STUDIES ON EFFECTS OF HORMONES ON CAMBIAL ACTIVITY AND ITS DERIVATIVES, AND NUCLEAR CHANGES DURING XYLEM VESSEL DIFFERENTIATION

by

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ABSTRACT

The thesis has two parts: The first part deals with the effects of exogenously applied hormones -- indoleacetic acid (IAA) and gibberellic acid (GA_3) at various concentrations, singly and in combination, on cambial activation and xylem and phloem differentiation in dormant twigs of poplar (*Populus trichocarpa* Torrey and Gray). Both hormones, IAA and GA_3, stimulate the cambium to divide, more so when they are applied in combination than singly. IAA promotes more cells to be formed toward the xylem and more cells to be differentiated as vessel elements per unit area, whereas GA promotes a greater production of cells toward the phloem. The second part deals with changes in the fine structure, and DNA and histone contents in nuclei of differentiating xylem vessel elements in corn (*Zea mays* L.) leaf. These changes are described with reference to 3 arbitrary stages. Early in cell differentiation the nucleus and nucleolus increase in size, the nucleolus shows an elaboration of granular and vacuolar regions and the DNA and histone contents are increased to twice their original amounts. Later the histone content continues to increase, the nucleolus reverts to its original size and shows loss of granular and vacuolar regions, and simultaneously some of the degenerative changes appear in the nucleus. The above mentioned changes in the nucleus and nucleolus are completed well before the complete deposition of secondary wall and final hydrolysis of the cell protoplast.

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List of Abbreviations Used in the Figures.

C -- cambial layers

Ch -- chromatin masses

D -- dictyosomes

FR -- fibrillar region of nucleolus

GR -- granular region of nucleolus

M -- mitochondria

Me -- mesophyll cells

N -- nucleus

NC -- nucleolus-associated chromatin

NE -- nuclear envelope

NPh -- new phloem

Nu -- nucleolus

NXy -- new xylem

P -- procambial cell

Pa -- parenchyma cells

Ph -- phloem

Pl -- plastid

RER -- rough endoplasmic reticulum

S -- spherule

Sc -- sclerenchyma band

SW -- secondary wall

T -- tracheid-like cells

V -- vacuole in cytoplasm

VE -- vessel element

VR -- vacuolar region of nucleolus

Xy -- xylem

1. INTRODUCTION

Woody dicotyledons and gymnosperms are characterized by lateral or secondary growth of xylem (wood) and phloem (bark). This secondary growth follows extension or apical growth and is due to the presence of a layer of meristematic cells, the cambium. In temperate climates the cambium shows seasonal activity: active growth takes place in spring and summer and is followed by a period of dormancy which is broken in the next spring. During the active period, the cambial cells divide periclinally and yield derivatives on either side -- those on the inside normally differentiate as xylem cells and those on the outside as phloem cells (Esau, 1965). The dormancy and activation of cambium have been related to photoperiod and temperature, and more specifically, to hormones such as indoleacetic acid (IAA) and gibberellic acid (GA). The differentiation of xylem and phloem derivatives also has been related to differential concentrations of these hormones. But nearly all this work has been done in one laboratory by Wareing and his students (Wareing, 1958a, b; Wareing, Hanney, and Digby, 1964; Digby and Wareing, 1966a, b).

The cytological changes during differentiation of vascular derivatives, particularly vessel elements in xylem and sieve elements in phloem, have been studied by many

authors and several reviews on the subject are available (Esau, 1969; Roberts, 1969; Singh and Srivastava, 1972; Srivastava and Singh, 1972). Nuclear changes, however, have been largely ignored, so much so that in one of the recent reviews on xylem differentiation (Roberts, 1969), there is not a single word on nuclear changes.

In this thesis, therefore, two topics are investigated:

1. Effects of exogenously applied hormones on induction of cambial activity and xylem and phloem differentiation in a selected tree species, and

2. Changes in the fine structure and DNA, RNA and histone contents in nuclei of differentiating xylem vessel elements.

It is appropriate at this point to indicate that most of the cytological studies on xylem (and phloem) differentiation have been done mainly with primary vascular tissues derived from procambium rather than with secondary vascular tissues derived from the cambium. The reason basically is the difficulty of extracting the cambium and secondary tissues from the tree and fixing them quickly enough for adequate preservation of cytological detail for electron microscopy (see Srivastava and O'Brien, 1966). As will become clear later, my attempt to study nuclear changes in vessel elements of secondary xylem had to be abandoned and

replaced by a study of these changes in the vessel elements of primary xylem. This is not a serious handicap, however, because studies that are available indicate a basic similarity in differentiation of the primary and secondary vascular tissues.

2. REVIEW OF LITERATURE

2-1. The role of hormones in cambial activity and vascular differentiation.

External applications of synthetic or natural hormones provide information on cambial activity and xylem and phloem differentiation. Application of indoleacetic acid (IAA) to decapitated, disbudded and non-dormant twigs of woody plants stimulated the cambial activity and production of new xylem tissue (Gouwentak, 1941). Likewise, application of gibberellic acid (GA, unspecified as to its type) stimulated cambial activity (Bradley and Crane, 1957; Wareing, 1958a, b). Wareing (1958a) applied IAA (100, 500 ppm) and GA (unspecified, 500, 1000 ppm) singly and in combination to non-dormant, disbudded shoots of _Acer pseudoplatanus_ and _Populus nigra_, both diffuse-porous species, and _Fraxinus excelsior_, a ring-porous species. His results showed that IAA alone promoted cambial divisions and production of undifferentiated parenchyma-like tissues in which new xylem cells differentiated in a narrow and discontinuous ring. With GA alone, new undifferentiated tissues were formed in the region pertaining to both xylem and phloem. With IAA and GA together, a wide zone of new vascular tissues was produced; the xylem consisted of differentiated vessel elements and fibres with thickened, lignified walls, but in the phloem tissue, the differentiation of cell types was not clear. Further experiments

(Wareing, Hanney and Digby, 1964) with varying concentrations (100, 500 and 1000 ppm) of IAA and GA on Populus robusta indicated that with IAA alone, the amount of xylem tissue did not further increase at any concentration above 100 ppm. Small amounts of undifferentiated xylem tissue were produced with GA alone; increased GA concentrations had little effect on further widening the zone of undifferentiated tissues. Combination of IAA and GA had a synergistic effect on cambial activity. Maximum production of xylem was obtained when IAA and GA were applied together each at 100 ppm. There was a small decrease in xylem production at higher concentrations of each substance, but the most 'normal' looking xylem, that is differentiated vessel elements with lignified tissue in between, was produced with IAA at 500 ppm and GA at 100 ppm. No phloem was formed with IAA alone at any concentration but a considerable amount of phloem was formed with GA alone at 100 ppm. As with xylem development, maximum phloem was formed with IAA at 100 ppm and GA at 100 ppm or 500 ppm. Since the newly differentiated phloem cells, following GA treatment, are difficult to distinguish from previous growth, Wareing used grapevine (Vitis vinifera) which has distinct annual rings in phloem -- the presence of annual rings helps a in the identification of new tissues. With the same treatments as before, similar results were obtained; additionally, the disposition of cells suggested the presence of

sieve elements and companion cells in the newly produced phloem. Similar results were also obtained with Larix decidua and with herbaceous species, Coleus blumei and Phaseolus multiflorus (Wareing, et al. 1964). Digby and Wareing (1966a) repeated these experiments with Populus robusta, Vitis vinifera, and Robinia pseudacacia shoots with nearly identical results. Production of xylem was maximal at IAA 100 ppm/ GA 100 ppm but the new tissues were incompletely differentiated; fully differentiated or 'normal'-looking xylem cells were produced at IAA 500 ppm/ GA 100 ppm, that is, at a relatively higher IAA and lower GA concentration. In contrast, the optimum production of phloem was at IAA 100 ppm/ GA 500 ppm, or at a higher GA and lower IAA concentration.

Digby and Wareing (1966a) also studied the effect of IAA and GA on the length of xylem fibres and xylem vessel elements and the diameter of xylem vessel elements. They found that IAA (100 ppm) increased the length of both fibres and vessel elements, but higher concentrations (up to 500 ppm) did not further increase the length. GA (100, 500 ppm) in the presence of IAA (100 ppm) increased the length of fibres but not vessel elements. Vessel elements increased in diameter with increased concentrations of IAA (500 ppm) in the presence of GA (100 ppm). With low concentration of IAA (100 ppm), the xylem cells produced were rectangular in transverse section; the new xylem tissue consisted mainly

of tracheids and very much resembled the summerwood. With a higher concentration of IAA (750 ppm), the xylem cells produced were larger in diameter and tended to be more nearly circular in transverse section, resembling the vessel elements of the springwood.

There is considerable evidence that endogenous hormones produced in the growing shoot apex and expanding buds stimulate the cambium to divide. For example, Digby and Wareing (1966b) extracted endogenous auxins and gibberellins from *Populus trichocarpa* (poplar) and *Ulmus glabra* (elm) stems at three different heights -- top, middle and bottom segments --, at three different times in the season -- before bud expansion, at the first sign of bud expansion and after bud expansion. In *P. trichocarpa*, which is a diffuse-porous wood, they did not find extractable amounts of IAA at any height of the trunk before bud expansion. At the time of bud expansion, there was an increase in IAA concentration in the top segment which was near the expanding apical bud. After bud expansion, IAA was detected at all heights of the tree. According to these authors, IAA was synthesized in the apical buds and transported down the trunk as a result of concentration gradient; commensurate with it, the cambial activity spread down the trunk. However, in *U. glabra*, which is a ring-porous wood, promotion of tryptophan, a precursor of IAA, was detected in all segments before bud expansion and as buds started to expand, tryptophan was converted to IAA.

In contrast to diffuse-porous species, there was no gradient of IAA down the trunk and the cambial activity started nearly simultaneously at all heights.

As for the gibberellins, Digby and Wareing (1966b) also subjected *Ailanthus* (a ring-porous wood) and *Betula* (birch, a diffuse-porous wood) to long-day, and long-day followed by short-day conditions. They found that 2 and 4 weeks of short-day treatment in *Ailanthus* gave high yields of GA in bioassay, whereas in *Betula*, there was high yield of GA only after long-day treatment and no significant or detectable amount after 2-4 weeks of short-day treatment. The decline in IAA level from long-day to short-day treatments was the same in both *Ailanthus* and *Betula*. Therefore, in *Ailanthus*, high levels of GA and low levels of IAA were present after short-day treatment, whereas in *Betula* both hormones were present in low or insignificant amounts after short-days.

2-2. Nuclear changes in differentiation of xylem vessel elements.

As indicated earlier, available studies indicate a basic similarity in differentiation of primary and secondary vascular elements. In the following review, therefore, the available literature on nuclear changes in differentiation of xylem vessel elements is discussed irrespective of their origin.

2-2-1. Structural changes.

There is virtually no published information on the fine structure of nuclei in the procambial cells and the few studies on the fine structure of nuclei in the procambial cells and the few studies on the fine structure of cambium (Srivastava, 1966a; Srivastava and O'Brien, 1966; Kidwai and Robards, 1969; Robards and Kidwai, 1969; Evert and Deshpande, 1970) provide little or no information on the nucleus.

It has been reported that the nuclei of the winter cambium in Pinus strobus show large chromatin masses near the periphery as well as in the center of the nucleus. But with the same fixation, the nuclei of summer cambium in Fraxinus americana do not show a separation of chromatin in distinct patches (Srivastava, 1966a; Srivastava and O'Brien, 1966). However, these observations must be interpreted with caution for the appearance of chromatin is known to be influenced by fixation, particularly aldehyde fixation (Sabatini, Bensch and Barrnett, 1963).

There is very little published information on the changes in structure, and DNA, RNA, and histones in nuclei during differentiation of xylem vessel elements. This despite the fact that vessel element differentiation has been studied in a variety of tissues such as primary xylem of wheat and corn leaves (Pickett-Heaps, 1966; Pickett-

Heaps and Northcote, 1966; Srivastava and Singh, 1972), pea roots (Cronshaw and Bouck, 1965), oat coleoptiles and young maple twigs (Cronshaw, 1965); and wound xylem in Coleus (Hepler and Newcomb, 1964). Whatever information is available comes from earlier light microscopic work.

As a first step, the procambial cells or cambial derivatives destined to become vessel elements enlarge and sometimes elongate. At the same time, both nuclei and nucleoli enlarge in volume (Bailey, 1920; List, 1963). The nuclei contain relatively large and bright Feulgen positive chromatin masses, which later disperse into smaller masses. Later, these masses appear coarsely granular. The nuclei are modified in shape, and then lose their staining ability (Bailey, 1920; Esau, 1965, pp. 261-264). Finally, there is a break down of the nuclear envelope and loss of nuclear contents (Esau, 1965, pp. 261-264). The nucleoli also enlarge but then decrease in volume. According to Bailey (1920), the large nucleoli are aggregations of smaller nucleolar masses with spaces or vacuoles between them. The nucleoli disintegrate at the last stage of differentiation (Bailey, 1920).

2-2-2. Changes in DNA, RNA and histones.

Swift (1950) observed an increase in the nuclear deoxyribonucleic acid (DNA) in the young xylem cells of Zea mays root. List (1963) measured the DNA content of individual nuclei in the first enlarged young metaxylem cells

in the roots of various monocotyledons. According to him, the nuclei enlarged during differentiation, and this increase in nuclear volume was correlated with an increase in DNA content of from 4 to 32 times that in the procambial cells. The increase in DNA content was believed to be due to doubling of chromosomes or endoduplication. The increase in DNA content was followed by an enlargement of the nucleoli and synthesis of proteins. Fosket (1968) extracted DNA from cultured Coleus stem slices and detected spectrophotometrically a progressive increase in DNA content per gm fresh weight of stem slices during wound xylem formation. Addition of 5-fluorodeoxyuridine to the medium blocked xylem differentiation which could be reversed by addition of thymidine. After addition of cytokinin to cultures and pea root segments, Torrey and Fosket (1970) and Libbenga and Torrey (1973), also noticed an increase in DNA to polyploid levels during differentiation of xylem elements from cortical cells. Corsi and Avanzi (1970) also observed a high DNA content in the nuclei of the differentiating vascular or xylem cells of Allium cepa root tips. In the first mm from the tip, the DNA content was approximately 2 times; in the next 0.5 mm segment, it was 3 to 4 times, even up to 8 times that in the other cells.

Studies on histone contents of nuclei of differentiating xylem cells are very few. Corsi and Avanzi (1970) found that in Allium cepa roots the procambial cells have a higher content of arginine-rich histone than differentiating

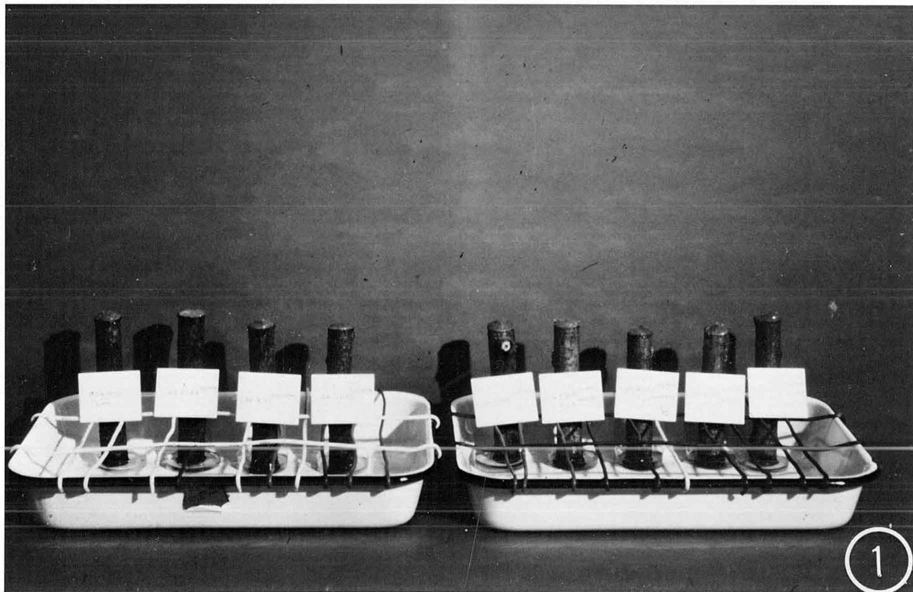
vascular cells. They also reported a high DNA/histone ratio in differentiating vascular cells, the DNA content had clearly increased (see previous paragraph) but histone synthesis may have been delayed or histone content lowered. Unfortunately, the authors did not specify their 'vascular cells' -- whether xylem or phloem cells -- and, furthermore, used 'provascular cells' instead of differentiating vascular cells.

3. MATERIALS AND METHODS

3-1. Application of hormones and other techniques for the study of cambial activity and its derivatives.

Twigs of <u>Populus</u> <u>trichocarpa</u> Torr. & Gray (poplar) were collected in late autumn after leaf fall, disbudded, chilled at 0-4°C for 8 weeks and then stored at 4°C until use. Six-inch long stumps were cut, placed erect with one end in water in a small petri dish and kept under constant conditions of temperature (22± 2°C) and photoperiod (12h)(Fig.1). Auxin (3 indoleacetic acid IAA, Fisher Company, no. 4271) and gibberellin (gibberellic acid GA_3, Grade III, Sigma G3250) in lanolin pastes were applied singly and in various combinations to the upper cut surfaces of stumps for a total of 21 days. The hormones were applied at the following concentrations: 0, 100, 500 ppm. Plain lanolin was applied to control stumps. Every 3 days, the lanolin paste was removed, the end of the stump (less than 1 mm) was retrimmed with a sharp razor blade, and fresh hormone(s) in lanolin paste was applied, so that the new lanolin paste was always in contact with fresh tissues. At the end of hormone treatment (21 days), the blocks were sampled 1 cm below the site of application, fixed in 6% glutaraldehyde (aqueous) for 12h,dehydrated in an ascending series of ethanol up to 70% ethanol and sectioned at 22-26μ thickness in 70% ethanol on a sliding microtome. Sections were routinely stained in

Fig. 1. Experimental set up for hormonal treatment of poplar twigs.



toluidine blue O (O'Brien, Feder and McCully, 1964; Feder and O'Brien, 1968). For lignification of new xylem cell walls, sections were stained with phloroglucinol-HCl (Srivastava, 1966b). For identification of callose in new sieve elements, the tannic acid-iron chloride and lacmoid schedule of Cheadle, Gifford and Esau (1953) was used.

3-2. Nuclear changes in differentiating xylem elements.

Bark samples including cambium and latest xylem tissues of *Populus trichocarpa* and *Robinia pseudoacacia* L. (black locust) were collected, treated as in part 3-1, sampled and fixed for both light and electron microscopy. However, since the activated cambial and new xylem tissues were very delicate, they were easily damaged during fixation and subsequent trimming. Despite several trials, with different fixatives and different osmolarities, a satisfactory fixation of hormone-treated poplar and black locust samples was not obtained. To complicate matters, glycol methacrylate used for embedment for light microscopy picked up background stain during cytochemical studies. Paraffin was eventually used, but this created difficulties in sectioning woody material. Thus, corn leaf, in which many vascular bundles run parallel to each other, was chosen as the material to study the nuclear changes in differentiation of xylem vessel elements.

Corn (Zea mays L.) seedlings were grown in a growth chamber (Controlled Environment Ltd.) at 15 h day-length, 400 foot-candles, and 23° ± 2 ° C temperature. Eight to ten cm lengths of young leaves including the youngest parts were dissected out from the sheath bases and fixed and processed in the following manners:

3-2-1. Light microscopy.

Paraffin embedding: The material was fixed in 10% acrolein in phosphate buffer for 24 h at 4°C, cut into 4 mm X 5 mm segments, dehydrated in an ascending series of ethanol with 2 changes of absolute ethanol for 24 h followed by 2 changes of toluene for 24 h, infiltrated with paraffin gradually at 40°C for 2 days, and then with pure paraffin for another 2 days, and finally cast into blocks. Paraffin blocks were trimmed to expose the tissue and soaked in 50% ethanol:glycerine (1:1) for 1-3 days before sectioning at 8μ.

Glycol methacrylate embedding: The material was fixed in 10% acrolein in xylene for 24 h at 4°C, cut into 1½ mm x 2 mm pieces, dehydrated sequentially in methanol: methoxyethanol (1:1), ethanol, N-propanol and N-butanol at 4°C, brought to room temperature, and embedded in glycol methacrylate (Feder, 1960). The sections were cut at 5μ

with a Porter Blum MT1 microtome and routinely stained with toluidine blue O (O'Brien et al., 1964; Feder and O'Brien, 1968).

Enzyme extraction, staining and cytophotometry for DNA, RNA and histones:

Paraffin sections were treated with DNAase (Sigma, DN 100, from beef pancreas), 0.25-1.50 mg/ ml in 0.03M $MgSO_4$ solution or 0.05M phosphate buffer at pH 6.8 at 37°C, but DNA was not removed completely even after 24 h treatment. However, 0.5N perchloric acid (2 h at 70°C) removed both nucleic acids. RNAase (Sigman, RNAase-A, no. R-4875, from bovine pancreas), 0.25 mg/ml in phosphate buffer at pH 6.8-7.0, removed RNA at 37°C after 3 h treatment.

Sections were deparaffinized, hydrolyzed in 1N HCl at 60°C for 20-40 m, stained with Schiff's reagent (basic Fuchsin, color index number 42510 from Harleco, Philadelphia) for 2 h, and washed in fresh bisulfite solution for 12 m following the method of Deitch (1966) and Feder and O'Brien (1968). Control sections were extracted with 0.5 N perchloric acid at 70°C for 2 h before staining with Schiff's reagent. Cytophotometric measurements (see below) were made at 570 nm.

Gallocyanin chrome alum staining schedule (Sandritter, Kiefer and Rick, 1966; Stange, 1970) for both DNA and RNA was also used. This schedule has the advantage that cell

walls, especially the lignified thickenings in xylem elements do not pick up a lot of stain which interferes with nuclear measurements. This is the chief disadvantage in using Azure B. A solution of 150 mg gallocyanin (Fluka Company, Switzerland) and 5 g chrome alum (chromium potassium sulfate) was freshly made up in 100 ml of distilled water and pH adjusted to 1.64. Slides were stained for 48 h and washed for 30 m in water. Slides of all treatments were stained at the same time. Unextracted slides gave combined value for both DNA and RNA. Slides extracted with RNAase gave values for DNA. The subtraction of DNA values from both DNA and RNA values gave the value for RNA. Despite the use of a series of wavelengths for the two treatments, with and without RNAase, and a lower wavelength at 510 nm, the extinction for some of the nucleoli could still not be measured since only those points which have a transmission between 5 and 95% are recorded with this program. The gallocyanin staining gave a deep purple color to nuclei and nucleoli, especially the latter, possibly because of the abundant presence of RNA.

Alkaline fast green was used for staining histones (Jensen, 1962; Deitch, 1966). Slides were extracted with 0.5 N perchloric acid at 70°C for 2 h to remove the nucleic acids, washed, stained in 0.1% fast green (FCF, Fisher Company, F-99, no. 42053) in 0.005M phosphate buffer at pH 8.1 for 1 h, and rinsed in water and 95% ethanol. Measurements were made at 640 nm.

All slides were mounted in immersion oil (refractive index 1.515) and measured under Carl Zeiss Scanning Microphotometer connected to a PDP 12-A digital computer. A CRT unit allowed a visual display of all measured points of various extinctions. With a small square box which could be increased or decreased in size and could be moved in any direction, unwanted measured points could be erased easily in the scanned area. All measurements were made with 0.5 μ step size and the diameter of the aperture was 1 μ. The program used is APAMOS (automatic photometric analysis of microscopic objects by scanning, Wied, Bahr and Bartels, 1970). 25-30 nuclei were measured in each group. The data obtained were analyzed statistically using analysis of variance.

