STUDIES ON THE RESPONSE OF ORCHID ROOTS TO 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D)

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ABSTRACT

Studies were conducted to elucidate the nature of the response of orchid roots to 2,4-dichlorophenoxyacetic acid [2,4-D] by approaching it through morphological, anatomical, and fine-structural investigations. The roots of orchids [*Dendrobium* Lady Hay and *Dendrobium* J. Thomas] either attached to plantlets or excised from plants responded to 2,4-D at 0.5-1.0 ppm level by forming tumorous growths at the tips.

The sequence of tumor formation was observed under light and electron microscopes. During the first 3 days, root tips expanded radially and mitotis in meristematic area decreased drastically. Then, the enlarged cortical cells degenerated and the cortex collapsed. Although cells in the epidermis, root cap, and initial zones persisted longer than cortical cells, they too eventually degenerated.

Mitotic cells at the periphery of the vascular cylinder replaced the cortical cells which degenerated and produced a population of new cells. After a prolonged lag period these cells started to proliferate at about 25 days after sub-culture in medium with 2,4-D. The rapid division of these cells resulted in tumor growth.

At the ultrastructural level, multivesicular bodies (MVB) and nucleoli were studied in relation to cell expansion growth and to RNA metabolism during the formation of tumors.

MVB appeared only after sub-culturing on 2,4-D containing medium, MVB frequently became associated with the cell wall and deposited internal vesicles in the wall space. The possible relation of MVB to cell expansion is discussed.

In the normal root tips, the area occupied by the granular region within the nucleolus and the degree of dispersion of granular components progressively increased within a vascular initial, dividing peripheral vascular cells, and elongating peripheral vascular cells. The surface intruded L-zone (lightly staining zone) of the nucleolus in the initial cells progressively moved into the nucleolus of the dividing and elongating cells.

During the first few days of culture in 2,4-D medium, the granular region in the nucleolus increased and the L-zone moved into the nucleolus. These changes may be due to increased RNA synthesis necessary for cell expansion.

Dedifferentiating cells occasionally contained multiple nucleoli. This was related to the acceleration of nucleolar reorganization of cells in the lag phase with a very low demand for ribosomal RNA.

Before the rapid cell proliferation phase, the granular region of the nucleolus increased rapidly. This was considered to be related to the increased production of ribosomal RNA which is a prerequisite for the rapid cell division in tumor formation.

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ACKNOWLEDGEMENT

To the St. John Plant Science Laboratory of the University of Hawaii, the author offers his gratitude for the use of its electron microscope facility.

INTRODUCTION

Although orchids have been successfully propagated clonally through aseptic cultures of shoot tips (Morel, 1960), leaf tips (Ball et al., 1971), and young inflorescences (Intuwong and Sagawa, 1973), orchids roots have not, as yet, been successfully utilized (Churchill et al., 1972). However, in other plants, roots have been grown aseptically and have produced plantlets (Thomas and Street, 1970). Besides, since in Phalaenopsis stuartiana (Orchidaceae) plantlets arise spontaneously from roots, propagation of orchids through root culture appeared feasible.

Therefore, plantlets with vigorous roots of Dendrobium Lady Hay produced through shoot tip culture and still in aseptic flasks were grown on modified Vacin and Went medium (Sagawa et al., 1966) with 1 ppm of 2,4-dichlorophenoxyacetic acid (2,4-D). The roots responded by production of a tumorous growth from which an occasional plantlet differentiated. These plantlets have been successfully transferred to the greenhouse and should flower within a year or two.

Although orchids have been grown in aseptic culture and in a variety of media for many years (Withner, 1959), tumorous growth has not been reported. Therefore, this thesis is an attempt to elucidate the nature of the response of orchid roots to 2,4-D by approaching it through morphological, anatomical, and fine structure studies.

MATERIALS AND METHODS

Flowering plants of Dendrobium Jacquelyn Thomas and plantlets of Dendrobium Lady Hay growing aseptically in flasks were both produced through the technique of shoot-tip culture (Sagawa and Shoji, 1967) and are a part of the orchid research collection of the Horticulture Department.

For excised root-tip cultures, 5-10 mm apical segments from actively growing aerial roots of Dendrobium J. Thomas were surface sterilized for 10 minutes in an Ultrasonic Cleaner containing 10% Clorox and grown on modified Vacin and Went medium (Sagawa et al., 1966) containing 0, 0.1, 0.5, 0.75, or 1.0 ppm of 2,4-D (2,4-dichlorophenoxyacetic acid, Eastman Organic Chemicals).

For attached root-tip cultures, plantlets, from aseptic cultures of Dendrobium Lady Hay, 3-5 cm in height, were grown on modified Vacin and Went medium containing 0, 0.1, 0.5, 0.75, 1.0 or 5.0 ppm of 2,4-D.

Cultures were grown in 50-ml erlenmeyer flasks containing 20 ml of medium and maintained at $26\pm3^{\circ}C$ and under continuous illumination (200 ft-c) with cool white fluorescent lamps.

Gross observations were made by use of a Wild stereomicroscope and recorded through a Wild camera attachment or a Nikon camera with a Micro-Nikkor lens.

For anatomical and fine structure observations, attached roots cultured on medium without 2,4-D or with 1.0 ppm 2,4-D were used. A minimum of 15 samples were taken

daily for the first 11 days and thereafter at intervals of 2-7 days. Samples were fixed at room temperature in 2% glutaraldehyde containing 0.02 M phosphate buffer (pH 7.2) for 3-5 hrs., rinsed in 0.2 M phosphate buffer (pH 7.2) three times, post fixed for 1-3 hrs. in 1% osmium tetroxide, in 0.02 M phosphate buffer (pH 7.2) at 4°C, dehydrated in a gradient series of acetone, and embedded in Epon or epoxy resin (Spurr, 1961).

For light microscopy, sections of 1.5-2.0 μ were cut with a glass or diamond knife on a Reichert Ultramicrotome and stained for 10 minutes with 0.05% toludine blue O in distilled water or 10 minutes in periodic acid Schiff reagent (Feder and O'Brien, 1968) followed by 10 minutes in aniline blue black in 1% acetic acid. Observations and photomicrographs were made with a Zeiss Photomicroscope.

The count of mitotic cells and the measurement of root diameter were made on photomicrographs (40 X) of median section of root. The photomicrographs were enlarged with a photographic enlarger to a total magnification of 200 X. Mitotic cell counts were made by initially drawing arcs 50, 100, 150, 200, and 300 μ from the cap junction which served as the center and then counting the mitotic cells in the area between two adjacent arcs. Root diameter was determined by measuring the width of the roots at distances of 50, 100, 150 μ from the cap junction. The epidermal layer was not included in the width measurements. A count of the number of cells at each diameter was then made.

For electron microscopy, silver to gray sections, cut with a diamond knife on a Reichert Ultramicrotome, were picked up on uncoated grids, double stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963), and examined with a Hitachi Model HS-8-1 electron microscope operated at 50 Kv.

RESULTS

The results are presented in three parts:

I. Morphological Observations,

II. Anatomical Observations, and

III. Fine Structural Observations.

I. Morphological Observations

Response of attached roots and excised root tips to different levels of 2,4-D.

Table I shows the responses of plantlets cultured for 60 days on media of varying 2,4-D concentrations. Plantlets on medium with 0.1 ppm of 2,4-D were normal. On media with 0.5, 0.75, and 1.0 ppm of 2,4-D, an increasing percentage, of plantlets--46.7, 60.0, 59.6--produced tumors on the roots. Besides the formation of tumors at root tips, these plantlets showed thickening of the leaf bases and greater number of secondary plantlets emerged in comparison with the control. Some plantlets continued to grow but others were dead 30-40 days after sub-culture. Figure 1 shows a plantlet grown for 60 days on medium with 1.0 ppm 2,4-D. Tumors formed at the tip of two root tips. At the highest level of 2,4-D (5.0 ppm) only 14.3% of the plantlets formed tumors.

In order to establish the frequency of tumor formation among the roots of a plantlet, 68 plantlets with 2-7 roots were sub-cultured on medium with 1.0 ppm 2,4-D. Table II shows the frequency of tumor formation among roots of plantlets with different numbers of roots. An average of 50.3%

TABLE I. TUMOR FORMATION ON ROOTS OF D. LADY HAY PLANTLETS CULTURED FOR 60 DAYS ON DIFFERENT CONCENTRATIONS OF 2,4-D

Concentration ppm 2,4-D	Number of Plantlets		Percent of Plantlets Forming Tumors
	Cultured	Forming Tumors	
0	25	0	-
0.1	25	0	-
0.5	30	14	46.7
0.75	55	33	60.0
1.0	94	56	59.6
5.0	7	1	14.3

TABLE II. FREQUENCY OF TUMOR FORMATION AMONG ROOTS ON PLANTLETS OF D. LADY HAY CULTURED ON MEDIUM WITH 1.0 PPM 2,4-D FOR 60 DAYS

	Number of roots/plantlet, before treatment							
	1	2	3	4	5	6	7	Total
Plantlets cultured	0	4	14	20	22	6	2	68
No. roots observed	0	8	42	80	110	36	14	290
No. roots forming tumors	0	4	22	56	54	6	4	146
Percent roots forming tumors	-	50.0	52.4	70.0	49.1	16.7	28.6	50.3

of the roots of an individual plantlet formed tumors. As the number of roots in a plantlet increased from 2 to 4, the percentage of roots forming tumors increased from 50.0 to 70.0. However, plantlets with more than 4 roots showed a decreasing frequency of tumor formation.

In order to establish whether root tips from flowering greenhouse plants would respond to 2,4-D in the same manner, root tips were surface sterilized and cultured for 60 days on medium in Table III. 11.8%, 18%, and 11.1% of the roots on 0.5, 0.75, 1.0 ppm of 2,4-D respectively formed tumors. At 0-0.1 ppm 2,4-D, the roots which survived elongated.

Morphological Observations

1. Response of roots attached to plantlets (attached roots).

During the first 3 weeks, the initially pointed green root tip progressively became round and yellowish. As shown in Fig. 2, after 3 weeks, the yellowish root tip is terminated by a spherical ball produced by swelling of the tip. Four weeks later, the spherical root apex turned green and proliferated.

In Fig. 3, the root tip was obliterated by the proliferating of tumor after 6 weeks of sub-culture. At this stage the tumor consisted of many protuberances with a smooth covering.

2. Excised root tips.

During the first 3 weeks, the initially pointed and green root tip gradually became yellowish and blunt. The

TABLE III. TUMOR FORMATION OF EXCISED ROOT TIP OF MATURE PLANTS OF D. J. THOMAS CULTURED FOR 60 DAYS ON MEDIA WITH VARYING LEVELS OF 2,4-D

Concentration ppm 2,4-D	Number of Excised Roots			Percent of Tumor Formation 1/
	Cultured	Uncontaminated	Forming Tumors	
0	72	66	0	-
0.1	52	47	0	-
0.5	80	68	8	11.8
0.75	101	89	16	18.0
1.0	118	108	12	11.1

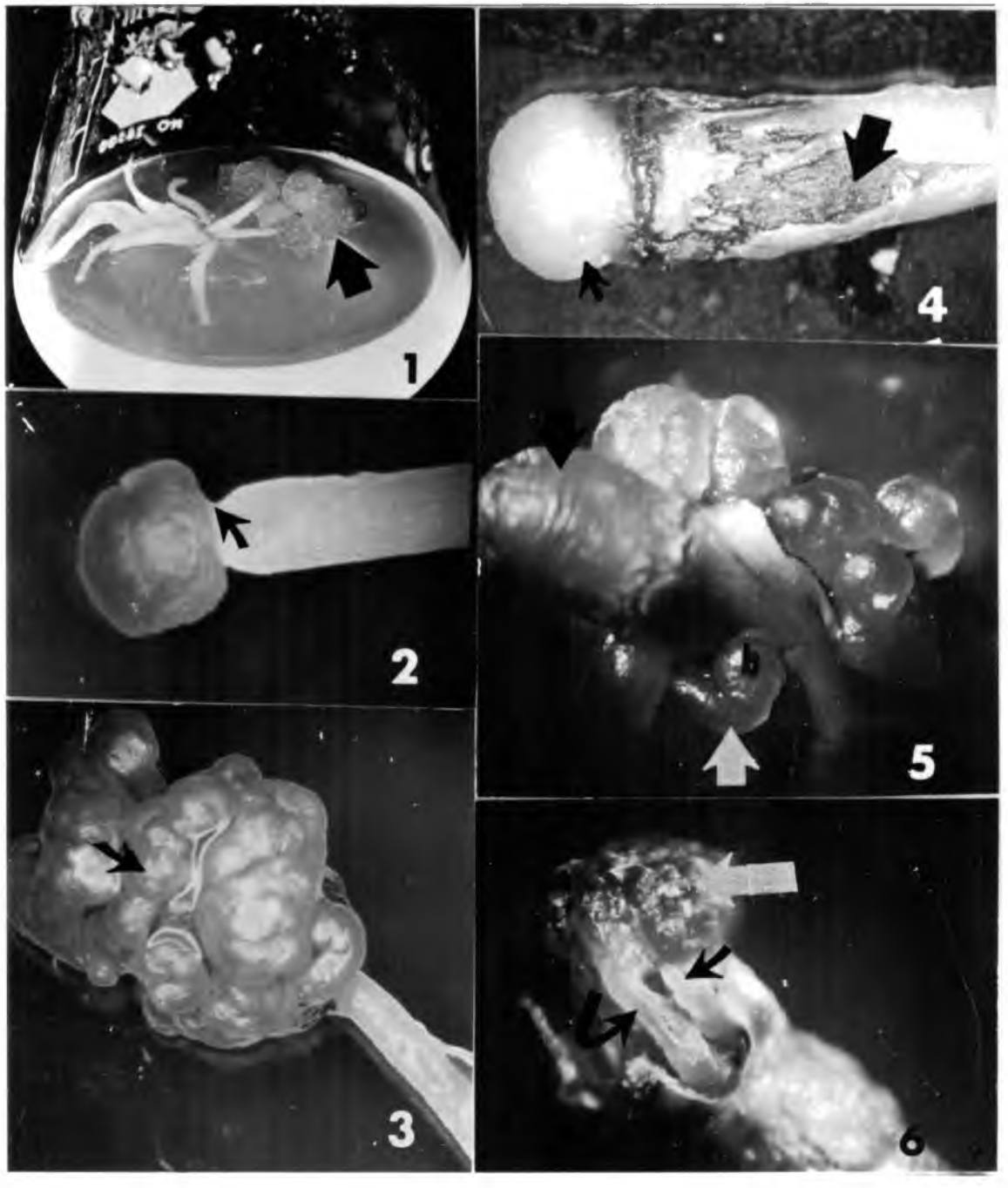
1/ Percentage was calculated from uncontaminated

discoloration, in this case, was less distinct when compared to attached roots. In Fig. 4, after 3 weeks, swelling was noticed and unlike the case of attached roots, it did not progress any further. Cracks or breaks appeared on the surface of the swollen root tip. Breaks also occurred below the constricted area where swelling was also evident.

After 7 weeks, tumors emerged from more than one location on the excised roots. Frequently, a tumor may initially resemble a root. However, with subsequent proliferation at the base, the root-like appearance is lost.

FIGURES 1-6

Figs 1-3. Response of attached root tips cultured on 1.0 ppm of 2,4-D. Fig. 1, a plantlet with tumors at root tips (arrow) after 60 days of sub-culture. 1.2 X. Fig. 2, an attached root with enlarged, spherical tip (arrow) after 3 weeks of culture. 8 X. Fig. 3, proliferation of root tumor of attached. root after 8 weeks of culture. Tumor shows many protuberances with a smooth covering (arrow). 4 X. Figs. 4-6, response of excised root tips cultured on 1.0 ppm 2,4-D. Fig. 4, an excised root tip after 3 weeks in culture showing breaks in the surface of the swollen tip (small arrow) as well as below the constricted area (large arrow). 5 X. Fig.5, an excised root tip after 7 weeks in sub-culture showing tumorous outgrowths, the original root tip (black arrow) and a root-like growth (white arrow) with thickened base (b). 8 X. Fig. 6, an excised root tip after 7 weeks in culture showing proliferating tumor (white arrow) attached to a stalk (crooked arrow) and a tumor resembling a root (black arrow). 8 X.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II. Anatomical Observations

Anatomy of root tip of Dendrobium Lady Hay plantlet

The root apex is shown in Fig. 7. Covering the root apex is a root cap which has developed from the cap initials. Cells of the cap are more vacuolated than the cap initials and contain conspicuous starch granules as seen by PAS reaction in this micrograph.

Underlying the root cap initials are 3-4 layers of initials: (a) epidermal initials, a layer of cells underlying the cap junction which is formed by the invagination of the epidermal initial layer; (b) cortical initials, one or two tiers of cells underlying the epidermal initials; and (c) vascular initials, a group of cells underlying the cortical initials.

The single layer of epidermal initials becomes biseriate at the vicinity of the rim of invagination and later develops rapidly into a multi-seriate condition approximately 200 μ from the cap junction.

From the initial layers of the cortex, 6-7 longitudinal files of cells develop. The exodermis which is the outer layer of the cortical file is derived from one independent layer of the cortical initials. About 200μ from the rim, the exodermis has a distinct pattern of long and short cells which can be easily distinguished from the epidermal layer in which cells are rich in cytoplasm. About 150μ from the rim, the cortex proper consists of 5-6 longitudinal files

of cells. The idioblasts with raphids are reliable indicators for identifying the differentiation of cortical cells in longitudinal files. They are distinctly differentiated at 150-200μ from the initials. The endodermis layer (Mejstrick, 1970) is not readily identifiable within 300μ from the cap junction where observations were made in this study.

From the vascular initials, the central cylinder develops. With 300μ from the cap junction, cells are undergoing differentiation, and therefore, the region of xylem, phloem, and pith cells is not easily distinguishable. However, because they are narrow and elongated, cells in the vascular cylinder are readily distinguished from cortical cells.

The distribution of mitotic cells in a given area in a root tip (Table V) shows that dividing cells are mainly located 50-150μ from the cap junction. Beyond 150μ, mitotic figures are much less. The initial cells located subterminally in the meristem within 50μ from the cap junction show a very low frequency of mitosis; in other studies, this area is defined as the quiescent center (Clowes, 1956).

The Categorization of Phases

Although the process of tumor formation of an attached root tip is a continuous morphological modification of the root tip, a categorization of the developmental events based on some major modifications of its morphology will be

made.

Figure 8 represents the sequence of tumor development in root tips cultured on medium with 1.0 ppm 2,4-D. The blackening by osmium tetroxide indicates the location of lipids including phopholipids (Jensen, 1962) and, in this case, also represents the density of metabolic cells.

The control root tip (Fig. 8a) is pointed and the apex is darkly stained. The location of the apical initials is indicated by a white arrow. The control root tip is called "Phase 0." On the third day (Fig. 8b) the apex has become blunt, and the initial layers are now near the surface of the tip. The stain is localized at the apex and the central cylinder. The period from the first to the third day constitutes "Phase I." On the 12th day (Fig. 8c), the apex is disrupted but is still densely stained, and the tip of the central cylinder is now swollen. The period between 4 to 12 days is "Phase II." On the 20th day (Fig. 8d), the tip of the central cylinder has greatly expanded and the apical initial zone has completely collapsed. The period between 13 to 20 days is "Phase III." On the 45th day, proliferation is evident (Fig. 8e). The period between 20-45 days is "Phase IV."

