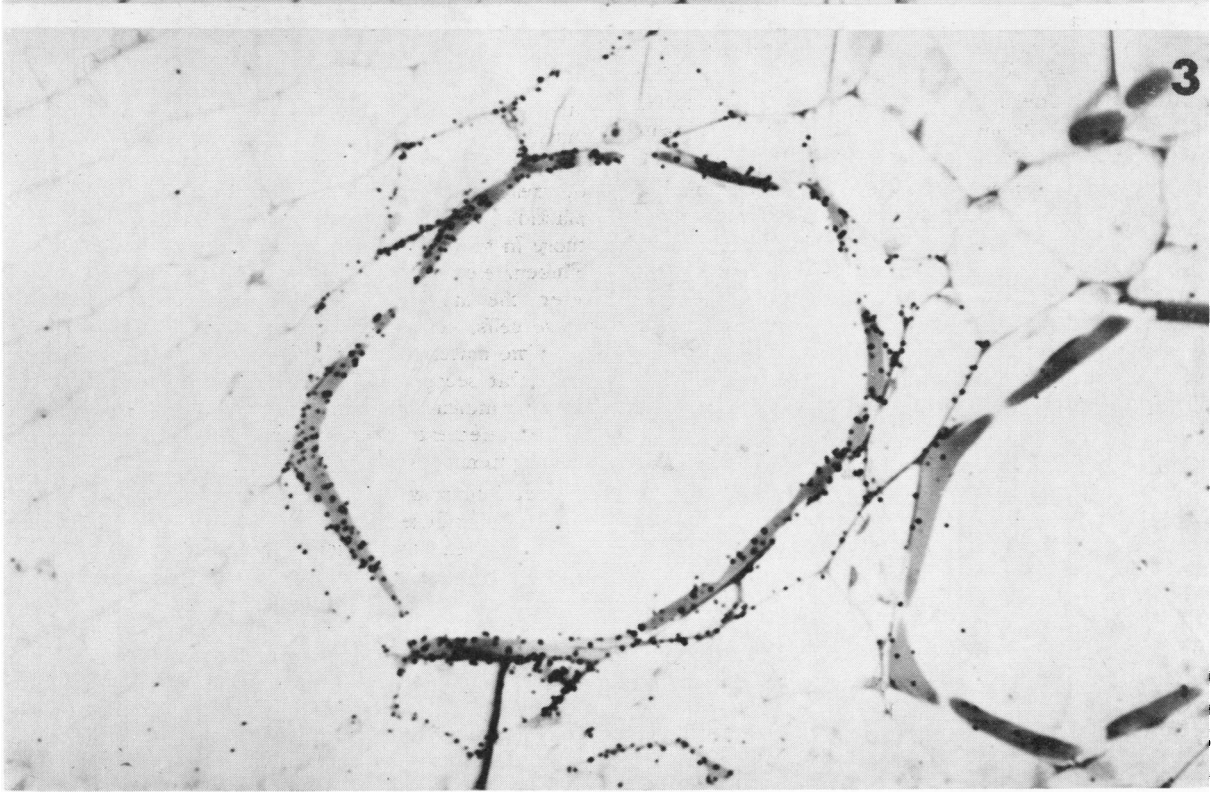


FIG. 2. Section from the apical end of internode treated with ^{3}H-IAA for 3 hr. The label is present in the thickened areas of the cell wall of young tracheary elements. Arrows indicate regions in which both secondary wall material and silver grains are absent.

FIG. 3. Section from the apical end of internode treated with ^{3}H-IAA for 24 hr. The walls of the young tracheary elements and adjacent parenchyma cells are heavily labeled. Considerably fewer grains are present over the walls of the oldest tracheary element to the right of the figure.

the solvent front was allowed to travel 15.0 cm. The silica gel from each R_F zone of the chromatograms was scraped off into scintillation fluid. Radioactivity was measured in a Tri-Carb liquid scintillation counter. The data from all tissue extracts were corrected for quenching, employing a correction factor derived from control (cold) tissue extracts to which known serial dilutions of ^{3}H-IAA were added.