3-2-2 Electron microscopy.

The material was fixed in 3% glutaraldehyde in 0.05M phosphate buffer, pH 6.8-7.0, for the first 2 h at room temperature and the next 22 h at 4°C. It was washed in phosphate buffer and cut into 1 mm X 1½ mm pieces, post-fixed in 2% osmium tetroxide in phosphate buffer at room temperature for 2 h, washed with 3-4 changes of buffer, dehydrated in an ascending series of ethanol, followed by propylene oxide, and embedded over several days with repeated

evacuation in araldite. Silver grey sections were cut on a Reichart OMU2 microtome with a diamond knife and examined under a Zeiss EM9A or a Phillips EM 300G after staining in uranyl acetate and lead citrate.

4. RESULTS

4-1. Effects of applied hormones on cambial activity and differentiation of xylem and phloem derivatives.

Cambial activity in poplar was measured by counting the number of cells in radial tiers. In any section the cambial layer was identified first. The new xylem was then measured as the number of newly differentiated or undifferentiated cells in a radial tier centripetally from the cambial layer to the annual ring of last year. New phloem cells were counted in the same manner from the cambial layer to the last phloem cell of the previous year, which was mostly a sieve element next to the phloem parenchyma. Fifty tiers were counted for each treatment. The results are expressed as mean of the 50 counts.

4-1-1. Cambium reactivation

The dormancy of cambium was broken as the stem segments were put in water dishes with summer photoperiod at room temperature (22°C), after 2 months of cold treatment. As indicated in Table 1, even in the controls, the cambium was activated -- it started to divide and produced a few undifferentiated cells on both sides (Fig. 4). Either hormone, IAA or GA_3, stimulated the cambium to produce more cells. However, both hormones applied together enhanced the activation of cambium (Table 1).

Table 1. Numbers of new xylem and phloem cells produced under different hormonal treatments.

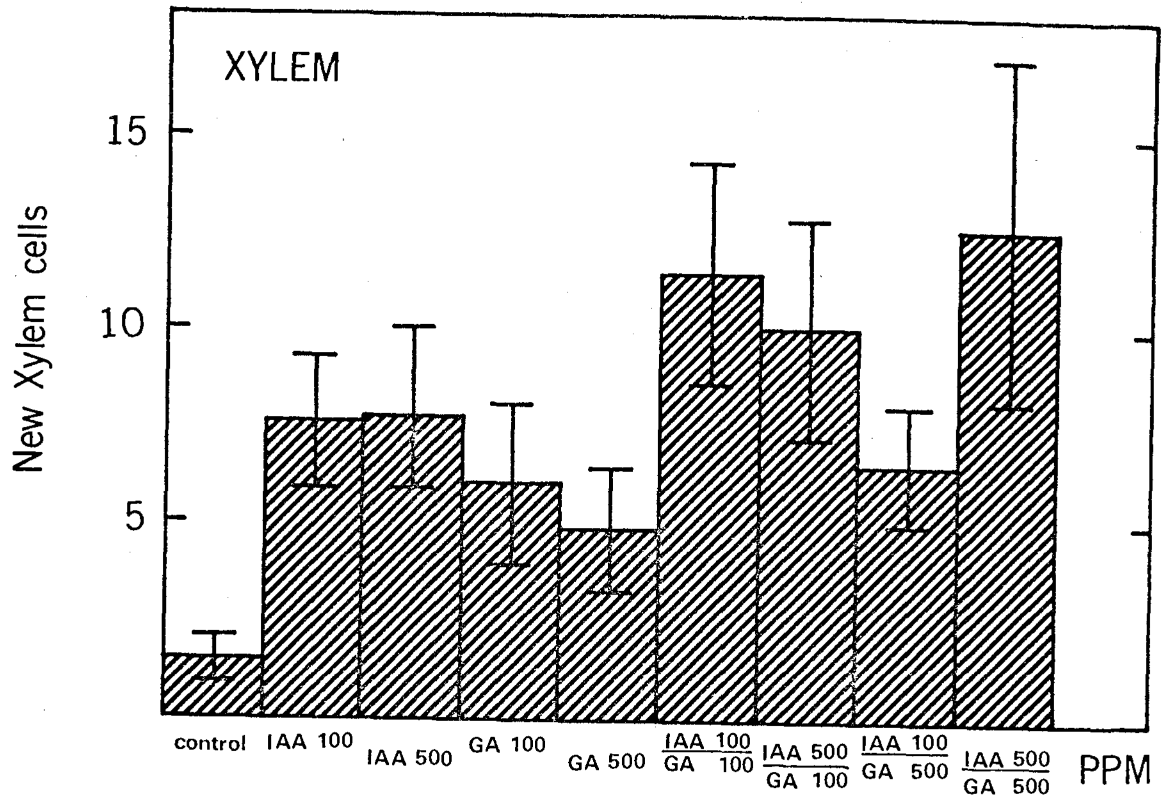
Treatment	Mean* ± S.D. New xylem cells	Mean ± S.D. New phloem cells	Mean ± S.D. Cambial cells	Mean ± S.D. Total cells in cambial zone
Control	1.52±0.61	7.68±1.33	1.38±0.53	10.58±1.44
IAA 100 ppm	7.60±1.74	10.43±1.64 ** (2.75)	2.86±0.85	20.88±3.16
IAA 500 ppm	7.80±2.32	9.92±1.98 (2.24)	3.06±0.62	20.76±2.78
GA 100 ppm	6.18±2.14	13.46±1.73 (5.78)	3.62±1.02	23.26±2.91
GA 500 ppm	4.92±1.58	15.42±2.30 (7.74)	3.04±0.66	23.38±2.79
IAA 100 ppm GA 100 ppm	11.50±2.78	13.60±1.90 (5.92)	3.74±0.83	28.84±3.75
IAA 500 ppm GA 100 ppm	10.06±2.77	10.28±2.53 (2.60)	3.12±0.62	23.44±4.70
IAA 100 ppm GA 500 ppm	6.52±1.64	13.88±2.47 (6.20)	2.66±0.50	23.06±3.21
IAA 500 ppm GA 500 ppm	12.68±4.42	14.54±3.28 (6.86)	2.68±0.76	29.70±6.70

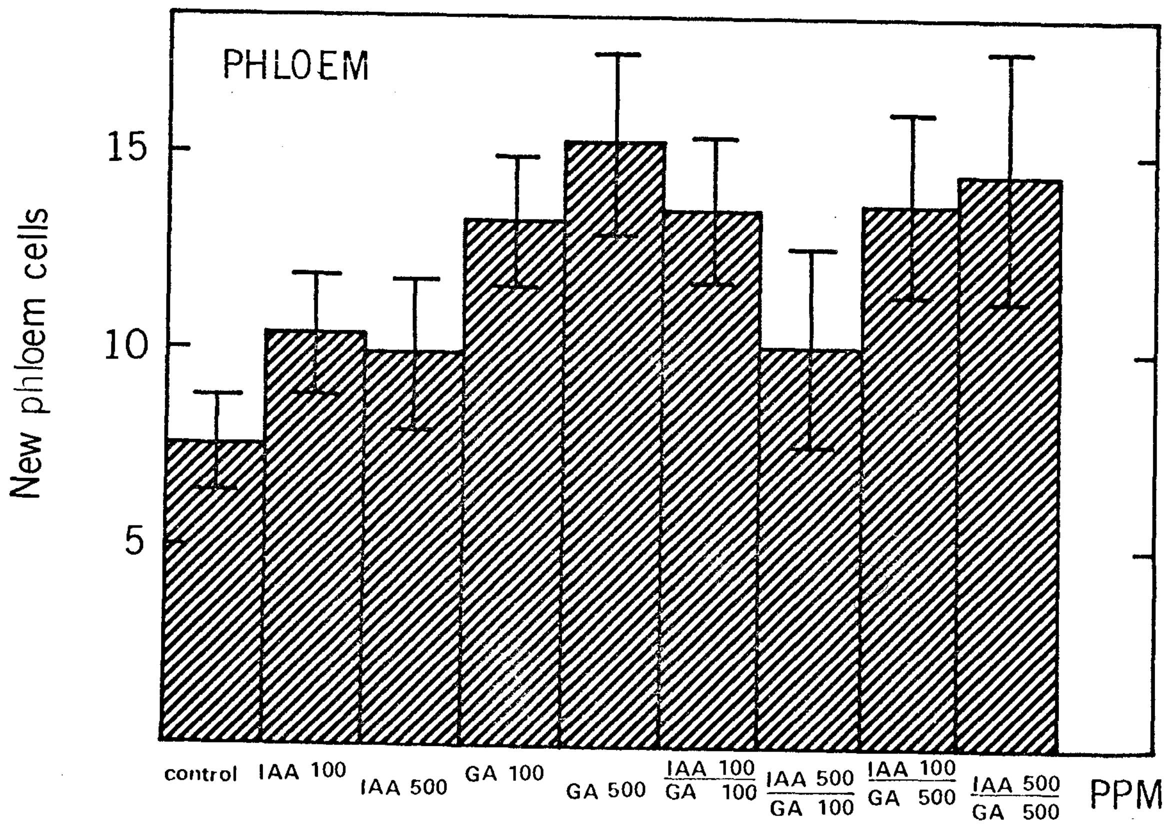
* Average of 50 radial tiers.

** Differences from the control.

Fig. 2. New xylem production under different hormonal treatments shown in bar histogram. Vertical bar represents twice the S.D.

Fig. 3. New phloem cells produced under different hormonal treatments shown in bar histogram.

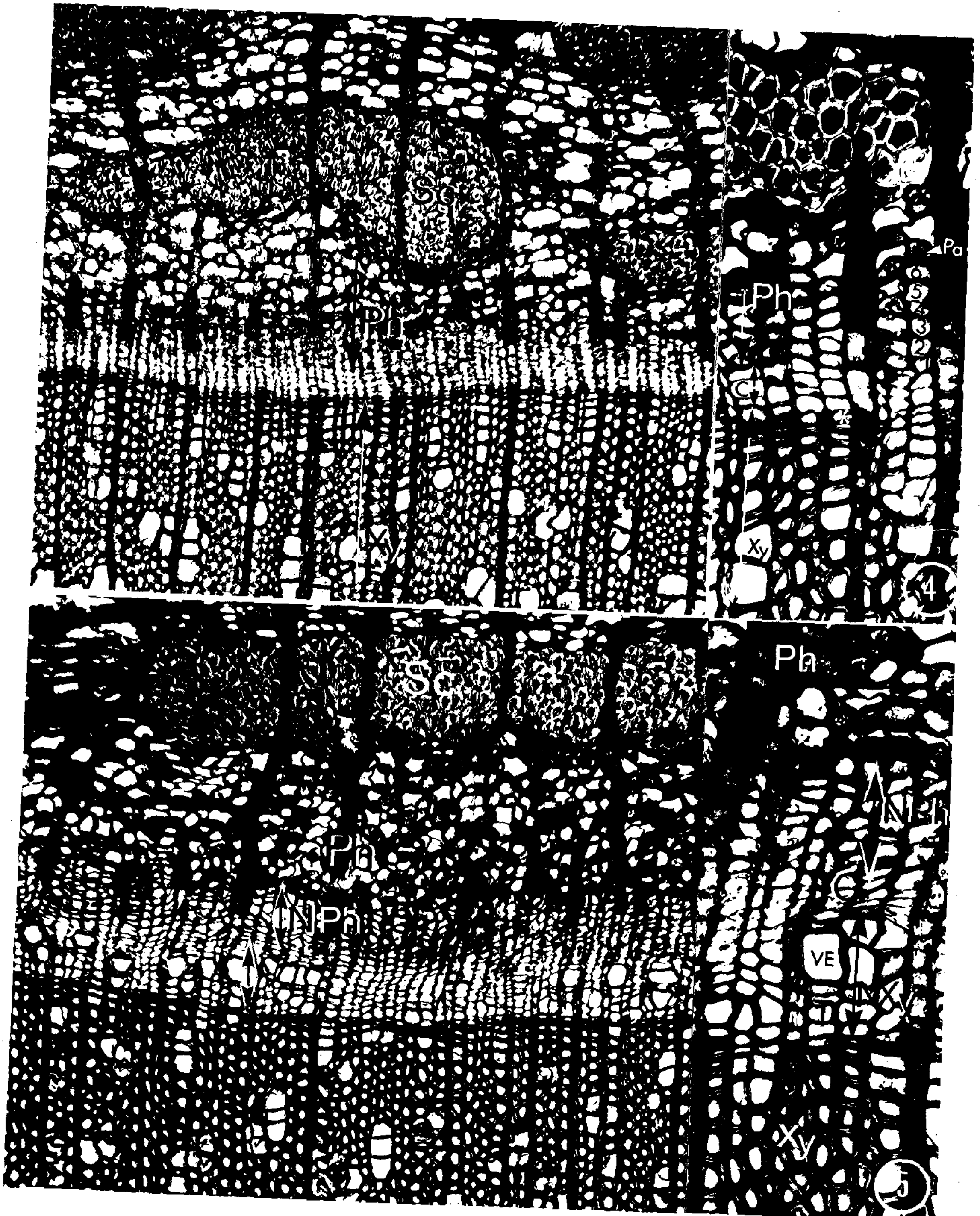

XYLEM
New Xylem cells
15
10
5
control
IAA 100
IAA 500
GA 100
GA 500
IAA 100 / GA 100
IAA 500 / GA 100
IAA 100 / GA 500
IAA 500 / GA 500
PPM


PHLOEM
New phloem cells
15
10
5
control
IAA 100
IAA 500
GA 100
GA 500
IAA 100 / GA 100
IAA 500 / GA 100
IAA 100 / GA 500
IAA 500 / GA 500
PPM

Figs. 4 - 12 are cross sections of poplar with different hormonal treatments at 120X and inserts at 300X.

Fig. 4. Control sample showing three layers of cambial cells which seem to be activated and have thin cell walls. Sometimes a xylem parenchyma cell (*) was produced. The numbers indicate how the measurement of phloem cells was done. Phloem parenchyma cell (pa, arrow) of the previous year was used as a marker in counting.

Fig. 5. IAA 100 ppm treated sample showing the new xylem tissue with groups of enlarged and lignified vessel elements and tracheids intervening with parenchyma cells.


Sc
Ph
Xy
Pa
Ph
C
Xy
4
Sc
Ph
NPh
Ph
NPh
C
VE
Xy
5

4-1-2. Xylem production.

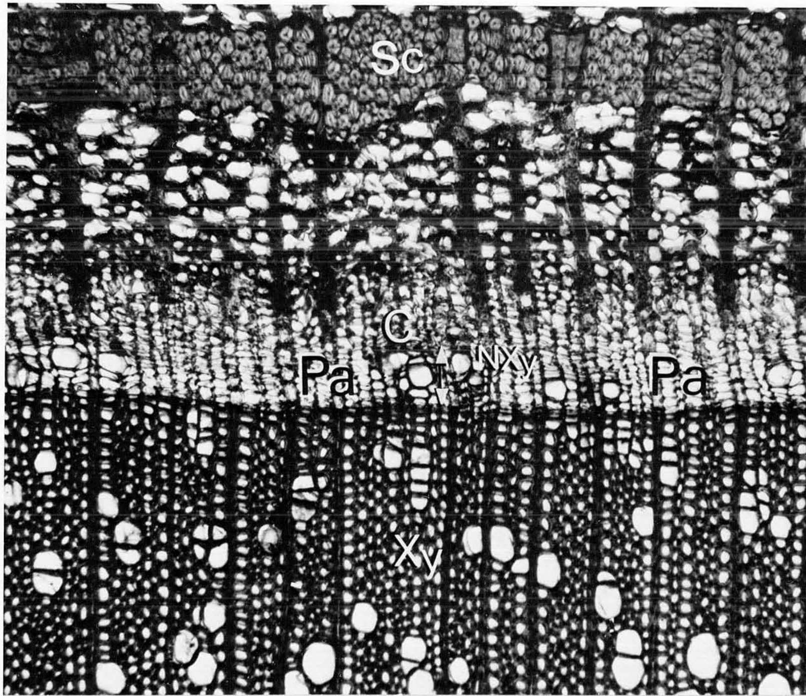
Application of IAA promoted xylem production (Table 1 and Fig. 2), but increasing the concentration of IAA from 100 ppm to 500 ppm did not seem to increase xylem production. The new xylem produced with IAA alone was made up of groups of differentiated vessel elements with some tracheid-like cells surrounding them and undifferentiated parenchyma cells in between (Figs. 5, 6). Vessel elements were often the first formed cells of a radial tier though sometimes they developed after a few small parenchyma cells and tracheid-like cells. As in the older wood, the differentiated vessel elements had enlarged diameter and had lignified walls, which showed positive phloroglucinol/HCl test, with bordered pits. Some cells surrounding the vessel element groups indicated a positive lignin content in their walls also. However, no detectable differences in lignin content of these cells, or in X-sectional diameter and angularity of vessel elements were noted with increased concentration of IAA (Table 2).

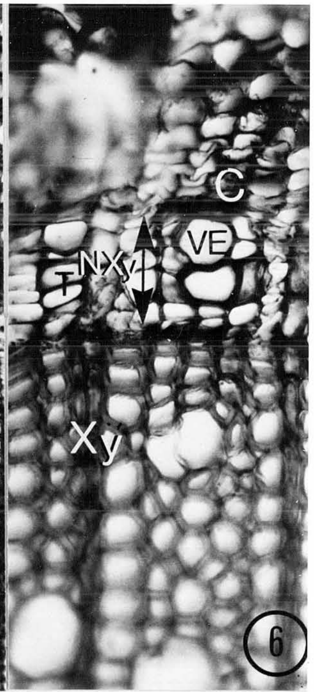
When GA alone was applied, some cells were produced on the xylem side (Fig. 2) but nearly all of them were undifferentiated and parenchymatous (Figs. 7, 8).

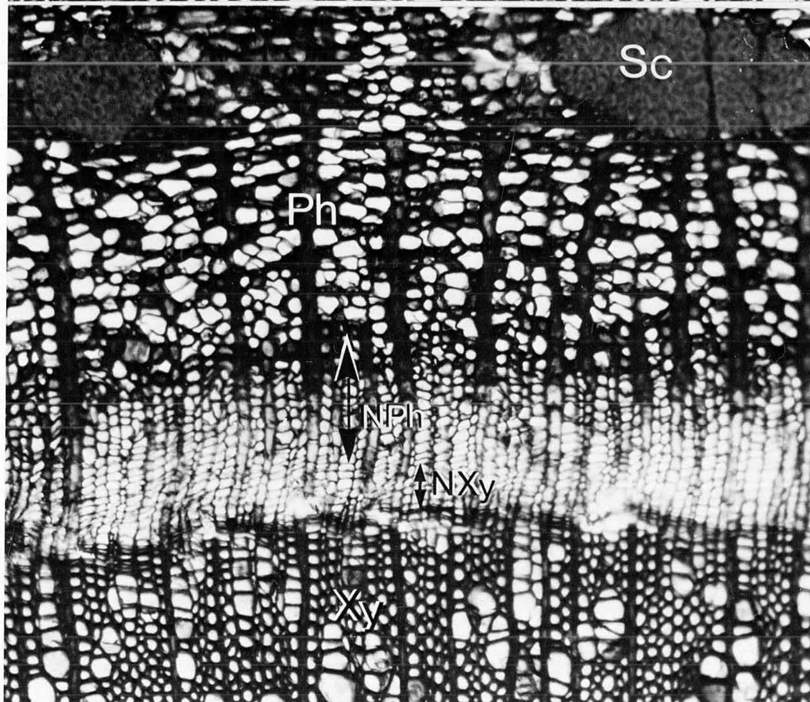
When IAA and GA were applied together, a higher production of xylem tissues was detected than with IAA alone. Maximum production of xylem occurred at IAA 100/GA 100 ppm

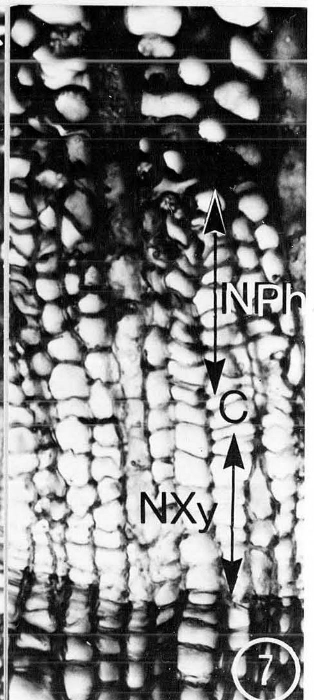
Fig. 6. IAA 500 ppm treated sample showing groups of vessel elements, similar to those in IAA 100 ppm treatment with intervening parenchyma cells.

Fig. 7. GA 100 ppm treated sample showing undifferentiated new xylem tissue and new phloem.


Sc
C
Pa
T
NXy
Pa
Xy


C
VE
T
NXy
Xy
6


Sc
Ph
NPh
NXy
Xy


NPh
C
NXy
7

and IAA 500/GA 500 ppm; it was slightly lower at IAA 500/ GA 100 ppm, but decreased sharply at IAA 100/GA 500 ppm (Table 1 and Fig. 2, see also Figs. 9 - 12). More vessel elements were produced per unit area at IAA/GA concentration of 100/100, 500/100 and 500/500 ppm than IAA alone and at concentration of IAA 100/GA 500 ppm (Table 2). There was no detectable difference in lignin staining in the newly formed vessel elements between different treatments. Cells of smaller diameter, surrounding the vessel element groups in IAA/GA treatments at 100/0, 500/0, 100/100, 500/100, 100/500 and 500/500 ppm were deeply stained with phloroglucinol-HCl. These cells may be tracheids. However, undifferentiated parenchyma cells were found between groups of lignified vessel elements in all the above treatments. This was especially true in IAA 500/GA 500 ppm treatment, where large numbers of undifferentiated parenchyma cells were produced before the differentiation of new lignified vessel elements and tracheids (Fig. 12). After an examination of 50 vessel elements for each treatment, no appreciable difference in the number of sides of vessel elements was detected between different treatments (Table 2).

Fig. 8. GA 500 ppm treated sample. No vessel element was produced although some of the new xylem cells were lignified. Companion cell (arrow head) and sieve plates (arrow heads in small insert) can be seen in the newly formed phloem cells. Section of small insert had been stained by lacmoid.

Fig. 9. IAA 100 ppm and GA 100 ppm treated sample. In comparison to IAA treated material, a wider zone of new xylem tissue was produced and a greater number of vessel elements differentiated per unit area.