Phase I

As shown in Fig. 9, the growth on the second day is marked by a conspicuous cell expansion, especially in the cortex. The root cap has stopped growing. Cells in the initial layers are vacuolated. In the cortex, the

previously orderly arrangement of exodermis has been disrupted by the expansion of the short cells. Other cells in the cortex have also greatly expanded. The cells in the vascular cylinder which are generally narrow and elongated have expanded radially. After the 3rd day, the cortical cells, further than 150μ from the cap junction do not expand further but undergo lysis.

The radial expansion of roots during Phase I at different distances from the cap junction is the result of cell expansion (Table IV). On the second day, radial expansion is conspicuous as shown by increase of 73.1-56.2% depending on the distance from the cap junction. On the 3rd day, the diameter does not increase any further.

Table V shows the number of mitotic cells in root tips during Phase I at various distances from cap junction. The average of mitotic figures decreases from 3.5 to 0.7 during the 3-day period of Phase I.

Phase II

This phase of growth is marked by the complete collapse of the cortex (Fig. 10). Cells of the root cap, epidermis, and within the central cylinder have expanded greatly. Cells in the initial layer are large and highly vacuolated. In the central cylinder, some of the vacuolated peripheral cells are in mitosis. Although mitotic cells have decreased greatly in number, they can be seen with regularity during this phase.

TABLE IV. WIDTH AND CELL NUMBERS OF ROOTS AT VARIOUS DISTANCE FROM THE CAP JUNCTION IN PHASE I.

Days in 2,4-D medium	Sections observed	Distance from cap junction toward root base					
		50 μ		100 μ		150 μ	
		Width 1/	No. cells	Width	No. cells	Width	No. cells
0	9	410.4 (0) 2/	28.6	501.0 (0)	31.2	586.0 (0)	32.0
1	10	422.4 (2.9)	28.0	512.2 (2.2)	30.0	587.8 (0.2)	34.0
2	9	710.4 (73.1)	29.0	807.2 (73.1)	32.0	916.0 (56.2)	34.0
3	12	720.0 (75.4)	30.0	864.0 (72.5)	34.0	889.0 (51.7)	*

1/ Epidermal layer not included.

2/ Percent increase from 0 day.

* Cell lysed.

TABLE V. NUMBER OF MITOTIC CELLS IN ROOT TIPS DURING PHASE I

Days	No. sections observed	Distance from the cap junction					Total	Avg.
		0-50 μ	50-100 μ	100-150 μ	150-200 μ	200-300 μ		
0	20	5	41	22	1	0	69	3.5
1	12	1	7	6	1	0	15	1.3
2	10	0	0	5	1	0	6	0.6
3	9	1	0	4	1	3	9	0.7

Phase III

Figure 11 is a section of a root tip on the 15th day of sub-culture. Cells of the root cap and epidermis have now collapsed. The initial cells remain enlarged and highly vacuolated. At the periphery of the vascular cylinder, a new population of vacuolated cells has emerged. In older areas of the vascular cylinder, some of the cells are degenerating.

Phase IV

This phase is characterized by large numbers of meristematic cells as shown in Fig. 12 on the 25th day of sub-culture. The space created by the collapse of the cortex has been filled by rapidly proliferating cells. On the 45th day, clusters of meristematic cells have formed among highly vacuolated cells within the tumor (Fig. 13).

FIGURE 7

Anatomical structure of root tip of Dendrobium Lady Hay. A median longitudinal section showing root cap (RC), vascular cylinder (V), cortex (C), and epidermis (E). Tiers of initial cells (I) are situated below the cap junction (Cj) which is formed by invagination of epidermal layer. Epidermis is biseriate at rim of invagination and multiseriate further away from the rim. The layer adjacent to the epidermis is the exodermis where short cells (arrows) alternate with large cells. The cortex (C) consists of 4-5 longitudinal files of vacuolated cells. Vascular cylinder (V) consists of longitudinally elongated cells without differentiated vascular elements. 210 X.

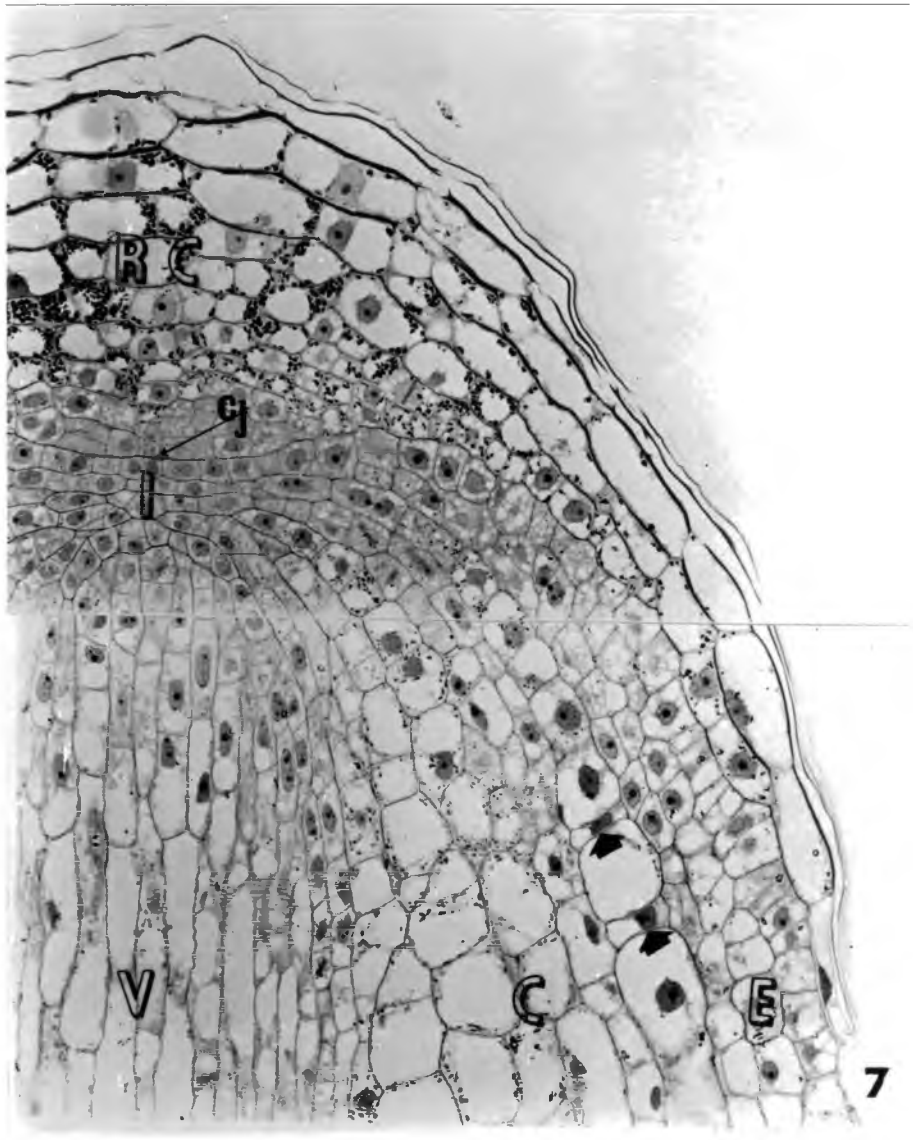

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FIGURE 8

Sequence of tumor development on attached root tips cultured on medium containing 1.0 ppm 2,4-D. Tips were stained with OsO4. Arrows indicate apical initials. 4 X. (a) Normal root tip with pointed apex before sub-culture (Phase 0). (b) Root tip with blunt apex on 3rd day of sub-culture (end of Phase I). (c) Root tip with a disrupted apex and broadened central cylinder on 12th day of sub-culture (end of Phase II). (d) Root tip with completely collapsed apex and broader central cylinder on 20th day of sub-culture (end of Phase III). (e) Root tip on 45th day of sub-culture (end of Phase IV) with a mass of disorganized tumor.

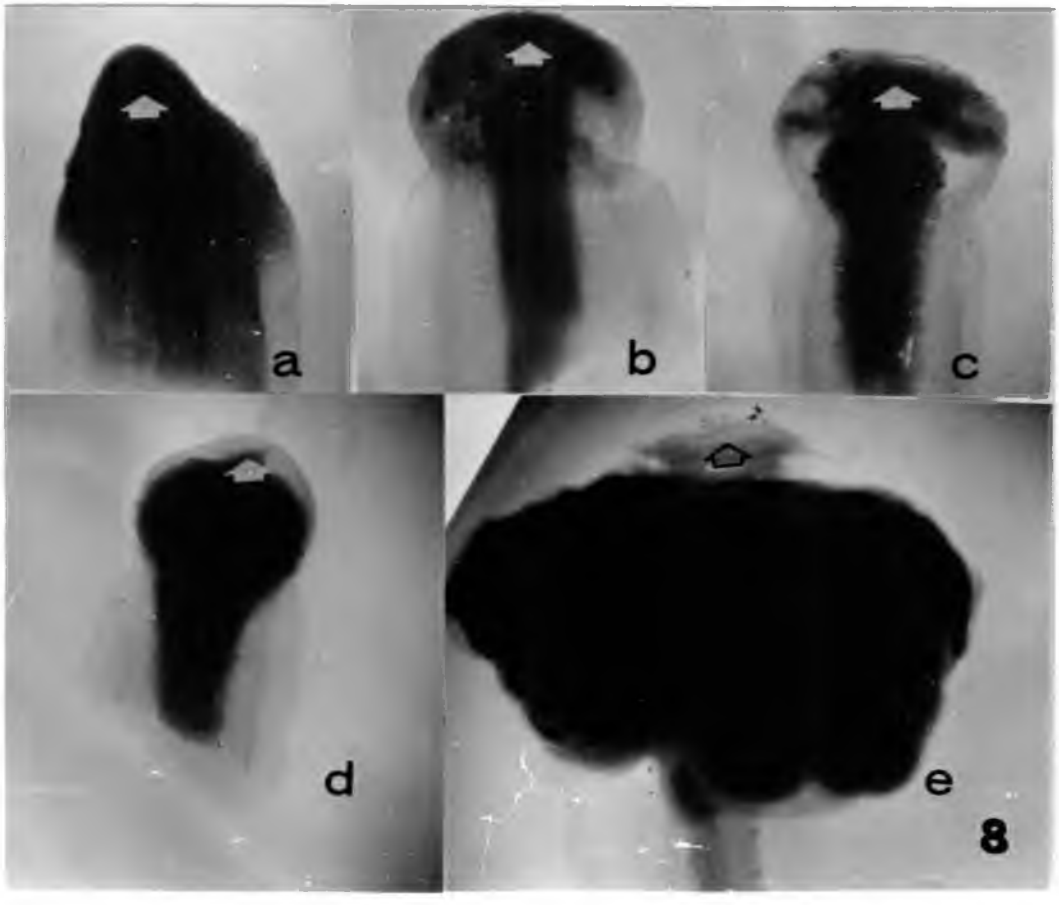
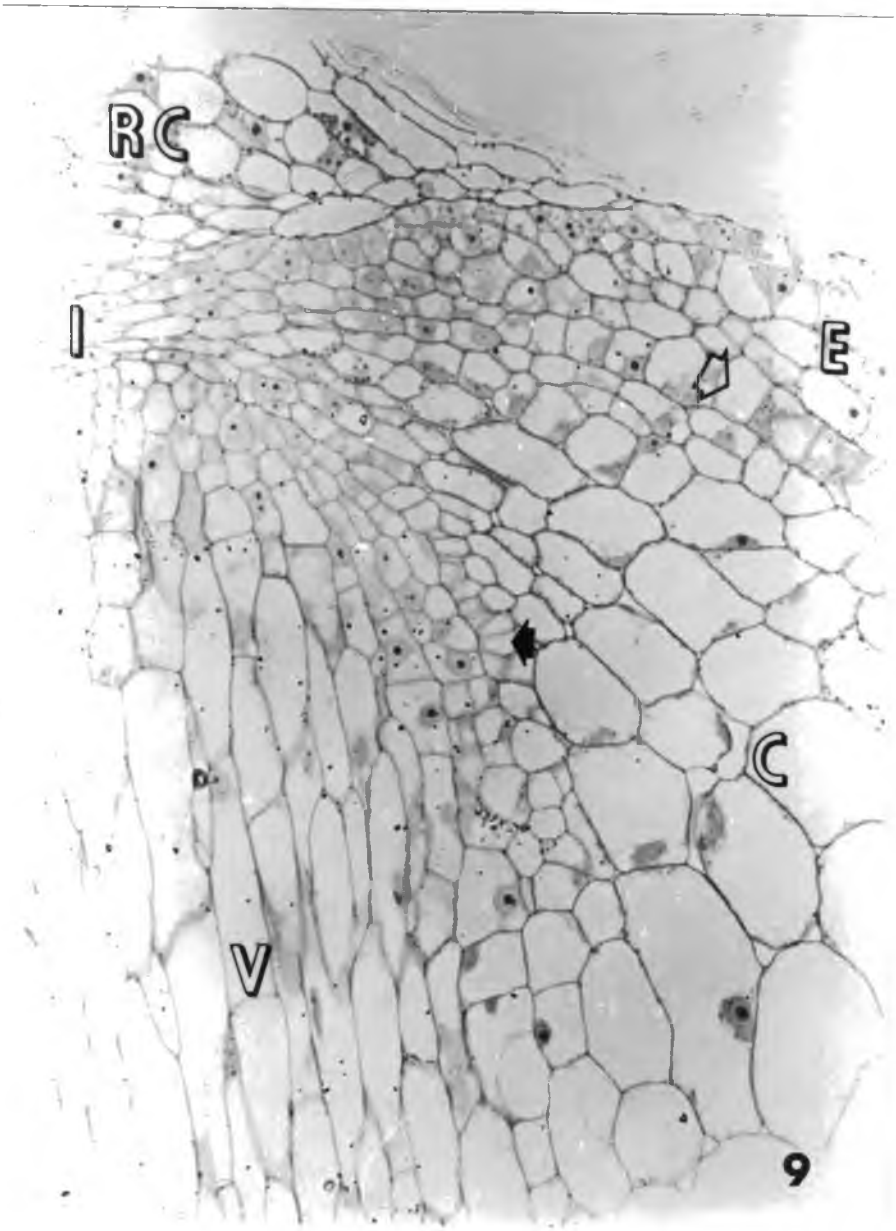

a
b
c
d
e
8

FIGURE 9

A median longitudinal section of root tip on 2nd day (Phase I) showing extensive radial growth. Apical initials (I) are highly vacuolated. Epidermal cells below the rim (E) and short cells of exodermis (hollow arrow) have expanded. In vascular cylinder (V), some peripheral cells (black arrow) have begun to expand radially. 160 X.


RC
I
E
C
V
9

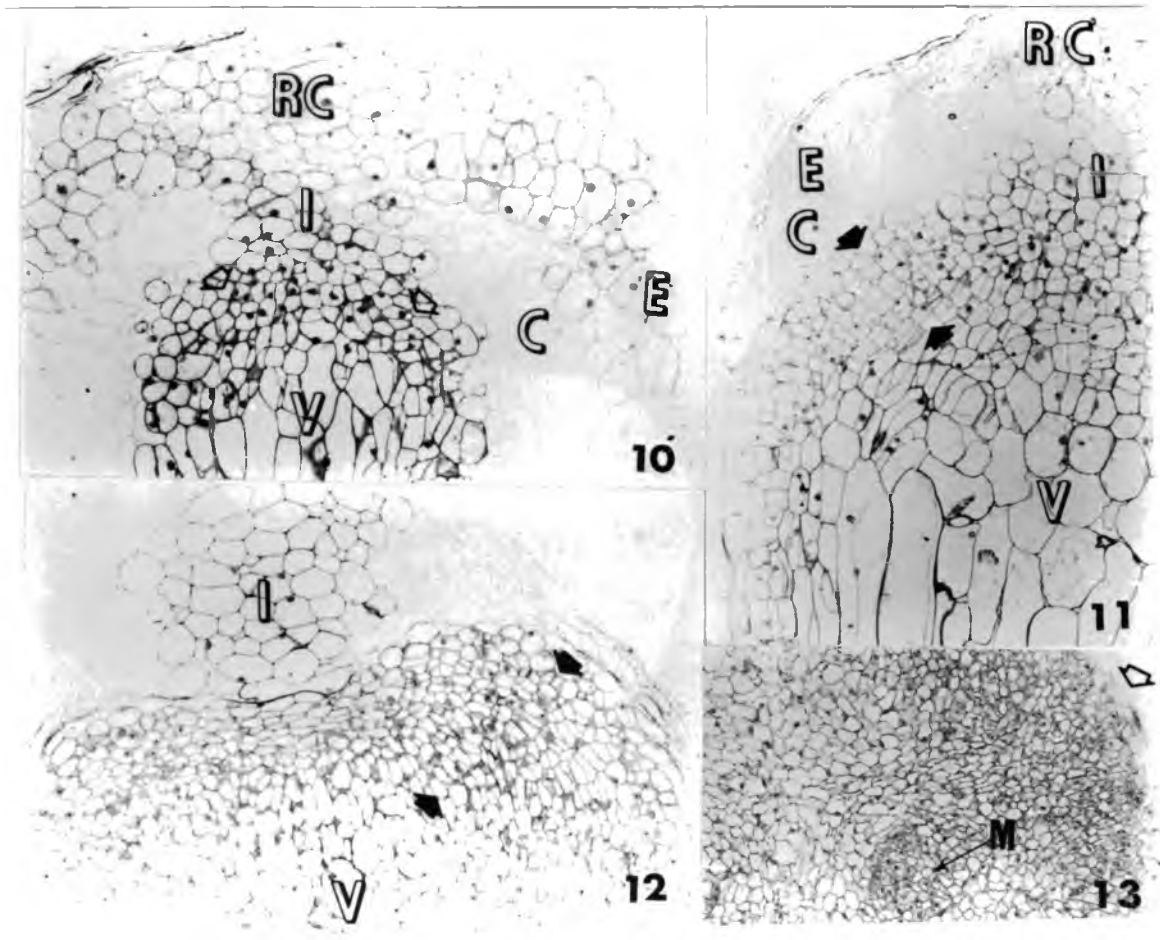
FIGURES 10-13

Fig. 10. A median longitudinal section of root tip on the 7th day showing completaly collapsed cortex and small persisting calls (arrows) in periphery of vascular cylinder (V). Also shown are greatly expanded root cap cells (RC) and apidermal cells (E). 120 X.

Fig. 11. A section of root tip on the 15th day (Phase III) showing an increase of calls in periphery of vascular cylinder (between arrows). Also shown are completely collapsed root cap (RC), epidermis (E), and cortex (C). Initial cells (I) are greatly expanded. 120 X.

Fig. 12. A section of root tip on 25th day (Phase IV) showing the emergence of new cells at periphery of vascular cylinder (between arrows) and the disorganization of vascular cylinder (V). The initial cells have become highly vacuolated (I). Only remains of the cell walls of root cap, epidermis, and cortex can be seen. 120 X.