Results

Distribution of Radioactivity. Analysis of the data representing the final activity in the donor blocks, receiver blocks, and the tissue extracts revealed that approximately 60 % of the radioactivity absorbed by the tissue was rendered insoluble in acetone during both the 3 hr and 24 hr treatments. The acetone extracts of fixed and unfixed tissue segments provided the radiochromatographic data in figure 1. After 3 hr treatment with ^{3}H-IAA, most of the activity was located at R_F 0 to 0.2, with a minor peak at R_F 0.8 to 0.9 (fig 1a, 1b). No significant activity was found at the position corresponding to IAA (R_F 0.5 to 0.7 on control chromatograms). The close agreement in the histograms of duplicate extracts (broken *vs.* unbroken lines) emphasizes the reproducibility of the results.

In the case of tissue treated with ^{3}H-IAA for 24 hr, the chromatograms revealed a broad zone of activity between R_F 0.5 to 0.9 (fig 1c, 1d). This zone may include both IAA and labeled derivatives of the auxin. The peak of activity at R_F 0.2, present after treatment for 3 hr, has virtually disappeared.

The low concentration of ^{3}H-IAA supplied (5×10^{-7} M) and the possibility that at this level it may be rapidly metabolized must be borne in mind. A point of importance, however, is the fact that the fixation procedures have no apparent effect on the pattern of distribution of the soluble label. One might, therefore, reasonably expect little, if any, effect on the bound fraction of the label supplied.

Radioautography: 3-Hr Treatment With ^{3}H-IAA. In the apical sections there was distinct labeling of the cell walls of the youngest tracheary elements (fig 2). The grains were restricted in location to the portions of the wall that showed secondary thickenings. (Note the absence of grains at the arrows where secondary thickenings were not present.) In some sections, xylem parenchyma cells adjoining the labeled tracheary cells also indicated some radioactivity in their walls. The silver grains were distributed at random over the cross-sectional area of the secondary wall thickenings, with no preferential accumulation towards either the primary wall or the lumen of the cell. No other tissues showed any incorporation of label. The restriction of labeling to the regions of wall deposition and the observation that plasmolyzed cells showed no grains over the cytoplasm, support the suggestion that the label was being specifically incorporated into secondary wall material.

Sections taken from midway down the internode segment after 3-hr apical treatment with ^{3}H-IAA showed no significant labeling of any tissue or cellular component, supporting the interpretation that the grains observed were due to the tritium label.

24-Hr Treatment with ^{3}H-IAA. Apical sections demonstrated heavy labeling of the secondary walls of the youngest tracheary cells and adjacent parenchyma cells (fig 3). There was considerably less, although still significant, labeling of the walls of older tracheary cells. In the case of xylem parenchyma cells the intensity of labeling was correlated with increased birefringence of the walls under polarized light. The grains tended to accumulate at the cell corners. Some localization of grains over plastids was also evident.

Sections taken midway down the internodes indicated that a heavy accumulation of grains was restricted to the walls of very young tracheary cells that were just beginning to differentiate and develop secondary wall thickenings. A suggestion of label also accumulating over phloem elements was displayed by some of the thicker sections. No activity was located over the cytoplasm or nuclei.

48-Hr Treatment with ^{3}H-IAA. In the apical sections very heavy labeling of young tracheary cells and adjacent parenchyma cells was displayed (fig 4). The walls of enlarged cells, in the process of differentiation into tracheary elements, were also heavily labeled. However, it was striking that other similarly enlarged cells in this region, that displayed no visible evidence of secondary wall deposition, were completely free of silver grains. After this period of treatment there was also prominent labeling of plastids within parenchymatous cells, particularly those in the xylem area and the inner cortex (fig 5). Phloem elements also demonstrated labeling. However, the label was not present over the walls of these cells, but was largely restricted to cytoplasmic or slime material. With the exception of companion cells that seemed to be relatively free of label, there was no indication that the grains were located over phloem elements of any particular type or stage of development.

Sections from the middle of the internodes showed grains distributed over the walls of both young tracheary cells and xylem parenchyma. Again, plastids in the cells of the xylem parenchyma were heavily labeled.