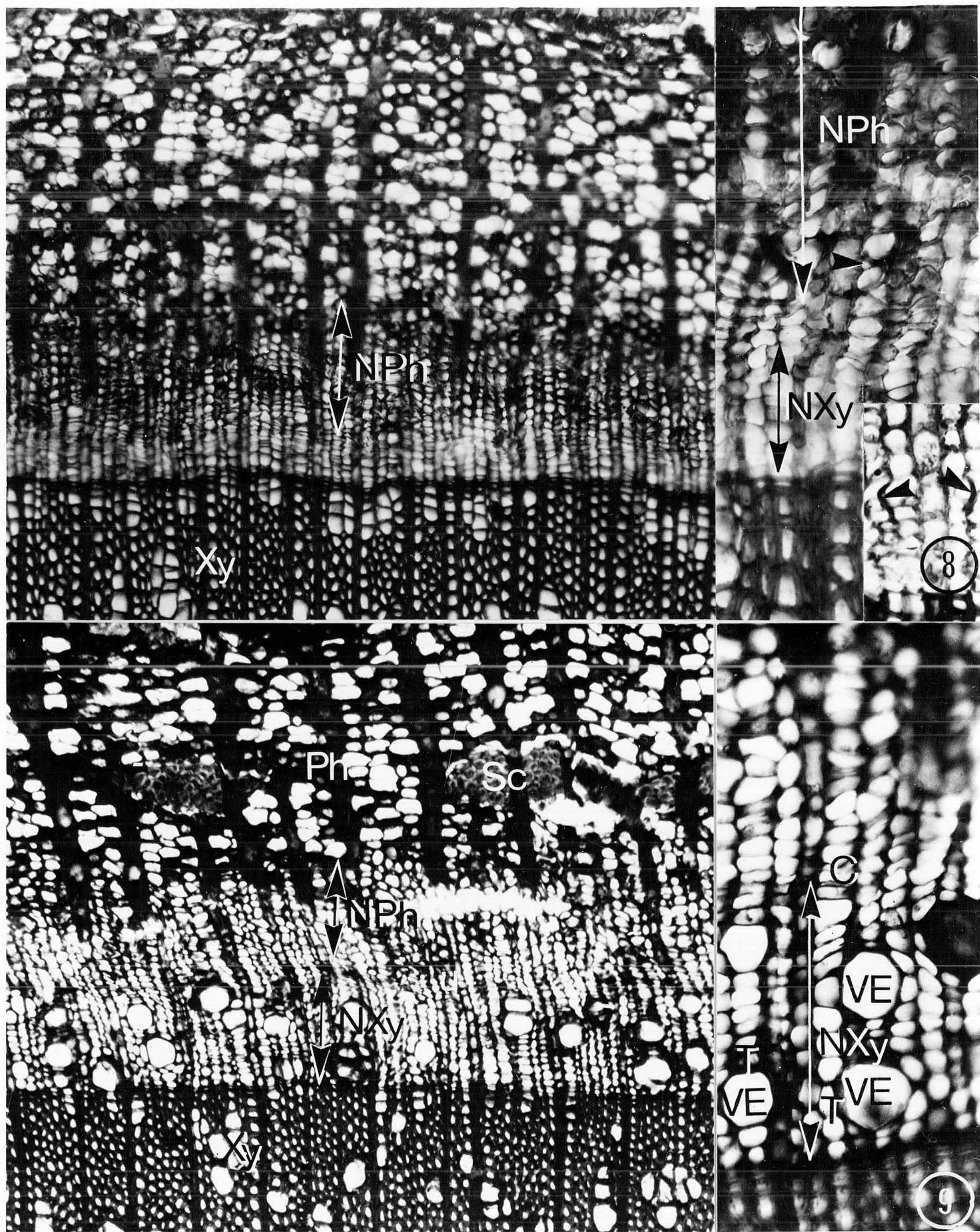

NPh
NPh
NXy
Xy
8
Ph
Sc
NPh
NXy
Xy
C
VE
NXy
T
VE
T
VE
9

Table 2. Number of vessel elements produced per unit area and number of sides in x.s. in differentiated vessel elements under different hormonal treatments.

Treatments	No. of vessel elements/mm^2	No. of sides
Control	0.62	4.28
IAA 100 ppm	27.37	5.70
IAA 500 ppm	22.75	6.32
GA 100 ppm	0.88	5.24
GA 500 ppm	2.25	5.22
IAA 100 ppm GA 100 ppm	36.37	7.56
IAA 500 ppm GA 100 ppm	36.00	7.44
IAA 100 ppm GA 500 ppm	21.10	5.46
IAA 500 ppm GA 500 ppm	34.50	6.58

Fig. 10. IAA 500 ppm and GA 100 ppm treated sample. Vessel elements do not seem to have increased in number nor in sides than in the IAA 100 ppm / GA 100 ppm treatment.

Fig. 11. IAA 100 ppm and GA 500 ppm treated sample shows a lesser production of vessel elements than in Figs. 9, 10. Bordered pits in vessel elements can be seen at the arrow head.

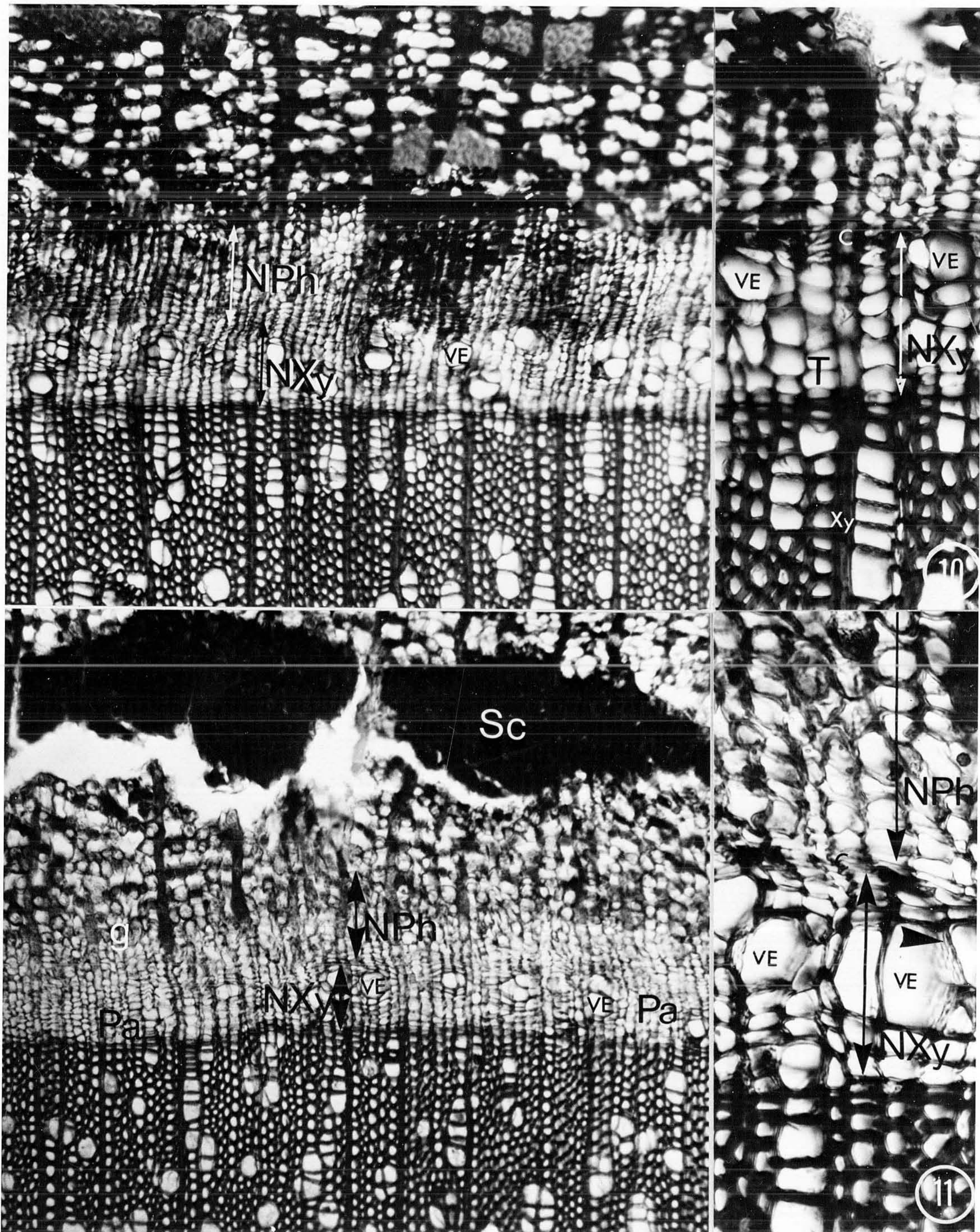
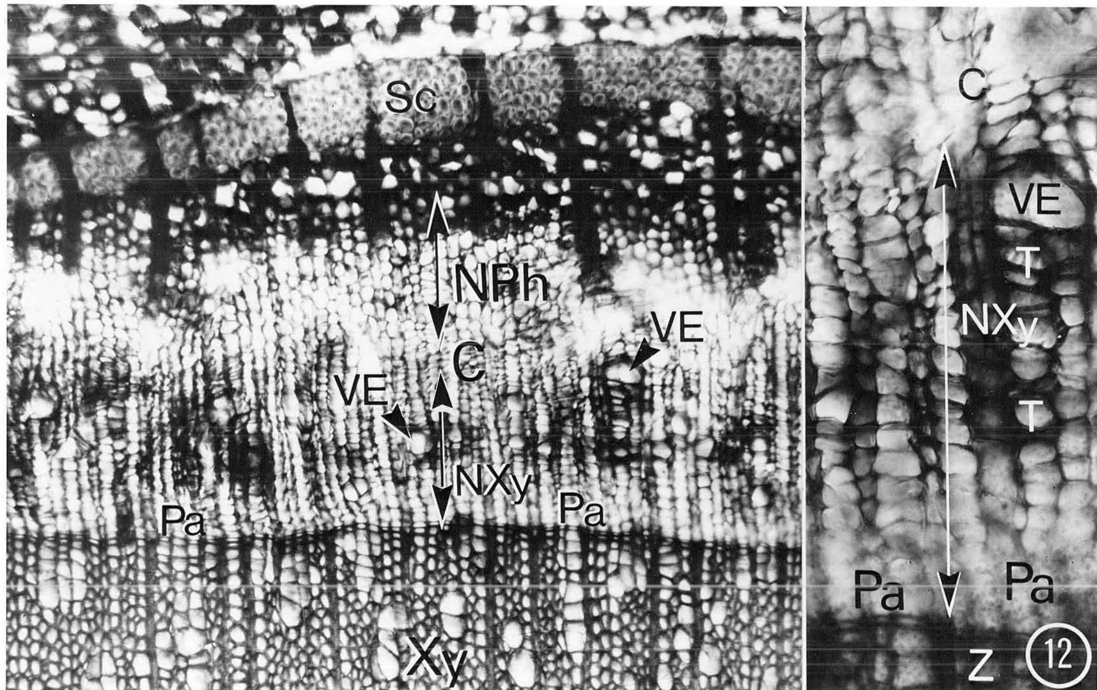

NPh
NXy
VE
C
VE
VE
T
NXy
Xy
10
Sc
NPh
g
NPh
NXy
VE
VE
Pa
Pa
c
VE
VE
NXy
11

Fig. 12. IAA 500 ppm and GA 500 ppm treated sample. Wide zones of new tissues were produced; however, parenchyma cells which were produced firstly toward xylem side stayed undifferentiated


Sc
NPh
C
VE
VE
NXy
Pa
Pa
Xy
C
VE
T
NXy
T
Pa
Pa
Z
12

4-1-3. Phloem production.

As is well known (see reviews by Esau, 1965; Srivastava, 1964), in many trees, phloem cells produced late in the growth season pass the winter months in a partially differentiated state and complete their differentiation early the next year. In Figure 4, these cells from the previous year's growth are numbered and served as a convenient marker for counting cells in radial tiers. In calculations of new phloem production under different hormonal treatments in Table 1, the number of these cells in the control tissue was subtracted from the total number in different treatments.

IAA alone produced some phloem cells but GA had a greater effect on phloem production (Figs. 3, 7, 8). An increase in concentration of GA to 500 ppm produced the maximum amount of phloem. Applications of IAA and GA in combination did not increase the phloem production any further than GA at 500 ppm. GA 500 ppm (Fig. 8), IAA 100/GA 500 ppm (Fig. 11), IAA 500/GA 500 ppm (Fig. 12) produced 6-7 layers of phloem cells, which was higher than the amounts of phloem produced by IAA 500/GA 100 ppm (Fig. 10). This indicated that GA was the main promotor for phloem production.

Some of the enlarged phloem cells further enlarged and differentiated into sieve elements with positive lacmoid

staining in sieve plates; at these locations companion cells were also present (Fig. 8).

4-2. Changes in nuclei of the differentiating xylem vessel elements.

The cytological changes during differentiation of xylem vessel elements in corn leaf have been described recently by Srivastava and Singh (1972) and are summarized below for orientation. According to these authors, the vessel elements pass through three distinct though continuous stages: 1. cell enlargement associated with cytoplasmic synthesis; 2. deposition of secondary wall in precise patterns and its lignification; and 3. autolysis of the protoplast and those parts of the primary wall including end wall, which are not overlaid by the secondary wall. These stages are characterized by marked changes in the amount (or number) and distribution of various organelles and membrane systems. In the following treatment, the structural and chemical changes in the nucleus are described with reference to the above stages but with this modification. Procambial cells are referred to as stage A; expanded vessel elements without secondary walls are referred to as Stage B; and vessel elements with secondary walls but end walls still intact are referred to as stage C. Under the light microscope it is nearly impossible to distinguish

between the vessel elements which are in late stage 2 and early stage 3 of Srivastava and Singh (1972). Consequently, in cytophotometric measurements, my stage C includes nuclei which belong to stage 2 and early stage 3 of Srivastava and Singh. It is assumed that after stage C nuclei are hydrolysed.

4-2-1. Structural changes in nuclei and nucleoli.

In the procambial cells, the nucleus is elongated and more or less cylindrical (Figs. 13, 19). Most of the chromatin is condensed into large rather irregular patches, which seem to be interconnected with each other (Figs. 20, 21). These patches lie in the nucleoplasm and against the inner membrane of the nuclear envelope but not against the pores. They also show a frequent and unmistakable association with the nucleolus, sometimes surrounding and being connected to it (Figs. 21, 22). Perichromatinic granules about 400-500 Å in diameter are present in the dense chromatin masses and are often surrounded by a light zone or halo from which dense chromatin is excluded (Figs. 20, 21).

The nucleolus is relatively small, composed mostly of densely packed fibrils and some granules on its outer margin. There is no distinct vacuolar region (Figs. 20,22). The lighter zone or halo around the nucleolus appears as an area of reduced density of fibrils and granules which in size and structure are similar to those in the nucleolus (Figs. 20, 22).

Fig. 13 - 39 are paradermal sections of corn leaf in the region of vessel element differentiation.

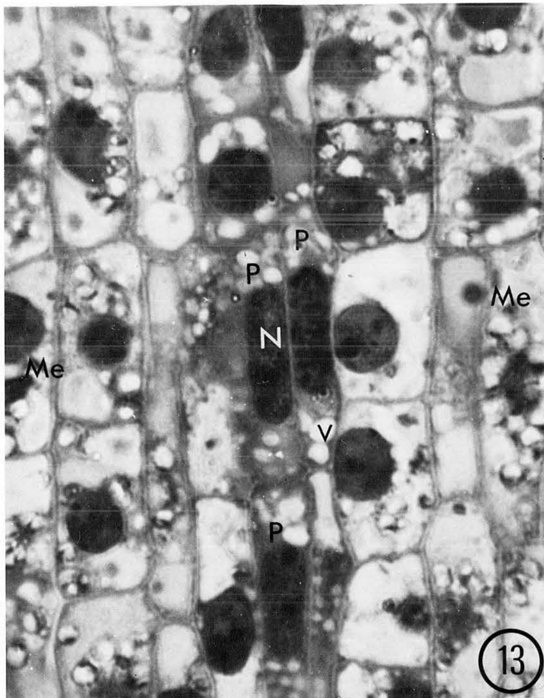
Fig. 13 - 18 are at 1200X.

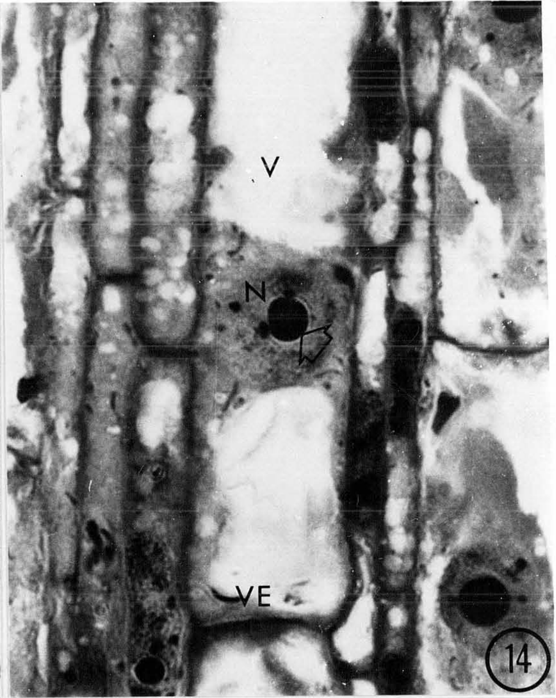
Fig. 13. Procambial cells (stage A) are narrow and elongated with thin walls. They have dense cytoplasm and small vacuoles. Nuclei are more or less cylindrical and contain dense chromatin masses.

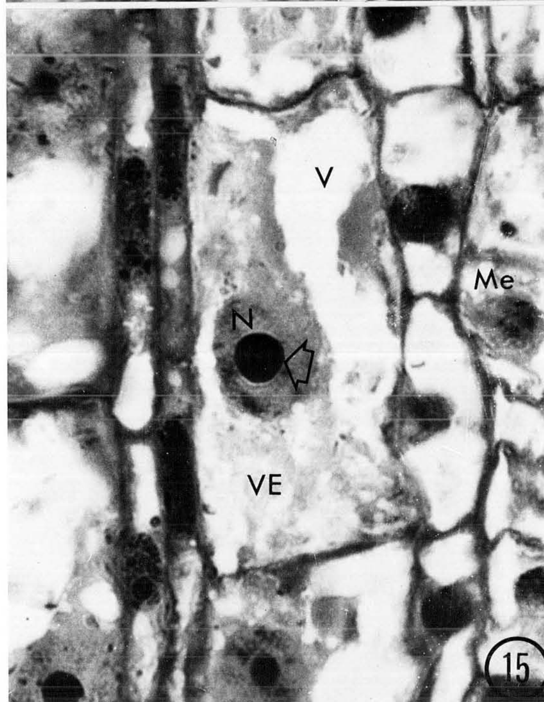
Fig. 14. The nucleus and nucleolus (arrow) of a young vessel element in stage B have increased to about twice their size in the procambial cells. Dense chromatin masses seem to have reduced in size and number. The vessel elements in this stage contain large vacuoles.

Fig. 15. Vessel element at a slightly older stage than in Fig. 14, but still in stage B. Nucleolus is much swollen.

Fig. 16. Vessel element at stage C with secondary wall deposited and end walls intact. Nucleus becomes lobed. Large clumps of chromatin (arrow) can be seen in the nucleoplasm.


P
P
N
Me
Me
V
P
13


V
N
VE
14


V
Me
N
VE
15

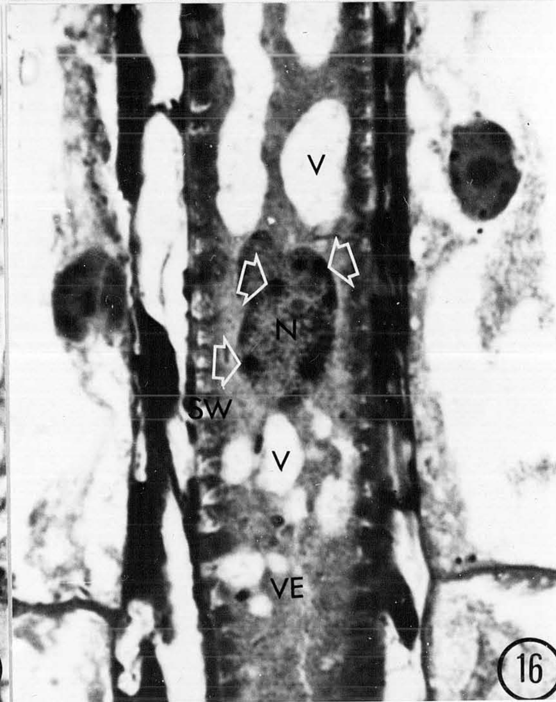

V
N
SW
V
VE
16

Fig. 17. A vessel element at stage C, slightly older than in Fig. 16. The nucleus is lobed and the nucleolus (arrow) is reduced to about the same size as in the procambial cells.

Fig. 18. A vessel element at much later stage C. Nearly all the cell contents are gone except the nucleus and nucleolus (arrow).

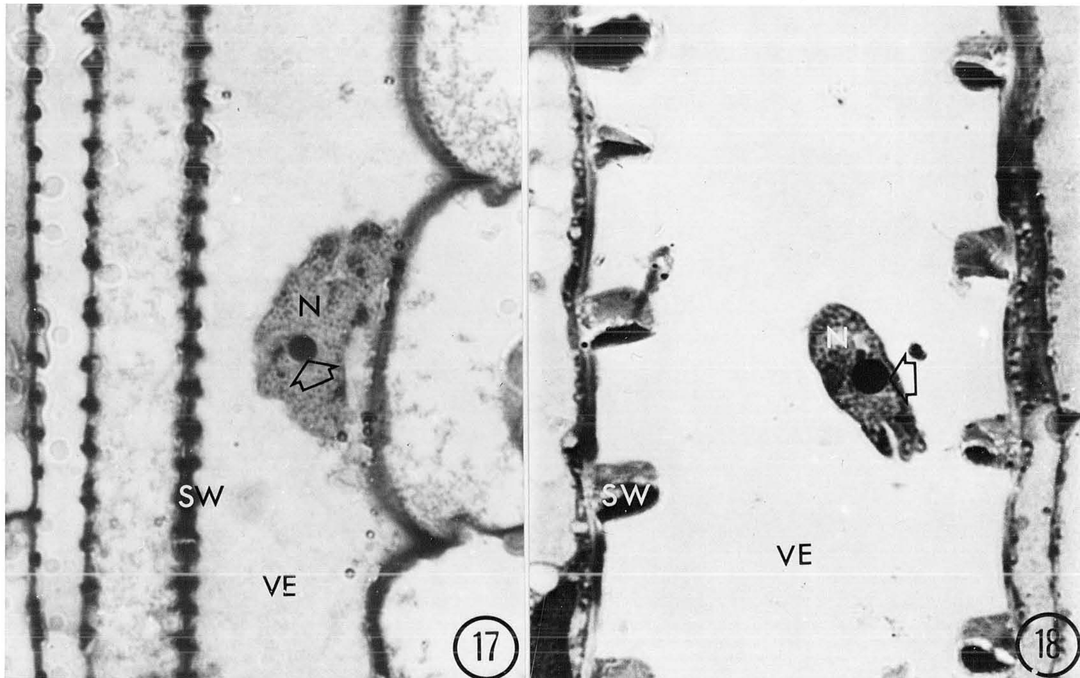

N
SW
VE
17
N
SW
VE
18

Fig. 19. Parts of two procambial cells (stage A) with their nuclei and nucleoli. The cells have thin walls, small vacuoles, abundant mitochondria, rough endoplasmic reticulum, ribosomes, and plastids. The nuclei are cylindrical. 6,500X.

Fig. 20. Part of a nucleus of procambial cell (stage A). Nucleolar halo is seen clearly at *. Chromatin is condensed into large irregular patches which seem to be interconnected. Perichromatinic granule can be seen at arrow. Nucleolus is relatively small, and compact. Some granules are seen at its outer margin, but no distinct vacuolar region can be found. 26,000X.