Fig. 13. Section of root tip on 45th day (Phase IV) showing a cluster of maristematic cells (M) surrounded by highly vacuolated cells of the tumor. 120 X.


RC
I
E
C
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10
RC
E
C
I
V
11
I
V
12
M
13

III. Fine Structural Observations

Multivesicular Bodies

Phase 0

Figure 14 is a thin section through vascular initial cell and the structures here are similar to meristematic cells of other plants. In the cytoplasm, mitochondria are still without distinct internal cristae, plastids are without distinct lamellae or starch grains and ribosomes and polyribosomes are dense. Golgi complex and rough endoplasmic reticulum are frequently present, and multivesicular godies (MVB) are not present as yet. In the nucleus, the nucleolus is small.

Phase I

Figure 15 shows vascular initial cells on the second day of sub-culture. The cytoplasm of these cells is marked by the appearance of MVB's. The number of Golgi complex has also increased. Figure 16 is a higher magnification of the portion within the inset on Fig. 15. MVB's are either in the cytoplasm or in association with the cell surface. When a MVB is fused to the cell wall its vesicles can be seen between the plasmalemma and the cell wall. The cytoplasm appears dense due to an increased number of polyribosomes.

Cells of cortex and periphery of vascular cylinder 10 cells below the initials are shown in Fig. 17. In the

cytoplasm of the peripheral cells, MVB are conspicuous along the cell wall. Polyribosomes are prominent in the cytoplasm. However, the adjacent cortical cells lack MVB and have extremely thin cell walls.

Phase II

By the 5th day there are numerous MVB in the cells of the vascular initial layer and, as shown in Fig. 18, numerous vesicles are included between the plasmalemma and the cell wall. Figure 19 shows the internal structure of an MVB located in the proximity of obliquely cut cell wall. The MVB, in this case, contains numerous spherical vesicles as well as some tubules both of which are delineated by unit membranes.

As previously described in the anatomical section, cells at the periphery of the vascular cylinder occasionally undergo mitosis. Figure 20 is a portion of such cell in late telophase on the 7th day and shows a cell plate with vesicular inclusions which are similar to the internal vesicles of MVB shown in Fig. 19.

Cells adjacent to the degenerating cortical cells and comparable to Fig. 17 in Phase I are shown in Fig. 21. The cortical cells have collapsed and are represented by only remnants of cell walls. The expanding peripheral vascular cells, in this case, do not have MVB although Golgi complex is conspicuous. Cell walls have thickened and starch granules are present in plastids.

Phase III

On the 15th day, cells located near the central cylinder (Fig. 22) have numerous Golgi complexes. Occasionally the Golgi vesicles are enclosed in a larger vesicle similar in structure to MVB. Figure 23 is a portion of another cell in the same region as Fig. 22 and shows a few MVB associated with cell wall. The number of internal vesicles is less than in the previous phase (Phase II). MVB are closely associated with Golgi complex as shown in Fig. 24 which is another portion of the same cell shown in Fig. 23. The internal vesicles of MVB show the same characteristics as that of Golgi vesicles in their stainability as well as their appearance.

Phase IV

Figure 25 shows a portion of a cell located within the tumorous growth on the 45th day. These cells are from the area next to the cluster of meristematic cells which is marked "M" in Fig. 13. The MVB associated with cell wall is the only one observed in this cell and does not show distinct internal vesicular structure. An obliquely cut cell wall shows microtubules in cell wall. Another MVB is shown in Fig. 26 which is a part of the cell in the same area as Fig. 25. MVB, in this case, is completely devoid of internal vesicles while some fibrillar materials are included. Golgi complex is also located near the MVB.

Nucleolus

Phase 0

(A) Vascular cylinder

Figure 27 is a portion of the nucleus of an initial cell showing the nucleolus. The granular zone with ribosome-like particles which are not clearly discernible is located at the periphery. The fibrillar region is composed of compact fibrils. The light zone (L-zone) intrudes into the surface of the nucleolus through the granular region and is embedded in the fibrillar region. The L-zone is connected to a mass of heterochromatin which is situated along the nuclear envelope (NE). Condensed chromatin masses are characteristic of initial cells.

Figure 30 shows peripheral cells in zone of frequently dividing cells. Granular region at the periphery of the nucleolus consists of more conspicuous granules than the initial cell. The L-zone is connected to heterochromatic mass. Chromatin is included in the L-zone. The internally located L-zones are dispersed and form lacunae which are electron transparent.

A portion of an elongating cell 5 cells from the initials is shown in Fig. 28. The nucleolus has a distinct granular region which is larger than fibrillar region. The L-zone is embedded in the fibrillar region.

(B) Cortex

Figure 31 is a portion of a cell in late telophase 4

cells below the initials with a cell plate and reorganizing nucleolus. The deeply intruding L-zone is conspicuous and the rest of nucleolus is dense fibrillar region.

Figure 29 shows a portion of a cortical cell 15 cells below the initials and located near a raphid cell. The nucleolus is compact with fibrillar and granular components intermingled within a dense matrix. Intruding L-zone is lacking but lacunae are scattered.

Phase I

Figure 32 shows a nucleolus in vascular initial cell on the 3rd day. The granular region has increased in area and the components are more readily visible when compared to Fig. 27 in Phase 0. A karyosome is embedded in fibrillar region. L-zone is located internally and associated with lacuna.

Figure 33 is a nucleolus of peripheral cell 4 cells below the vascular initials. The granular region is better defined than in Fig. 32. L-zone is located internally and associated with lacuna. Karyosome is located near the peripheral granular region.

Figure 34 shows the structure of nucleolus in a cortical cell 4 cells from the initials. The granular region is greatly dispersed, L-zone is internal and scattered in the fibrillar region, and karyosome is attached to the peripheral granular region.

A portion of degenerating cortical cell located 10

cells from the initials is shown in Fig. 35. The nuclear envelope is invaginated. The nucleolus is compact and the components are not distinguishable. The karyosome is not attached to the nucleolus.

Phase II

A portion of a cell in late telophase from the periphery of vascular cylinder is shown in Fig. 36. The nucleolar components are scattered, the largest nucleolus showing intruding L-zone, some being associated with chromatin and all composed of only fibrillar materials.

Figure 37 is a portion of nucleus of a cell in the periphery of vascular cylinder on the 9th day. The L-zone is intruding at two different sites, internal L-zones and lacuna are located in the fibrillar region. Granular region is somewhat distinct. The karyosome is free within the nucleus.

Phase III

Figure 38 shows a portion of a cell among recently divided cells located near the disorganizing central cylinder on the 15th day. The fibrillar and granular zones are distinct. Some of the granular components are extended to the nuclear envelope. The L-zones are not present but a lacuna is located in fibrillar region. Ribosomes are abundant in cytoplasm.

Figure 39 is another cell in the same area on the 15th day showing the fibrillar and granular region of nucleolus on separate sides of the nucleolus. The granular region is greatly extended and dispersed. L-zones and lacuna are absent.

The cell adjacent to the one in Fig. 39 is shown in Fig. 40. The segregation of the granular and the fibrillar regions are less distinct and lacuna is irregular and within fibrillar region.

Phase IV

A portion of a cell in meristematic area as indicated in Fig. 12 is shown in Fig. 41. The granular regions are not well differentiated. L-zone is present, either intruding at the surface or in fibrillar region. In the cytoplasm, polyribosomes are abundant.

Figure 42 is a portion of a cell in telophase in a meristematic area of tumor on the 45th day. One of the daughter cells shows many nucleoli. Two have characteristic intruding L-zones. A portion of the nucleus of the other daughter cell has a nucleolus with L-zone intruding at two different sites; one passing through the nucleolus.

The observations of fine structure as related to each phase are presented in Table VI.

TABLE VI. SUMMARIZATION OF OBSERVATIONS OF FINE STRUCTURE

Phase	Anatomical remarks of cells	Nucleolus				MVB
		Granular region	L-zone	Lacuna	Karyosome	
0	Initials	+ 1/	S 2/	+ 3/	+ 4/	0 5/
	Meristematic	++	S,I	++	+++	0
	Elongating	+++	I	+++	+	0
	Cortical	0	0	++	+	0
I	Slow expanding	++++	I	+++	++++	+++
	Rapid expanding	0	0	+	+	0
II	Dedifferentiating	* ++	S,I	++	++	++++
	Degenerating	0	0	+	+	0
III	Vacuolated	+++++	0	++++	0	++
IV	Meristematic	++	S,I	++	++	+
	Elongating	+++	I	+++	++	+

1/ Granular space and dispersion of components of granular region, 0 - +++++

2/ Presence of L-zone and its location, 0 = absent; I = internal; S = surface

3/ Space occupied by lacuna, + - ++++

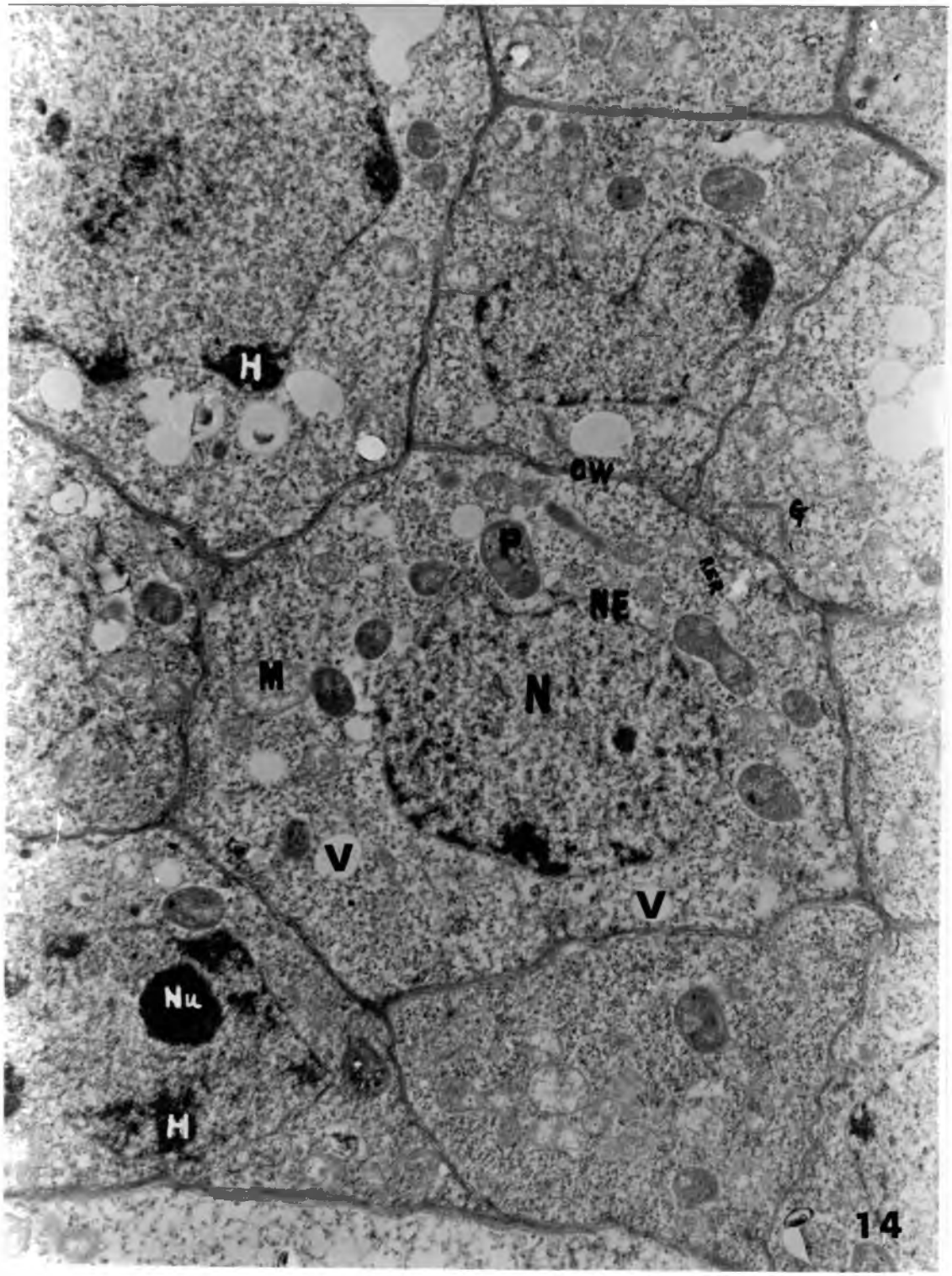
4/ Frequency of karyosome attachment to nucleolus, 0 - ++++

5/ Frequency of occurrence of MVB, 0 - ++++

* Multiple nucleoli occurrence

FIGURE 14

Vascular initial cell in Phase 0. Plastids (P) are without distinct lamellae or starch grains, mitochondria (M) are without internal cristae, ribosomes and polyribosomes are dense, Golgi complex (G) and rough endoplasmic reticulum (RER) are sparse, nucleoli (Nu) are small, and masses of heterochromatin (H) are attached to nuclear envelope. 10,000 X.


H
CW
P
NE
M
N
V
V
Nu
H
14

FIGURES 15, 16

Vascular initials on 2nd day of Phase I.

Fig. 15. Section through vascular initials showing numerous multivesicular bodies (arrows) in the cytoplasm and cell surface. Golgi complex (G) are also abundant. V = vacuole, P = plastid, N = nucleus. 10,000 X.

Fig. 16. A higher magnification of inset on Fig. 15 showing MVB (MV) in cytoplasm as well as inclusion of vesicles between plasmalemma and cell wall (arrow) and polyribosomes (P_o). V = vacuole, P = plastid. 45,000 X.

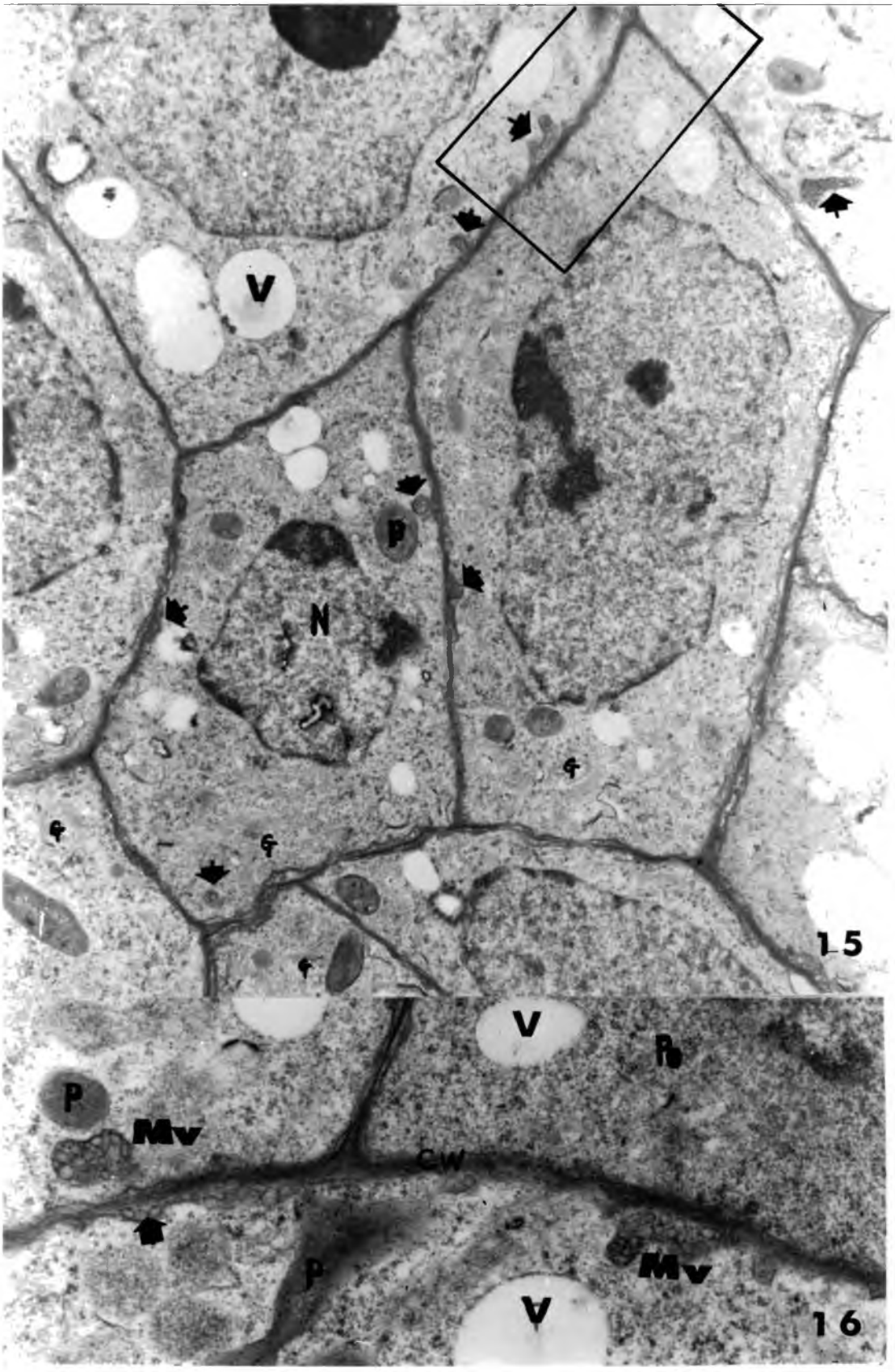
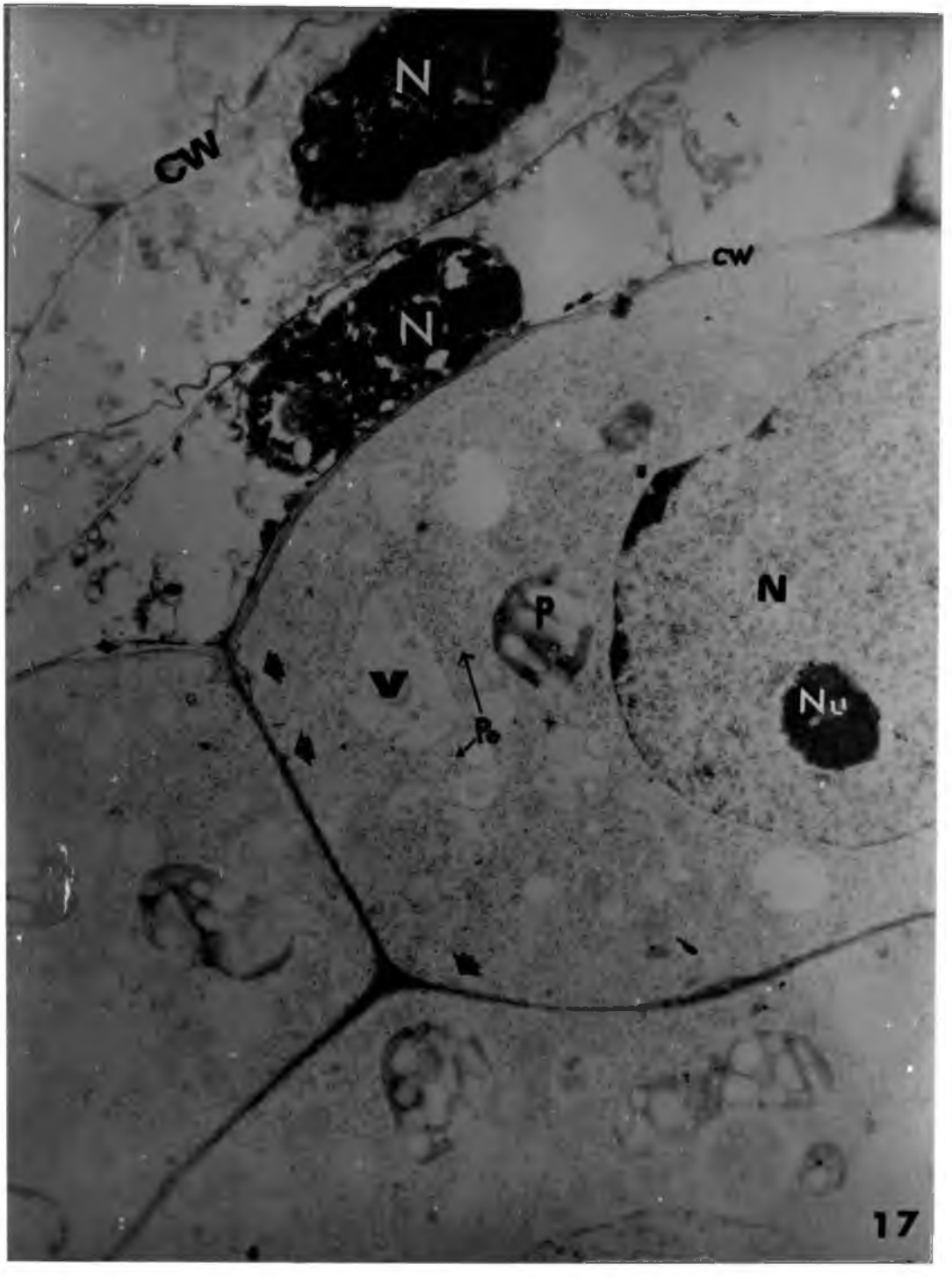

V
P
N
G
G
G
G
15
V
P
Mv
CW
P
V
Mv
16

FIGURE 17

Portion of cells of cortex and of the periphery of vascular cylinder in Phase I. Cell of periphery of vascular cylinder shows numerous MVB (arrows) and prominant polyribosomes (Po). Cells of degenerating cortex show dense nuclei (N) and thin cell walls (CW). P = plastid, Nu = nucleolus. 10,000 X.