A thick section (*ca.* 1–1.5 microns) taken from the basal end of an internode segment displayed intense labeling of phloem elements (fig 7). Figure 8 shows a thin section of the same group of cells demonstrating an identical pattern of grain distribution. Whereas the companion cells appeared to be free of grains, all other phloem elements displayed intracellular accumulation of radioactivity. The grains appeared to be located over both cytoplasm and slime. Plastids in the surrounding parenchyma-

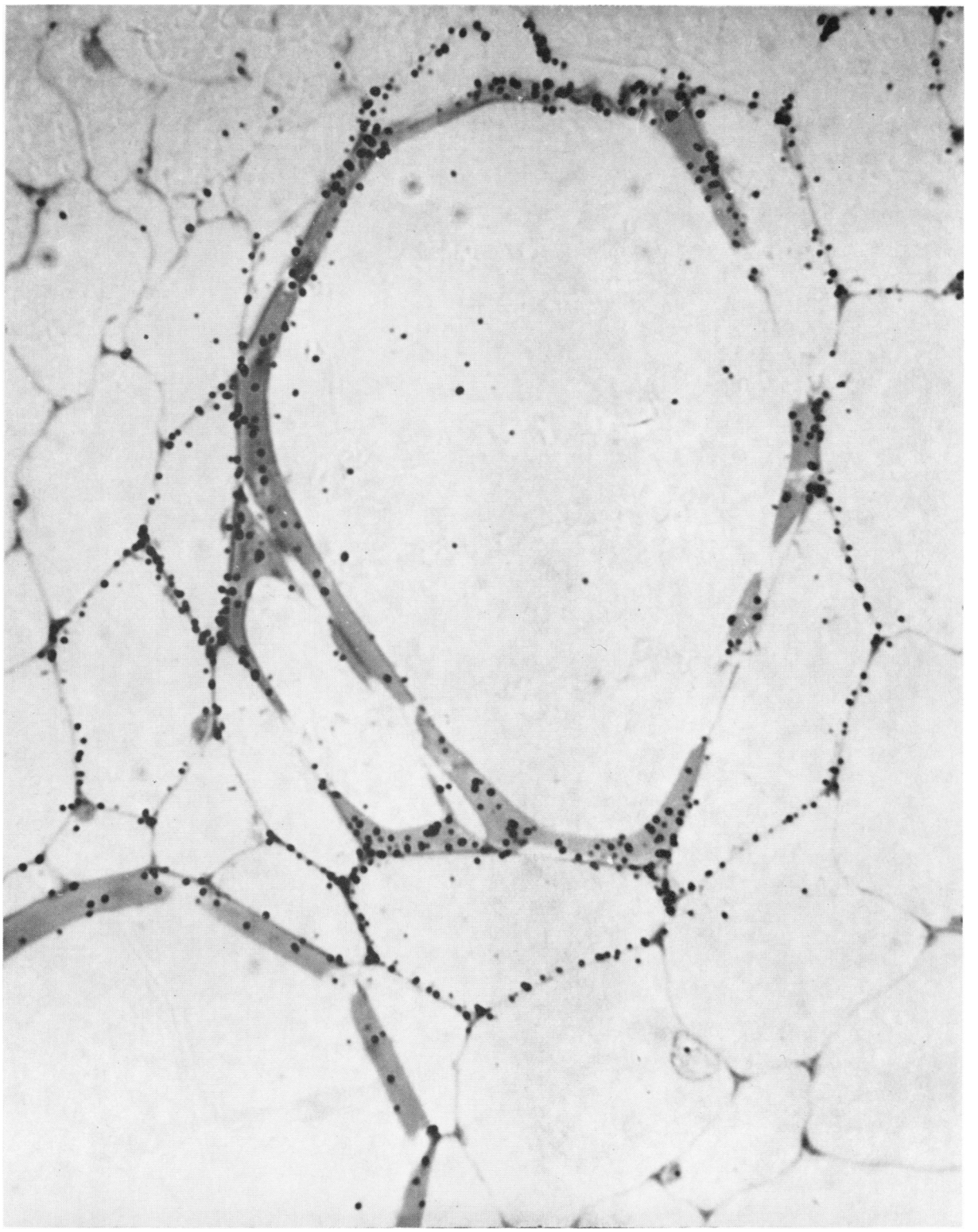


FIG. 4. Section from the apical end of internode treated with ^{3}H-IAA for 48 hr. Xylem parenchyma cells, adjacent to the young labeled tracheary element, also indicate radioactivity in their walls correlated with increased birefringence under polarized light.

