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Cytoplasmic segregation in onion root tip cells and formation of vacuoles

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Abstract

Onion root tips were studied both with transmission and scanning electron microscope. The cells in the meristematic region of initials were found to be little vacuolated. At early stages of cell differentiation areas free of ribosomes are segregated in the cytoplasm. No (or only incomplete) membranes delimiting such areas could be distinguished in most cases. Seemingly, the membraneous structures may form later or simultaneously with the progress of breakdown of biostructure of the cytoplasm in the segregated area. The microscopic image of cytoplasm in the segregated areas (and even of the vacuolar sap at early stages of vacuole formation) resembles the matrix of cytoplasm populated with ribosomes. The cytoplasm in the whole central area is segregated at early stages of differentiation of root cap cells. The process of autophagic vacuoles formation in which the preexisting endomembranes seemingly are involved is described. It is proposed that such vacuoles may form after separation of two unit membranes of a cisternae surrounding a portion of the cytoplasm or an organelle, followed by independent growth of an outer unit membrane.

INTRODUCTION

The formation of an extensive vacuolar system in most cells of higher plants is a conspicuous developmental process occurring in the course of cell maturation and specialization. Plant vacuoles are segregated compartments within the protoplast with various content (Pisek 1955), characteristically enclosed in the vacuolar membrane, the origin of which is still controversial. Most authors today agree that vacuoles form either by local dilatation of the cisternae (Buvat 1961; Poux 1962; Bowes 1965; Picket-Heaps 1967; Gifford and Stewart 1967) or by fusion of small vesicles (Matile and Moor 1968, Mesquita 1969; Berjak 1972) derived from endoplasmic reticulum and from dictiosomes (Marinos 1963; Ueda 1966). Formation of vacuoles from degenerating mitochondria and plastids is also suggested (Bell and Mühlethaler 1962, 1964). These observations challenge the early con-

cept that vacuoles may originate only by fragmentation of preexisting ones (Went 1888; Bailey 1930; Zirkle 1932). The old hypothesis (Pfeffer 1890/91; Priestley 1929) concerning the formation of vacuoles by local hydration and segregation of a portion of cytoplasm by the *de novo* formed vacuolar membrane is recently only infrequently suggested (Sitte 1958; Mühlethaler 1960). Although not contradictory to the mechanisms of vacuole formation first described, the segregation phenomena in the cytoplasm presented in this paper seem to add some support to the latter concept.

MATERIAL AND METHODS

Tips 1-mm long were cut from a few-days-old 5-7 mm long adventitous roots of onion bulbs placed with their bottom in cold 2% glutaraldehyde in 0.1 M sodium cacodylate pH 7.2. After initial fixation for 2 hrs, glutaraldehyde was replaced with an analogously buffered solution of osmium tetroxide and fixation continued for 2 hours in the cold. Subsequently, the tips were either dehydrated in ehtanol series followed by propylene oxide and embedded in maraglas (Freeman and Spurlock 1962), or prepared for investigation with a scanning electron microscope according to one of the three methods already used on other occasions (Humphreys and Wodzicki 1972; Wodzicki and Humphreys 1972, Wodzicki and Brown 1973, 1974): a) the tips were washed in bidistilled water, frozen and fractured under liquid nitrogen and deep freeze-dried under high-vacuum (10^{-6} torr) overnight; b) dehydrated in ehtanol series, the tips were frozen and fractured under liquid nitrogen, and dried by Anderson's (1969) critical point method; c) the tips were processed by the critical point method and sectioned afterwards with razor blade. A layer of gold 200—400 Å thick was evaporated upon the surface of the specimens before viewing with the Cambridge Mark 2A scanning electron microscope. Ultrathin sections obtained by means of a diamond knife and a Sorvall MT-2 Ultramicrotome were stained with lead citrate for studying with a Philips 200 transmission electron microscope.

Legend to abbreviations on photographs: Cytoplasm (C), Nucleus (N), Vacuole (V), Cell Wall (CW), Segregated area in cytoplasm (S).

RESULTS AND OBSERVATIONS

Little segregation can be seen in the dense cytoplasm of meristematic cells in the region of initials (Fig. 1). Only in few places small vacuoles with fibrous and membraneous contents are visible. Segregation phenomena in the ground cytoplasm are already in progress in somewhat more differentiated cells just below the initial region. Numerous areas free of ribosomes have segregated (Fig. 2). The density of the cytoplasmic matrix in these regions (long black arrows) is not visibly different from that populated with ribosomes. In a few places, however, segregated spaces

filled with more diluted material are seen (long white arrows). No separating membrane can be distinguished around most of the segregated areas. A thin membrane surrounds completely only a few spaces free of ribosomes (short black arrows), but incomplete membraneous structures associated with such areas are also observed (short white arrows). The origin of these membranes is uncertain. They may represent transformed endomembranes already present in the cell, but spontaneous formation of the lamellar structures at the boundary of two reseparated colloidal phases cannot be excluded. An interesting phenomenon suggesting one of the possible mechanisms of membrane formation at the boundary of segregated spaces in the cytoplasm is presented in Figs. 3-5. Small vesicles of uniform size migrate towards the segregated area and open at its proximal side seemingly adding also their content to the pool (Fig. 5). The process resembles mechanism of cell plate formation. No continuous membrane is seen as yet, however, the whole region is separated from the ribosome--rich cytoplasm by fibrous material of greater density, probably accumulated from the residual membranes of the vesicles. This may serve as "ready to use" material for constructing or organizing the membrane of the segregated area.

Segregation involves also the cytoplasmic regions densely populated with ribosomes (Fig. 6). Segregated cytoplasm surrounded with membrane is seen enclosed in small vacuolar compartments. Size relationships of the cytoplasmic contents and the vacuole do not, in this case, suggest engulfment of the material by the preexisting vacuole. Isolation by the cisternae of double-membrane endoplasmic reticulum followed by separation of two unit membranes is rather envisaged. This type of segregation may be represented in case indicated by white arrows (Fig. 6 and 7). Independent growth of the outer unit membrane may result in formation of an autophagic vacuole. Thin lamellae of cytoplasm separate frequently numerous small vacuoles seen at this stage of cell differentiation, preventing the contact of tonoplasts.