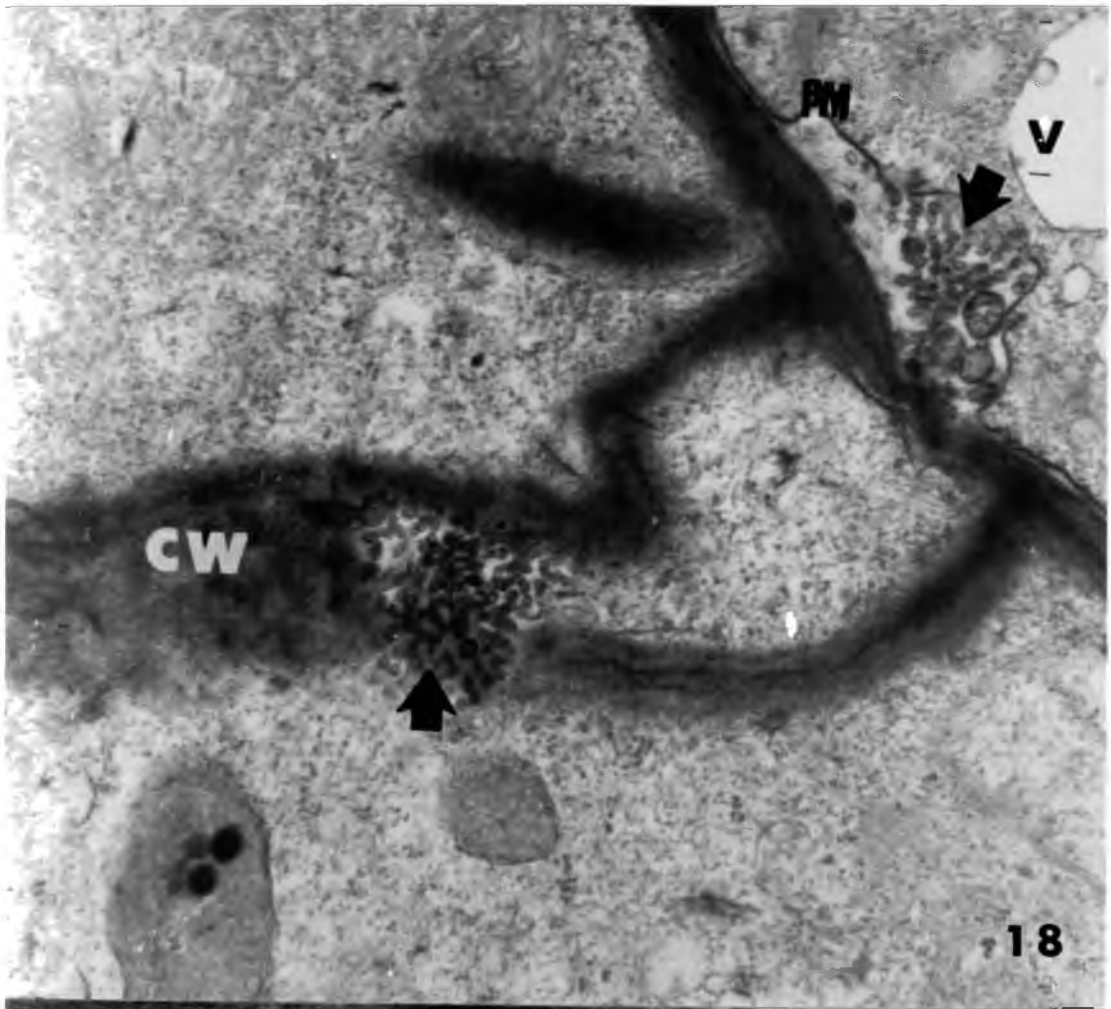

N
CW
CW
N
N
P
V
Nu
17

FIGURES 18, 19

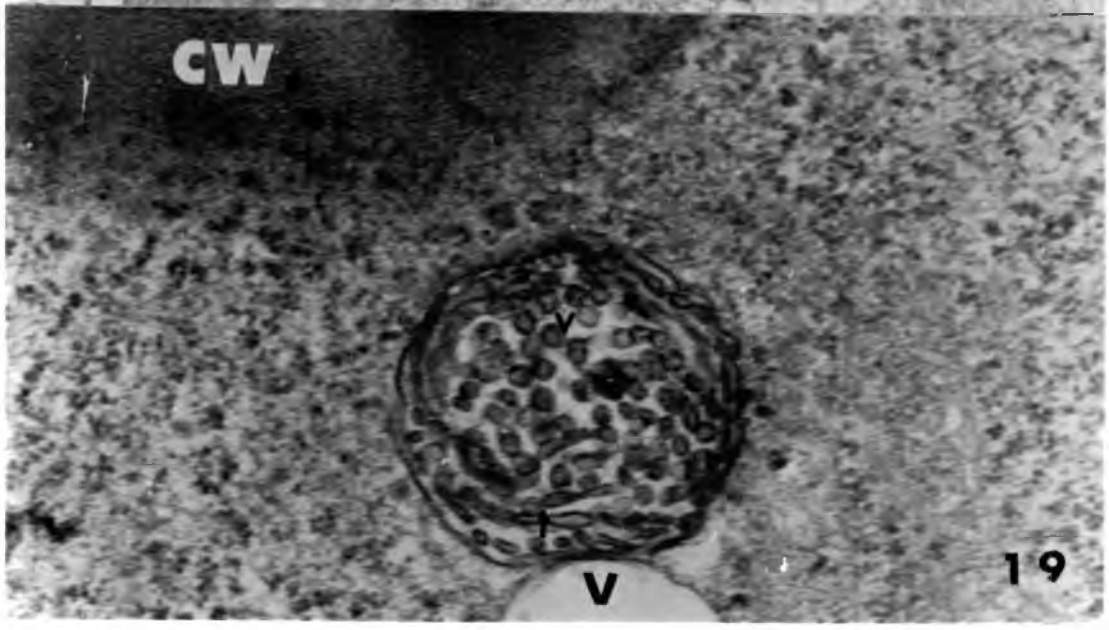
Portion of vascular initials in Phase II.

Fig. 18. A part of vascular initial showing MVB (arrows) between cell wall (CW) and plasma membrane (PM). V = vacuole. 30,000 X.

Fig. 19. Another portion of vascular initial showing internal structure of MVB consisting of spherical vesicles (v) and tubules (t). MVB is in proximity of obliquely cut cell wall (CW). V = vacuole. 45,000 X.



18



19

FIGURE 20

Cell plate in vascular cylinder cell in Phase II. A portion of cell in late telophase at the periphery of vascular cylinder on 7th day (Phase II) shows vesicles (arrows) in cell plate (CP). N = nucleus, V = vacuole. 30,000 X.

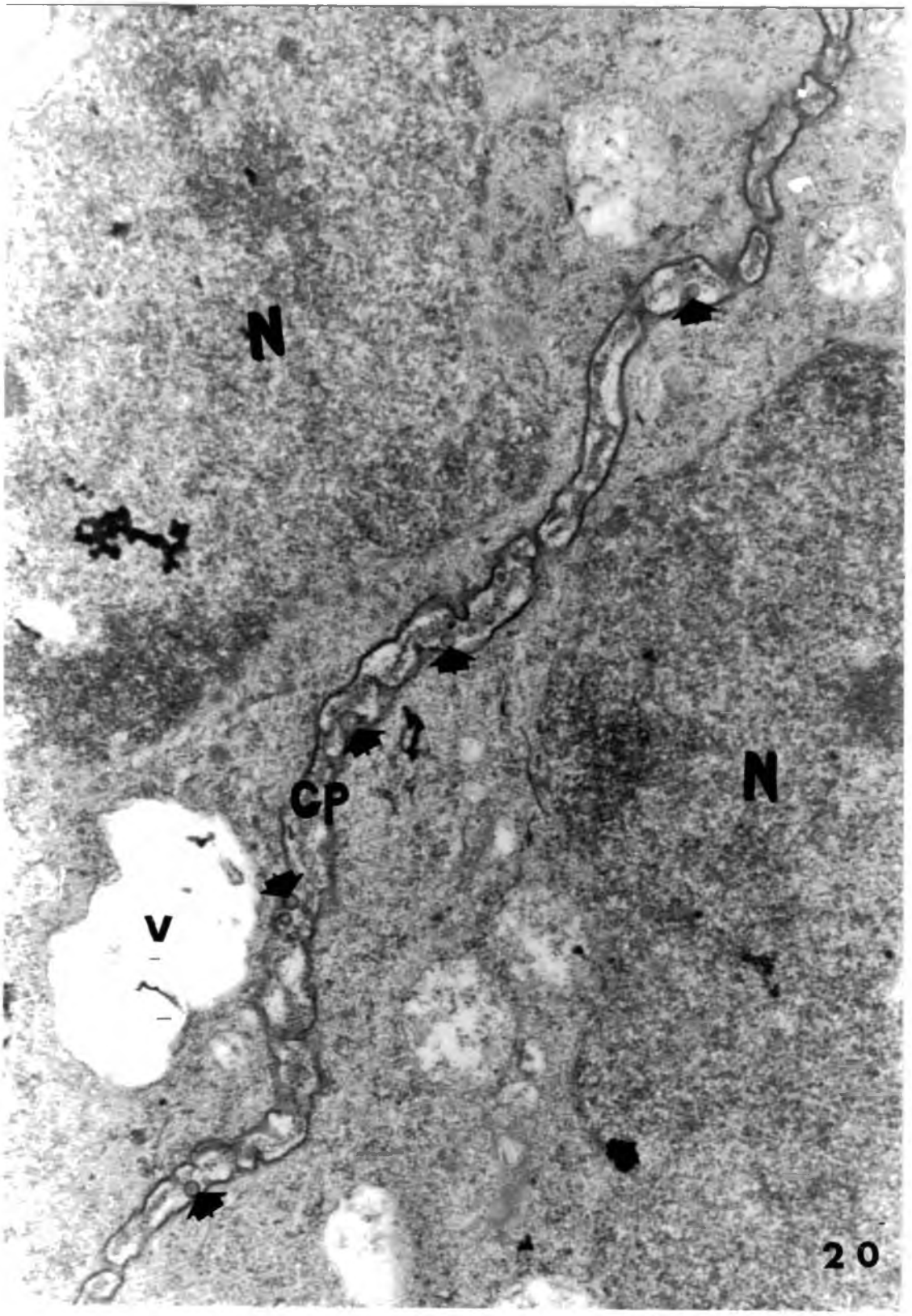
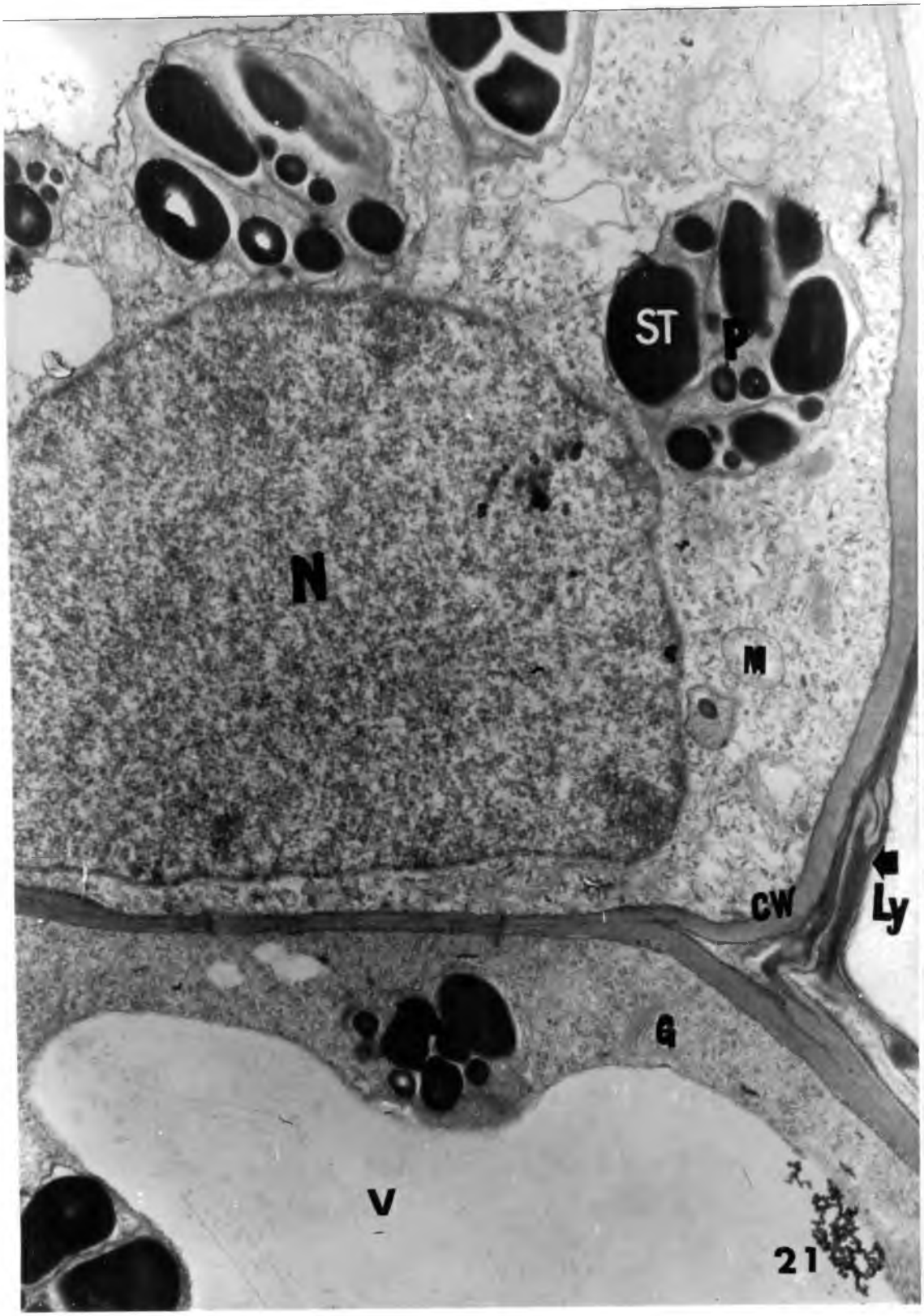

N
CP
N
V
20

FIGURE 21

Cells at periphery of vascular cylinder in Phase II. MVB's are lacking, cell walls (CW) have thickened, starch grains (ST) are prominent in plastids (P), and Golgi complexes are present (G). In collapsed cortical cell (LY), only cell wall remains (arrow). M = mitochondria, N = vacuole. 18,300 X.


ST
P
N
M
CW
Ly
G
V
21

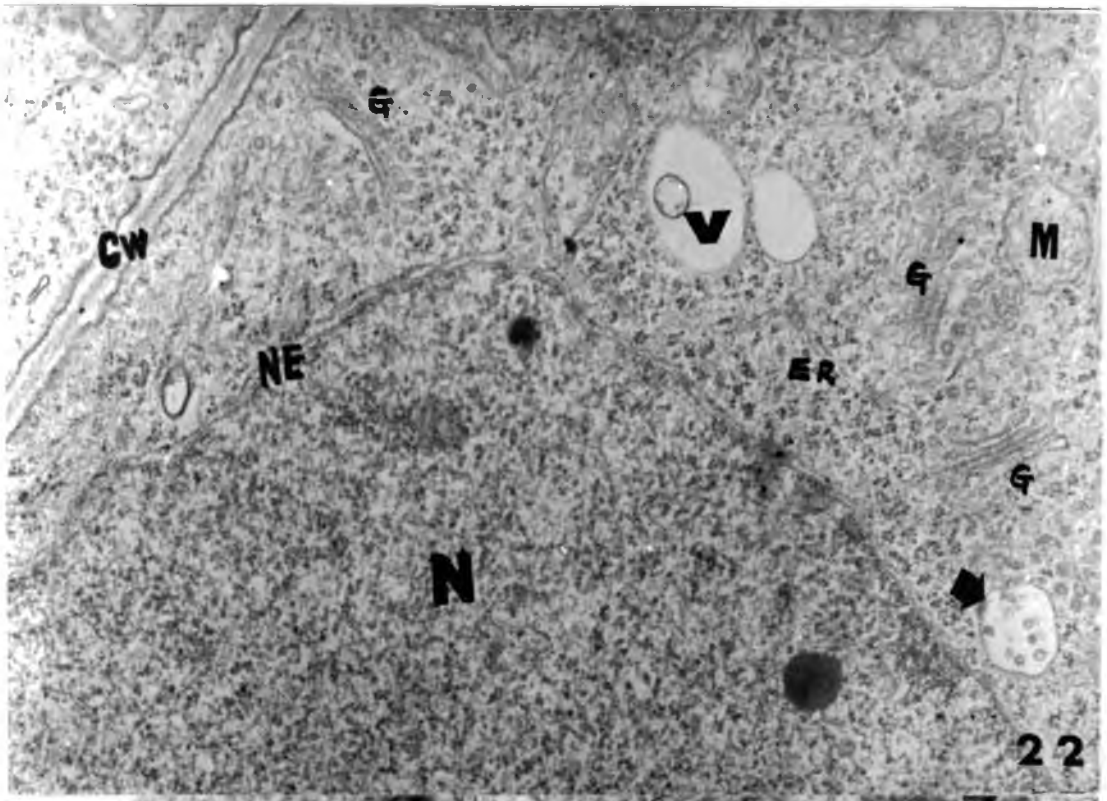
FIGURES 22 - 24

Peripheral central cylinder cells in Phase III.