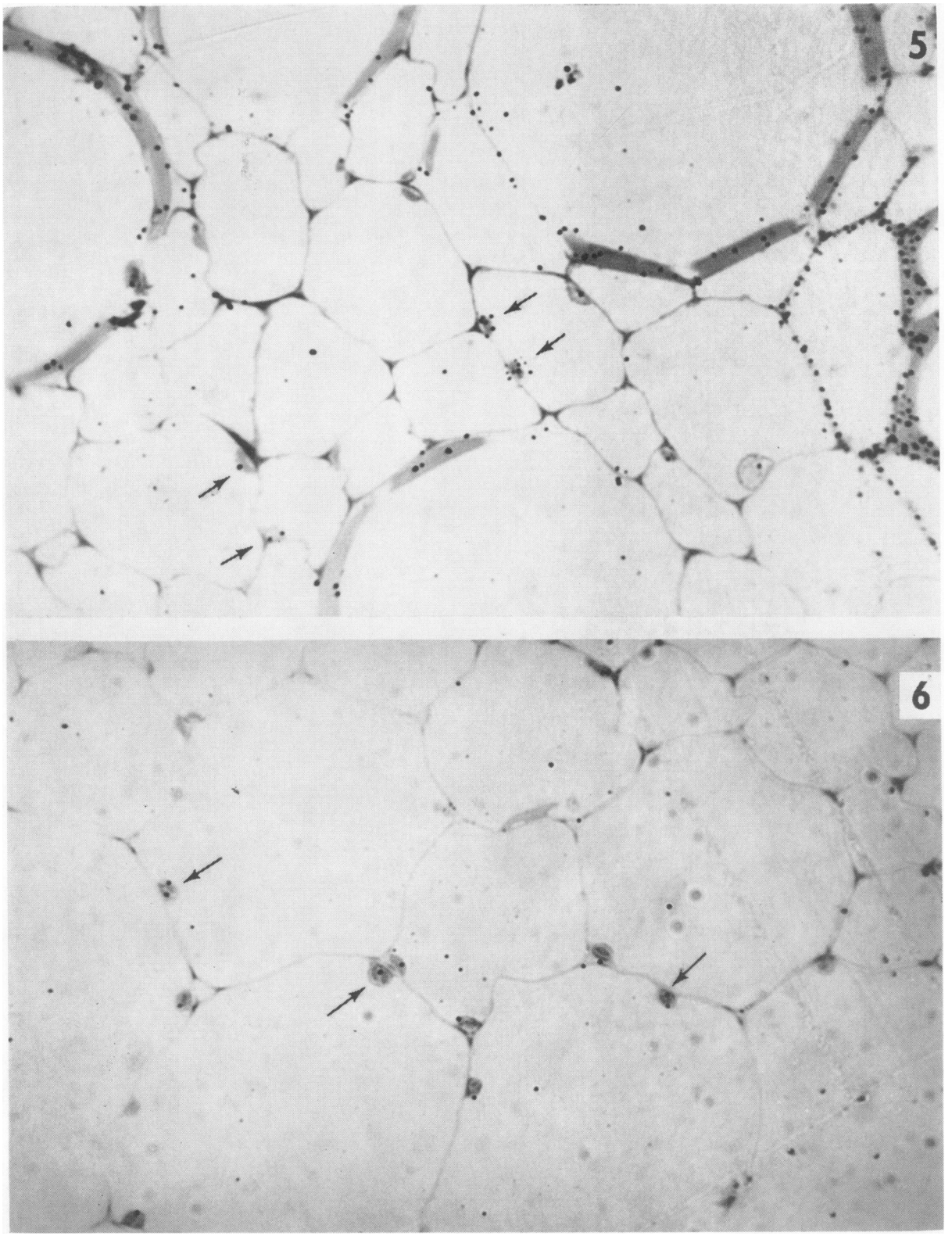


FIG. 5. Section from the apical end of internode treated with ^{3}H-IAA for 48 hr. Silver grains are clustered over the plastids (arrows).