The cytoplasm of segregated areas seemingly degenerates, forming the fibrous, granular or membraneous content of new vacuoles (Fig. 8). At earlier stages of cell vacuolation the microscopic image of the matrix of ground cytoplasm and vacuolar sap is but little different (Fig. 20). Progressive changes in the cytoplasm forming a vacuolar compartment, seemingly without separating of the portion of cytoplasm by preexisting endomembranes, may be traced in Fig. 8. The ground cytoplasm populated with ribosomes (*a*) is separated from the area free of ribosomes (*b*) by incomplete membraneous structures. Separated by a continuous membraneous layer is the inner less dense portion (*c*) of this segregated compartment. Fibrous material is still seen in this region. The areas with no visible structural material are indicated by (*d*).

Segregation phenomena in the root meristematic region can be conveniently studied by the scanning electron microscope. Regions of various density in the cytoplasm may be revealed and the contents of vacuoles exposed. Cytoplasm of the cell in the region of initials seen in Fig. 9 is but little segregated. All vacuoles in somewhat more differentiated cells contain dense material (Figs. 11 and 12). The segregated area of the cytoplasm (arrow) may be distinguished in the cell presented

in Fig. 10. The segregated compartments revealed in Fig. 11 may represent further steps of developmental changes leading to vacuole formation. Vacuoles of the more differentiated provascular cells below the region of meristematic initials (Figs. 13 and 14) are apparently free of reticulate contents.

Segregation phenomena in the cytoplasm are well seen in differentiating cells of the root cap. The central portion of cytoplasm of highly differentiated cells is segregated as revealed by three different techniques (Figs. 16—18). Fig. 15 allows orientation in the region studied (arrow). At a late stage of differentiation, the cytoplasm of the segregated central area of the cell is organized in a loose reticulate structure and remains in contact with denser parietal cytoplasm. Early stages of cytoplasmic segregation are seen in the cells underlying the outer layer (Fig. 19). In spite of some damage seen as ruptured areas produced by exposure of soft material to high-vacuum and electron beam in the microscope column, segregation is evident. This type of formation of vacuole does not conform with the commonly suggested fusion of preexisting small vacuoles or provacuoles in the process of cell vacuolation.

Early stages of segregation in the cytoplasm of differentiating root cap cells, as revealed by the transmission electron microscope, are essentially similar to those described previously. Segregated areas of cytoplasm free of ribosomes, incompletely surrounded by the membranes are visible (Figs. 21 and 22). Inside these areas the cytoplasm degenerates forming membraneous inclusions, often of myelin-like configuration which are particularly conspicuous at later stages of differentiation. Membranes forming at the boundary between the ribosomal and non-ribosomal regions of the cytoplasm (arrows) do not seem to be associated with endoplasmic reticulum.

DISCUSSION

The described phenomena of segregation in the cytoplasm of differentiating cells of onion root tips fall into two categories. No attempt was made to investigate other possible processes of vacuole formation known from the literature already discussed (Wodzicki 1974). The first of the two categories conforms to the mechanism of autophagic vacuoles formation described by Ashford and Porter (1962), Napolitano (1963), and Winborn and Bockman (1968) for animal cells. It consists in engulfment of a portion of the cytoplasm or organelle in the envelope formed from the cisternae of endoplasmic reticulum or dictiosome. Separation of the two component leaflets of the cisternae, followed by independent growth of the outer unit membrane may be proposed as a mechanism of this type of vacuole formation in the cells of the onion root meristematic region. Secondly, segregation of a portion of cytoplasm may occur without previous formation of a separating membrane. The process may culminate in vacuole formation similar to that suggested by Sitte (1958) and Mühlethaler (1960). Segregation of some areas of cytoplasm free of ribosomes seems to be the first step of cytodifferentiation resulting in vacuole formation. Interestingly, this process has been observed in cells entering diverse patterns of specialization in which the vacuoles formed by cytosegregation are most probably not functionally equivalent. These observations raise a question whether ribosomes play

any direct role in maintenance of the framework of the dynamic structure of the cytoplasm.

The microscopic image of the vacuolar sap at early stages of cell differentiation resembles that of the matrix of ground cytoplasm when the segregated vacuole is already clearly delimited by the membrane. The difficulty in definition of the ground cytoplasm as compared with vacuolar sap has been already discussed by Esau and Cheadle (1962). An incomplete membrane observed at the boundary of the areas of cytoplasm free of ribosomes may indicate delimitation of such areas by a membrane formed in an as yet little understood process after (or during) cytoplasmic segregation. Their origin may possibly be related to spontaneous formation of membraneous lamellae from macromolecules released during breakdown of the dynamic biostructure of cytoplasm. The process may be compared to the ageing of colloidal sol. Only in some cases could the association of the cisternae of preexisting endomembranes with segregated areas be observed. Another possibility is provided by the observed migration of small vesicles of unknown origin which open at the boundary of the segregated area. The delicate membranes of such vesicles do not seem to form a continuous membrane by fusion. The membranes of these vesicles after rupture seem to add, to a deposit of fibrous material at the boundary of the segregated area. This would be unusual behavior of cytoplasmic membranes which are known to reorganize in vesicular entities after rupture, and it does not suggest an origin of the observed vesicles from preexisting cell membranes.

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Fig. 1. Little-vacuolated cells in the region of initials of onion root apical meristem. TEM × 8000.