Fig. 22. Portion of cell on 15th day showing numerous Golgi complexes (G), and Golgi vesicles forming MVB-like structure (arrow). CW = cell wall, N = nucleus, NE = nuclear envelope, V = vacuole, ER = endoplasmic reticulum, M = mitochondria. 30,000 X.

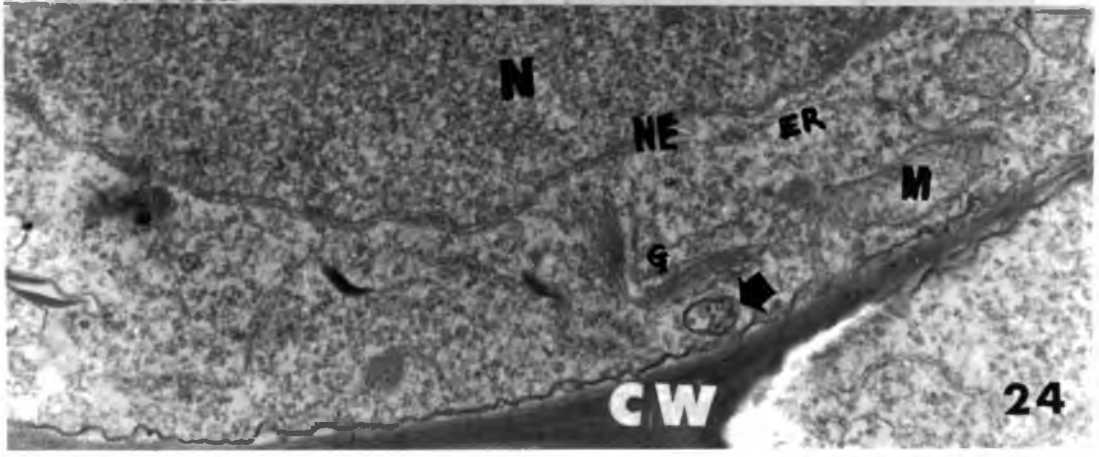
Fig. 23. A portion of another cell showing MVB (arrows) associated with cell wall (CW) but with fewer internal vesicles than in Phase II. V = vacuole. 23,200 X.

Fig. 24. Another portion of same cell as in Fig. 23 showing the proximity of MVB (arrow) to Golgi complex (G). N = nucleus, NE = nuclear envelope, ER = endoplasmic reticulum. 23,200 X.



22

23



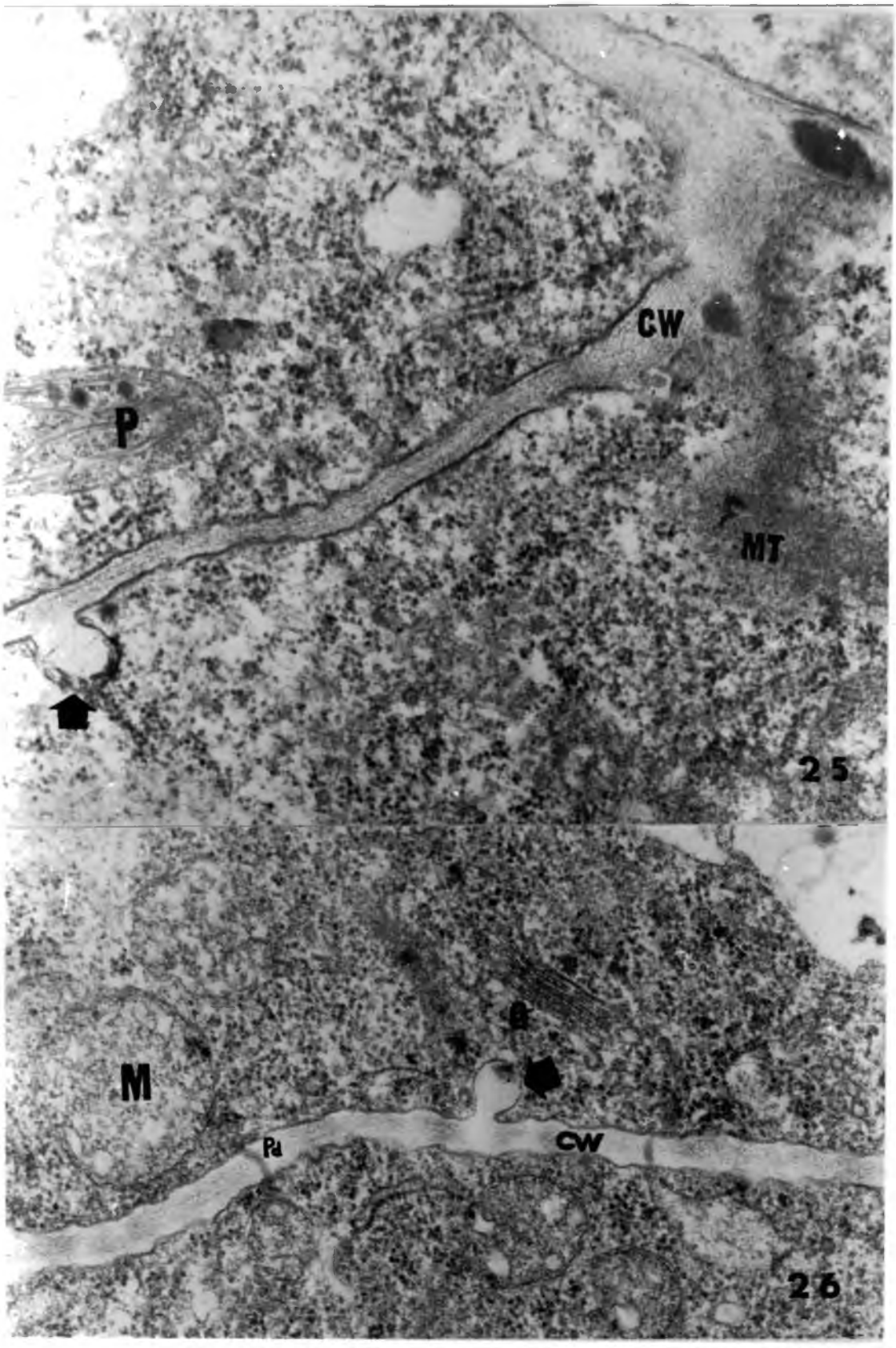
24

FIGURES 25, 26

Cells within tumorous growth in Phase IV.

Fig. 25. Portion of cell showing MVB (arrow) associated with cell wall but without distinct internal vesicles and microtubules (MT) in obliquely cut cell wall (CW). P = plastid. 45,000 X.

Fig. 26. Portion of cell showing MVB (arrow) lacking internal vesicles but possessing fibrillar structures and Golgi complex (G) nearby. M = mitochondria, Pd = plasmodesmata, CW = cell wall. 45,000 X.


CW
P
MT
25
M
Pd
CW
26

FIGURES 27-29

Structure of nucleolus in Phase 0.

Fig. 27. A portion of nucleus of vascular initial showing granular (G) region, fibrillar (F) region and L-zone (L). L-zone is associated externally with heterochromatin mass (H) which borders nuclear envelope (NE). 30,000 X.

Fig. 28. Nucleolus in elongating cell situated 5 cells below vascular initials. Granular region (G) is dispersed and defined, and L-zone (L) is located within fibrillar region (F). Nu = another nucleolus, NE = nuclear envelope. 30,000 X.

Fig. 29. Nucleolus in cortical cell situated 15 cells below initials. Nucleolus is compact and indenting. L-zone is lacking but lacunae (Lc) are located in central part of nucleolus. 30,000 X.

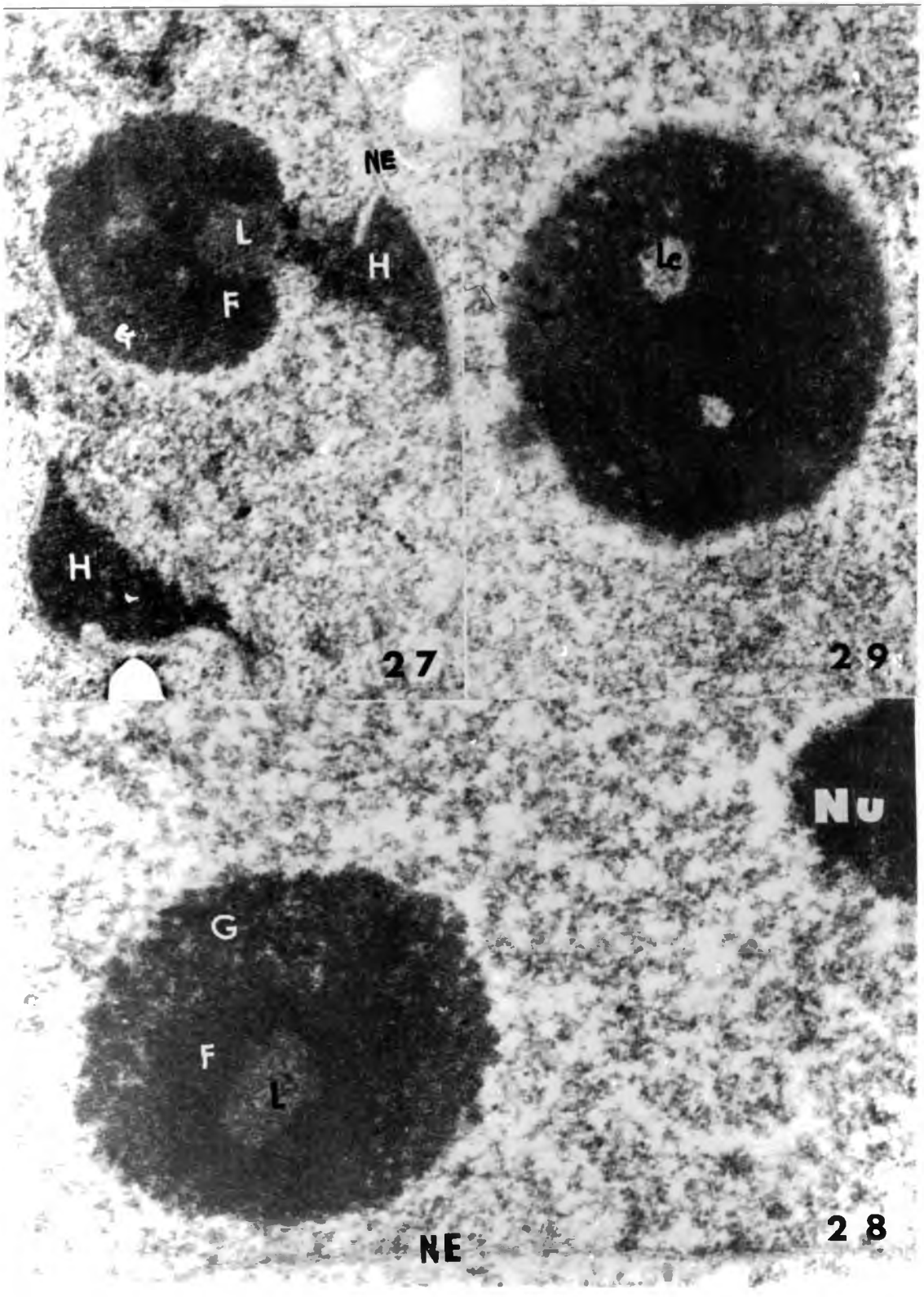

NE
L
H
F
G
H
27
Le
29
Nu
G
F
L
NE
28

FIGURE 30

Nucleolus in a cell of dividing zone at periphery of vascular cylinder in Phase 0. Granular region (G) with ribosome-like particles is at the periphery, and fibrillar region (F) with compact fibrils is in the central part. The L-zone intrudes the surface and contains chromatic materials (white arrows). The dispersed L-zones are lacuna (black arrows) which are electron transparent. H = heterochromatin. 45,000 X.

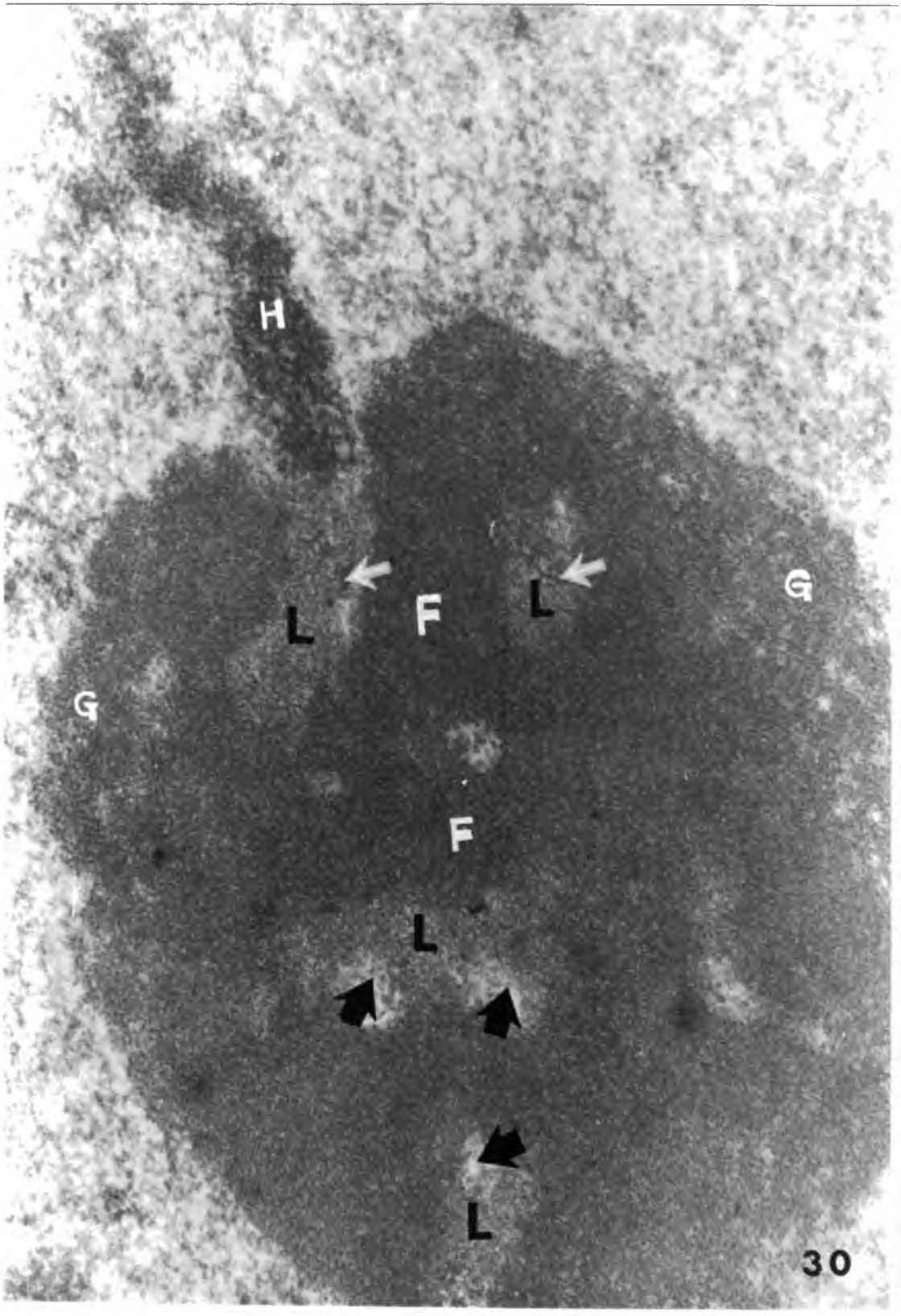
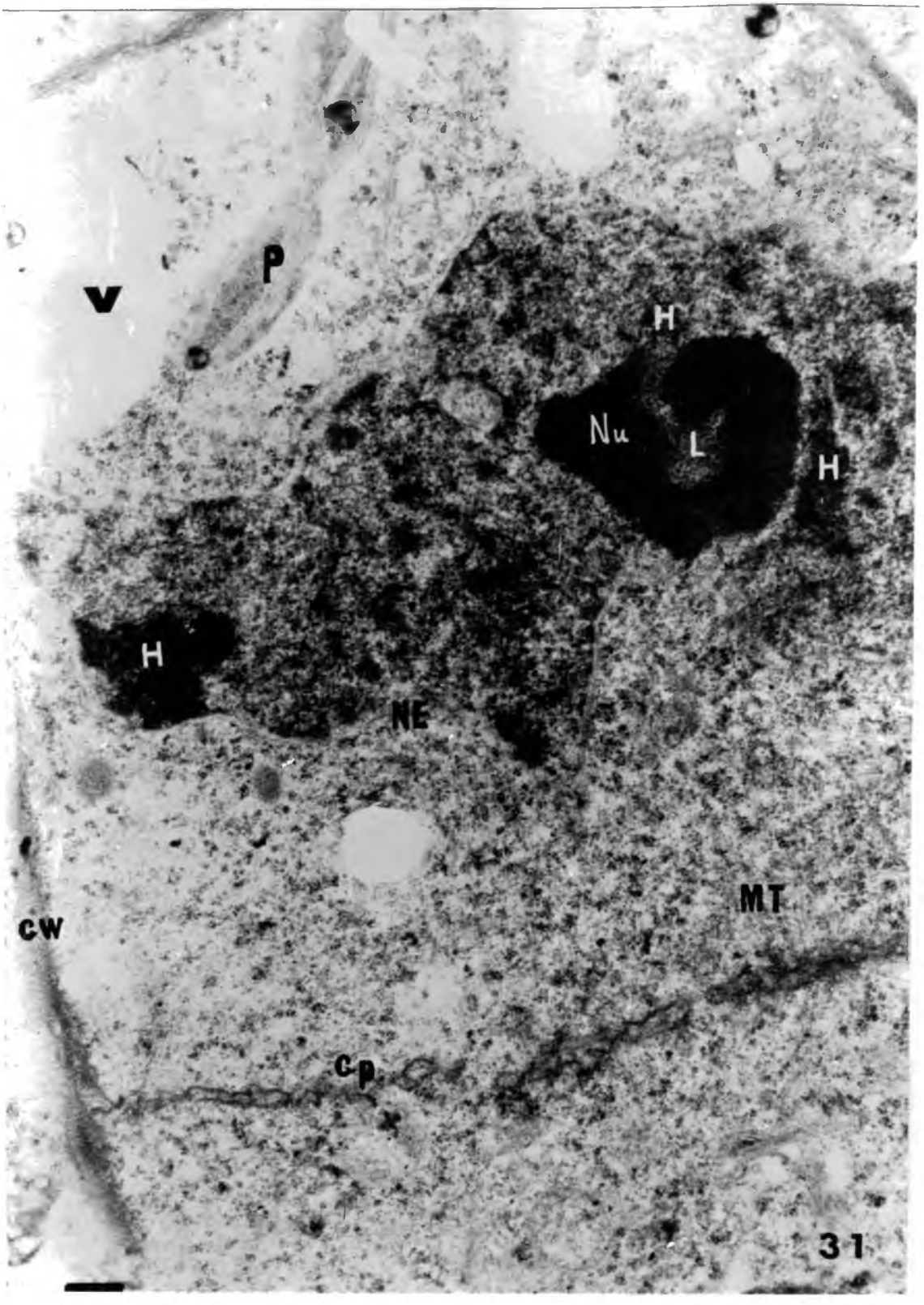

H
L
F
L
G
G
F
L
L
30

FIGURE 31

Portion of cortex cell in late telophase, Phase D. The reorganizing nucleolus (Nu) consists of deeply intruding L-zone (L) associated externally with heterochromatin (H) and compact fibrillar materials. MT = microtubules, NE = nuclear envelope, P = plastid, Cp = cell plate, V = vacuole, CW = cell wall. 18,300 X.