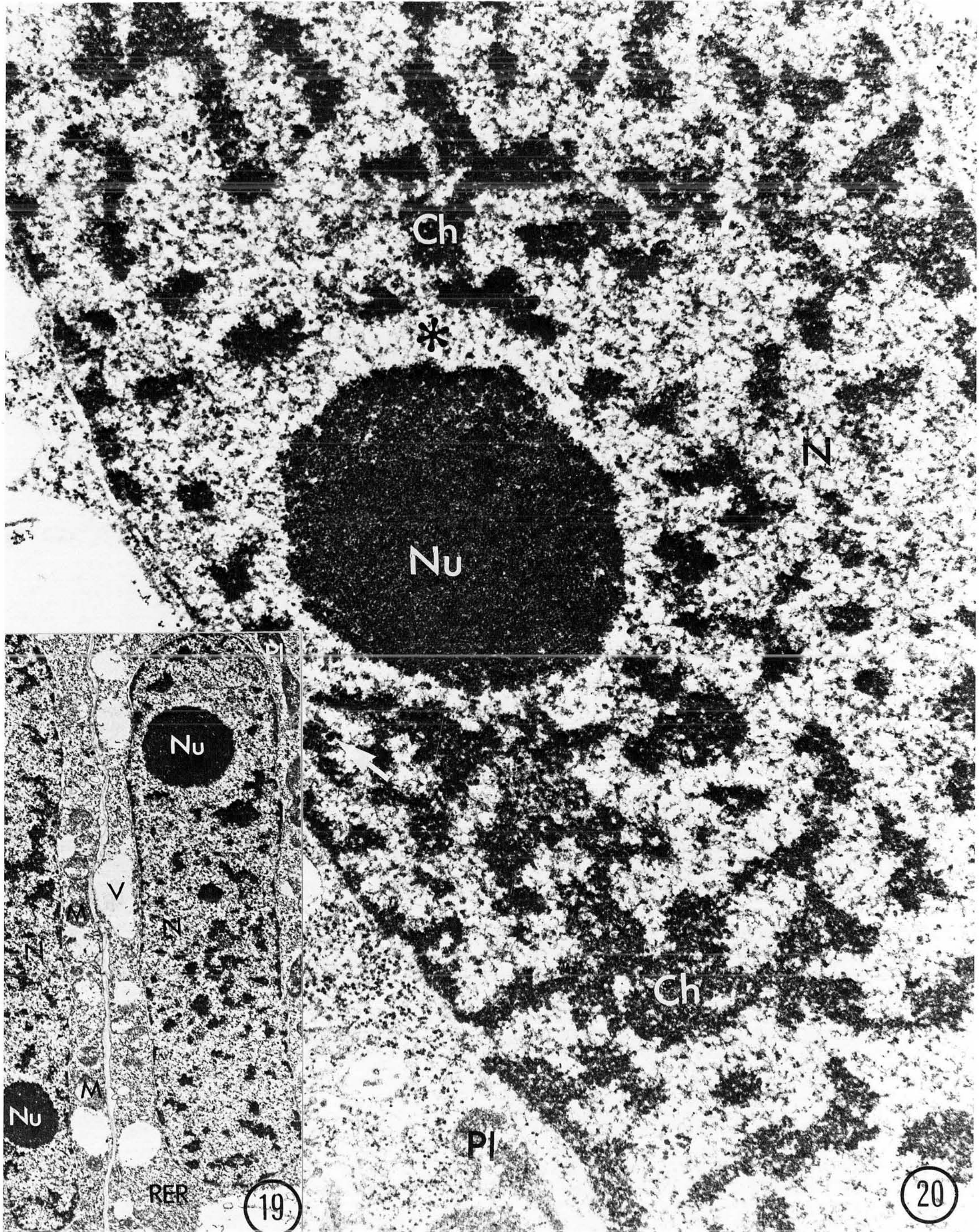
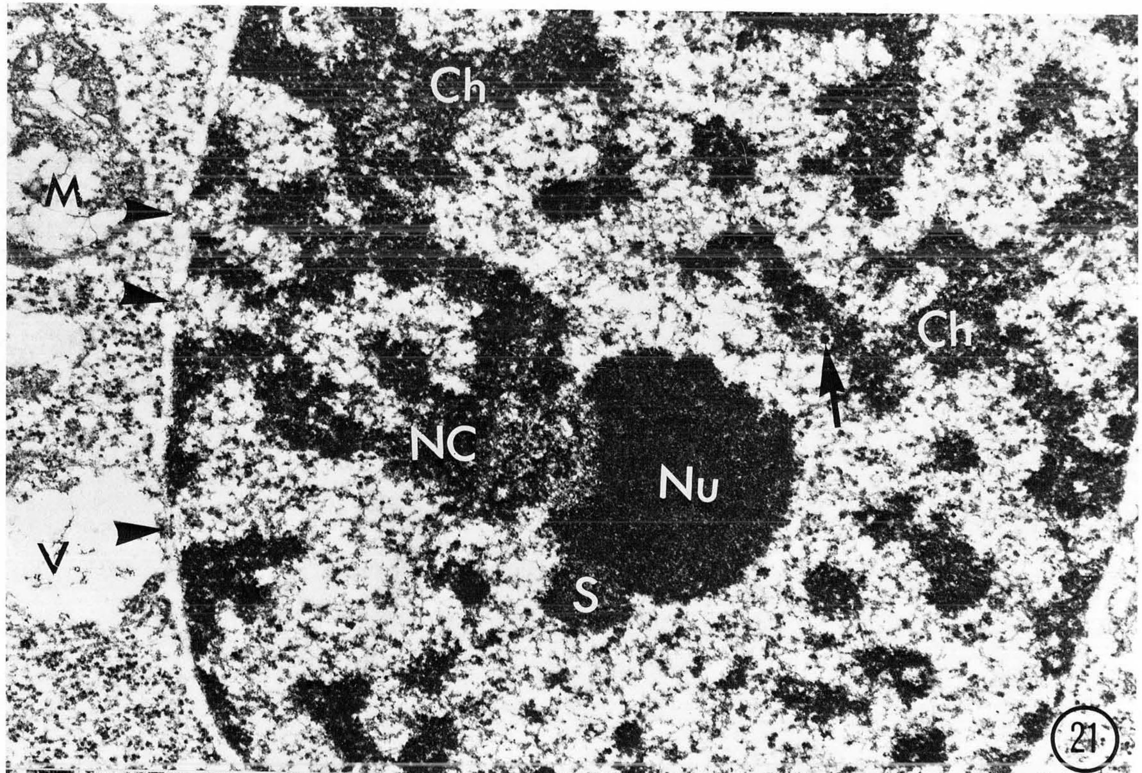
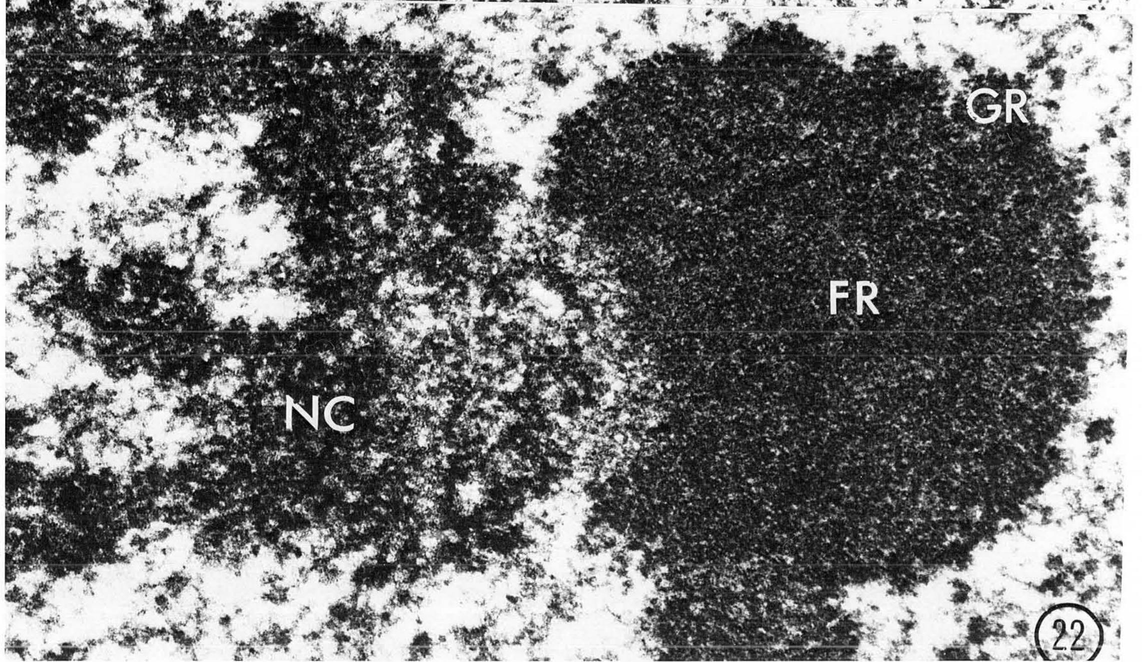

Ch
*
N
Nu
Ch
Pl
20
Pl
Nu
V
M
N
N
M
Nu
RER
19

Fig. 21. Dense chromatin masses in a procambial nucleus (stage A) are interconnected, lie against the nuclear membrane except around the nuclear pores (arrow heads), and are often associated with the nucleolus. Perichromatinic granule is indicated by arrow. A spherule on the surface of the nucleolus is present. 26,000X.

Fig. 22. Higher magnification of nucleolus of Fig. 21 showing the central portion of fibrillar region and a few granular regions at the periphery. The nucleolus-associated chromatin is connected to the nucleolus through a restricted area. 60,000X.


Ch
M
NC
Nu
Ch
V
S
21

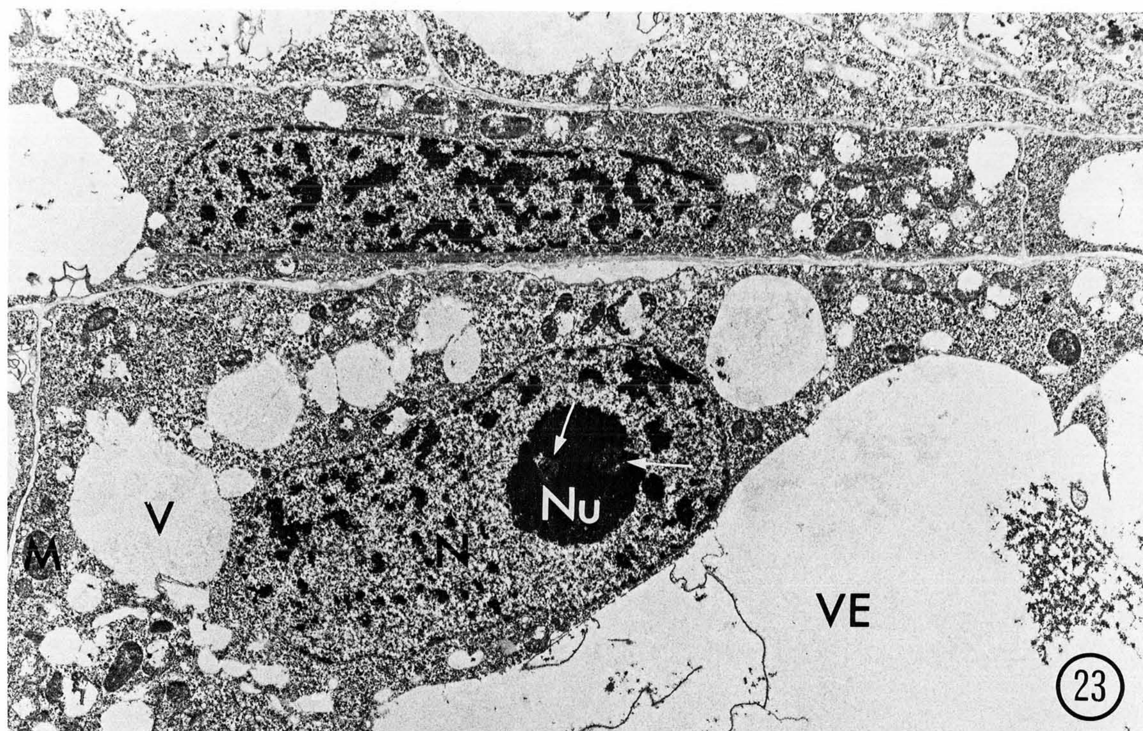

GR
FR
NC
22

In stage B, there is a dramatic increase in nuclear volume. The nuclei are at least 2-3 times as large as those in the procambial cells (Figs. 14, 15). At the same time, the large irregular patches of chromatin are replaced by smaller clumps which are rather evenly dispersed in the nucleoplasm (Figs. 23, 24). Perichromatinic granules are seen more frequently than in the procambial cell stage (cf. Figs. 20, 21 with 26, 27). Some of them are surrounded by dense chromatinic masses whereas others are in dispersed chromatin masses (Figs. 26, 27). Large granules ranging from 1000-5000 Å in diameter occur in the nucleoplasm (Fig. 28). They are irregular in shape and highly electron dense with no substructure.

The changes in nucleolus are very marked. The nucleolus increases in volume, becoming 2-3 times as large as in the procambial cells (Figs. 29, 30, 31). Nucleolar halo is present (Figs. 30, 31). The nucleoli show distinct granular, fibrillar and vacuolar regions (Figs. 25, 29, 30, 31). The granular regions are composed of granules of 150-200 Å in diameter which are occasionally strung in chains (Fig. 30, insert). They occupy the outer parts of the nucleolus and line the vacuolar regions, and consequently, appear to form a coarse network (Figs. 30, 31). The fibrillar regions are composed of tightly packed fibrils of a smaller diameter than the granules. At first the fibrillar

Fig. 23. Part of a young vessle element at early stage B, shows enlarged vacuoles and cell volume. Nucleus and nucleolus increase to about twice the size as in procambial cells. Dense chromatin masses are reduced in size. Vacuolar regions (arrows) of nucleolus appear and contain some chromatin-like material. 6,500X.

Fig. 24. A nucleus of a young expanded vessel element (stage B) at a slightly older stage than in Fig. 23. Chromatin is more dispersed and the nucleolus-associated chromatin has more or less disappeared. 6,500X.


M
V
N
Nu
VE
23

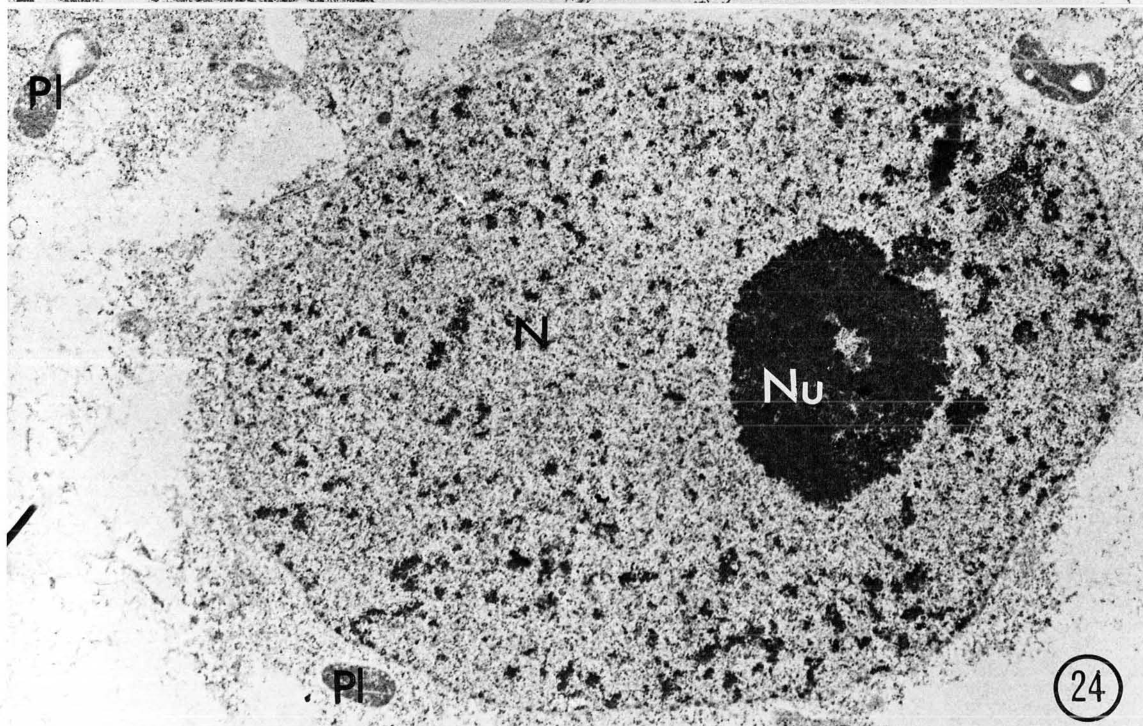

Pl
N
Nu
Pl
24

Fig. 25. The enlarged nucleolus of stage B shows clear zonations -- Granular regions at the margins, and fibrillar region in the center. Vacuolar regions are found connected to the nucleoplasm (black arrow heads). Chromatin like material can be seen inside some of the vacuolar regions (white arrow head). 26,000X. Insert at 1,800X.

Fig. 26. Perichromatinic granules (arrow heads) about 400-500 Å in diameter are present in the dense chromatin masses at all stages but become more common in stage B. They are surrounded by a halo. 60,000X.

Fig. 27. Perichromatinic granules found in dispersed chromatin often occur in clusters in stages B and C. 60,000X.

Fig. 28. Large irregular granules ranging from 1000-5000 Å in diameter are found in stages B and C in the nucleoplasm; they are highly electron dense with no substructure. 60,000X.

Fig. 29. Vacuolar regions in stage B nucleolus are well developed and contain material stained similarly to chromatin (white arrow heads). Granular regions increase very much at the margin. A spherule is indicated. 26,000X.

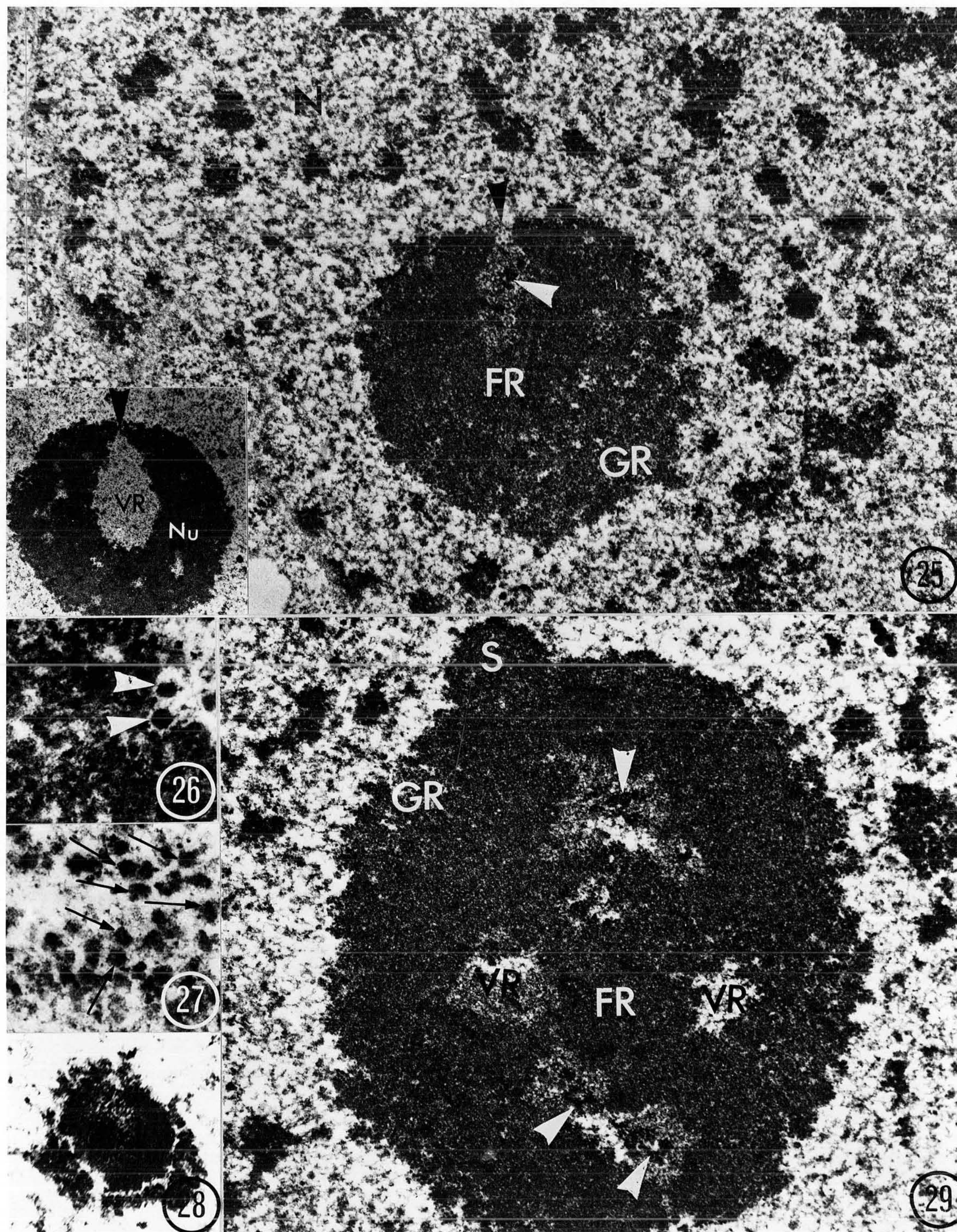

N
FR
GR
VR
Nu
25
S
GR
VR
FR
VR
26
27
28
29

Fig. 30. Nucleolus at stage B shows large vacuolar region which is lined with granular region and is composed of fibrils and granules in low density. Perinucleolar halo is indicated by *. 26,000X.

Higher magnification in the insert shows amorphous and densely packed fibrils of fibrillar region, and granules strung at places in chains of granular regions. 65,000X.