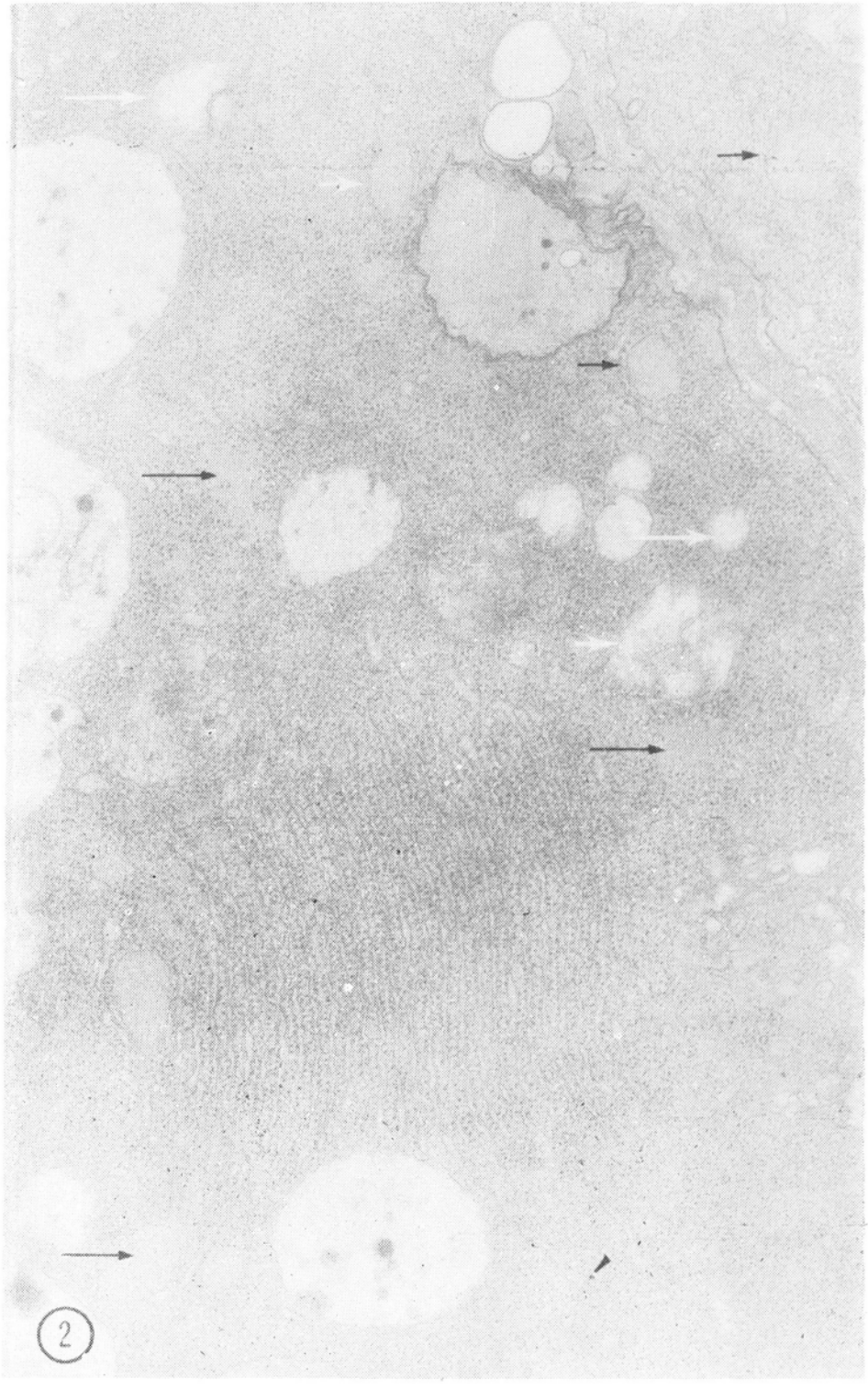
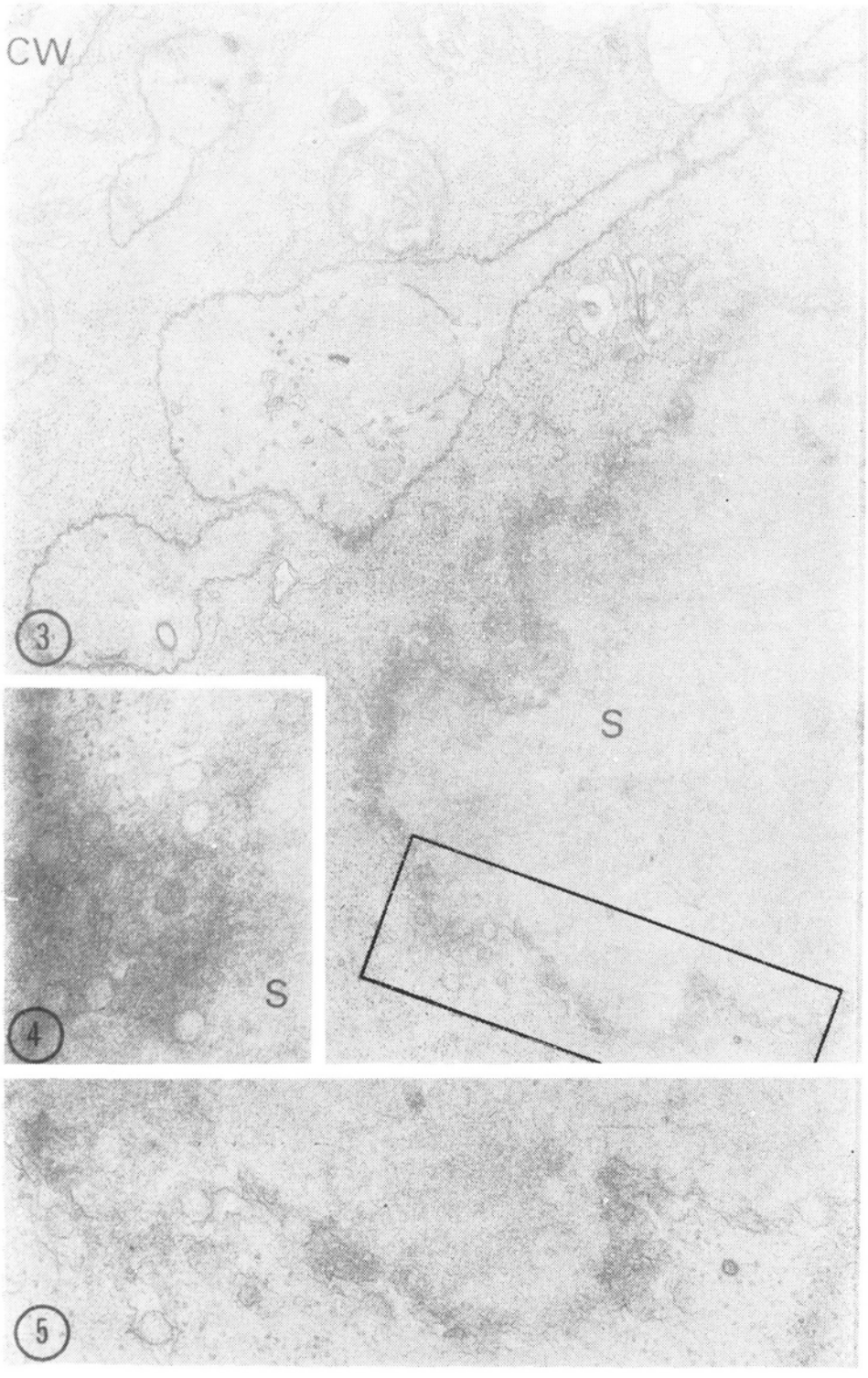


Fig. 2. Meristematic cell just below the region of root apical initials.