V
P
H
Nu
L
H
H
NE
MT
CW
CP
31

FIGURES 32-34

Nucleoli in cells in Phase I.

Fig. 32. Nucleolus in vascular initial cell showing karyosome (K) embedded in fibrillar region (F), internal L-zone (L), and lacunae (Lc) in fibrillar region. G = granular region. 45,000 X.

Fig. 33. Nucleolus of peripheral cell situated 4 cells below the vascular initials. Karyosome (K) is nearly separated, granular region (G) is better defined, and lacunae (Lc) and L-zone (L) are within fibrillar region (F). 30,000 X.

Fig. 34. Nucleolus in a cortical cell situated 4 cells below the initials. The granular region (G) is widely dispersed. Karyosome (K) is attached to granular region, L-zone (L) is internal and scattered, Lacunae (LC) is in fibrillar region (F). 45,000 X.

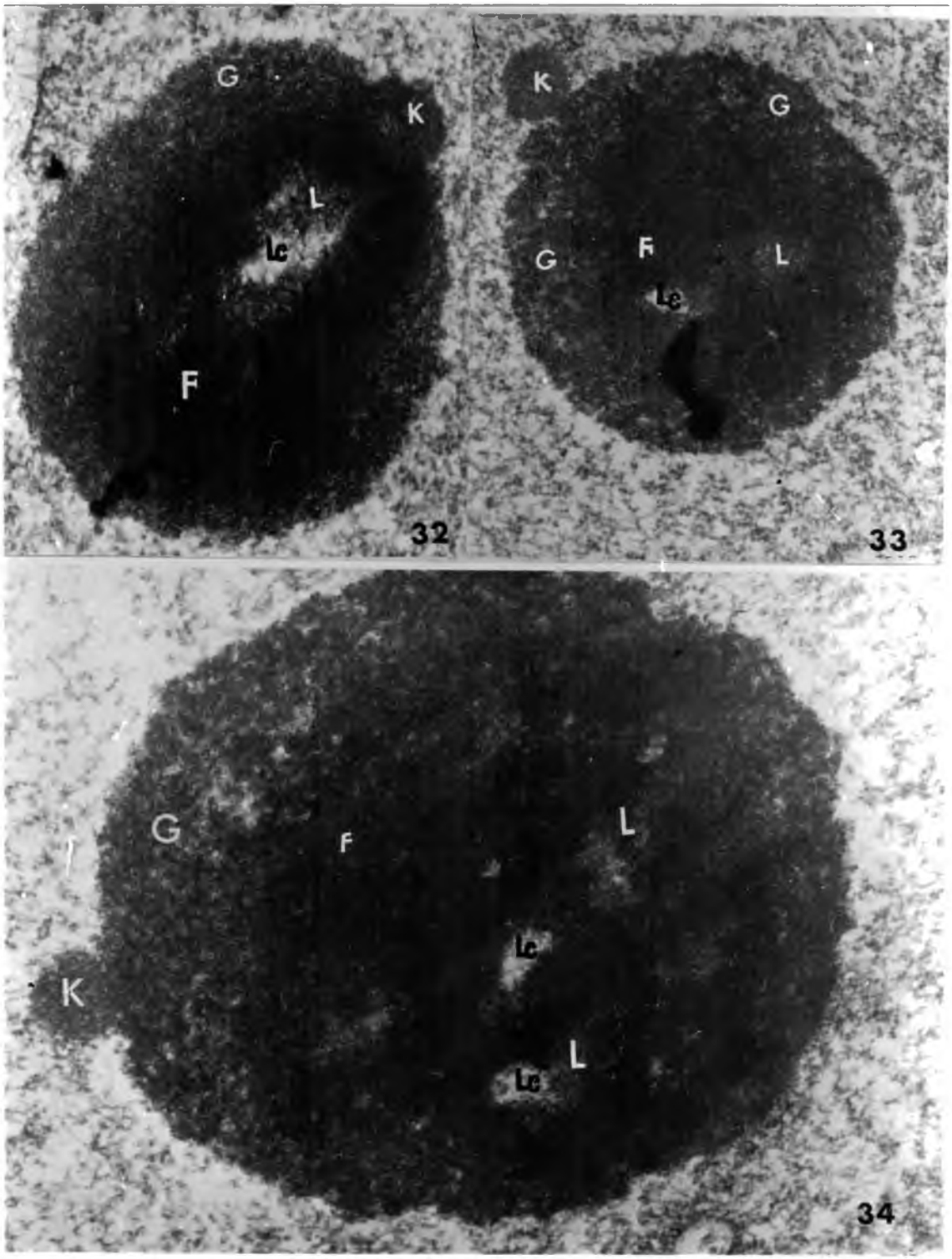

G
K
L
Lc
F
32
K
G
G
F
L
Lc
33
G
F
L
Lc
K
L
Lc
34

FIGURE 35

Nucleolus in degenerating cortical cell, Phase I. Nucleolus (Nu) is compact and components are not distinguishable. Karyosome (K) is not attached to the nucleolus. The nuclear envelope (NE) is invaginated. V = vacuole, CW = cell wall. 30,000 X.

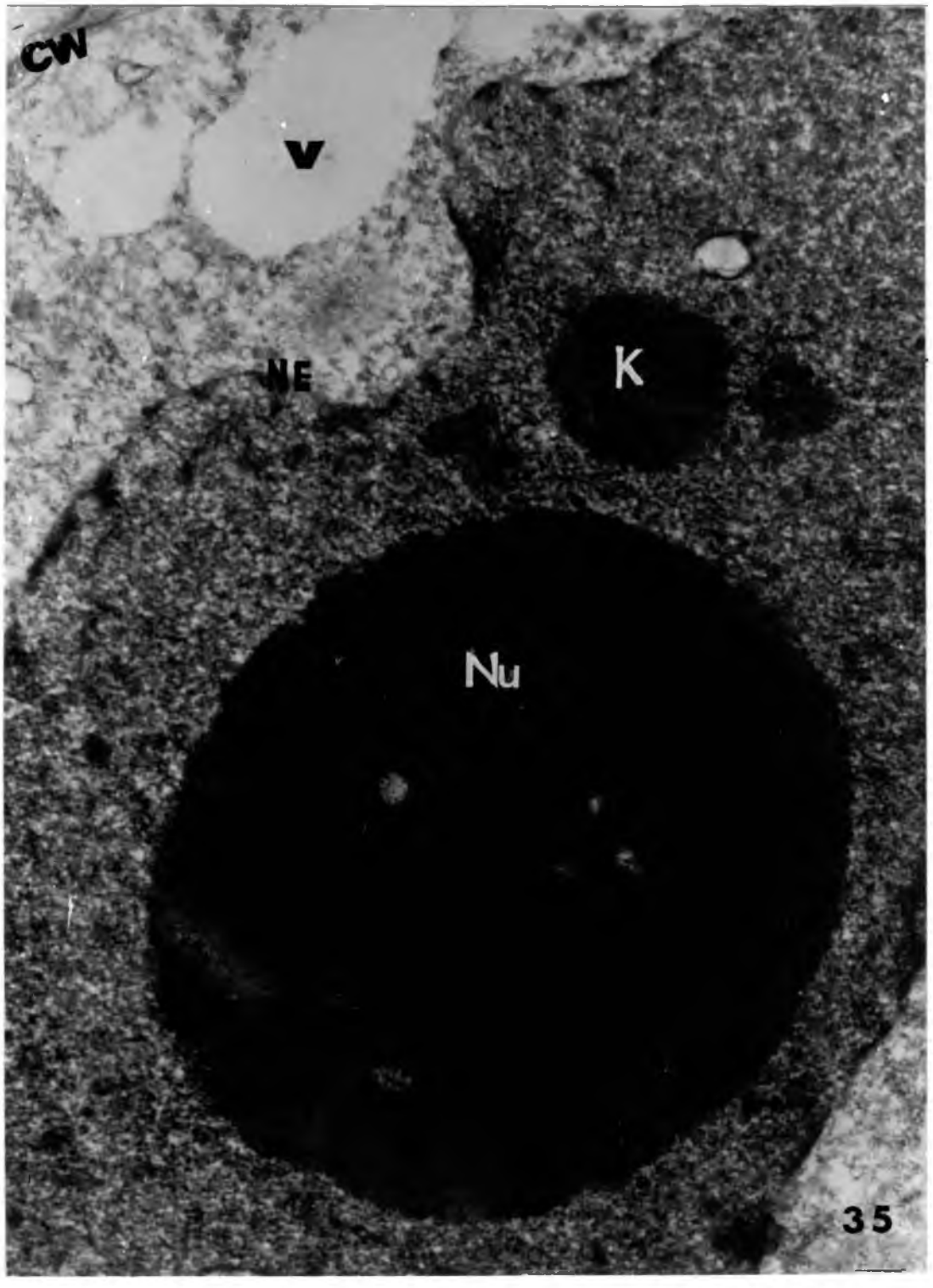

CW
V
NE
K
Nu
35

FIGURE 36

Portion of cell in telophase at periphery of vascular cylinder, Phase II. Numerous, small nucleoli (Nu) are scattered in nucleoplasm. The largest nucleolus (black arrow) has intruding L-zone (L) while others are associated with chromatin (white arrow). NE = nuclear envelope, Cp = cell plate. 30,000 X.

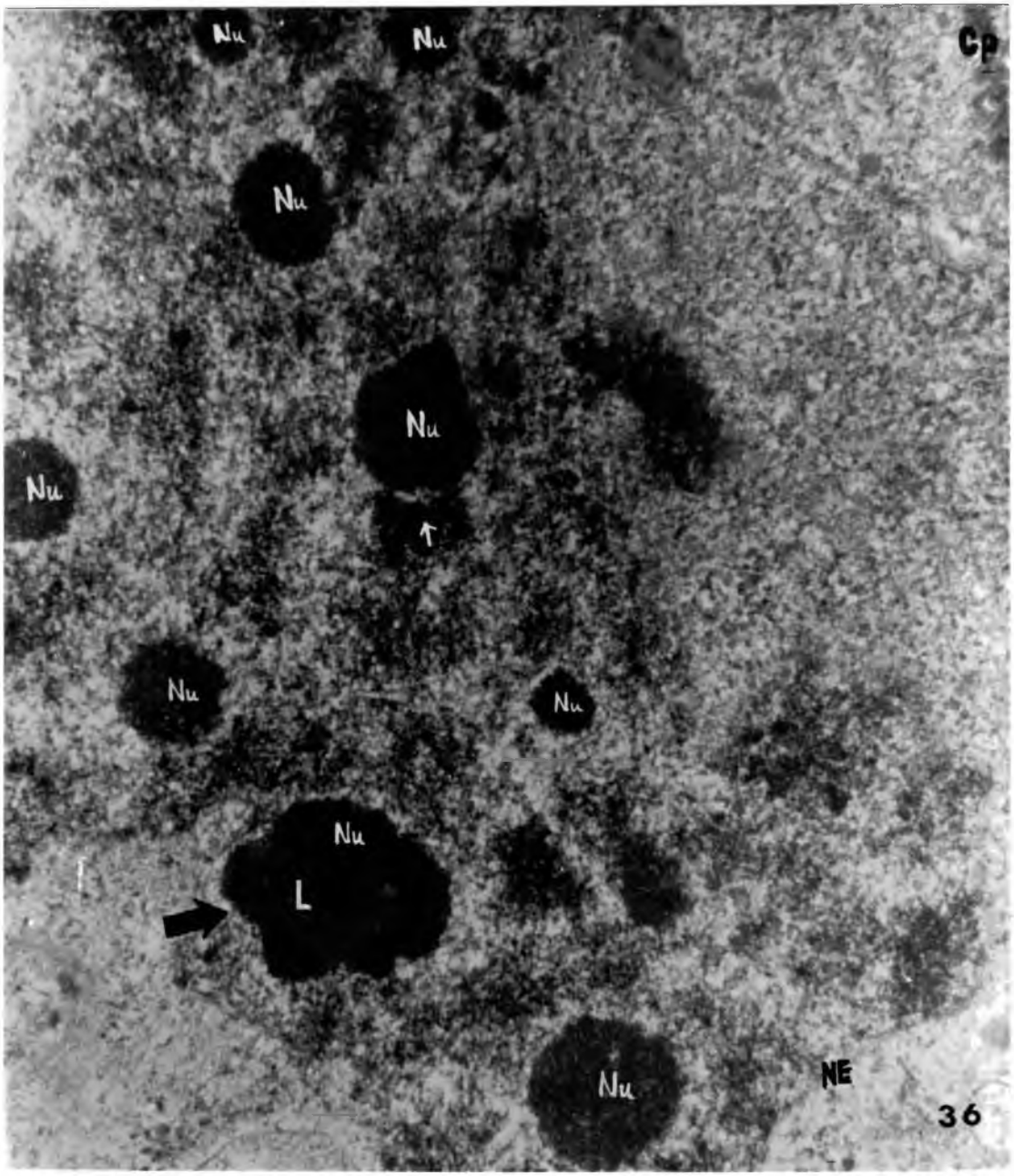

Nu
Nu
Cp
Nu
Nu
Nu
Nu
Nu
Nu
L
Nu
NE
36

FIGURE 37

A portion of nucleus of cell in periphery of vascular cylinder, Phase II. L-zone (L) is intruding at two different sites. Some internal L-zones and lacunae (Lc) are located in fibrillar region (F). Granular region (G) is somewhat distinct and karyosome (K) is free within the nucleus. P = plastid, NE = nuclear envelope, V = vacuole, CW = cell wall. 23,200 X.

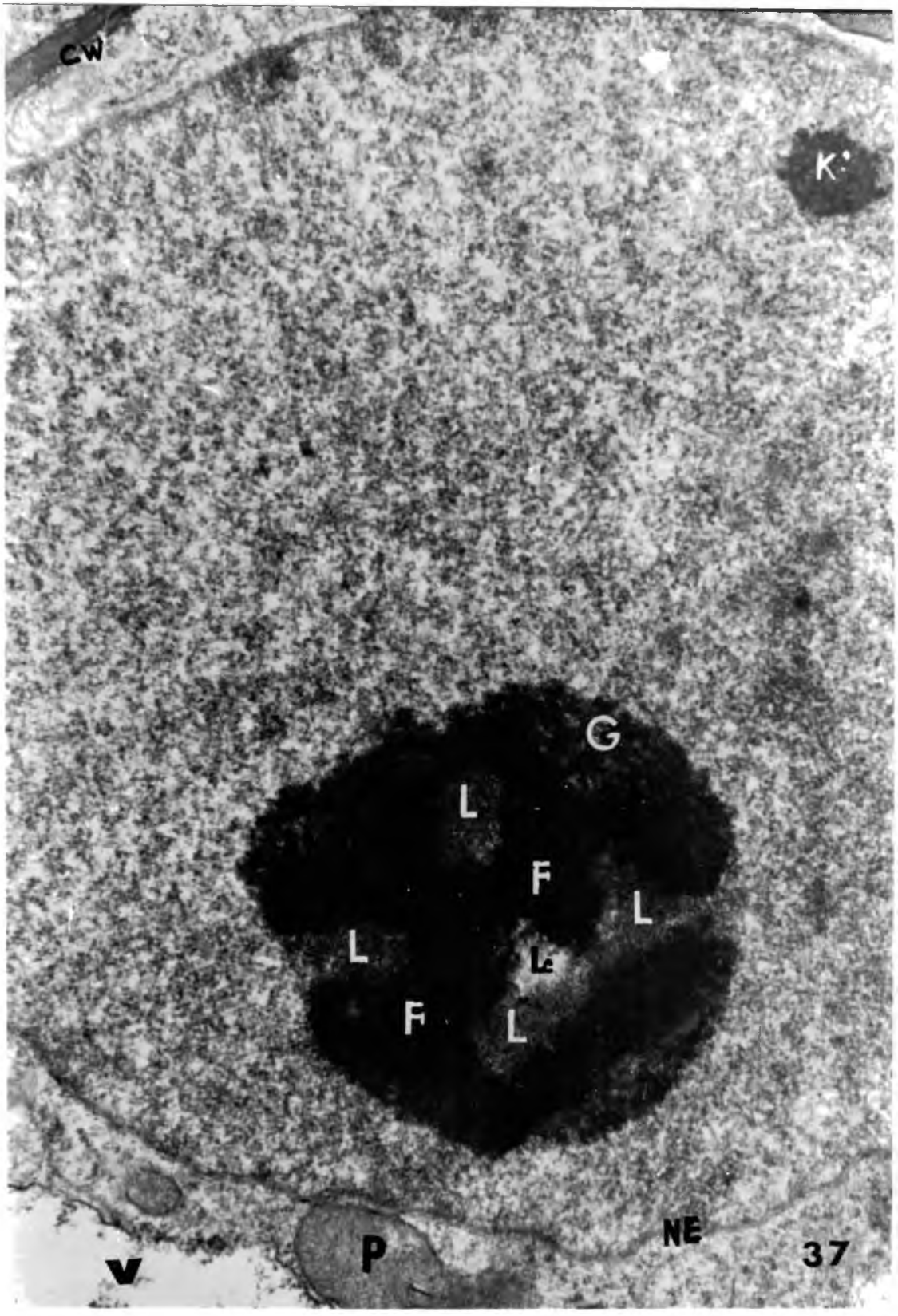
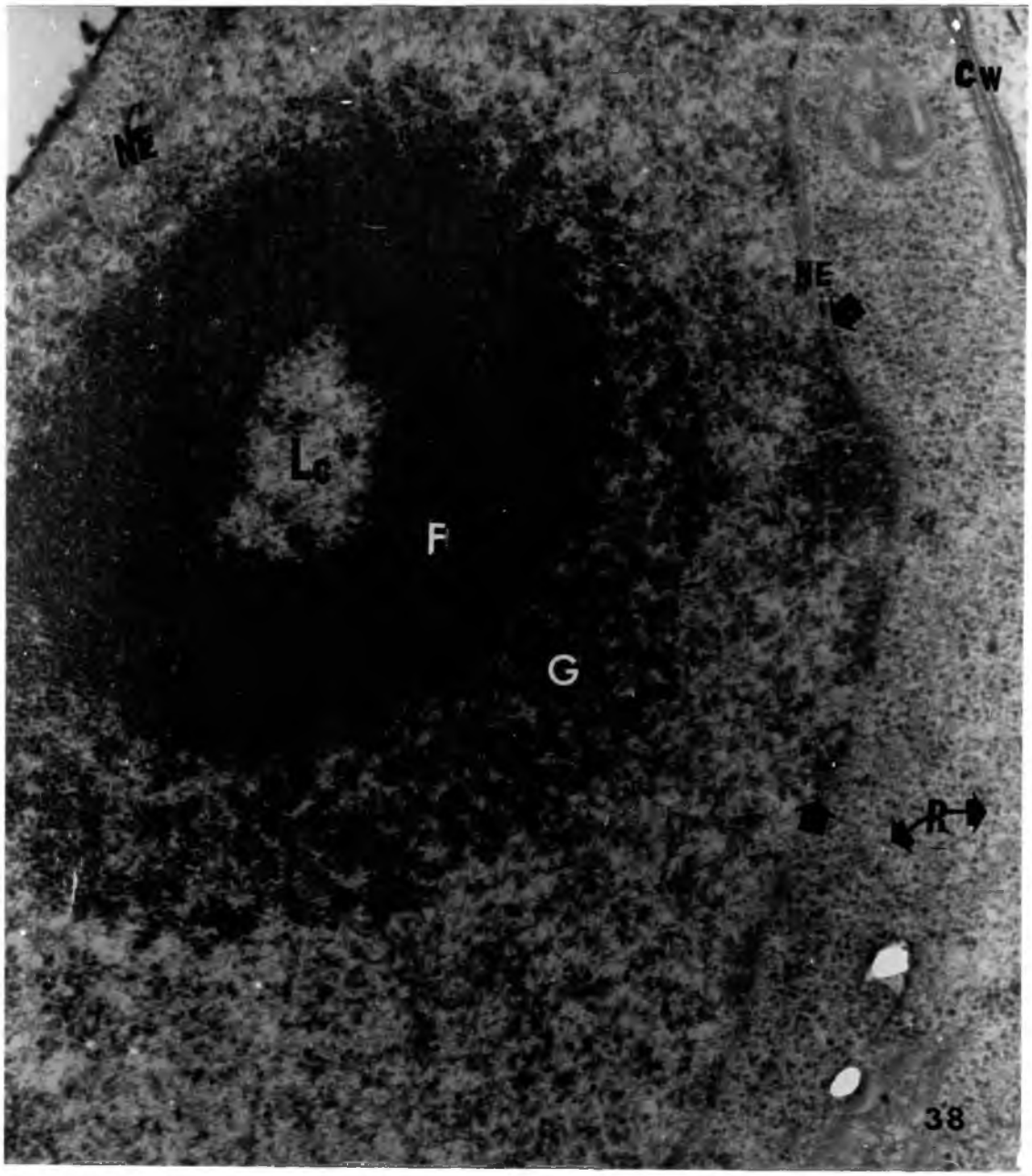