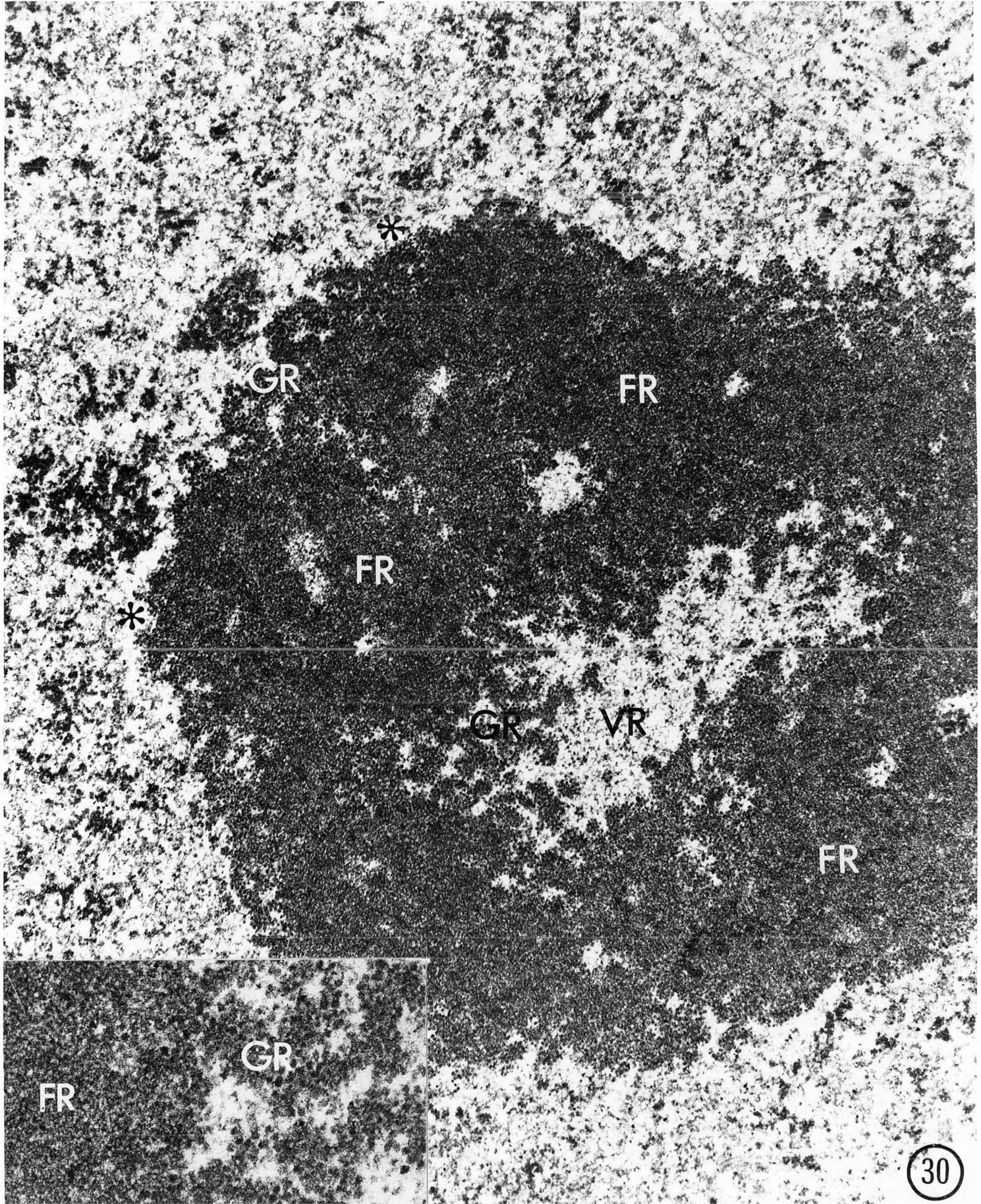
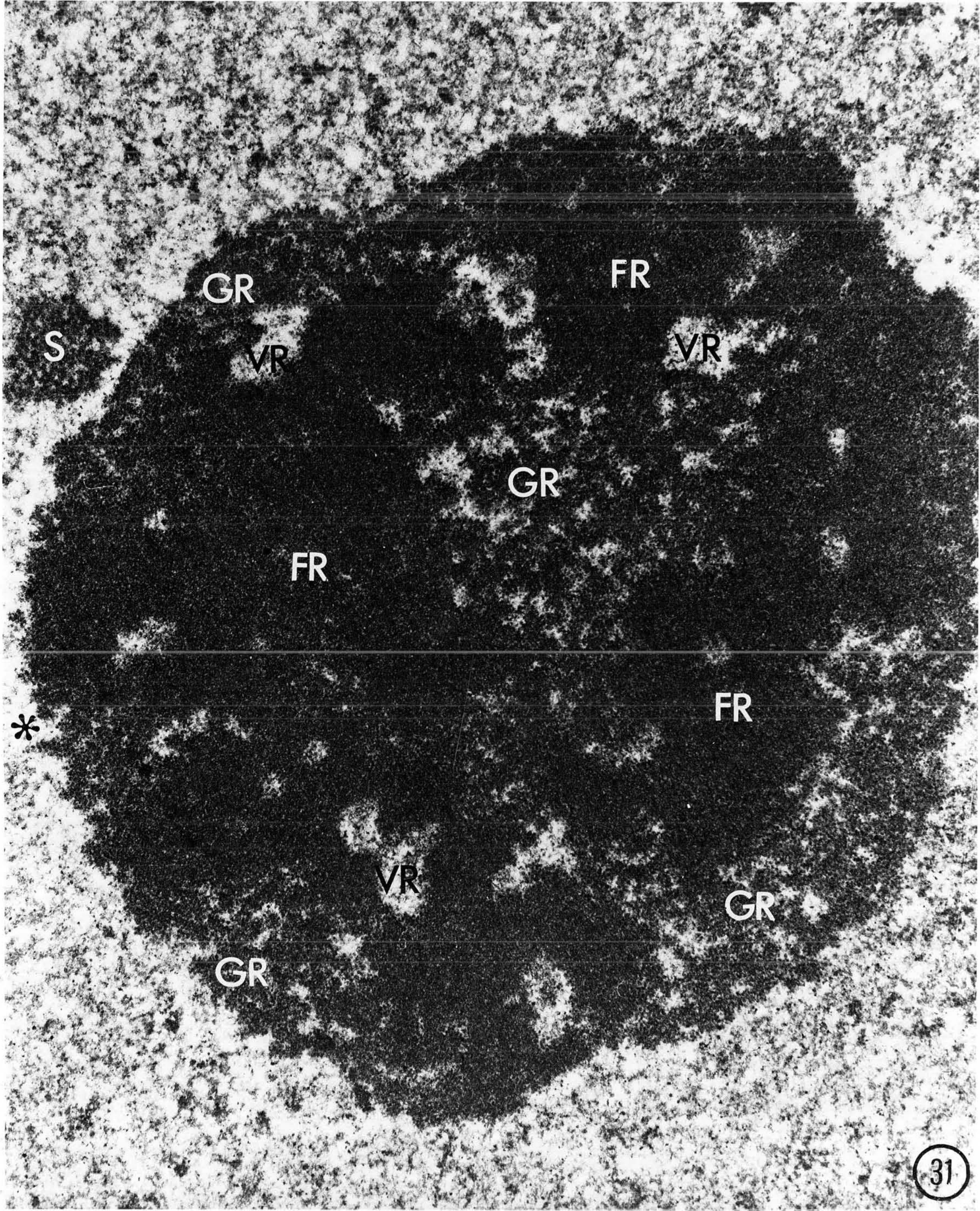

*
GR
FR
FR
*
GR
VR
FR
GR
FR
30

Fig. 31. Nucleolus at late stage B vessel element demonstrates a different arrangement of granular and fibrillar regions. Vacuolar and granular regions are abundant and the fibrillar region is no longer in the center of the nucleolus. Nucleolar halo can be seen at *. 26,000X.


GR
S
VR
FR
VR
GR
FR
FR
*
VR
GR
GR
31

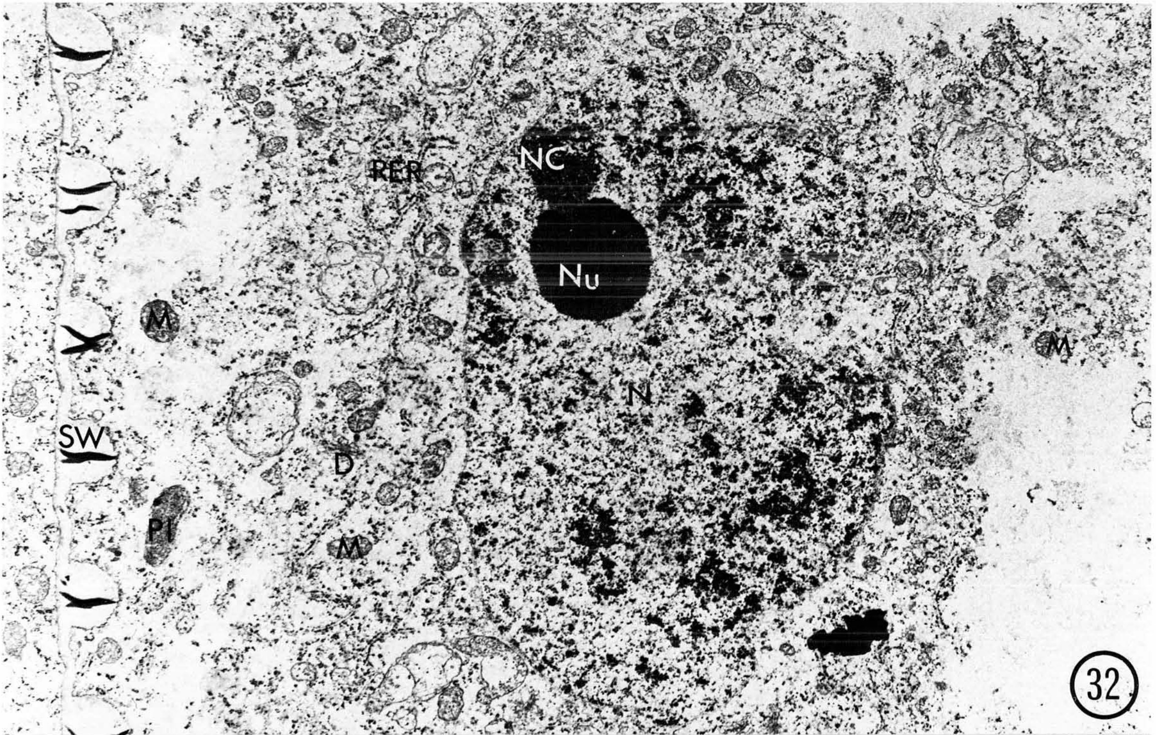
region occupies the more central portion of the nucleolus (Figs. 21, 22) but later with the appearance of vacuolar and granular regions it is broken up into several masses (Figs. 25, 29, 30, 31). The nucleolar vacuoles vary in size and are generally lined by granules, sometimes by fibrils (Figs. 30, 31). These regions are not empty but have a low density of fibrils and granules, and in favourable views are connected to the nucleoplasm (Figs. 25 insert, 30, 31).

The nucleolus-associated chromatin which was typical of stage A disappears in this stage. However, contents of some of the vacuolar regions have electron dense material similar to chromatin (Figs. 25, 29) and it seems as if the nucleolus-associated chromatin is transferred to the inside of the nucleolus at this stage.

During the secondary wall deposition (stage C), the nuclei become much lobed (Fibs. 16, 17, 32, 33) and the outer membrane of the nuclear envelope is often connected to the rough endoplasmic reticulum (Fig. 33). Chromatin aggregates into larger masses, some of which lie against the inner membrane of the nuclear envelope, but its general appearance becomes coarsely granular (Figs. 33-35). At the same time, the nucleoplasm becomes increasingly electron light (Figs. 35, 36, 37). In distinction to the previous stages, large granules ranging from 1000-5000 Å in diameter

Fig. 32. Vessel element at stage C shows secondary wall and lobed nucleus. Shrinkage of nucleolus and reappearance of nucleolus-associated chromatin are characteristic of this stage. Cytoplasmic organelles, like rough endoplasmic reticulum, mitochondria and plastids are present. 6,500X.

Fig. 33. Part of a lobed nucleus at stage C indicates the connections of nuclear envelope and the rough endoplasmic reticulum (arrow heads). Chromatin aggregates into large masses and lies against the envelope. 26,000X.


RER
NC
Nu
M
M
N
SW
D
Pl
M
32

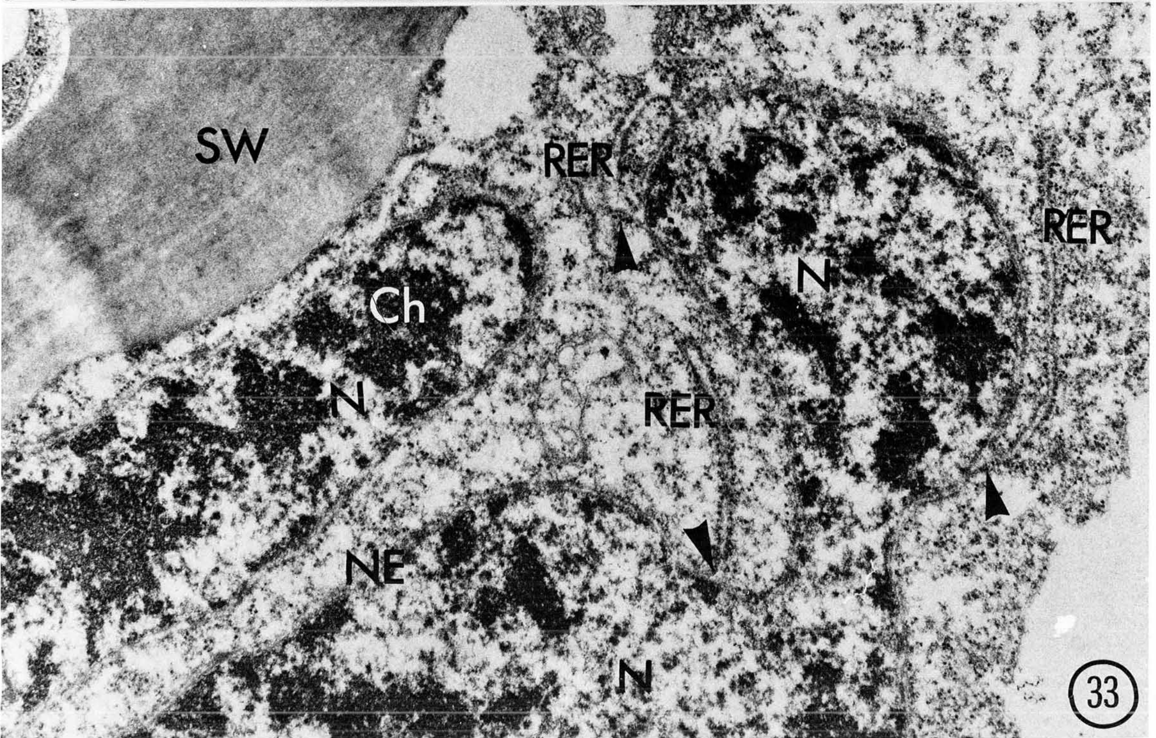
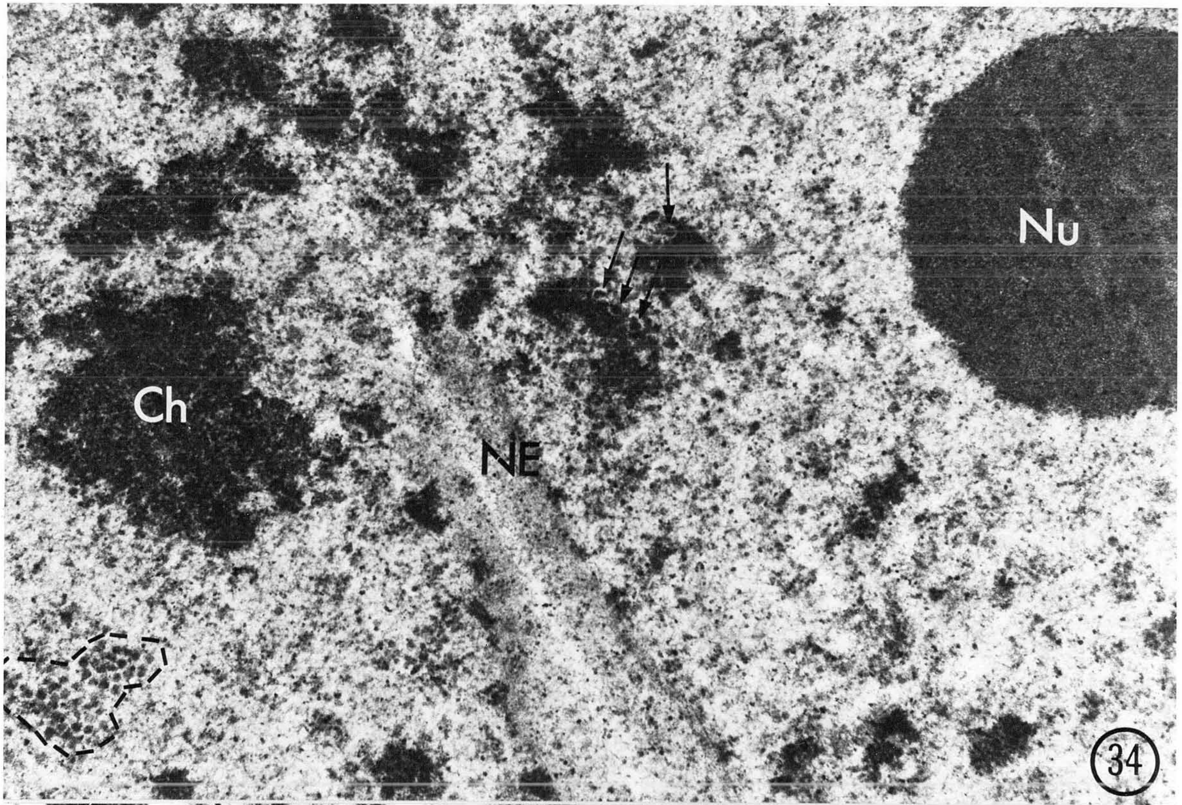
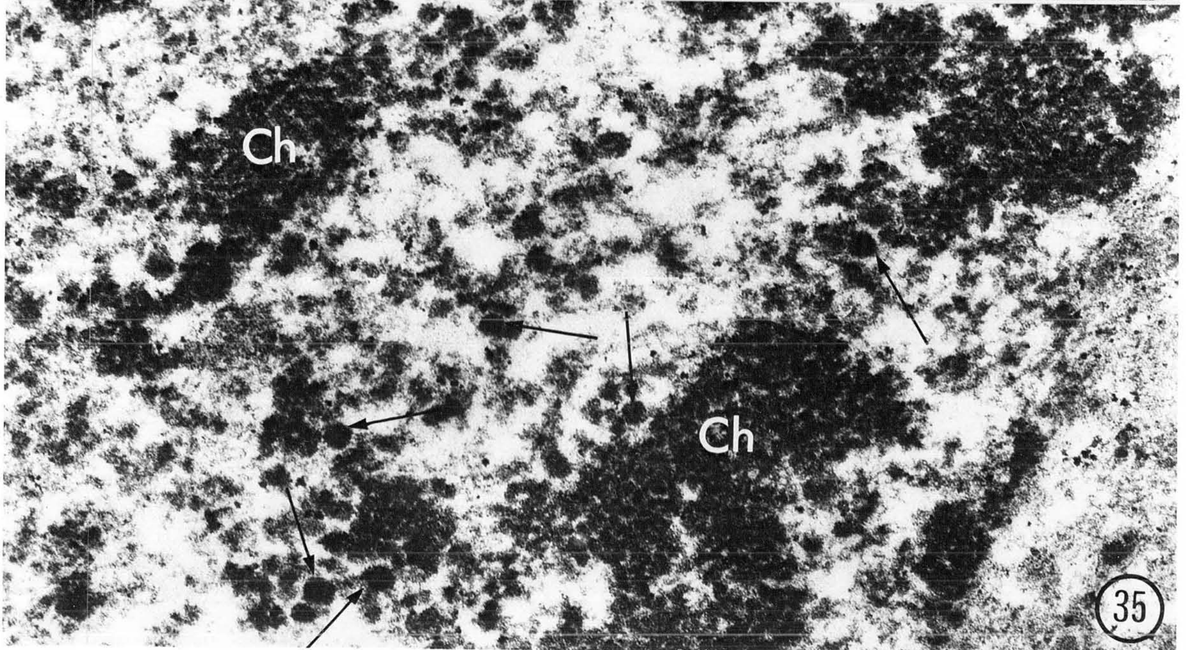

SW
RER
RER
N
Ch
N
RER
NE
N
33

Fig. 34. Nucleolus of stage C vessel element is much reduced in size and compact with a central fibrillar region and a few granules at the edge. Perichromatinic granules in dense chromatin masses (arrows) and in dispersed chromatin (circles by dashed line) are frequent. 26,000X.

Fig. 35. Chromatin in stage C appears very coarse and granular with many perichromatinic granules (arrows). At the same time, the nucleoplasm becomes relatively electron light. 65,000X.


Nu
Ch
NE
34


Ch
Ch
35

increase in number in the nucleoplasm, and are sometimes surrounded by the chromatin and nucleolus-associated chromatin (Figs. 36, 37). Perichromatinic granules (400-500 Å) surrounded by halos persist within the chromatin masses and they also appear in large numbers singly or often in clusters in dispersed chromatin (Figs. 34, 35, 38). The decrease in the electron density of the nucleoplasm and the clumping of chromatin probably represent a loss of the proteinaceous matrix of the nucleoplasm. Associated with the increase in number of large 1000-5000 Å granules and the 400-500 Å perichromatinic granules and the nucleolar changes (see below) they represent a degeneration of nuclear machinery.

Nucleoli are again greatly reduced in size and become very compact (Figs. 34, 36, 37, 38). They show little substructure and appear to be composed mostly of tightly packed fibrils and a few granules around the edge but the large granular regions of stage B are mostly gone (Fig. 38). The vacuolar regions also are greatly reduced in number and the chromatin-like material present inside them in stage B is no longer observed (Fig. 36, 37). However, the nucleolus-associated chromatin reappears in the nucleoplasm and is attached to the surface of nucleolus through a restricted region (Fig. 37).

Fig. 36. Nucleus in stage C vessel element contains electron light nucleoplasm. The reduced nucleolus contains two vacuolar regions which are seldom seen at this stage. A large granule appears inside the dense chromatin (arrow). 26,000X.