Arrows : segregated areas of cytoplasm free of ribosomes (long black); areas with diluted material (long white); areas free of ribosomes delimited by membrane (short black); areas incompletely separated by the membrane (short white). TEM × 31000.



Figs. 3 — 5. Segregated compartment in the cell near the region of root apical initials.

Small vesicles of uniform size are seen migrating towards and opening onto the proximal side of the segregated area. TEM Fig. 3×31000; Figs 4,5×58000. Fig. 5 Higher resolution of the area indicated in Fig. 3.

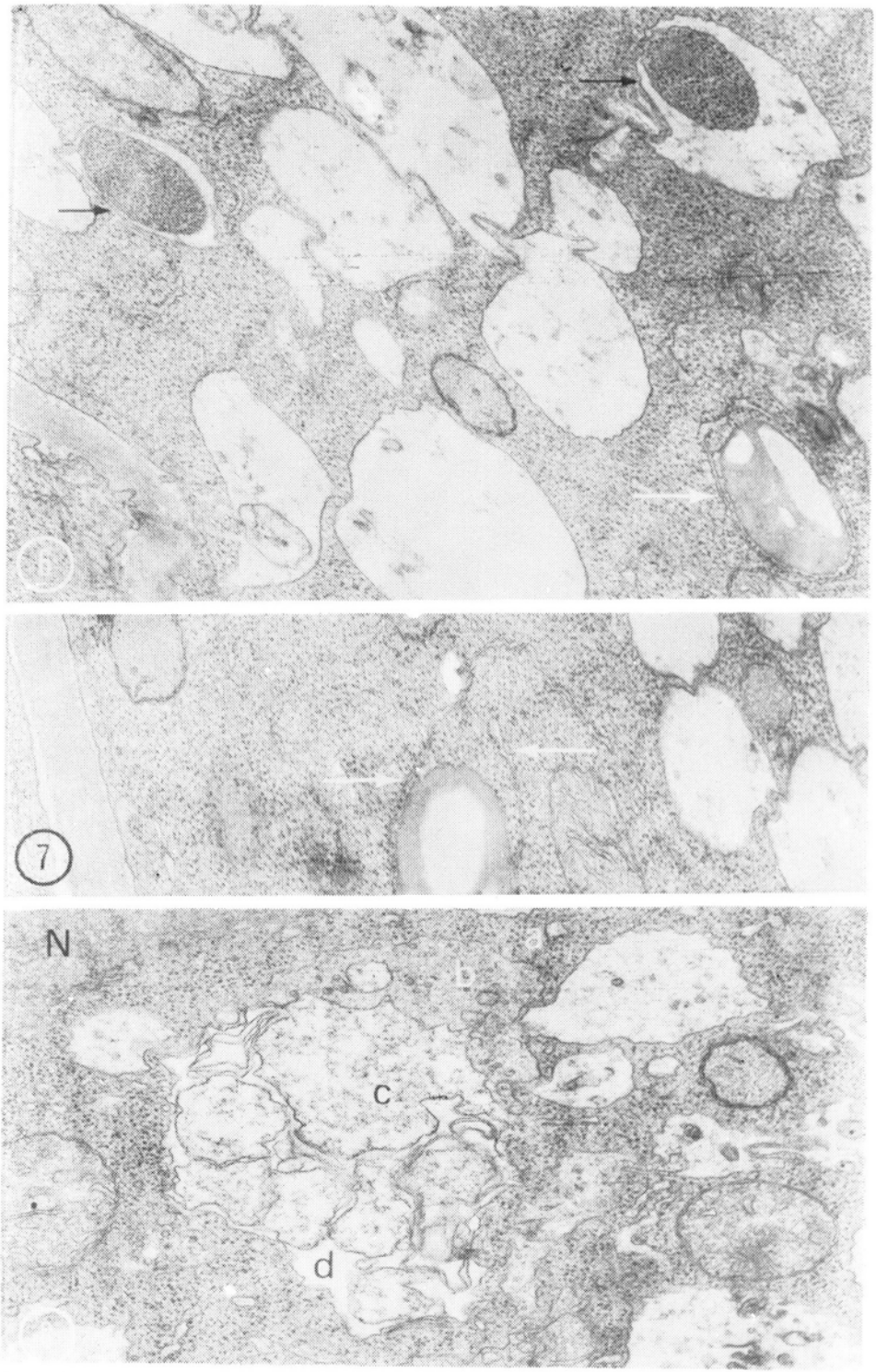


Fig. 6. Segregated cytoplasm (black arrows) in autophagic vacuole. An organelle surrounded completely with a cisternae of ER (white arrow).

Fig. 7. ER cisternae seemingly in the process of enclosing a plastid (arrows).

Fig. 8. Formation of vacuole seemingly without involvement of preexisiting endomembranes. For description see text p. 000. All microphotographs TEM × 31000.