CW
K
G
L
F
L
L
Le
F
L
V
P
NE
37

FIGURE 38

Portion of a cell near disorganizing central cylinder, Phase III. The granular (G) and fibrillar regions (F) are distinct. Some granular components (arrows) have extended to the nuclear envelope (NE). L-zones are not present but a lacuna (Lc) is located in fibrillar region. Ribosomes (R) are abundant in cytoplasm. CW = cell wall. 45,000 X.


NE
Cw
NE
Lc
F
G
R
38

FIGURES 39-40

Portions of cells near disorganizing central cylinder, Phase III.

Fig. 39. Portion of nucleus showing well separated fibrillar (F) and granular (G) regions. Numerous ribosomes (R) are present in cytoplasm. NE = nuclear envelops. 30,000 X.

Fig. 40. Portion of the nucleus in a cell adjacent to the one in Fig. 39 showing a nucleolus with developing granular region (G). Lacuna (Lc) is irregular and within fibrillar region (F). NE = nuclear envelope. 30,000 X.

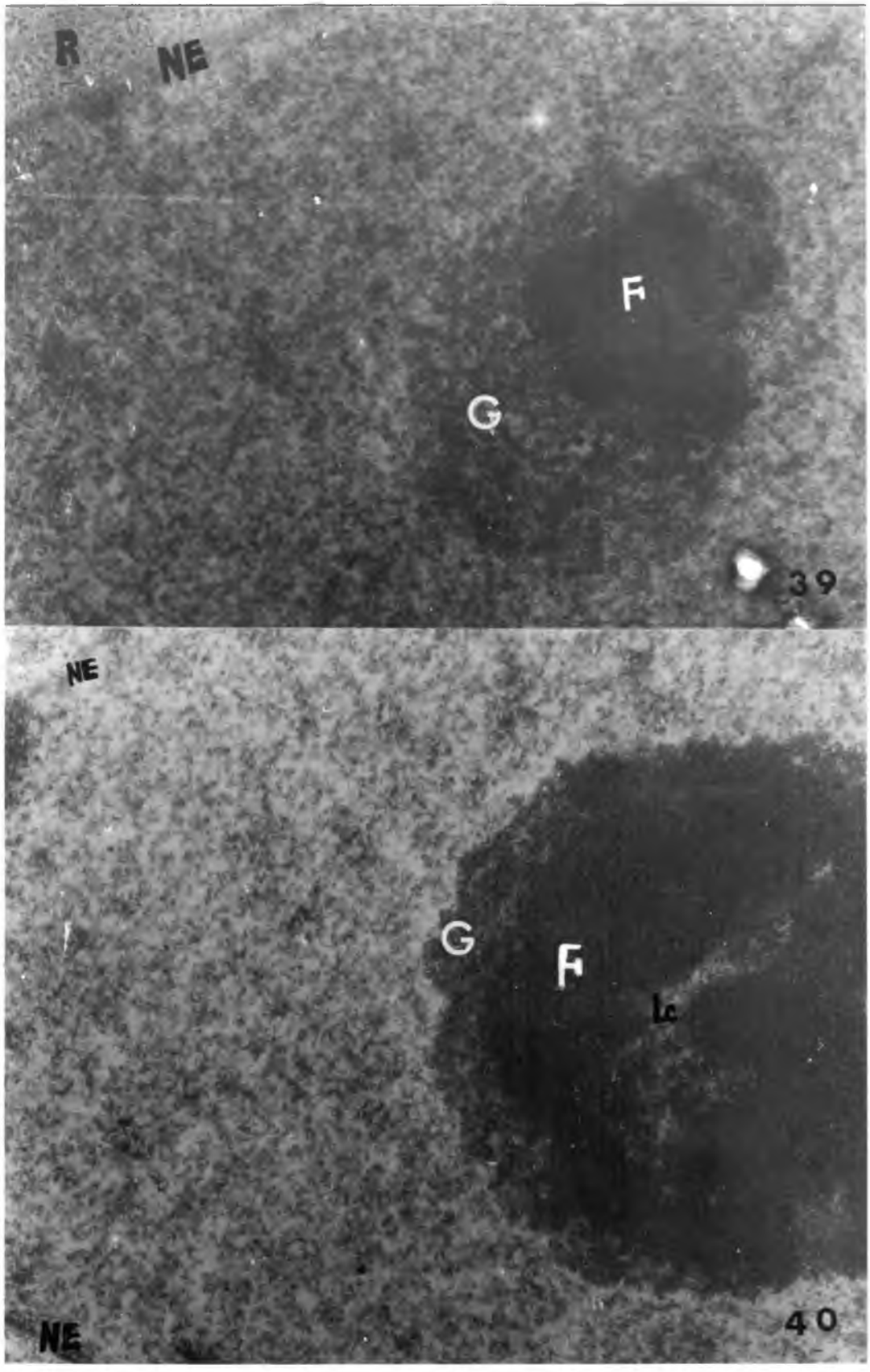

R
NE
F
G
39
NE
G
F
Lc
NE
40

FIGURE 41

A portion of cell in meristematic area on 25th day, Phase IV. Polyribosomes (Po) are abundant in cytoplasm. The nucleolus has intruding and internal L-zones (L). Granular region (G) is not well differentiated. NE = Nuclear envelope, M = mitochondria, V = vacuole, P = plastid, CW = cell wall. 30,000 X.

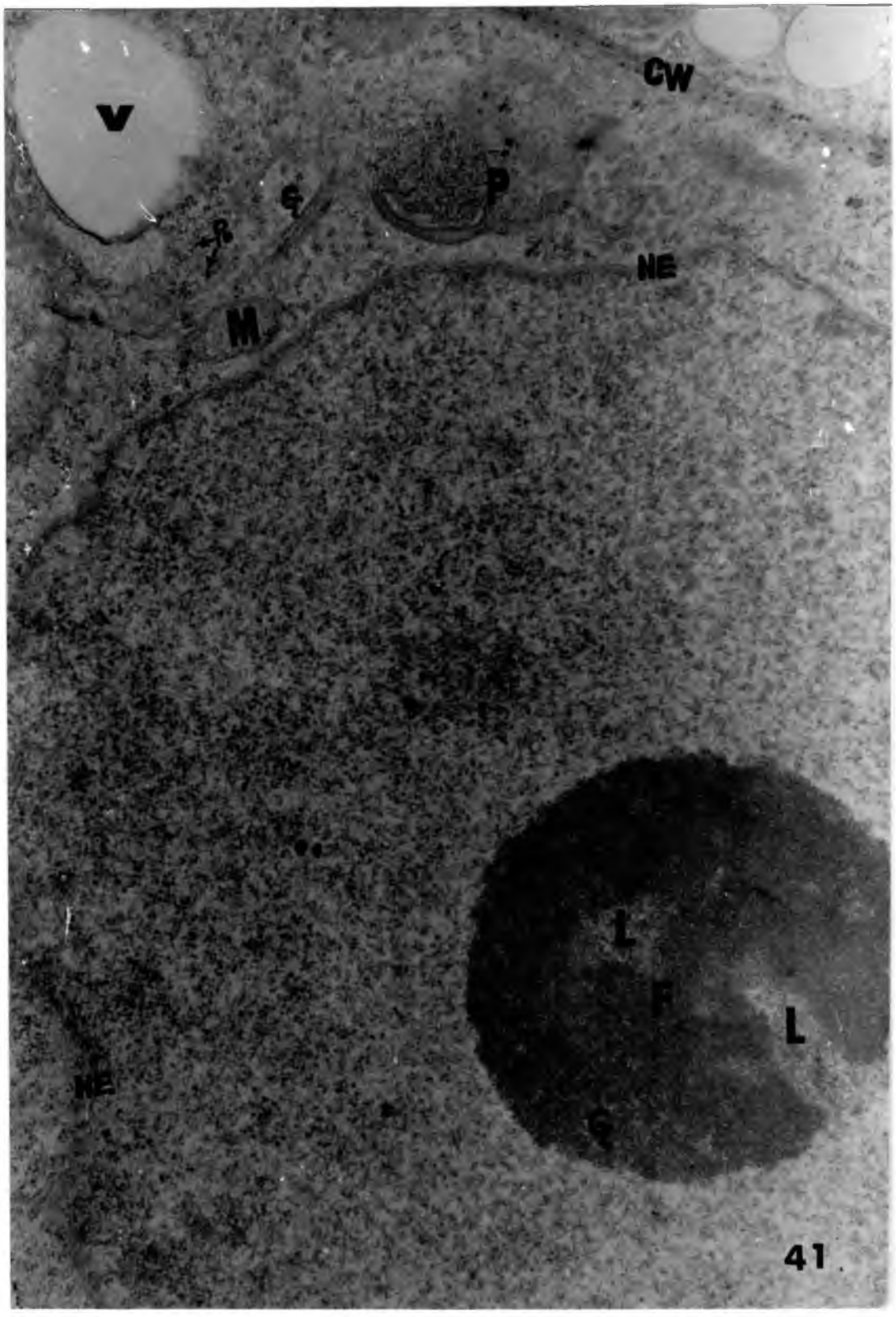
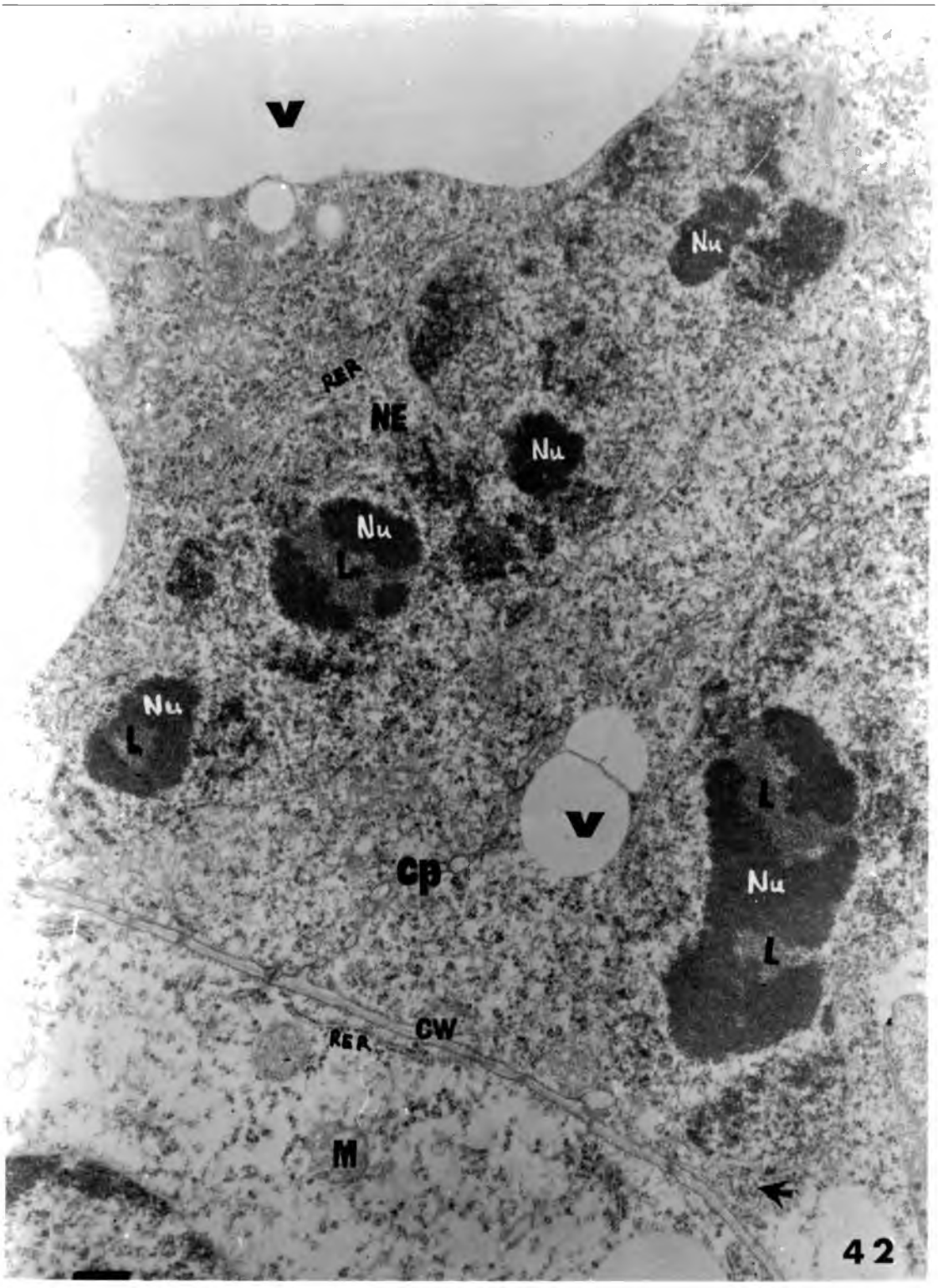

V
CW
P
NE
M
L
F
L
NE
41

FIGURE 42

A portion of cell in telophase in meristematic area on 45th day, Phase IV. The daughter cell, on the left, shows four nucleoli (Nu) in restituting nucleus. Two have characteristic intruding L-zone (L). The daughter cell, on the right, has a single nucleolus with two intruding L-zones (L), one passing through the nucleolus (Nu). The cell plate (Cp) is present as well as occasional MVB (arrow). RER = rough endoplasmic reticulum, NE = nuclear envelope, V = vacuole, CW = cell wall, M = mitochondria. 18,300 X.


V
Nu
RER
NE
L
Nu
Nu
L
Nu
L
V
L
Cp
Nu
L
RER
CW
M
42

DISCUSSION

Although in aseptic culture of orchid plantlets, seeds, and shoots, medium containing 2,4-D or other type of auxins have been used (Bergman, 1972), this is the first time that tumors have been reported to be induced which in turn gave rise to limited numbers of plantlets. However, the number of plantlets produced are too few to make this method commercially feasible as a technique for rapid clonal propagation. Other attempts to culture orchid root tips without auxins have failed (Churchill et al., 1971). In some other plants, abnormal growth at root tips has been observed in response to applied auxin-type herbicides (Gorter and Zweep, 1964; Taylor, 1946; Wilde, 1951; Beal, 1944).

The root tips of orchids, either attached to plantlets or excised, respond differently to various level of 2,4-D. At a low level (0.1 ppm) they grew normally; while at a higher level (5.0 ppm), they died. At an intermediate level (0.75-1.0 ppm), they mostly reacted by forming tumors.

Although auxins including 2,4-D are generally known to inhibit elongation of roots, in dilute solutions (10^{-4} ppm), elongation of root can be promoted (Kiermayer, 1964). The lowest level tested in this study is far higher than that reported by Kiermayer (1964). Since growth of orchid roots is very slow, measurement was difficult. However, at 0.1

ppm 2,4-D the level of auxin is still too low to disturb the normal fluctuation of auxin in the root, therefore, the normal growth of the root was not distrubed.

The addition of 0.75-1.0 ppm of 2,4-D starts to saturate the calls with auxin and prevents the normal regulation necessary for orderly growth as suggested by Van Overbaek (1964). The disruption of the control of apical organization, in turn, will induce random cell division resulting in tumors.

At a given level of 2,4-D (1.0 ppm), about half of the roots in a plantlet formed tumors. Plantlets having four roots showed the highest rate of tumor formation. Further increase in number of roots per plant resulted in a decrease in the rate of tumor formation. This can be attributed to the competition for metabolites among the roots. One of the physiological effects of 2,4-D is to form a dominant metabolic sink in the stem-root axis (Hanson and Slife, 1969). Orchid plantlets, in this case root tips, may be considered to be the main metabolic sink since in many cases after tumor formation the plantlets died. Hence, the smaller the number of roots per plantlets, the less is the competition among the roots. A lower rate of tumor formation in plantlets with less than 4 roots can be attributed to the less vigorous condition of these plantlets. Although selection was made on the basis of plantlet height in this study, the plantlets with fewer roots were apparently weaker.