FIG. 6. Section from the basal end of internode treated with ^{3}H-IAA for 48 hr. Plastids in the parenchymatous pith cells are distinctly labeled (arrows).

tous cells also indicated radioactivity (fig 6). There was, of course, pronounced label over the walls of young tracheary elements. Occasional indications of some nuclear and cytoplasmic labeling were also present.

Discussion

The current observation that adding ^{3}H-IAA to *Coleus* stems results in rapid and very specific labeling of the walls of young, differentiating tracheary cells ties in well with earlier evidence from corresponding internodes of the same clone that the amount of available IAA controls the number of tracheary cells differentiated (23). The slow basipetal progression of this specific labeling over 48 hr. correlated with the basipetal development of normal cambial activity, further supports the view that the labeling is a direct reflection of the action of IAA (rather than of random exchange of tritium).

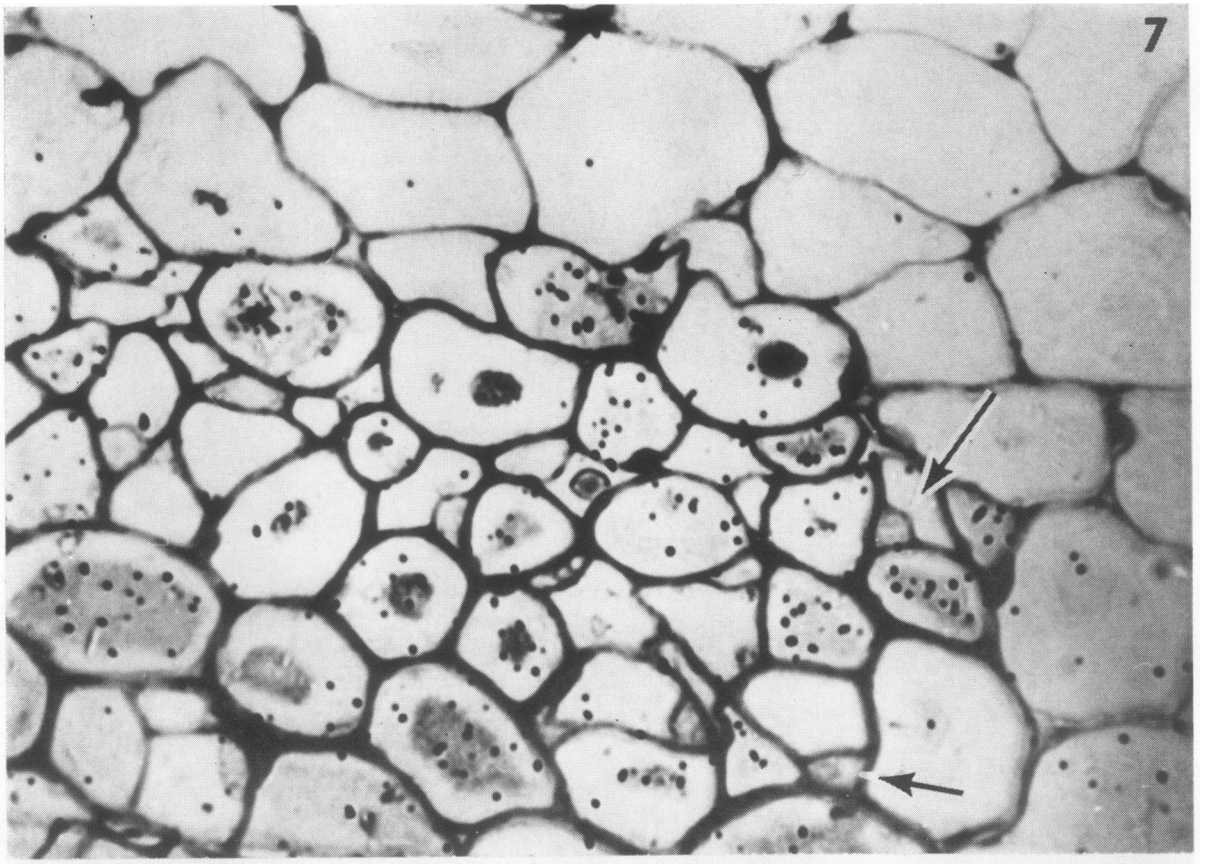
The results of earlier workers are quite diverse. Liao and Hamilton (13) examined squash preparations of excised onion root tips treated with 5.7 μM ^{14}C-IAA for 6 hr. They reported that "most of the meristematic cells" showed grains in both nucleus and cytoplasm. Rasmussen and Bukovac (16) added very high concentrations of ^{14}C-NAA (10^{-3} M and 10^{-5} M) to debladed bean petioles and concluded from the heavy labeling of xylem elements in their 58 μ sections that "conductive tissue appeared to be a pathway for basipetal movement." Preliminary experiments by Wangermann (26), using ^{14}C-IAA applied to young internodes of *Coleus*, showed that when thick sections of fresh tissue were applied directly against X-ray film the blackening on the inner margin of the bundles was co-extensive with the general xylem regions of the vascular bundles. The technique was not refined enough, according to Wangermann, to decide whether the phloem was active or not. Experiments still in progress, using frozen sections and liquid emulsion film, were cited as showing some activity "concentrated also in some cells of the phloem." The only authors to use tritium-labeled IAA (although at 10^{-4} M) and reasonably thin sections (14 μ) reported greatest blackening in the epidermis and cambial regions. However, the figures from this paper (31) are particularly unimpressive and unconvincing. Veen (25) added the relatively high concentration of 4×10^{-4} M NAA labeled with ^{14}C to *Coleus* internode segments for 24 hr. The tissue was frozen before being embedded and was sectioned at 5 to 10 μ. At the treated end of the segment grains were present in the cytoplasm (rather than the wall) of pith cells. Veen reported that blackening was more intense over cortical than over pith tissue.