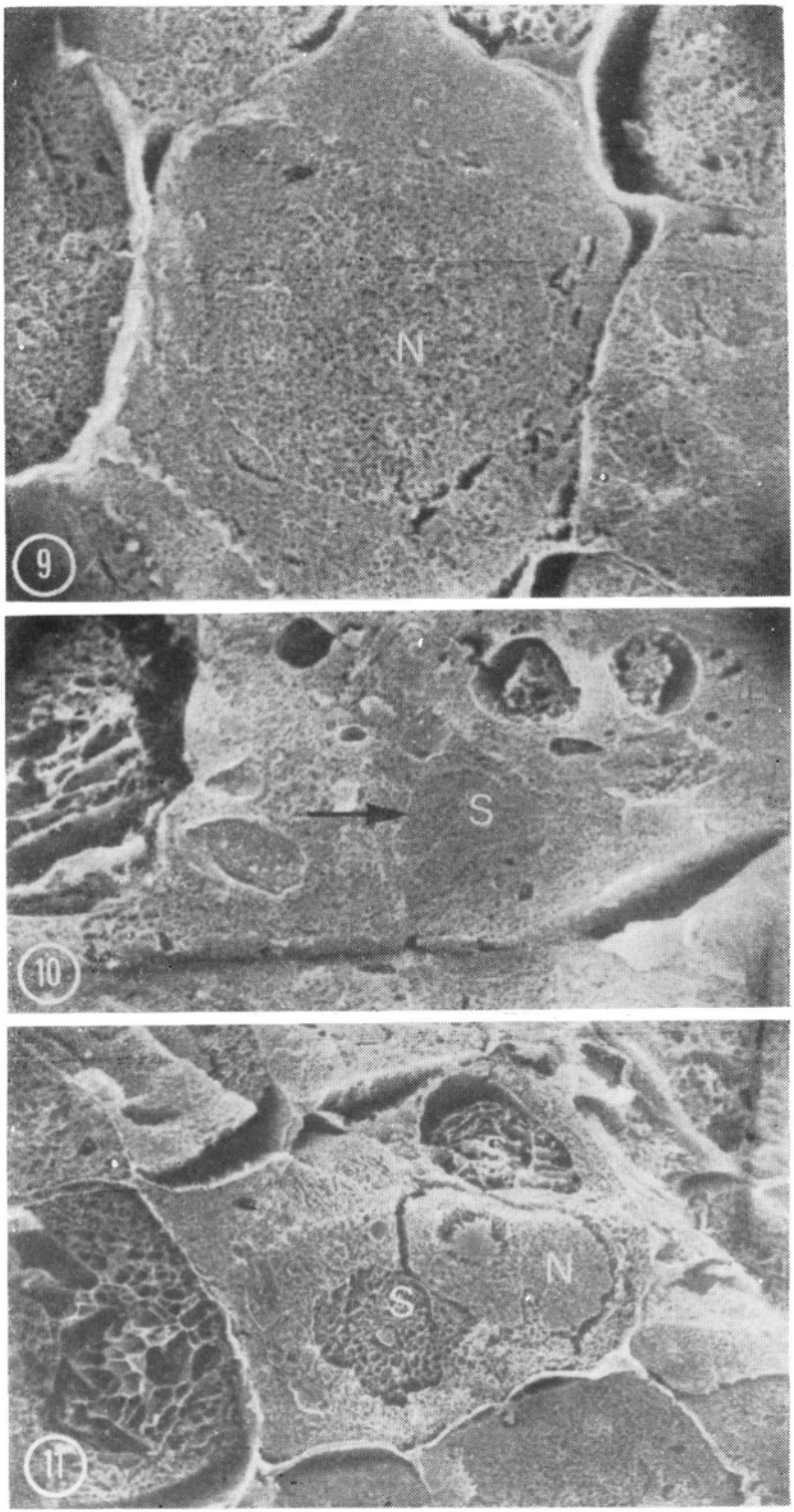


Fig. 9 Cell in the region of root apical initials.

Fig. 10. Segregated area in the vacuolating cell near the region of root apical initials.

Fig. 11. Vacuoles forming from segregated areas in cytoplasm. All specimens freeze-fractured freeze--dried. SEM Fig. 9, 10×5000, Fig. 11×2000.

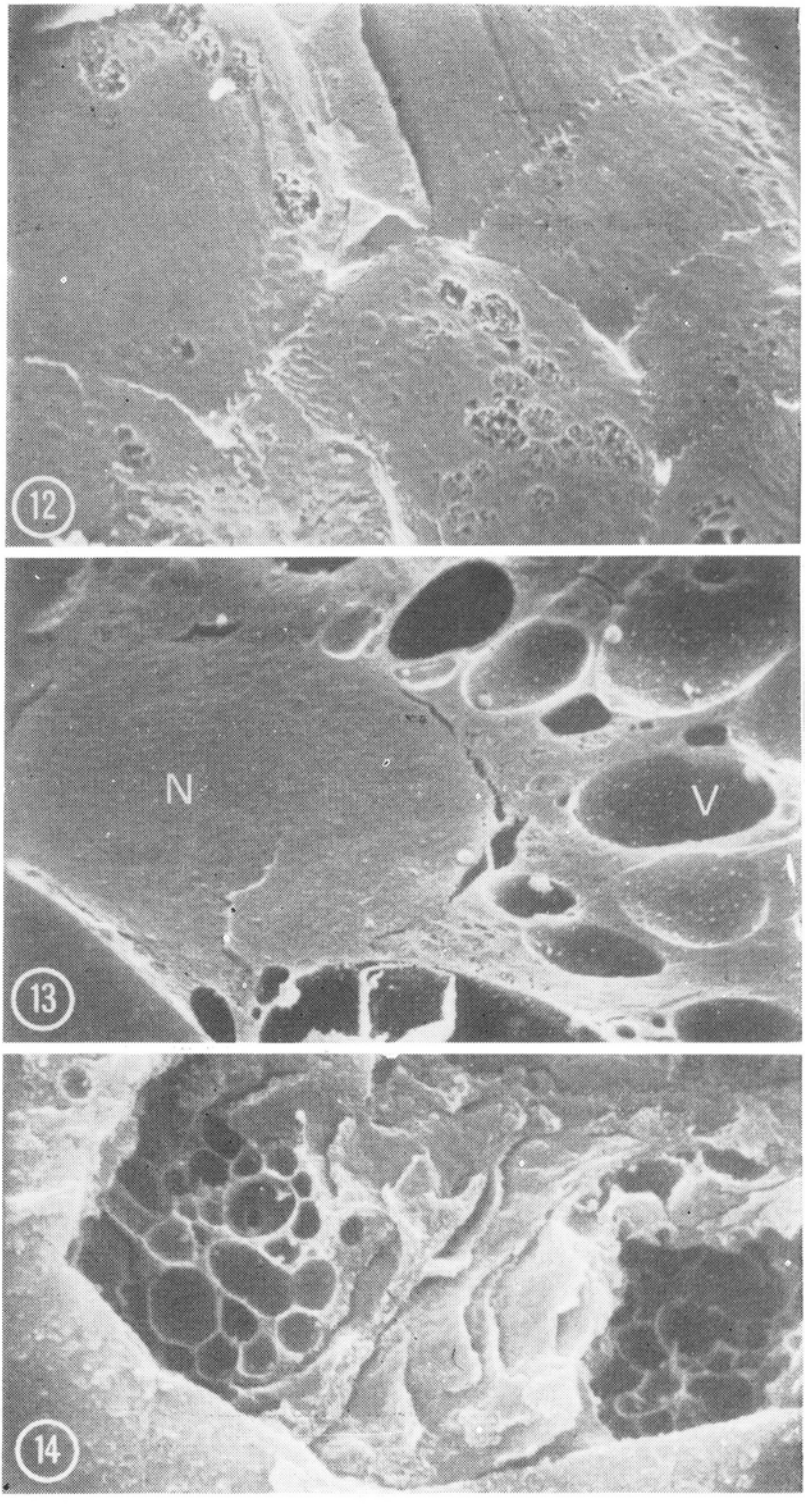


Fig. 12. Vacuolating cell near the region of root apical initials.