The morphological observations made here pose an

interesting point with regard to the origin and the pattern of tumor development. Frequently, the initial growth of a tumor is a root-like appendage. Since the lateral root is generally induced by auxin (Gorter and Zweep, 1954), this may indicate that the tumor shares the same origin as the lateral root primordia. Therefore, the proliferation of certain tissues of the root may either produce a tumor or lateral root depending on the situation. In this case, 2,4-D may initially activate cell division in a potential site for root primordia and produce a root-like structure. However, the continued exposure to 2,4-D induces further proliferation of this root-like structure and converts it into a tumorous growth.

What changes, then, occur in cells during abnormal growth? Anatomical and ultrastructural investigations were conducted in an attempt to answer this question.

Anatomically, the root apex is organized from four different initial layers from which all other cells are derived in an orderly manner. The addition of cells by division in the meristamatic area (50-150μ from the cap junction) and subsequent elongation of derived cells result in growth of the root.

Upon transferring to medium with 2,4-D, root growth is disturbed through irregular expansion of cells and decrease in mitotic activity. The expansion of cells is very rapid in the cortex and cells in that area start to degenerate

acropetally within the apex. The continuous degeneration of cells in the cortex and expansion of the apical initial cells result in a complete loss of organization within the root. Cells in the vascular periphery and to a lesser degree in the initial layers dedifferentiate and slowly replace the degenerating cells; these cells expand and remain without undergoing further division for a prolonged period.

Upon reaching a certain point, Phase IV, the lag phase proceeds to rapid proliferation resulting in an accumulation of meristematic cells. From this point on, some cells expand while other cells divide resulting in the formation of tumors.

Early Cell Expansion and Mitotic Disturbance

Soon after culture on 2,4-D (1-2 days) cell expansion is general among the cells in the root tip. After 3 days, a different pattern can be seen -- cortical cells are first to degenerate.

Multivesicular bodies (MVB) appear as early as on the 2nd day especially in the cytoplasm of vascular cylinder initials. The structure of MVB is very similar to those reported in studies of suspension culture of carrot cells (Halperin and Jensen, 1967), in that they were bounded by a single membrane with numerous internal vesicles. The frequent association of MVB to cell wall or deposition of internal vesicles into wall space is a characteristic of MVB here and in other studies (Fowke and Setterfield, 1969;

Halperin and Jensen, 1967; Marchant and Robards, 1968].

MVB are frequently located in the vicinity of Golgi complex. In some areas Golgi vesicles are enclosed in large vesicles which are very similar to MVB [Fig. 22]. Although no evidence towards the origin of MVB is available, at least they appear to be related to Golgi complex in their function. The early inclusion of vesicles in cell plates at late telophase further indicates the possible relation of the MVB to Golgi complex since phragmoplasts have been related to Golgi complex [Mollenhauer, 1965].

The possible sequence of events in which MVB are involved in the synthesis or deposition of certain cell wall materials is explained by Walker and Bisalputra [1967]: 1] the formation of the structure in the cytoplasm, 2] the movement of the structures to the plasma membrane, 3] their fusion with the plasmalemma, and 4] the final reduction in size and content of the vesicles at the cell wall. The observations made here adhere closely to the sequence.

MVB in their frequency and number of internal vesicles fluctuate during each phase of growth in this study. Starting with none in Phase 0, they first appear in the 2nd day of Phase I and is followed by higher frequency in Phase II where increasing numbers of cells are undergoing extension growth. In Phase II the cells also have thickened cell walls. Where cell extension is less conspicuous as in

Phase III and IV, MVB become less frequent. Hence, the involvement of MVB in cell extension which was initially induced by 2,4-D appears to be likely.

A question might be raised, then, as to why MVB are not present in rapidly expanding cortical cells which are involved in cell extension growth? At this point, the role of the nucleolus should be discussed.

The nucleolus is known as the site of ribosomal RNA synthesis and produces more than 70-90% of cellular RNA (Busch and Smetana, 1970). Many studies on nucleolar structure and its activity consistently mention the relation between the structure of the nucleolus and its cellular metabolic activity (Barlow, 1970; Hyde, 1967; Jordan and Chapman, 1971).

Among the four different nucleoli in Phase 0 root cells of the vascular initial, dividing and elongating vascular cells, and differentiated cortical cells, the nucleolus in the cortical cell is different from the other three. This nucleolus is characterized by a compact shape without distinct nucleolar components. The other nucleoli consist of granular and fibrillar regions, and light zones (L-zone).

The presence of functional nucleolar components is important in relation to nucleolar activity. The granular components are related to the formation of ribosomal RNA (r-RNA) and may be either their precursors or mature ribosomes (Jones, 1965). The area occupied by the granular region within the nucleolus and the degree of dispersion of

granular components progressively increase within a vascular initial, dividing peripheral vascular cell, and elongating peripheral vascular cell. This relates well with increases in amount of RNA in these cells as established by histochemical studies (Jensen, 1956). A similar pattern was observed in the cells of the Plantago root tip (Hyde, 1967).

The L-zone, named for its lightly staining character, is the nucleolar organizing region (Hyde, 1967; Chouinard, 1970). The frequent association of the L-zone with heterochromatin and the presence of chromatin in the L-zone (Fig. 30) support the nucleolar organizing function of the L-zone.

The distribution of the L-zone in the nucleolus is another variable among different cells in the root tip. The surface intruded L-zone in the initial cells progressively moves into the nucleolus of the dividing and elongating cells. Since the fibrillar region is the initial site of r-RNA synthesis as shown in autoradiographic studies (Karasaki, 1965; Geusken and Bernhard, 1966), the chromosomal region (L-zone) should move in as the requirement for r-RNA synthesis increases. The movement of the L-zone from the external position in the initial cells to the internal position is associated with the template activity of r-RNA of the L-zone. DNA is localized at the edge of inactive nucleoli and spread through the core of active nucleoli (Ebstein, 1969). Likewise, the surface intruding L-zone is considered as low in r-RNA synthesis (Jordan and Chapman, 1971).

As a consequence, the compact nucleolus in differentiated cortical cells may represent an inactive nucleolus lacking in granular components which are directly related to cytoplasmic RNA synthesis and functional L-zone which template the r-RNA. The other cells possess the functional components although they vary in structures.

The contrast between the functional and inactive nucleoli becomes clear on the 3rd day in Phase I. The nucleolus in the cortical cell becomes more dense and extremely compact. A similar nucleolar structure has been observed in autolysing animal cells (Bernhard, 1964). However, in the other cells including dividing cells, the granular region increased considerably from Phase 0. In these cells, the nucleoli are characterized by having internally located L-zones which relate to active template activity for r-RNA. The increased nucleolar activity, therefore, supports the expanding growth of these cells. The compact type nucleolus represents the inhibition of formation of a new structure or not reacting to the demands for r-RNA for expansion growth.

The differences in nucleolar structure and in presence of MVB among growing or rapidly expanding cells followed by degeneration may be combined to explain the early response of cells to 2,4-D. Auxin exerts a strong influence on cell elongation by increasing the extensibility of the cell wall (Galston and Davis, 1969). Upon receiving the stimulus the nature of which is still in controversy (Scott, 1972),

cells in different positions in the root react differently. Cortical cells (may be the only differentiated cells) having a compact type of nucleolus fail to react by growth but the increased extensibility of the cell wall results in an extension of the cell followed by cell lysis. The other cells -- vascular initial, dividing, and elongating cells -- have nucleoli with active structural components, react to the stimulus by modifying the nucleolar structure to the active state and synthesize r-RNA for cell growth. MVB appear in these cells for deposition of cell wall materials.

Although the resolution of the mechanism of activation of nucleolar activity is beyond the scope of this study, the association of karyosome in activation of the nucleolus should be noted. Karyosome associated with nucleolus is frequently observed in cells in Phase I. The karyosomes are either attached to the fibrillar or granular region of the nucleolus. The karyosome which is embedded in the fibrillar region might repress nucleolar RNA synthesis as suggested by Smetana et al. (1971) in animal cells where a similar association of karyosomes to nucleolus was observed. Since nucleolar growth is through increase of protein in the fibrillar region (Hyde, 1967), the repression of nucleolar RNA synthesis in the area of fibrillar region where karyosome is embedded will impair the nucleolar growth. Nucleolar organizing genes have the double function of synthesizing r-RNA and/or reorganizing nucleolar material

itself (De La Torre and Clowes, 1973). The repression of nucleolar RNA for the nucleolar growth itself may, in turn, activate r-RNA synthesis which results in the increased granular region. The karyosomes associated with the granular region, therefore, may result from newly produced granular components. Consequently, the further development of the granular region results in the detachment of karyosome from the nucleolus.

Although the decrease in mitotic activity in Phase I is conspicuous in this study as well as in other studies (Hanson and Slife, 1969; Kiermayer, 1964), there is no explanation for this at the present time. Observations show that the meristematic cells also expanding. The increased polyribosomes in these cells are also conspicuous. As already discussed, the 2,4-D initially affects the cell wall to increase extensibility and cells react to synthesize cell wall materials through polyribosomal biosynthesis. The expenditure of cellular energy for cell growth results in the cessation of mitotic activity since cell division is an energy-requiring process (Webster and Van't Hof, 1973). If this is the case, the effect on mitotic disturbance is not the initial action but rather mitotic disturbance follows the expansion growth caused initially by 2,4-D.

Disorganization and Dedifferentiation

Initially, the degeneration of cells starts from differentiated cortical cells in Phase I. The process of

degeneration of cells extends and eventually, the whole cortical region is collapsed in Phase II. Although cells in the initial layers, epidermal layer, and cap cells persist for a longer time [until the 10th day of Phase II], they also eventually degenerate. In terms of the organization, the loss of the organized apex occurs as early as on the 3rd or 4th day of Phase I which is indicated by the modifications of individual cells. However, a complete disruption of organized growth occurs about the 10th day of Phase II.

The dedifferentiation of cells mainly occur in enlarged cells at the periphery of vascular cylinder when the root tip becomes disorganized. Lysis of the cortical cells which mainly occur prior to dedifferentiation of cells at the periphery of vascular cylinder may relate dedifferentiation to cell lysis. Autolysing cells have frequently been attributed to producing mitotic stimulus [Habeshaw and Heyes, 1971; Yemon and Mitchel, 1970]. It is also known that a stimulus such as wounding is essential to induce crown gall tumor [Kupila-Ahevenniemi and Therman, 1968]. Although intact roots have not been exposed to external wounding during the experiments, the lysis of cells may provide the same stimulus as autolysing cells which are caused from excision or some other external wounding. The sloughing of cells in cortex have also been observed in other root cultures [Torrey and Fosket, 1970; Webstar and Radin, 1972]. The occurrence of cell lysis

before the distinct increase in dedifferentiated cells may suggest the possible existence of a mitotic stimulus on the cells at the periphery of vascular cylinder. The frequent cell division in vascular peripheral cells, therefore, may be related to the autolysing cells. It has been observed that a burst of cell divisions occur on the surface or near the surface of excision (Yamon et al., 1968).

In a dedifferentiating cell in late telophase, multiple nucleoli are observed. Multiple nucleoli seem to have the same function as the normal nucleoli (Stevens, 1964), showing the same developmental pattern with compact fibrillar materials. The wide distribution of small developing nucleoli in restituting nucleus indicates that ribosomal DNA (r-DNA) (Busch and Smetana, 1970) is very widely spread in the chromosome. The appearance of the characteristic L-zone in only one of these nucleoli indicates that the other part of the nucleolar organizing region may activate formation of many small nucleoli in a nucleus.

Normally the gene potential of chromosome for production of nucleoli is initially inhibited and may be repressed so that only one nucleolus is formed. Since multiple nucleoli are observed only in late telophase, the small nucleoli may later fuse to form a larger structure. Hence the significance of the occurrence of multiple nucleoli in dedifferentiating cells may lie in the acceleration of restitution of nucleoli.

The accelerated nucleolar restitution is known to

occur when protein synthesis is artificially inhibited by using cyclohexmide (Fernandez-Gomez et al., 1972). Cells in quiescent center which are known as having less cytoplasmic and nucleolar RNA and a lower rate of RNA synthesis (Hyde, 1967) also showed accelerated nucleolar reorganization (De La Torre and Clowes, 1973). In this connection, it is interesting to note that dedifferentiated cells in this study enter a prolonged lag phase (Phase II). As shown in anatomical observations, those cells become highly vacuolated until they reach the proliferative phase (Phase IV) which starts about the 25th day. A number of biological systems have been described in which there is little or no r-RNA synthesis such as in germinating wheat embryo in resting state (Chen et al., 1971) and in stationary phase of plant cell culture (Varma and Marcus, 1973).

If the phenomenon of accelerated nucleolar reorganization is truly caused by the low level of cytoplasmic r-RNA (De La Torre and Clowes, 1973), the observation of multiple nucleoli here may indicate that there is very low demand for r-RNA in cytoplasm in cells during the lag period (Phase II) and this in turn can be related to the other systems described.

Proliferation of Cells

The degeneration of root cells and the substitution of these by newly divided cells result in a different aspect of growth. The lag phase (Phase II) is linked to the

proliferative phase (Phase IV) by preproliferative phase (Phase III) which is established characteristic cell population at periphery of vascular cylinder but without active mitosis.

The nucleolus in Phase III undergoes a remarkable transformation from the hypofunctional state in the lag phase to a state of great activity as indicated by the profusion of granular activity. The predominant increase in granular components in animal cells are also shown by the effect of thioacetamide which is known as the stimulant for RNA synthesis (Bernhard and Granboulan, 1968). Another characteristic of the nucleolus in this phase which is the distinct segregation of nucleolar components observed here cannot be explained at the present time. The segregation of nucleolar components are mainly reported in animal cells as a typical drug effect (Thomas, 1972; Journey and Goldstein, 1981; Koulish and Kleinfeld, 1964). So far, this type of nucleolus has not been observed in plant cells.

The activity of the nucleolus as indicated by the extreme profusion of granular components coincide well with the increased ribosomes in the cytoplasm. The lack of functional L-zone in this nucleolus indicates that the heterochromatin of reduced density in L-zone (Hyde, 1967) may have further dispersed into lacuna giving a different configurational stage of chromatin for the active templating for RNA which is encountered in this phase (Phase III).

The rapid synthesis of r-RNA indicated by granular activity of the nucleolus and the sudden increase of ribosomes in cytoplasm characterize well this phase which is in transition to the proliferative phase (Phase IV). In many cases, during the transition from resting state to an active state of growth, r-RNA synthesis occurs very actively prior to rapid cell division (Varma and Marcus, 1973; Chen and Osborne, 1970). Since r-RNA accumulation is a prerequisite for cell division (Webster and Van't Hof, 1973), as a transitional stage, this phase plays an important role through nucleolar activity.

Upon entering the proliferative phase which is defined by the accumulation of small meristematic cells, the structure of the nucleolus more or less is restored to that in Phase 0. Occasionally, multiple nucleoli are observed but they are less frequent when compared with the lag phase. The ribosomes change from monomeric in the previous stage into polyribosomes. This is general phenomenon in proliferating cells. It has been shown that proliferative and non-proliferative cells differ by greater number of polyribosomes in the proliferative cells (Webster and Van't Hof, 1973). One of the main characteristics of this phase of growth, then, may lie in the formation of polyribosomes from monomeric ribosomes which are produced in the pre-proliferative phase (Phase III).

Among the meristematic cell populations, some cells elongate resulting in isolation of patches of meristematic cells. These patches undergo rapid cell division resulting in formation of tumors.

SUMMARY

The roots of orchids (Dendrobium Lady Hay and Dendrobium Jacqualyn Thomas) either attached to plantlets or excised and in aseptic culture responded to 2,4-D by formation of tumors. Among various concentrations tested (0-5 ppm), tumors were most frequently formed at 0.75 - 1.0 ppm.

The sequence of tumor formation was observed under the light and electron microscopes from 0 to 45th day when tumor proliferation was conspicuous.

Anatomically, the root apex is organized from four different initial layers from which all other cells are derived in an orderly manner. The addition of cells by division in the meristematic area (50 - 150 μ from cap junction) and subsequent elongation of derived cells result in growth of the root.

The anatomical modifications of root tips during tumor development were observed according to the categorized phases; Phase I, 1st-3rd day; Phase II, 4th-12th day; Phase III, 13th-20th day; Phase IV, 21st-45th day. The major modifications of anatomical structure of root tips in each phase are summarized as follows:

1. During Phase I, root growth was affected by irregular expansion of cells especially in the cortex and a drastic decrease in mitotic activity.

2. Expansion of cortical cells was followd by cell

lysis in Phase II resulting in the collapse of cortex. Cells mainly at the periphery of vascular cylinder continued to divide while other cells degenerated resulting in a complete loss of organization of root apex by the end of Phase II.

3. During Phase II and Phase III cells at periphery of vascular cylinder dedifferentiated, divided, and filled the gaps produced by lysis of cortex.

4. The proliferation of cells began in Phase IV. In the process of elongation of some cells, groups of meristematic cells became isolated. Further meristematic activity of these isolated cells resulted in rapid tumor growth.

At the ultrastructural level, multivesicular bodies (MVB) and nucleoli were the main concerns of this study.

MVB consisting of clusters of vesicles bounded by a single membrane appeared only after 2,4-D treatment and were related to cell expansion as shown by the following observations:

1. MVB appeared on the 2nd day when cell expansion was considerable.

2. The frequency of internal vesicles in MVB increased as cell expansion increased.

3. MVB were not present in cortical cells which expanded rapidly and proceeded immediately into lysis.

4. MVB, frequently associated with cell wall, deposited their internal vesicles in wall space.

The observations on nucleolar structure were based on the components of nucleolus and are summarized as follows:

1. In root tips in Phase 0, there were variations in nucleolar structure of cells in different tissues. The area occupied by the granular region and the degree of dispersion of the granular region were the least in initial cells and increased in the dividing and the elongating cells. The intruding light zone (L-zone) of initial cell progressed internally as cells toward the base of the root apex were examined. The differentiated cortical cell, however, had a compact nucleolus.

2. In Phase I when cells were expanding, two different types of nucleoli were observed--one which had extensive granular region in association with karyosome and the other which was compact. Except for the differentiated cortical cells, all cells observed had the first type of nucleolus in this phase. This structural modification may be an indication of activated r-RNA synthesis. The compact type lacked the functional structure to respond to the stimulus.

3. Multiple nucleoli were observed in dedifferentiating cells in late telophase at Phase II. The small and numerous nucleoli scattered in restituting nuclei may be considered as accelerated nucleolar reorganization which may be related to transition from a very low demand of r-RNA in cytoplasm in lag phase (Phase II) to a burst of r-RNA synthesis in proliferative Phase (Phase III) which

followed.

4. A transformation of nucleolar structure from compact and hypofunctional state in Phase II to an elevated state of activity was observed in Phase III (15th day). The increase in granules can be related to the increased production r-RNA which is a prerequisite for cell division.

This study demonstrated that root tips can be cultured for the clonal propagation of orchids. However, because the number of plantlets is very few, this method is not commercially feasible as a technique for rapid clonal propagation at the present time.

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