The radioautograms in the literature would therefore not lead one to expect results like ours. This is partly due, no doubt, to the fact that earlier workers used thick sections, ^{14}C, or high concentrations of auxin, in contrast to the ultrathin sections, tritium, and physiological concentrations of auxin of very high specific activity that we used. We had the additional advantage of using a system in which auxin had been shown to be controlling vascular regeneration. However, part of the differences in results is undoubtedly due to the fact that some of the earlier workers used techniques that would give a measure of *total* radioactivity in the tissue (in contrast to our studies of only that tritium that was insoluble in acetone).

Our results do not fit easily into the hypotheses (3) involving a direct action of auxins on cell walls in general. However, auxins have been shown to be responsible for initiating the differentiation of procambial or cambial derivatives into tracheary cells in a variety of tissues (6, 7, 9, 24, 27, 29, 30). Our radioautographic studies indicate that the secondary wall material of differentiating tracheary cells is indeed the earliest detectable locus of bound label from ^{3}H-IAA. This would seem to support the postulated xylogenic role of auxin. The pattern and rate of incorporation suggest that much of the labeled secondary wall material may be deposited during the course of the experiment.

The mechanism whereby the label from ^{3}H-IAA is thus incorporated into the wall remains obscure. One possibility lies in the induction of peroxidase activity by auxin leading to lignogenesis (5, 21). Jensen (8) has demonstrated the high peroxidase activity of cells in the vascular bundles and epidermis. Nevertheless, one has to assume in this case the degradation of the labeled IAA itself into phenolic derivatives that are subsequently incorporated into lignin. A second possibility arises from the work of Roberts (17, 18) who histochemically localized protein-bound sulfhydryl groups in the cell walls of redifferentiating pith parenchyma cells and xylem vessels of wounded *Coleus* internodes. In view of the reported interactions between growth substances and SH-enzymes *in vitro* (12, 19) it remains conceivable that IAA couples with SH-groups in the xylem walls.