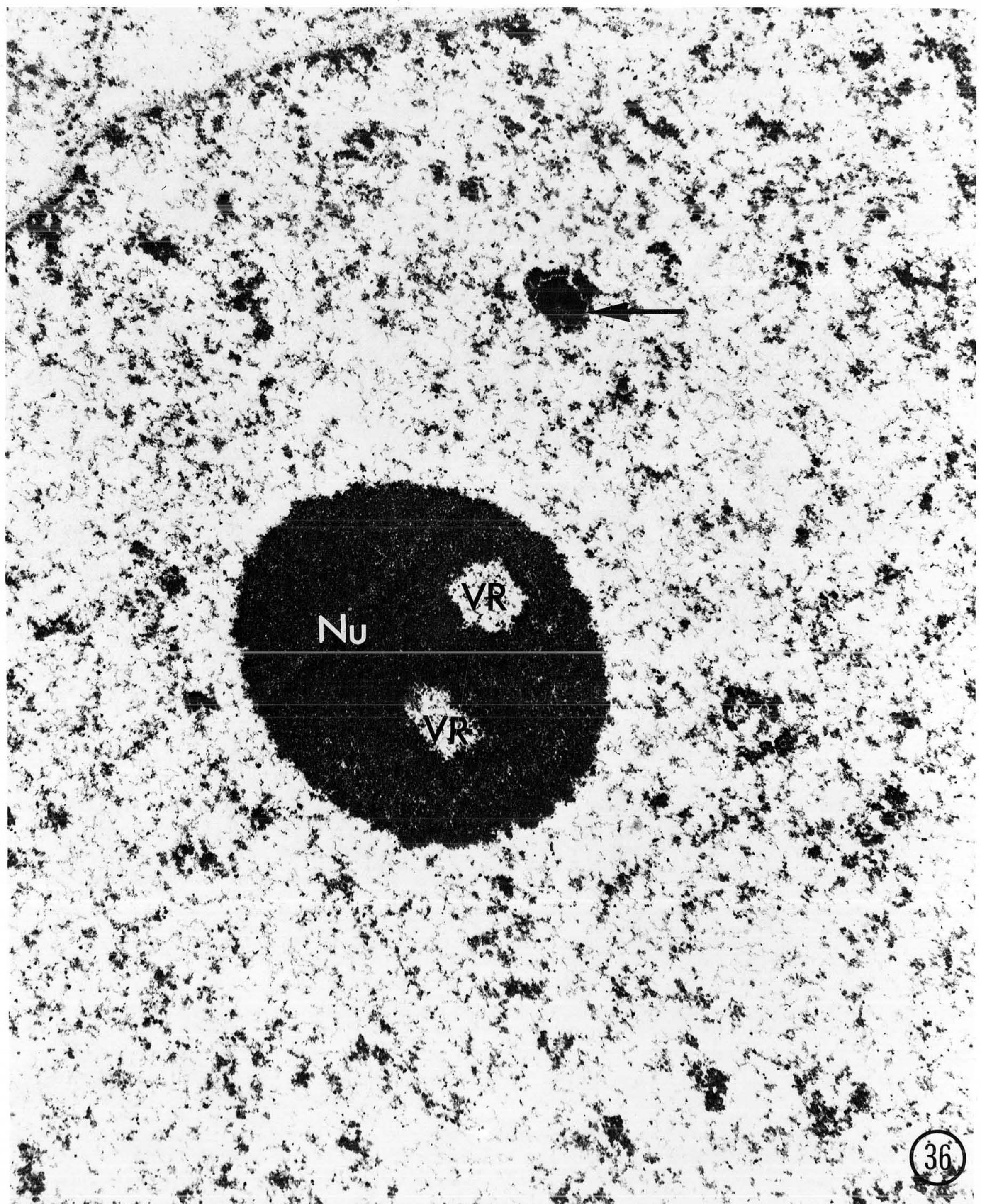
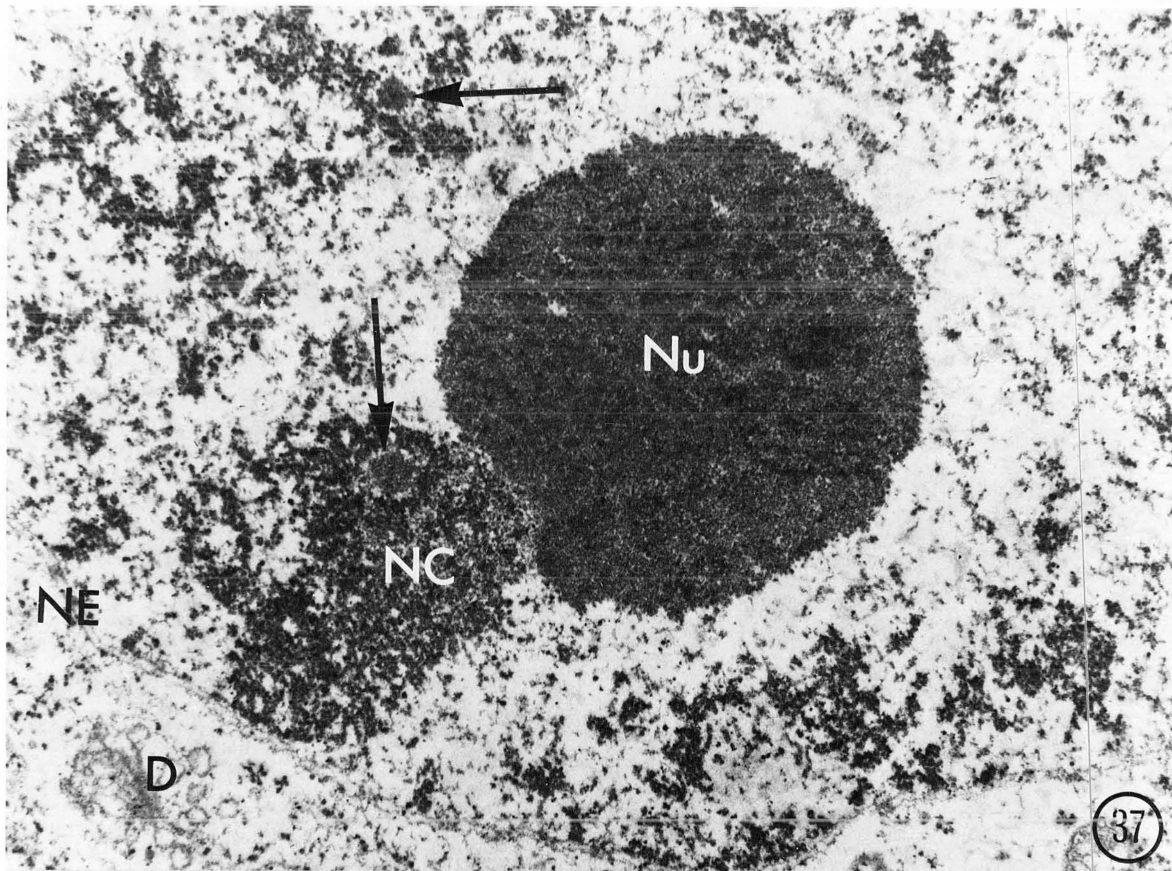
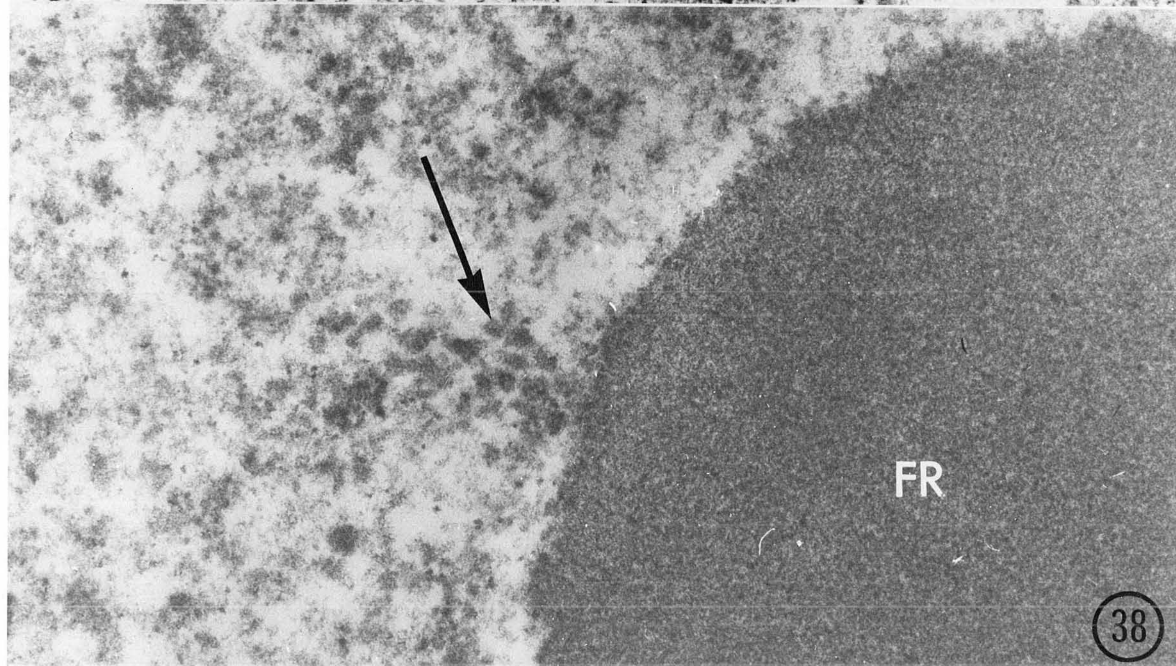

VR
Nu
VR
36

Fig. 37. Nucleolus-associated chromatin reappears in stage C and again is connected to the nucleolus through a restricted area. Large granules occur in the nucleoplasm and nucleolus-associated chromatin (arrows). 26,000X.

Fig. 38. Higher magnification of part of a nucleolus at stage C shows a highly compact nucleolus with densely packed fibrils in the center and a few granules at the edge. Granules in cluster (arrow) similar to perichromatinic granules in Fig. 27 are seen next to the nucleolus. 65,000X.


Nu
NC
NE
D
37


FR
38

In the oldest vessel elements studied here which still had a nucleus (Fig. 18), the nucleus was lobed with the nuclear envelope and pores still mostly intact (Fig. 39). The nucleoplasm was mostly electron light with scattered clumps of coarse chromatin. The nucleolus was highly compact and showed little substructure. In addition some crystalline aggregates appeared associated with it.

Throughout the three stages, but more often in stages A and B, a structure known as spherule has been seen attached or close to the surface of the nucleoli (Figs. 21, 29, 31, 39). It is different in structure from the nucleolus-associated chromatin and appears to consist of coarsely coiled thick fibres (Fig. 39, insert).

4-2-2. Changes in DNA, RNA and histones --

Cytophotometric measurements were made on nuclei of cells at the three stages of differentiation: (1) procambial cells, (A); (2) young expanded vessel elements without secondary wall thickening, (B); and (3) older vessel elements with secondary wall but with the end walls still intact, (C). The results were highly significant at 5% level.

As indicated in Table 3 and Figure 40, in differentiating vessel elements (stages B and C), the DNA content measured after Feulgen staining was nearly double that in the procambial cells (stage A). Histone content increased

Fig. 39. Same vessel element as in Fig. 18 shows a degenerating nucleus; the only portion of cytoplasm still present appears at the upper right corner. Some areas (*) are void of chromatin or nucleoplasm. Nuclear pores (arrows) and most of the envelope are still intact. Spherule persists. Unknown crystalline structures (?) appear close to the nucleolus which is highly dense and compact with little evidence of substructure. 26,000X. Insert at 65,000X shows the pattern of the crystalline structures(?) and spherule.

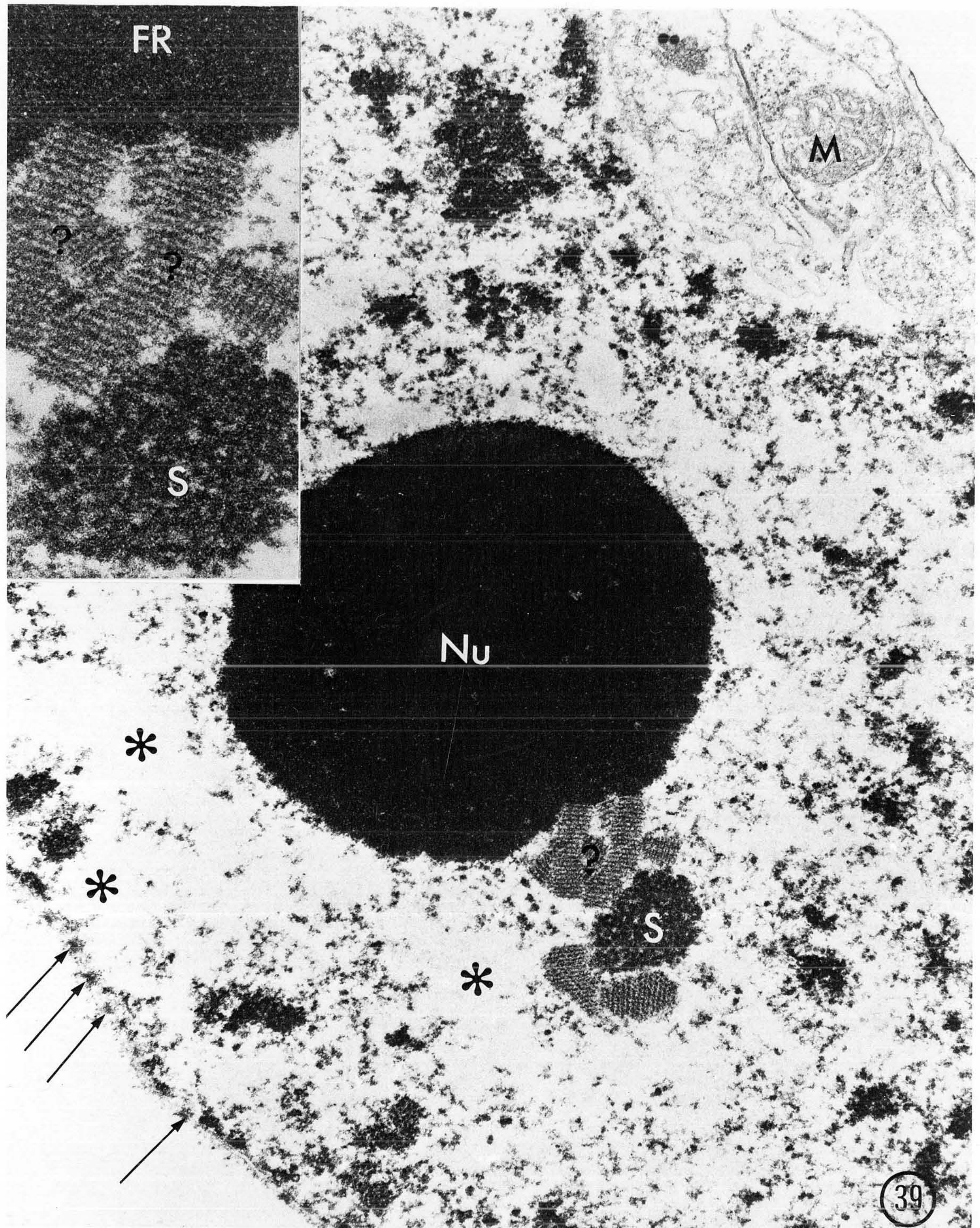
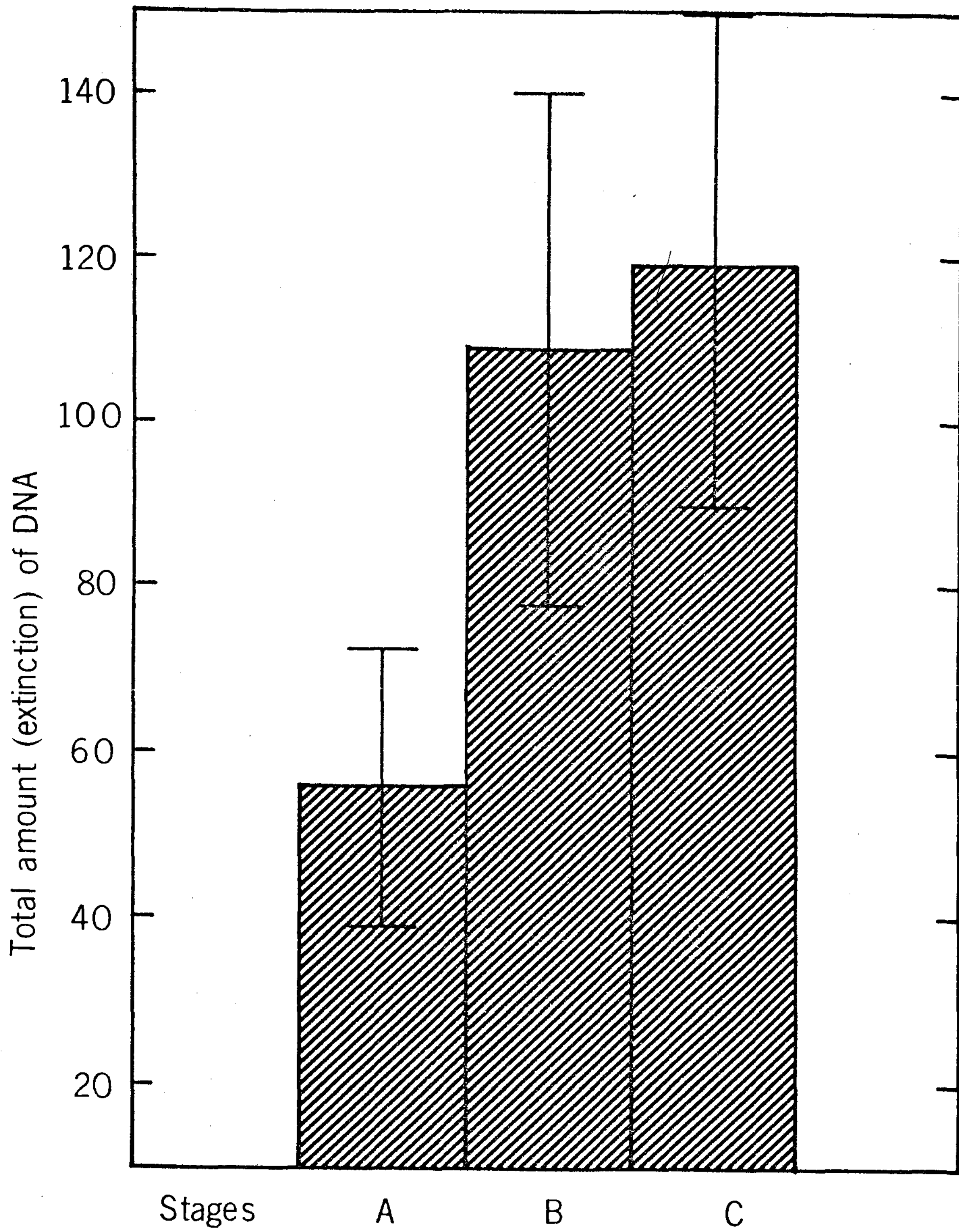

FR
?
?
S
M
Nu
*
*
*
?
S
39

Table 3. The total amounts of DNA and histones in the three stages: (A) procambial cells, (B) young vessel elements, and (C) older vessel elements expressed as total extinction.

Stages	DNA (Feulgen)	Histone (Alkaline fast green)	DNA/histone
A	56.09 ± 16.75	22.91 ± 6.75	2.45
B	109.31 ± 37.83	47.95 ± 15.03	2.28
C	119.38 ± 35.54	94.42 ± 29.63	1.26


140
120
100
80
60
40
20
Total amount (extinction) of DNA
Stages
A
B
C

from procambial cells to young vessel elements in stage B, but as differentiation continued to stage C, the histone content continued to increase to about 4.5 times the amounts in the procambial cells (Fig. 41).

Frequency distribution of DNA content in the three stages following gallocyanin and Feulgen staining is shown in Fig. 42. It is clear that in their differentiation from procambial cells, the vessel elements show a shift toward a higher DNA content. Indeed some of the nuclei show as much as 4-8 times higher DNA content than those in the procambial cells. The frequency distribution of histones followed the same pattern as DNA (Fig. 42).

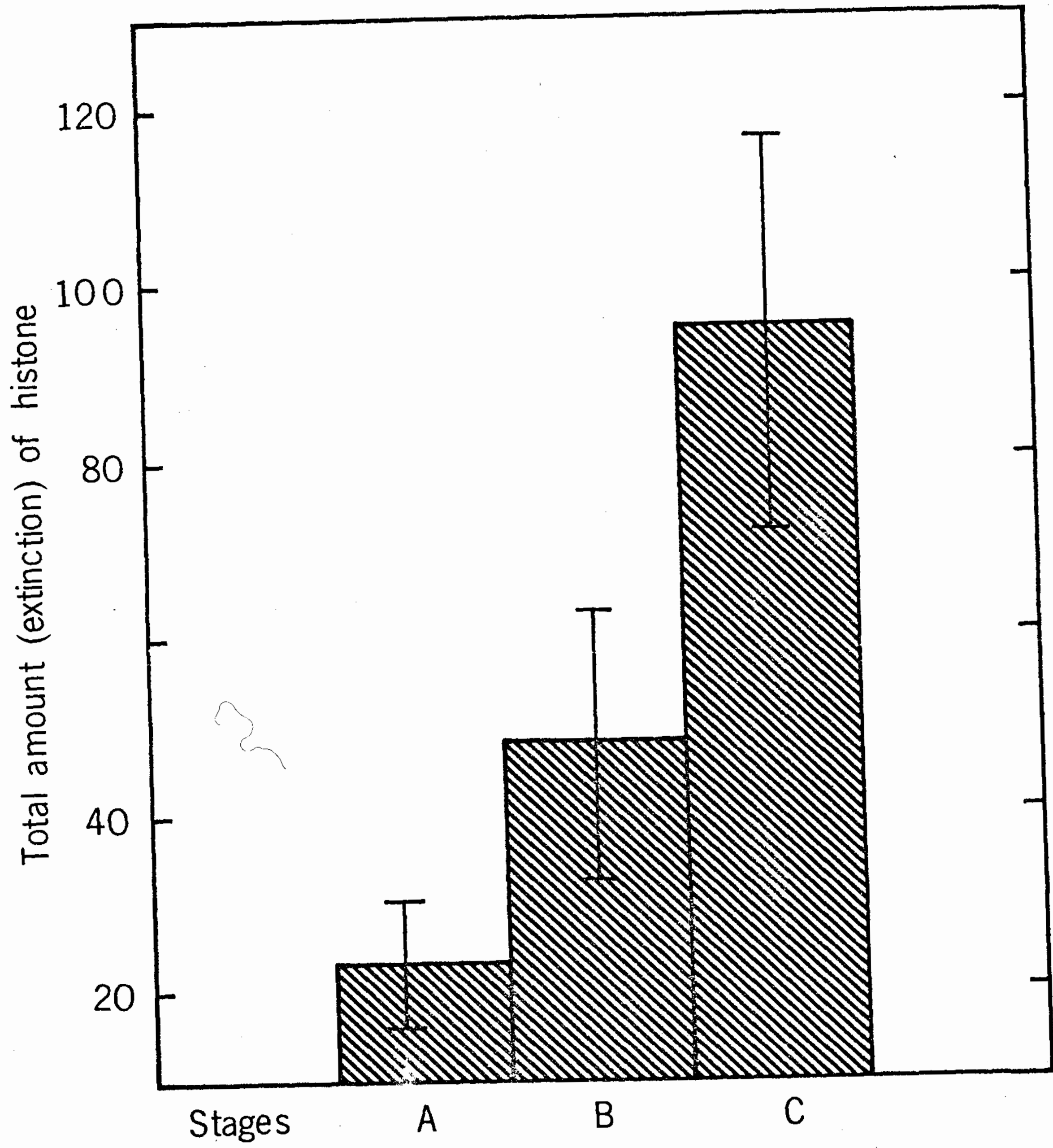
Expanded vessel elements in stage B with large nuclei and nucleoli are expected to contain more RNA for building up cell components. As shown in Table 4, however, RNA values in vessel elements in stage B did not exceed -- indeed were lower than -- the values for RNA in both the procambial cells (A) and older vessel elements (C). Since the microspectrophotometer does not detect any measurements which are under 5% transmission, this may account for the lower total values for RNA, particularly in stage B.

Table 4. Total amounts of DNA and RNA values in the three stages: (A) procambial cells, (B) young vessel elements, and (C) older vessel elements expressed as total extinction

Stages	Total nucleic acids (DNA+RNA)	RNAase treated (DNA)	Subtraction from DNA values (RNA)
A	63.08 ± 18.42	38.02 ± 10.18	25.06
B	121.80 ?± 57.01	120.04 ± 24.17	1.76 ?
C	176.73 ± 48.58	160.27 ± 71.08	16.45

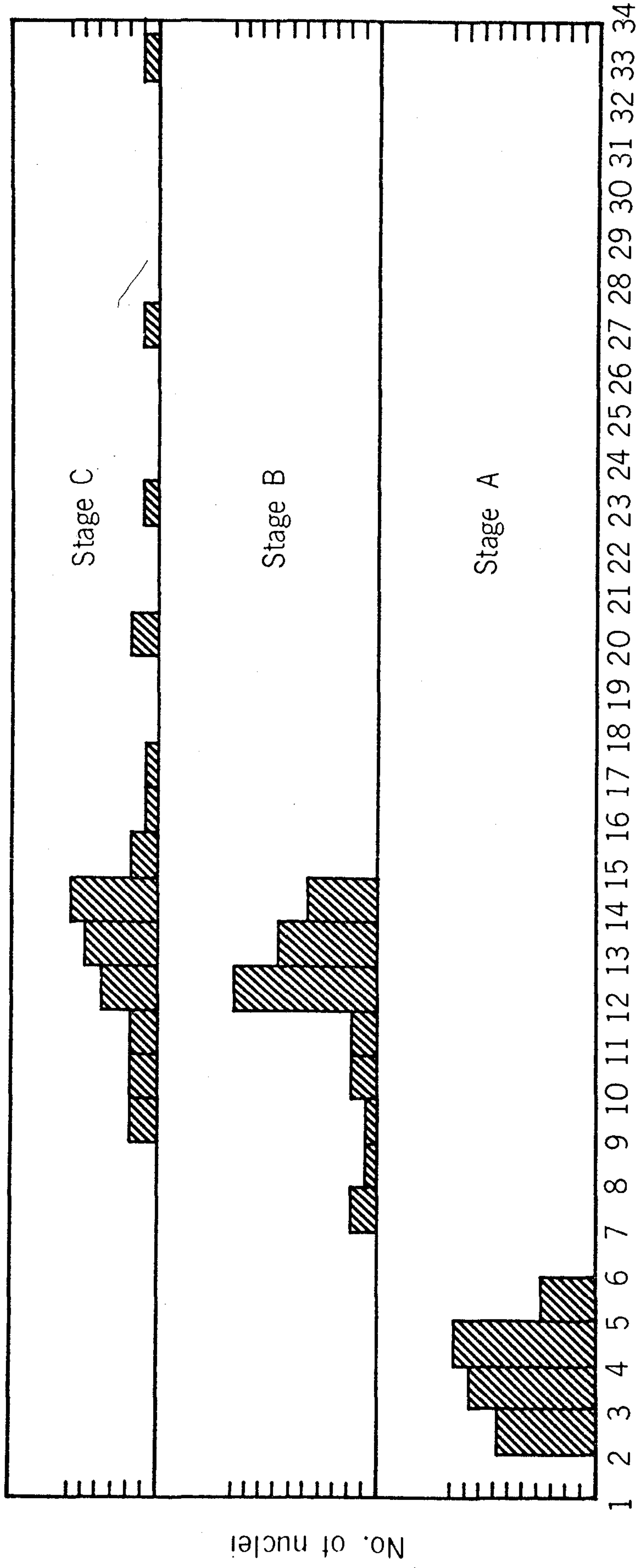
? low value due to loss in counting. See text.