Figs. 13, 14. Provascular cell of onion root apical meristem. All specimeans processed by critical point method. Figs. 12, 13 Sfecimens pactured frozen in abs. EtOH before drying. Fig. 14 Specimen sectioned and ruptured after drying. SEM × 5000.

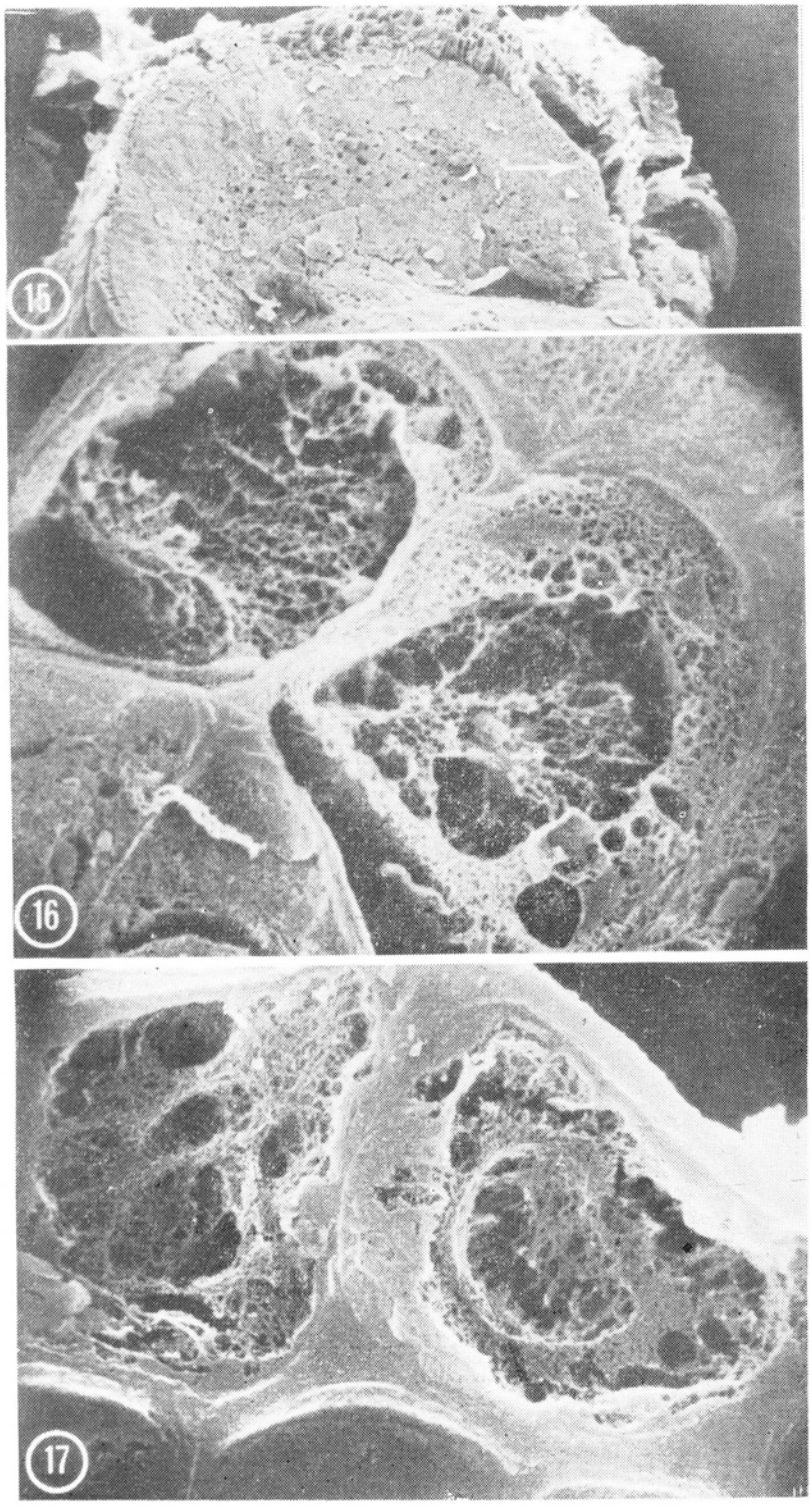
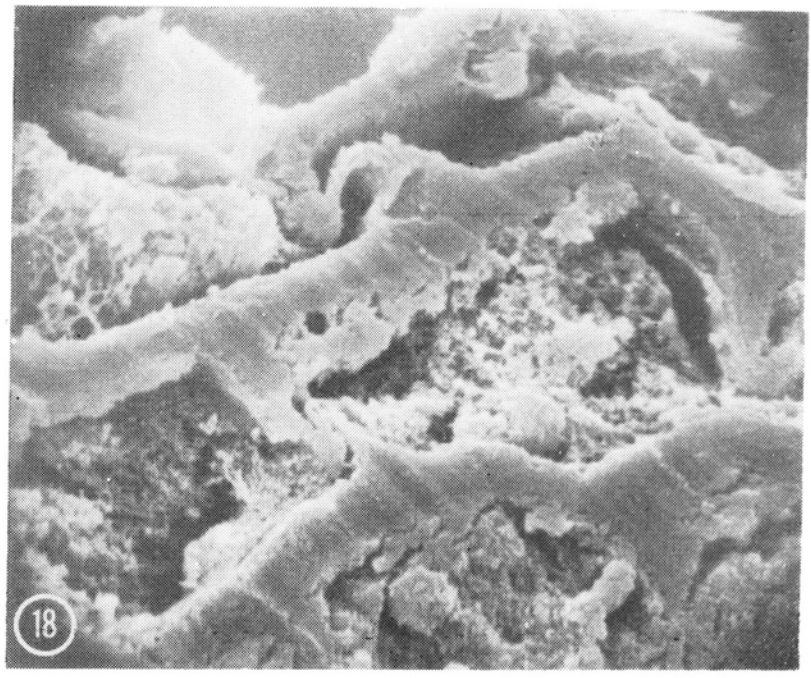


Fig. 15. Onion roct tip freeze-fractured transversally just below the apex. SEM × 100. Specimen freeze-dried.

Figs. 16, 17. Cells of root cap advanced in differentiation. SEM × 5000.