Our results provide no similar evidence to either support or negate a direct involvement of IAA in phloem development (10, 11, 23). Whereas tracheary cells were clearly labeled after 3 hr of treatment with ^{3}H-IAA, distinct labeling of phloem occurred only after 24 to 48 hr of treatment. Furthermore, the label is not present in the walls of phloem elements, nor is it restricted to differentiating phloem mother cells. Nevertheless, the pattern of phloem labeling is specific and suggestive from another viewpoint. Whereas companion cells are virtually free of label, the sieve tubes, phloem, parenchyma, and enlarged cambial derivatives on the phloem side are all impartially radioactive. The results of Eschrich (4) strongly support the view that phloem is involved in the basipetal translocation of IAA. Our data indicate that when the tissue is supplied with ^{3}H-IAA, labeled compounds may be detected in the phloem in water- and acetone-insoluble forms.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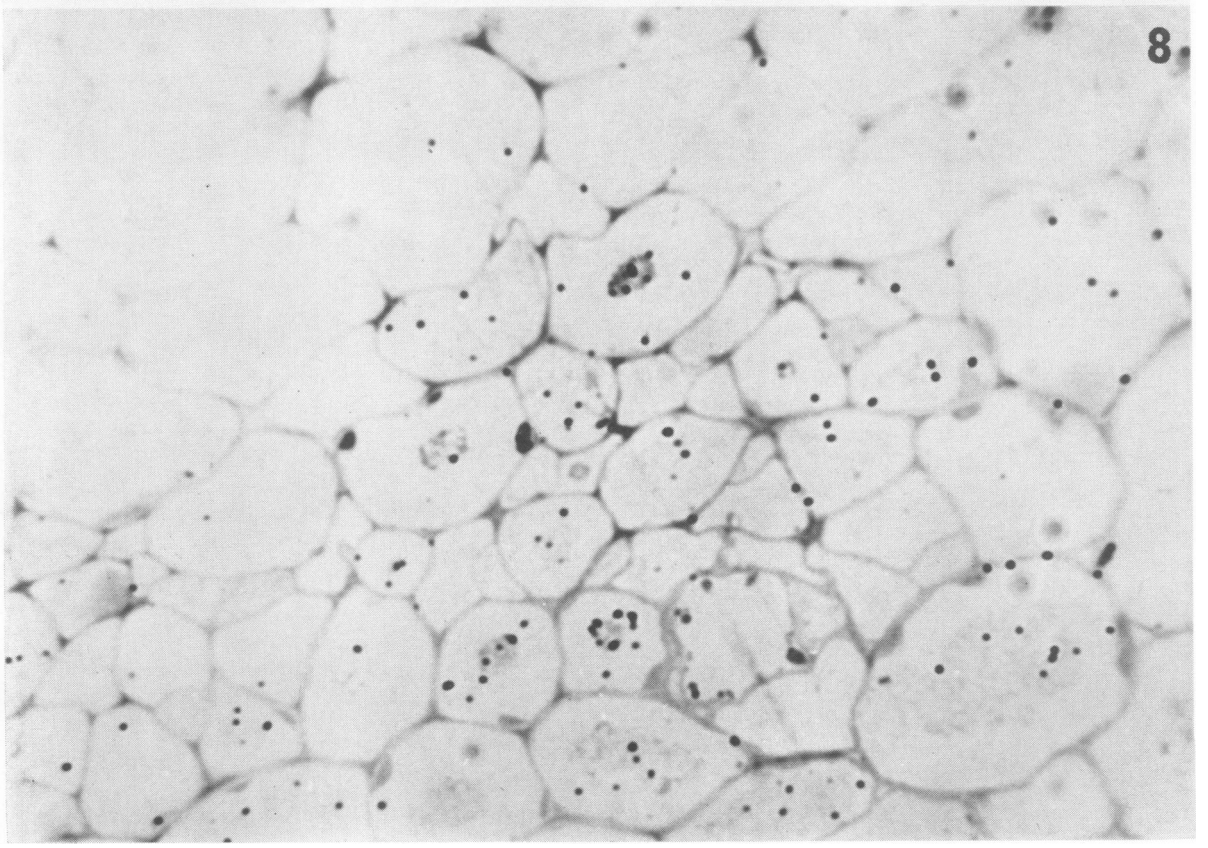
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It appears possible that certain bound complexes or derivatives of IAA may be transported in the same system.

Considering the low concentrations of IAA supplied to our tissue and the distinct patterns of labeling, it would seem difficult to dismiss these bound fractions of auxin as merely products of detoxication mechanisms.

Acknowledgments

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FIG. 7. Section from the basal end of internode treated with ^{3}H-IAA for 48 hr. Whereas companion cells (arrows) appear to be free of grains, all other elements in the phloem area are labeled.

FIG. 8. Thinner section through the same cells seen in the preceding figure demonstrating a similar distribution of label.

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