Fig. 41. Total amounts of histones in the 3 stages: (A) procambial cells, (B) young vessel elements, and (C) older vessel elements. Vertical bars represent twice the S.D.

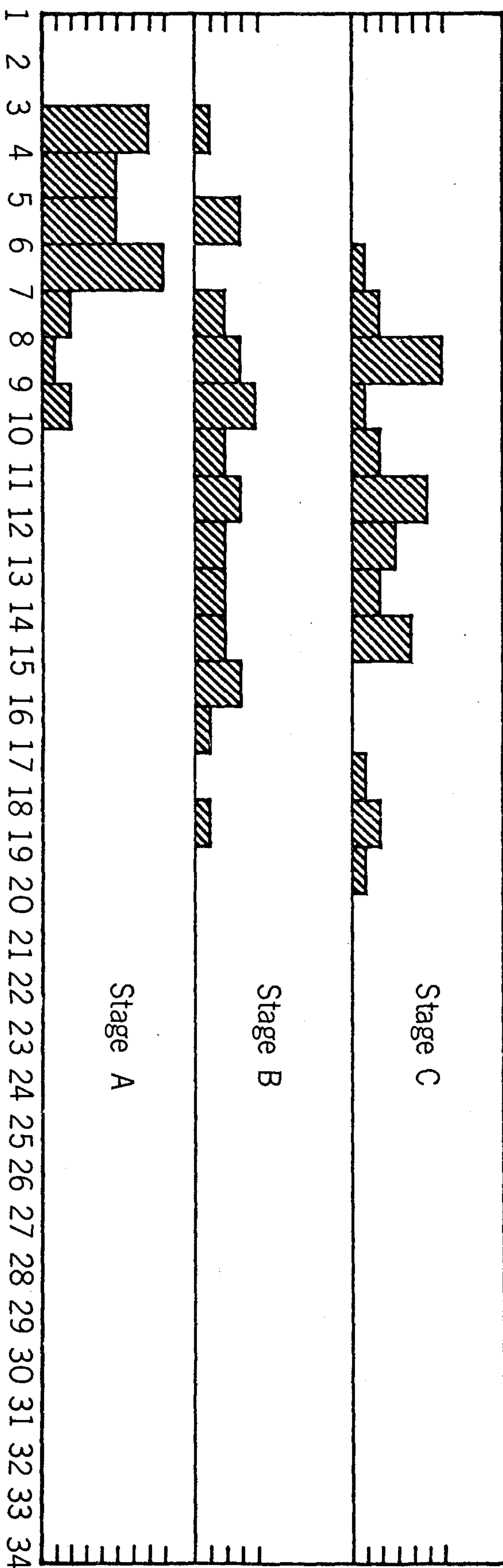


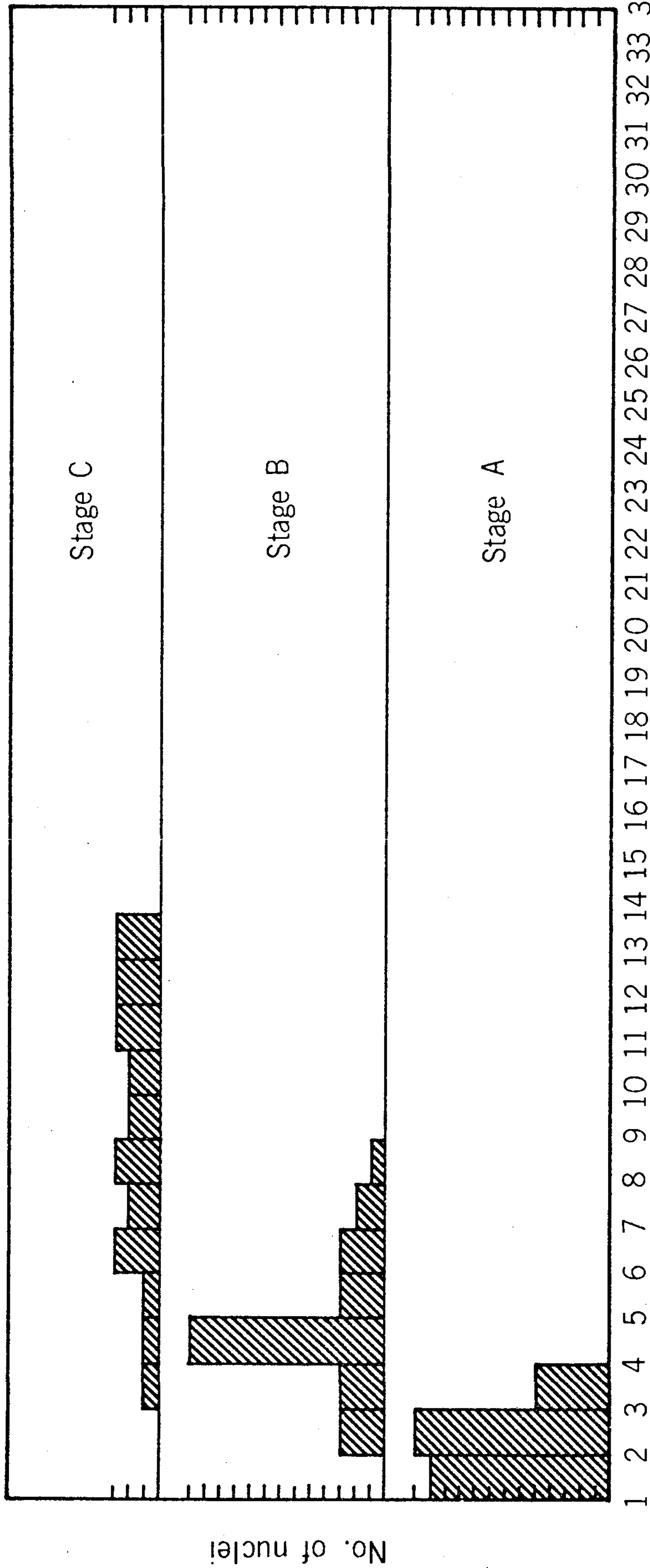
120
100
80
40
20
Total amount (extinction) of histone
Stages
A
B
C

Fig. 42. Frequency distribution of DNA and histones in individual nuclei of the 3 stages: (A) procambial cells, (B) young vessel elements, and (C) older vessel elements. DNA values are given for gallocyanin staining, DNA (a); and Feulgen staining, DNA (b).



Stage C
Stage B
Stage A
No. of nuclei
Total extinction of DNA (a) values
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34


No. of nuclei
Stage A
Stage B
Stage C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34
Total extinction of DNA (b) values


Stage C
Stage B
Stage A
No. of nuclei
Total extinction of histone values
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34

5. DISCUSSION

An attempt was made in this thesis to study the hormone-induced activation of cambium and xylem and phloem differentiation in poplar and to investigate the nuclear changes during differentiation of xylem vessel elements. Unfortunately due to technical difficulties it proved impossible to study the nuclear changes in poplar and hence that part of the work was done on the more easily processed material of corn leaf. Here too, problems were encountered in cytophotometric measurements of nuclei due to contamination staining of lignified walls, in enzymatic extraction of DNA, and finally in gallocyanin staining of RNA. These limitations notwithstanding, there are several conclusions which are discussed in the following paragraphs.

The results on hormone-induced cambial activation and xylem and phloem differentiation are in basic agreement with those of Wareing, et al. (1964) and Digby and Wareing (1966a), though there are differences in detail. Both hormones, IAA and GA, singly and in combination, stimulate the cambium to divide and produce derivatives on both sides. IAA promotes xylem differentiation and GA promotes phloem differentiation, but whereas Wareing, et al. (1964) and Digby and Wareing (1966a) reported that IAA alone did not produce phloem cells, in my material IAA alone produced

small amounts of phloem derivatives (Fig. 3, 5 and Table 1).

In conformity with the observations of Wareing, et al. (1964), I also found that an increase in the IAA concentration alone, from 100 to 500 ppm, does not lead to increased xylem production. But in contrast to the observations of Wareing (1958a) on *Fraxinus* and Digby and Wareing (1966a) on *Robinia*, I found no significant increase in number or diameter of the vessel elements with increased IAA concentration. This may be related to the fact that poplar is a diffuse-porous species, whereas *Fraxinus* and *Robinia* are both ring-porous. Also, in contrast to the situation in *Fraxinus* and *Robinia*, a higher IAA concentration (500 ppm) does not induce the vessel elements to become more sided and circular in transverse sections (cf. Table 2, Figs. 5, 6).

Combination of the two hormones has a synergistic effect on xylem production and number of differentiated vessel elements. Greater amount of xylem production and vessel element differentiation occur when IAA/GA concentrations are the same (100/100 ppm, 500/500 ppm), or when IAA concentration is higher (500/100 ppm). Xylem produced in low IAA and high GA concentration (100/500 ppm) has a lesser number of differentiated vessel elements (Table 2). Roberts and Fosket (1966) reported that xylem produced at low IAA concentrations is low in lignin content and the tracheary cells have rather

thin lignified walls. Wareing et al. (1964) and Digby and Wareing (1966a) claimed that most "normal-looking" xylem tissue is produced at an IAA/GA concentration of 500/100 ppm. However, in my results, none of the new xylem produced under any treatment is ever "normal-looking" to me. Also there was no appreciable difference in lignin staining the vessel elements in different treatments. As a whole, the new xylem produced in these experiments consisted of lignified groups of vessel elements and tracheid-like cells in a discontinuous ring with undifferentiated parenchyma cells intervening or between the new lignified groups and old wood of last year (see Figs. 9, 10, 12). This discrepancy may be because of minor differences in experimental set up, for example, the photoperiod, light intensity, temperature, etc. It does not seem to be due to duration of experiment for Wareing's experiments were also conducted for 3 weeks.

As far as the phloem production is concerned, a combination of the two hormones does not increase phloem production any further than is obtained by GA 500 ppm alone. In my experiments, whenever GA concentration was lower than IAA concentration (100/500 ppm) there was a decrease in phloem production.

Finally, the peaks of xylem and phloem production

in my material are different from those obtained by Wareing et al. (1964) and Digby and Wareing (1966a). These discrepancies may be due to two reasons: (1) Unknown amounts of endogenous hormones are present in poplar stems. For instance, it has been reported that disbudded stems contain small amounts of endogenous auxins which are insufficient to initiate cambial division but sufficient to bring about division or differentiation in the presence of small amounts of other hormones (Digby and Wareing, 1966a). (2) A different method of counting was done on new xylem and phloem production. In all of Wareing's work, the new xylem was determined by measuring the radial distance in mm from the center of the cambial zone to the last annual ring. For phloem, the radial distance from the center of the cambial zone to the last sclerenchyma band at the outer edge of the phloem was measured, and the value for newly produced phloem was obtained by subtracting the phloem width of the lanolin control from the phloem width in an experiment. In the present study, however, the actual number of new cells produced toward the xylem or phloem was counted. This method gave different results from those obtained by measuring the radial distance because the latter is affected by enlargement of the derivatives and is not a true measurement of increase in cell production. Furthermore, I

have also noticed that the distance from the last sclerenchyma band to the cambium is very variable from tree to tree, even from twig to twig.

As has been described in detail, there are marked changes in the structure of nucleus and nucleolus and in DNA and histones of the nucleus during xylem vessel differentiation. Among the first changes are an increase in nuclear size and dispersal of chromatin, an increase in DNA and histone content, and an increase in size and clarity of zonation of the nucleolus.

The increase in nuclear size is associated with the increase in cell size of young vessel elements. This has been observed by Bailey (1920) and List (1963) and according to them, the increase ratio is 1:1. The increase in nuclear size is accompanied by an amplification of DNA and histone to at least double the original amount in procambial cells; later, DNA stays about the same but histone increases further during stage C to a value 4 times that in the procambial cells. An increase in nuclear DNA content has been reported by List (1963), Corsi and Avanzi (1970), Innocenti and Avanzi (1971) in xylem cells of root tips of various plants, and by Torrey, Fosket and Hepler (1971) in differentiating tracheary elements of cultured root cortical cells. Parallel increases of DNA and histones have also been reported by Rasch and Woodward (1959) in other kinds of tissues such as root meristem and microspores of _Tradescantia paludosa_ and tumor growth in _Vicia faba_

stem. These authors claimed that the amount of histone per nucleus varied widely from species to species but appeared in a relatively constant proportion to DNA; in other words, the DNA/histone ratios remained unchanged in tissues of a given species. However, Gifford and Tepper (1962) found a decrease in histone staining but no change in DNA staining during the development of inflorescence in Chenopodium album. The decrease in histone staining with alkaline fast green is not a reliable criterion for a decrease in histone content, because some changes in histones, such as acetylation, reduce histone stainability. Acetylation occurs mostly in the arginine- rather than the lysine-rich fraction of histones and precedes the increase in nuclear ribonucleic acid synthesis (Pogo, Allfrey, and Mirsky, 1966; Allfrey, Pogo, Littau, Gershey and Mirsky, 1968). Hence, staining of histones by alkaline fast green following acetylation is more an indication of arginine residues than total histones. In my material, the first increase in histone content may indicate the simultaneous duplication of a nucleohistone complex. The later increase in the older vessel elements may be due to an actual increase in amount, or may simply reflect a change in histone compositon, particularly a decrease in the arginine-rich and an increase in the lysine-rich fraction.

The exact reason for the increase in DNA content is unclear. It may be that some specific genes which are

needed to build large amounts of a particular substance or substances necessary for differentiation of vessel elements have to be multiplied. For instance, the enlargement of vessel elements is accompanied by an increase in polyribosomes and cytoplasmic ground substance, later wall polysaccharides and lignin are synthesized and deposited at specific locations, and finally, there is an extensive but controlled hydrolysis of cytoplasm, organelles and parts of cell wall. These changes occur at specific times and in precisely controlled order and suggest a high degree of integration of cell activities. They may result at least partly from endoduplication of DNA.

Although it has not been shown in this thesis, others, using electron microscopic autoradiography, have demonstrated RNA synthesis in areas of dispersed fibrils between dense chromatin masses (Littau, Burdick, Allfrey, and Mirsky, 1964; Kemp, 1966). The chromatin fibres in these areas are thicker, irregular, and contain non-histone protein and RNA (Ris, 1965; Ris and Kubai, 1970; Bram and Ris, 1971), or often appear as chains of granules (Monneron and Bernhard, 1969; Bernhard, 1966). I have shown that in stage B the chromatin breaks up into small masses and occurs in a dispersed state. It is possible, therefore, that this stage not only reflects the time for duplication of DNA and histone complex but also a period of intensive RNA synthesis.

The enlargement and elaboration of the various zones of the nucleolus are equally dramatic. The central fibrillar region in the procambial cells does not seem to increase very much but the granular and vacuolar regions appear and increase in size and are responsible for the several fold increase in nucleolar volume in stage B. These regions have been found in different tissues and are well described by many authors (Lin, 1955; Lafontaine and Chouinard, 1963; Hyde, Sankaranarayanan, and Birnstiel, 1965; Bernhard and Granboulan, 1968; Hay, 1968; Lafontaine, 1968; Busch and Smetana, 1970; Chouinard, 1970; Kuroiwa and Tanaka, 1971). Both granular and fibrillar regions have been reported to contain RNA and protein (Leduc and Bernhard, 1965; Bernhard, 1966; Bernhard and Granboulan, 1968; Busch and Smetana, 1970). The vacuoles described here in stage B are similar to the lacunae reported by Chouinard and Leblond (1967) and Chouinard (1970) which contain masses of dense material staining with the same intensity as the chromatin. These authors claimed that the lacunae are the cross-sectional views of an invaginated channel which contains the chromatin as shown by the fact that the latter is digestible with DNAase. This chromatin normally occurs outside the nucleolus partly or entirely surrounding it and is referred to as the nucleolus-associated chromatin. By combining enzymatic digestion and electron autoradiography on cultured monkey

kidney cells labeled with short pulses of uridine ^3H, Bernhard and Granboulan (1968) demonstrated that the synthesis of nucleolar RNA started in nucleolus-associated chromatin then extended to the fibrillar and eventually to the granular regions. In my material, the nucleolus-associated chromatin disappeared from the nucleoplasm during enlargement of the vessel elements (stage B) and at the same time chromatin material appeared within the nucleolar vacuoles. Later in stage C, the granular and vacuolar regions diminished in size or disappeared, and at the same time the nucleolus-associated chromatin reappeared in the nucleoplasm. These processes of the penetration of nucleolus-associated chromatin into the nucleolus body and the increased elaboration of granular regions suggest an increased synthesis of ribosomal RNA during stage B. It is noteworthy that these changes occur already while the cell is expanding before any secondary wall deposition.

The large granules, 1000-5000 Å in diameter, in the nucleoplasm, have been noticed by Sun (1961) and Simard (1970) and possibly are the nuclear bodies of Dupuy-Coin, Kalifat and Bouteille (1972). Their function is unknown. Since cytochemistry of thin sections has not been performed, their composition also remains unknown. In the literature, various types of granules have been reported in the nucleoplasm. However, only one type of granule found here is

similar to the perichromatinic granules of about 300-400 Å in diameter, which are believed to contain both DNA and RNA (Swift, 1965; Bernhard and Granboulan, 1968; Bernhard, 1966; Hay, 1968; Monneron and Bernhard, 1969). Other structures-- spherules, also have been described by Hay (1968), but their function and composition are also unknown. Spherules appear quite often in the differentiating vessel elements but their relationship with the differentiation process is unclear.

The lobing of the nucleus reflects an increased surface area in stage C. The modified histone content, clumping of chromatin (also reported by Bailey, 1920; Esau, 1965; and Innocenti and Avanzi, 1971), possible loss of protein matrix of nucleoplasm, and the shrinkage of the nucleolus, all suggest that the nucleus is not very active in transcription at this stage. The increased number of perichromatinic granules and the large granules may also suggest the beginning of degeneration or autolysis. The nucleus seems to be the very last organelle to disappear during autolysis, for in the oldest vessel element studied here which had little cytoplasmic contents, the nuclear membrane was still more or less intact and, although the nucleoplasm showed signs of degeneration, a nucleolus was still present (Fig. 39). In the literature, Esau (1965) has reported a break down of nuclear membrane and a leaking of some of the nuclear contents and Innocenti and Avanzi (1971) have reported an extrusion of the nucleolus.

It is well known that hormones play an important role in differentiation. Auxins, gibberellins and cytokinins apparently increase DNA and RNA synthesis, whereas abscissic acid inhibits their synthesis (Silberger and Skoog, 1953; Nitsan and Lang, 1966; Key, 1969; Torrey and Fosket, 1970; Torrey, et al. 1971; Piesco and Alvarez, 1972; Libbenga and Torrey, 1973). Liao and Hamilton (1966) fed the root tip cells of _Allium cernuum_ and _Vicia faba_ with ^{14}C-IAA or ^{14}C-2,4-D and found that the nuclei are one of the binding sites for auxin. Grieshaber-Scheubel and Fellenberg (1972) reported that auxin at low concentrations, loosened the binding of lysine-rich histones to DNA; whereas at higher concentrations, it loosened the binding of arginine-rich histones to DNA. Gibberellic acid (unspecified) stabilized the binding of lysine-rich histones to DNA at high concentrations but at the same concentrations, loosened the binding of arginine-rich histones to DNA. These results suggest that different growth substances may be able to loosen selectively the binding of special histone components to DNA. Piesco and Alvarez (1972) investigated the stimulation of chromatin responses to kinetin. Three major responses have been found: Reduced deoxyribonucleoprotein (DNP) thermal stability; increased Feulgen dye binding; and altered lysine to arginine ratios in histones bound to DNA. This may be the first step in transformation of DNA to a state where

special m-RNA may be transcribed.

Many interesting questions involving cell differentiation in various specific organs still need to be answered. Auxins, together with gibberellins move basipetally from the buds down the trunk through cambial zone or late phloem or late xylem tissue though they may travel at different rates. But the question is what controls the different gradients of hormones on the two sides of the cambial layer, which factors favor phloem production and differentiation, and which favor xylem production and differentiation; in other words, what determines these differentiation patterns in such a precise fashion? What subtle variations in control cause different cell types to differentiate within the xylem tissue: the enlarged and distinct vessel element, the narrow thick-walled xylem fiber, the thick-walled tracheid, the living, thin-walled xylem parenchyma cell or ray cells? During differentiation of a xylem vessel which is a tube of many vessel elements, one vessel element may be fully mature whereas the neighboring vessel element may still be in the early stage of differentiation. Does an individual xylem cell react and respond to hormonal stimuli differently?

This thesis only presents some of the facts about differentiation; however, further experiments are needed before the differentiation of vessel elements can be understood in total.

6. SUMMARY

Both hormones, IAA and GA_3, stimulate the cambium to divide, more so when they are applied in combination than singly. IAA alone promotes more cells to be formed toward the xylem, whereas GA alone promotes a greater production of cells toward the phloem. When applied alone, the IAA promotes differentiation of vessel elements in the new xylem, but an increase in IAA concentration from 100 to 500 ppm does not promote additional xylem production or the number of vessel elements in the new xylem per unit area. In combination with GA, however, IAA promotes the production of new xylem as well as number of new vessel elements per unit area. Among the concentrations used, maximum phloem production is obtained by 500 ppm GA. Addition of IAA increases cambial activity but not net production of phloem. In the new phloem sieve elements and companion cells are formed.

The changes in nuclei and nucleoli during differentiation of xylem vessel elements are described with reference to 3 arbitrary stages. In the procambial cells (Stage A), nuclei are elongated and cylindrical and possess dense chromatin masses. The nucleoli are

small, composed mainly of fibrillar structures, and are connected to the nucleolar associated chromatin. In the young enlarged vessel element with no secondary wall (Stage B), there is an increase in nuclear size. A dispersal of chromatin, an increase in DNA and histone contents to double their original amounts, and an increase in size and clarity of zonation of the nucleolus into granular, fibrillar and vacuolar regions. Simultaneously the nucleolus-associated chromatin disappears from the nucleoplasm and occurs inside the vacuolar regions of nucleolus. In the older vessel elements with secondary walls but end walls still intact (Stage C) there is an increase in lobing of the nuclei and clumping of chromatin. The DNA amount stays the same, but histone content is again doubled. At the same time, there is a shrinkage of nucleolus, disappearance of nucleolar zonation, and the reappearance of nucleolar associated chromatin in the nucleoplasm. In progressively older vessel elements, there is a loss in stainability of the nucleoplasm, an increased clumping of chromatin, and a loss of structural detail in the nucleolus. These results indicate that the vessel element differentiation involves high DNA activity both in the nucleoplasm and the nucleolus. These activities are reached very early in differentiation (Stage B)

and subsequently, while the cell is differentiating, the nucleus seems to enter degenerative changes.

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Appendix A. Number of cells in cambial zone, cambium, new phloem and xylem in 50 radial tiers in poplar twigs under control conditions.