Fig. 16 Cells situated on specimen shown in Fig. 15 at arrow. Fig. 17 Analogous specimen processed by critical point method following fracture when frozen in abs. EtOH.



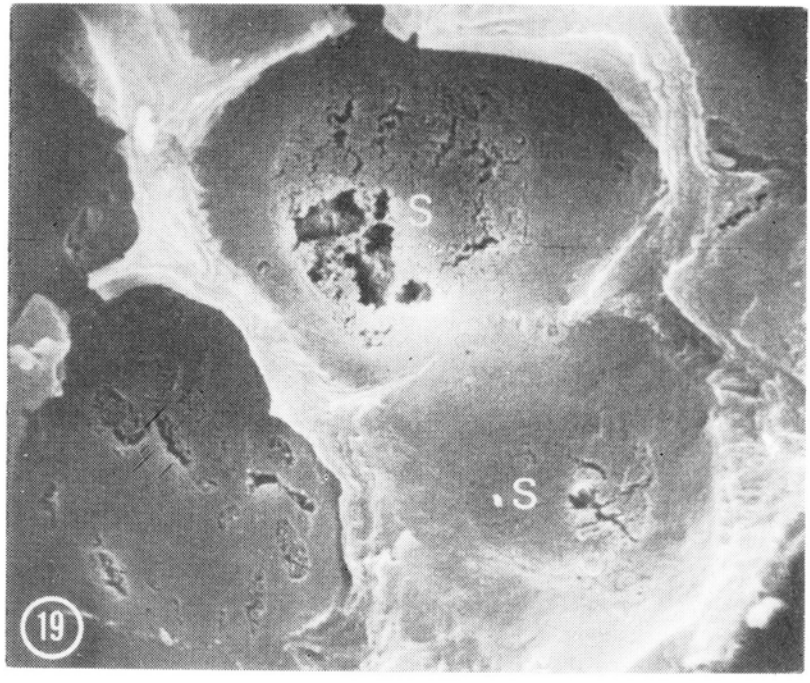
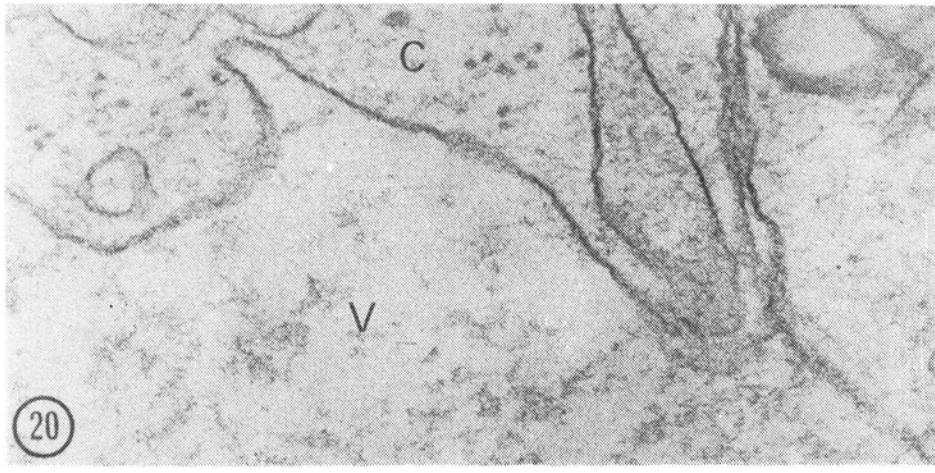


Fig. 18. Cells analogous to those seen in Figs. 16 and 17, but specimen was sectioned after processing by critical point method. Similar cell organization is seen in spite of plastic deformations.

Fig. 19. Root cap cells at early stages of differentiation. Specimen fractured frozen in abs. EtOH and processed by critical point method. SEM. Both microphotographs × 5000.



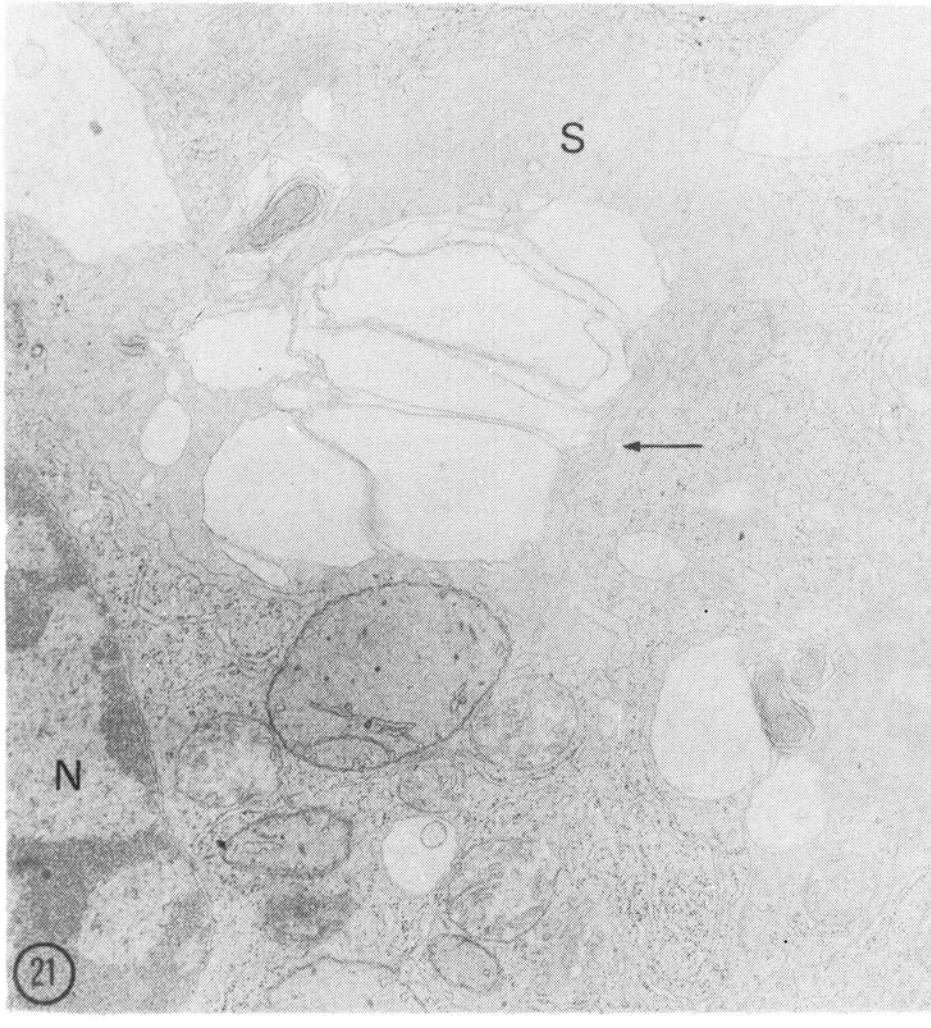


Fig. 20. Developing vacuole in the cell near the region of root apical initials. TEM × 102000.