Cambial zone		Cambium	New phloem	New xylem
1.	11	2	7	2
2.	11	2	7	2
3.	11	1	8	2
4.	11	1	7	3
5.	10	1	7	2
6.	12	2	9	1
7.	11	1	9	1
8.	10	1	8	1
9.	11	2	7	2
10.	13	3	9	2
11.	12	2	7	1
12.	11	2	6	3
13.	13	2	10	3
14.	13	2	10	1
15.	11	2	9	1
16.	10	2	8	1
17.	10	1	8	1
18.	11	2	7	1
19.	10	1	9	1
20.	10	1	7	1
21.	9	2	7	2
22.	13	1	7	1
23.	10	1	8	1
24.	13	1	10	1
25.	12	2	8	2
26.	11	1	8	2
27.	10	1	7	2
28.	10	2	6	2
29.	7	1	5	2
30.	10	2	7	1
31.	10	1	8	1
32.	10	1	8	1
33.	9	1	6	1
34.	8	1	6	2
35.	11	1	9	1
36.	9	1	7	1
37.	11	2	8	1
38.	10	1	7	1
39.	8	1	5	2
40.	7	1	4	2
41.	11	1	8	2
42.	13	1	10	2
43.	10	1	8	2
44.	10	2	7	1
45.	12	1	9	1
46.	9	1	7	2
47.	11	1	9	1
48.	11	1	9	1
49.	10	1	8	1
50.	12	1	9	1
Mean	10.58±1.44	1.38±0.53	7.68±1.33	1.52±0.61

Appendix B. Number of cells in cambial zone, cambium, new phloem and xylem in 50 radial tiers in poplar twigs under IAA 100 ppm treatment.

	Cambial zone	Cambium	New phloem	New xylem
1.	25	3	13	9
2.	23	2	11	9
3.	24	3	12	10
4.	20	2	10	8
5.	22	4	11	7
6.	22	2	11	9
7.	25	3	13	9
8.	28	5	12	8
9.	25	5	11	9
10.	21	3	12	13
11.	25	3	11	7
12.	17	4	12	9
13.	15	2	9	6
14.	21	2	7	6
15.	20	3	11	7
16.	19	3	11	6
17.	23	2	11	6
18.	16	3	13	7
19.	20	2	7	7
20.	21	3	8	9
21.	25	3	13	5
22.	17	4	8	5
23.	19	2	8	9
24.	19	3	8	8
25.	26	5	12	9
26.	20	4	9	7
27.	21	3	10	8
28.	19	2	10	7
29.	18	2	10	6
30.	20	3	10	7
31.	22	3	13	9
32.	25	3	13	9
33.	28	3	10	12
34.	22	3	10	9
35.	20	2	10	8
36.	24	3	11	7
37.	20	4	8	9
38.	22	3	12	9
39.	23	2	12	8
40.	19	4	9	7
41.	18	3	10	7
42.	20	3	10	5
43.	17	2	11	5
44.	22	2	10	9
45.	19	3	10	6
46.	17	2	8	5
47.	15	2	10	5
48.	18	2	9	6
49.	17	2	11	6
50.	20	2	10	7
Mean	20.88±3.16	2.86±0.85	10.43±1.64	7.60±1.74

APPENDIX C. Number of cells in cambial zone, cambium, new phloem and xylem in 50 radial tiers in poplar twigs under GA 100 ppm treatment.

Cambial zone		Cambium	New phloem	New xylem
1.	24	2	13	9
2.	30	2	17	11
3.	27	3	14	10
4.	23	3	10	10
5.	28	3	14	11
6.	30	3	15	12
7.	23	4	13	6
8.	25	5	14	6
9.	27	2	15	10
10.	21	2	11	8
11.	25	5	13	7
12.	23	3	13	7
13.	23	5	14	4
14.	23	4	13	6
15.	26	4	15	7
16.	22	4	13	5
17.	22	7	12	3
18.	22	5	13	4
19.	20	4	12	4
20.	23	4	14	5
21.	24	4	14	6
22.	27	3	17	7
23.	26	3	15	8
24.	19	2	12	5
25.	22	3	14	5
26.	20	3	11	6
27.	20	4	12	4
28.	24	4	16	4
29.	22	4	13	5
30.	21	2	14	5
31.	24	5	13	6
32.	19	4	11	4
33.	25	5	15	5
34.	27	4	15	8
35.	20	3	12	5
36.	25	4	16	5
37.	24	4	15	5
38.	21	3	12	6
39.	20	3	13	4
40.	19	3	12	4
41.	21	3	12	6
42.	22	4	14	4
43.	21	3	13	5
44.	22	5	12	5
45.	20	3	10	7
46.	25	3	16	6
47.	18	3	11	4
48.	26	5	16	5
49.	24	4	13	7
50.	28	4	16	8
Mean	23.26±2.91	3.62±1.02	13.46±1.74	6.18±2.14

APPENDIX D. Number of cells in cambial zone, cambium, new phloem and xylem in 50 radial tiers in poplar twigs under IAA/GA, 100/100 ppm treatment.

Cambial zone		Cambium	New phloem	New xylem
1.	26	4	11	11
2.	27	5	11	11
3.	28	3	13	12
4.	29	4	14	11
5.	29	4	14	11
6.	31	6	11	14
7.	31	5	13	13
8.	32	4	17	11
9.	30	3	15	12
10.	34	3	17	14
11.	27	3	14	10
12.	28	3	14	11
13.	27	5	15	7
14.	28	3	14	11
15.	25	3	12	10
16.	27	5	12	10
17.	28	5	13	10
18.	27	4	14	9
19.	29	4	12	13
20.	30	3	14	13
21.	31	3	14	14
22.	27	3	12	12
23.	29	4	11	14
24.	28	4	15	16
25.	35	4	16	13
26.	33	5	15	18
27.	39	4	16	14
28.	33	4	16	13
29.	33	3	12	13
30.	26	3	10	13
31.	22	3	10	9
32.	22	2	14	6
33.	22	4	12	6
34.	27	5	12	10
35.	25	5	12	8
36.	25	4	12	9
37.	29	4	15	10
38.	22	3	13	6
39.	23	3	13	7
40.	25	4	15	6
41.	35	4	18	13
42.	29	4	16	14
43.	34	3	13	15
44.	31	3	11	15
45.	35	3	16	16
46.	28	3	14	11
47.	30	4	14	12
48.	29	3	14	12
49.	32	4	16	12
50.	30	3	13	14
Mean	28.84±3.75	3.74±0.82	13.6±1.90	11.50±2.77

APPENDIX E. Number of cells in cambial zone, cambium, new phloem and xylem in 50 radial tiers in poplar twigs under IAA 500 ppm treatment.

Cambial zone		Cambium	New phloem	New xylem
1.	20	4	11	5
2.	17	4	8	5
3.	20	4	10	6
4.	20	3	10	7
5.	17	3	9	5
6.	19	2	11	6
7.	21	3	12	6
8.	20	4	10	6
9.	20	5	9	6
10.	22	3	13	6
11.	22	3	12	7
12.	20	2	13	4
13.	20	2	13	5
14.	22	3	13	5
15.	18	2	11	6
16.	22	4	12	5
17.	23	3	11	6
18.	26	2	13	9
19.	28	3	11	11
20.	24	3	13	14
21.	24	3	12	8
22.	25	3	11	9
23.	21	3	11	11
24.	18	3	7	7
25.	23	3	8	8
26.	21	3	10	12
27.	19	3	10	8
28.	21	3	9	6
29.	18	3	10	9
30.	22	3	9	10
31.	21	3	9	9
32.	20	3	9	8
33.	18	3	10	5
34.	20	3	8	9
35.	19	3	7	9
36.	22	3	8	11
37.	22	4	10	8
38.	26	3	12	11
39.	20	3	10	7
40.	18	2	7	9
41.	20	3	8	9
42.	26	4	9	13
43.	24	3	10	11
44.	17	3	6	10
45.	25	3	13	8
46.	17	3	9	6
47.	17	3	6	8
48.	19	3	8	8
49.	16	2	8	6
50.	18	4	7	7
Mean	20.76±2.78	3.06±0.61	9.92±2.00	7.80±2.32

APPENDIX F. Number of cells in cambial zone, cambium, new phloem and xylem in 50 radial tiers in poplar twigs, under GA 500 ppm treatment.

	Cambial zone	Cambium	New phloem	New xylem
1.	21	3	12	6
2.	25	2	18	5
3.	26	3	15	8
4.	25	3	17	5
5.	24	2	15	7
6.	26	2	19	6
7.	19	2	13	5
8.	21	2	16	4
9.	22	3	13	3
10.	20	3	13	4
11.	21	3	15	3
12.	20	2	16	2
13.	23	3	16	4
14.	22	3	17	2
15.	24	3	14	7
16.	27	2	17	8
17.	22	3	13	6
18.	26	3	16	7
19.	24	4	14	6
20.	25	3	16	6
21.	27	4	18	5
22.	27	4	18	5
23.	29	4	18	7
24.	26	3	17	6
25.	21	2	14	5
26.	25	3	18	4
27.	24	3	18	3
28.	23	3	14	6
29.	25	4	15	6
30.	24	3	17	4
31.	27	4	16	7
32.	23	3	15	5
33.	24	4	12	8
34.	26	4	15	7
35.	23	3	15	5
36.	27	2	15	5
37.	25	3	19	3
38.	19	4	12	4
39.	27	3	18	5
40.	18	3	12	3
41.	24	4	16	4
42.	24	3	17	4
43.	22	3	13	6
44.	25	3	19	3
45.	18	3	11	4
46.	24	3	17	4
47.	22	4	15	3
48.	21	4	12	5
49.	19	3	13	3
50.	17	2	12	3
Mean	23.38±2.79	3.04±0.66	15.42±2.30	4.92±1.58

APPENDIX G. Number of cells in cambial zone, cambium, new phloem and xylem in 50 radial tiers in poplar twigs under IAA/GA, 500/100 ppm.

Cambial zone		Cambium	New phloem	New xylem
1.	16	2	7	7
2.	22	4	10	8
3.	22	3	8	11
4.	19	3	10	6
5.	25	4	13	8
6.	18	3	8	7
7.	21	4	6	11
8.	21	3	10	8
9.	14	2	6	6
10.	21	4	8	9
11.	18	4	7	7
12.	20	4	9	8
13.	27	3	10	13
14.	29	3	15	11
15.	28	2	10	16
16.	34	3	16	15
17.	28	3	10	15
18.	33	4	15	14
19.	28	3	14	11
20.	26	2	10	10
21.	31	3	14	15
22.	31	3	13	15
23.	28	4	13	14
24.	27	3	10	12
25.	28	4	12	12
26.	19	3	12	8
27.	20	3	8	8
28.	20	3	9	9
29.	20	4	8	8
30.	22	2	8	9
31.	19	3	10	8
32.	24	4	8	8
33.	19	3	12	6
34.	18	3	10	8
35.	18	3	9	8
36.	22	3	8	9
37.	26	3	7	7
38.	21	3	8	8
39.	19	3	11	11
40.	29	3	12	8
41.	20	3	13	13
42.	19	4	12	13
43.	29	3	9	14
44.	25	3	13	11
45.	27	3	13	11
46.	26	3	10	12
47.	25	2	7	9
48.	20	2	9	9
49.	22	4	10	8
50.	28	3	14	11
Mean	23.44±4.70	3.12±0.62	10.28±2.53	10.06±2.77

APPENDIX H. Number of cells in cambial zone, cambium, new phloem and xylem in 50 radial tiers in poplar twigs under IAA/GA, 100/500 ppm treatment.

	Cambial zone	Cambium	New phloem	New xylem
1.	21	2	13	6
2.	21	2	14	5
3.	26	3	16	7
4.	21	2	13	6
5.	25	2	17	6
6.	22	3	15	4
7.	21	2	14	6
8.	24	4	10	10
9.	20	3	13	6
10.	26	3	11	8
11.	25	3	15	7
12.	18	3	15	5
13.	24	2	10	8
14.	23	3	12	8
15.	21	3	11	7
16.	20	3	11	6
17.	21	3	12	6
18.	19	2	14	3
19.	21	3	12	6
20.	20	3	13	4
21.	25	3	16	6
22.	27	3	16	9
23.	25	3	15	9
24.	28	3	13	7
25.	26	2	18	9
26.	26	3	15	8
27.	26	3	15	10
28.	28	3	15	7
29.	31	2	20	9
30.	27	3	15	9
31.	19	2	10	7
32.	19	3	11	5
33.	20	2	11	7
34.	22	2	12	8
35.	27	3	19	5
36.	20	3	12	5
37.	25	3	12	4
38.	19	3	12	4
39.	19	3	16	4
40.	24	3	17	6
41.	24	2	15	7
42.	22	3	13	6
43.	22	2	14	6
44.	21	3	13	5
45.	23	3	14	6
46.	32	3	21	8
47.	22	2	13	7
48.	22	2	13	7
49.	21	2	13	6
50.	22	2	14	6
Mean	23.06±3.21	2.66±0.52	13.88±2.47	6.52±1.64

APPENDIX I. Number of cells in cambial zone, cambium, new phloem and xylem in 50 radial tiers in poplar twigs under IAA/GA, 500/500 ppm treatment.

	Cambial zone	Cambium	New phloem	New xylem
1.	31	4	16	11
2.	22	2	11	9
3.	25	4	12	9
4.	25	2	14	10
5.	26	4	10	7
6.	21	4	10	7
7.	21	3	11	10
8.	24	3	13	5
9.	20	2	11	6
10.	20	2	13	9
11.	24	3	9	7
12.	19	2	14	8
13.	24	3	9	8
14.	20	3	10	7
15.	20	4	17	13
16.	34	3	13	16
17.	32	2	18	16
18.	36	2	15	19
19.	36	2	14	18
20.	34	4	23	17
21.	44	2	20	14
22.	36	3	16	14
23.	33	3	15	14
24.	32	3	19	17
25.	39	3	19	15
26.	37	4	21	17
27.	42	2	17	18
28.	37	2	14	19
29.	35	2	13	21
30.	36	2	13	13
31.	28	3	16	17
32.	36	2	19	15
33.	36	3	16	14
34.	33	2	19	19
35.	40	2	19	14
36.	31	2	13	16
37.	39	4	15	20
38.	34	3	18	13
39.	35	2	17	16
40.	30	2	14	14
41.	30	2	11	17
42.	29	3	11	15
43.	28	3	12	13
44.	26	2	14	10
45.	22	2	12	8
46.	24	4	14	6
47.	24	2	16	6
48.	24	2	12	10
49.	24	3	13	8
50.	27	2	16	9
Mean	29.70±6.70	2.68±0.76	14.54±3.28	12.68±4.42
F value	106.66	9.57	8.47	97.15

APPENDIX J. Number of vessel elements produced per unit area under different treatments.

	(over 0.8 mm)										mean over 1 mm
	1	2	3	4	5	6	7	8	9	10	
Control	0	3	0	0	0	1	0	0	1	0	0.62
IAA 100 ppm	16	21	32	19	38	20	20	15	22	16	27.37
GA 100 ppm	0	0	0	2	0	0	0	0	5	0	0.88
IAA/GA 100/100 ppm	34	39	33	23	40	24	25	25	27	21	36.37
IAA 500 ppm	16	22	13	18	17	19	17	23	14	23	22.75
GA 500 ppm	1	1	1	1	0	1	3	2	2	6	2.25
IAA/GA 500/100 ppm	26	19	29	20	31	35	23	29	38	38	36.00
IAA/GA 100/500 ppm	19	15	30	10	18	24	10	11	20	12	21.10
IAA/GA 500/500 ppm	28	30	27	33	31	30	20	30	26	21	34.50

APPENDIX K. Number of sides of vessel elements differentiated with different hormonal treatments in ppm.

	Control	IAA 100	GA 100	IAA/GA 100/100	IAA 500	GA 500	IAA/GA 500/100	IAA/GA 100/500	IAA/GA 500/500
1.	4	6	4	9	5	4	9	7	5
2.	5	5	6	7	6	5	6	4	5
3.	4	4	5	8	6	4	8	6	6
4.	4	5	7	10	6	9	7	6	6
5.	5	6	7	8	6	6	8	6	5
6.	5	7	7	8	6	6	9	4	7
7.	4	6	7	6	8	6	9	6	7
8.	4	7	5	8	6	5	7	6	9
9.	5	7	5	6	6	5	7	6	5
10.	5	7	4	7	7	5	8	4	7
11.	4	5	5	7	7	7	5	7	7
12.	4	5	5	8	9	6	7	6	9
13.	4	8	4	8	5	6	8	7	6
14.	4	6	4	6	5	4	8	5	7
15.	4	5	5	7	6	4	8	5	7
16.	4	6	4	5	6	4	9	4	6
17.	4	7	7	8	4	5	7	5	6
18.	4	5	5	6	6	5	9	5	6
19.	5	6	4	7	6	6	8	4	7
20.	4	5	4	8	6	5	7	6	6
21.	4	4	4	7	6	6	7	6	4
22.	4	5	4	7	8	5	8	4	7
23.	4	7	5	7	7	6	7	6	8
24.	4	6	4	7	6	4	8	5	6
25.	5	6	5	8	7	6	6	7	5
26.	0	5	5	6	5	4	7	5	7
27.	0	5	6	7	8	5	7	6	7
28.	0	6	5	10	8	4	7	6	6
29.	0	6	7	8	6	4	5	4	6
30.	0	4	5	9	6	6	8	6	6
31.	0	6	6	6	7	5	9	5	6
32.	0	5	5	11	5	6	9	5	8
33.	0	4	5	6	7	6	7	6	8
34.	0	6	5	7	6	5	8	6	7
35.	0	7	5	6	6	4	8	4	8
36.	0	5	6	6	6	6	7	6	8
37.	0	6	4	7	6	4	8	6	6
38.	0	5	6	8	5	6	8	5	4
39.	0	4	4	8	7	6	6	4	5
40.	0	6	6	7	8	7	7	7	8
41.	0	6	5	10	4	5	7	7	6
42.	0	7	7	9	8	5	8	5	7
43.	0	6	4	8	6	5	9	6	6
44.	0	6	8	7	7	4	8	6	7
45.	0	5	6	9	7	4	6	5	6
46.	0	4	0	7	5	6	5	4	9
47.	0	6	0	8	6	6	8	6	6
48.	0	7	0	10	6	5	7	6	9
49.	0	5	0	7	8	4	6	4	8
50.	0	7	0	8	7	5	7	6	6
Mean	4.28	5.70	5.24	7.56	6.32	5.22	7.44	5.46	6.58

APPENDIX L. Total intensity of DNA after Feulgen staining in each stage: (A) procambial cells, (B) young vessel elements and (C) older vessel elements.

	Stage A	Stage B	Stage C
1.	32.93	36.41	67.23
2.	34.56	51.51	71.64
3.	34.66	52.05	78.93
4.	37.37	53.89	80.80
5.	38.12	73.96	82.05
6.	38.22	76.62	82.93
7.	38.35	84.72	83.54
8.	40.45	85.20	86.92
9.	40.83	86.87	89.89
10.	43.84	90.84	94.34
11.	48.36	92.01	105.34
12.	48.63	95.53	107.76
13.	56.79	97.90	115.08
14.	56.84	104.48	116.19
15.	56.84	109.56	116.29
16.	57.37	115.72	117.04
17.	58.28	116.62	118.99
18.	60.38	117.36	120.43
19.	60.55	121.45	125.12
20.	61.03	127.59	126.04
21.	61.52	133.60	134.16
22.	62.68	137.70	137.78
23.	63.69	141.78	140.54
24.	64.81	144.70	141.22
25.	68.11	154.92	145.37
26.	72.96	157.49	146.86
27.	76.66	157.75	178.77
28.	80.32	168.07	185.97
29.	93.12	183.80	188.36
30.	95.60		195.97
Mean	56.09±16.75	109.31±37.84	119.38±35.54
F value	35.05		

APPENDIX M. Total intensity of histone after alkaline fast green staining in each stage.

	Stage A*	Stage B*	Stage C*
1.	11.56	23.27	39.25
2.	12.89	25.11	45.45
3.	13.06	27.61	52.10
4.	16.58	32.61	62.07
5.	17.55	33.33	62.60
6.	17.57	36.43	69.00
7.	17.69	40.00	73.34
8.	17.90	40.75	77.70
9.	18.49	41.07	83.30
10.	18.53	41.30	87.42
11.	18.58	41.48	89.03
12.	19.52	43.38	92.82
13.	20.38	45.40	99.15
14.	20.91	46.26	100.20
15.	21.72	46.28	109.03
16.	22.19	46.42	112.51
17.	22.51	48.17	113.92
18.	23.62	48.41	116.28
19.	24.56	48.53	122.92
20.	24.83	52.08	124.91
21.	25.41	52.64	126.83
22.	25.56	55.24	137.03
23.	27.24	62.70	131.39
24.	28.01	64.40	137.98
25.	29.71	66.58	
26.	32.25	71.23	
27.	32.48	76.70	
28.	33.84	85.37	
29.	34.46		
30.	37.81		
Mean	22.91±6.75	47.95±15.03	94.42±29.63
F value	98.7		

* Stages A, B, C are the same as stated in Appendix L.

APPENDIX N. Total intensity of DNA after extraction with RNAase and staining by gallocyanin chrome alum.

	Stage A*	Stage B*	Stage C*
1.	21.39	77.15	90.14
2.	23.15	77.69	97.21
3.	23.64	80.24	100.69
4.	25.88	96.16	104.88
5.	26.52	102.26	112.09
6.	28.11	103.86	117.63
7.	29.30	118.25	122.60
8.	30.20	119.43	124.24
9.	31.06	122.09	126.55
10.	31.53	123.18	129.67
11.	31.87	123.43	134.21
12.	32.62	123.88	134.31
13.	32.75	124.88	135.53
14.	35.39	125.53	135.92
15.	35.69	128.39	138.46
16.	36.69	128.40	141.17
17.	41.57	128.48	141.37
18.	42.63	128.86	143.67
19.	42.65	131.11	143.85
20.	43.69	132.51	147.94
21.	44.38	133.40	148.55
22.	45.35	133.54	154.03
23.	45.69	134.92	155.37
24.	45.76	137.16	168.07
25.	47.71	138.60	172.90
26.	48.31	141.24	208.19
27.	51.47	142.37	209.11
28.	51.77	144.83	234.44
29.	55.51	144.84	272.72
30.	58.42	145.10	332.01
Mean	38.02±10.18	120.04±24.17	160.27±71.08
F value	62.43		

* Stages A, B, C, are the same as stated in Appendix L.

APPENDIX O. Total intensity of DNA + RNA stained with gallocyanin chrome alum.

	Stage A*	Stage B*	Stage C*
1.	45.09	116.73	113.33
2.	45.09	117.38	119.87
3.	46.83	117.79	123.50
4.	47.05	122.58	126.89
5.	48.79	123.04	133.57
6.	49.38	125.05	135.46
7.	50.47	127.33	144.57
8.	50.51	134.08	147.90
9.	51.60	134.95	159.17
10.	51.62	135.76	168.03
11.	51.65	137.74	168.50
12.	52.10	139.99	174.39
13.	53.35	142.43	177.64
14.	56.91	143.08	179.52
15.	57.34	143.53	181.10
16.	57.99	150.94	188.09
17.	59.23	152.96	190.51
18.	60.28	155.60	193.86
19.	60.75	160.69	197.59
20.	63.88	170.51	202.09
21.	68.49	170.78	202.59
22.	68.93	174.66	203.82
23.	74.17	176.13	206.77
24.	77.38	185.24	221.00
25.	78.61	189.33	223.42
26.	87.12	190.32	251.81
27.	91.27	190.89	273.40
28.	92.35	197.87	278.77
29.	102.46	204.97	
30.	112.25	221.88	
Mean	63.08±18.42	121.80±57.01	176.73±48.58
F value 46.99			

* Stages A, B, C, are the same as stated in Appendix L.