Fig. 21. Segregated area with degenerating cytoplasm incompletely limited by membranes in differentiating cells of root cap. Arrow points to seemingly developing membrane. TEM × 25000.

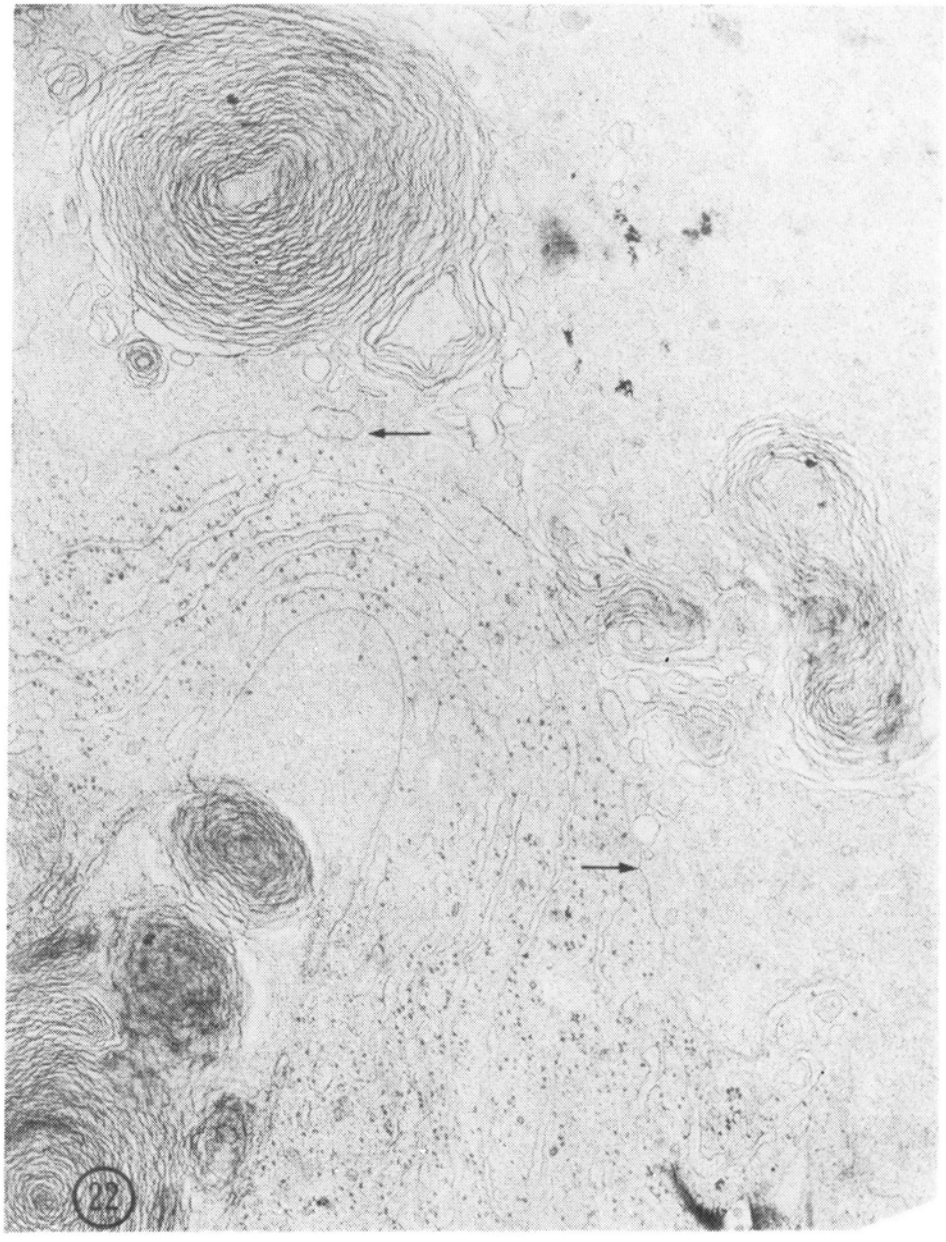


Fig. 22. Differentiating cell of root cap. Myelin-like figures formed by cytoplasmic material after functional biostructure has been disorganized. Segregated areas with degenerating cytoplasm incompletely limited by membranes. Arrows point to seemingly developing membranes. TEM ×25000.

Streszczenie

Cytosegregacja i tworzenie wakuol w komórkach wierzchołka korzenia cebuli

Wierzchołki korzenia cebuli badano przy pomocy transmisyjnego i skaningowego mikroskopu elektronowego. W regionie inicjałów merystemu wierzchołkowego komórki były mało zwakuolizowane. We wczesnych fazach różnicowania obserwowano segregację obszarów cytoplazmy pozbawionych rybosomów. W większości przypadków obszary takie nie były rozdzielone membraną, lub obserwowano brak ciągłości otaczających membran. Wydaje się, że takie struktury membranowe mogą tworzyć się później w miarę postępu procesu degradacji dynamicznej biostruktury cytoplazmy w odsegregowanym obszarze. Obraz mikroskopowy cytoplazmy w obszarach odsegregowanych (a nawet obraz soku wakuolarnego we wczesnych stadiach powstających przez odsegregowanie wakuoli) przypomina obraz cytoplazmy podstawowej zawierającej rybosomy. We wczesnych stadiach różnicowania komórek czepka korzenia cebuli, cytoplazma całej centralnej części komórki zostaje odsegregowana co prowadzi do utworzenia wakuoli. Opisano też proces tworzenia typowych wakuoli autofagicznych, w którym udział biorą istniejące już w komórce membrany cytoplazmatyczne (prawdopodobnie ER). Wydaje się że wakuole takie mogą powstawać po rozdzieleniu się dwu membran elementarnych cysterny otaczającej część cytoplazmy lub organellę po czym następuje wzrost powierzchniowy tylko membrany zewnętrznej.