Chromoplasts in *Rosa rugosa*: Development and Chemical Characterization of Tubular Elements

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Rosa, Chromoplast, Ultrastructure, Development, Chemical Composition

1. Development and fine structure of the chromoplasts in hips of *Rosa rugosa* have been investigated by electron and polarizing microscopy.

2. The chromoplasts develop from chloroplasts. During disorganization of the thylakoid system characteristic strutures become visible: 'oblique' grana, U-shaped thylakoids, and occassionally 'thylakoid teeth' as well as thylakoid anastomoses. — In this early stage, tubules appear in the plastid matrix. They are sometimes connected with membranes, especially with thylakoids.

3. In ripe hips the chromoplasts are packed with tubules. These chromoplast tubules, which attain lengths of up to at least 1.5 μm, are non-ramified and lie parallel to each other. Their diameter is variable, with a mean of 18 nm. They are polygonal in cross-section with electron-dense walls and electron-transparent cores. Association of ellipsoidal osmiophilic globules with the tubules can often be seen. Irregularly swollen remnants of former thylakoids are found in connection with these tubules. In spindle-shaped chromoplasts, the tubules are oriented parallel to the longitudinal axis. Positive birefringence and positive dichroism are observed.

4. The chemical composition of tubule fractions has been analyzed. The tubules contain appreciable amounts of carotenoids, phospho- and glycolipids, and proteins.

5. The origin and development of the tubules is discussed. It appears likely that reorganization of part of the thylakoid system and synthesis of carotenoids are involved in the formation of tubules.

6. The plastids in the yellow autumn leaves of *Rosa rugosa* contain globules but not tubules.

1. Introduction

Chromoplasts (*i. e.* the yellow- to red-colored plastids) occur in several forms, which differ in their main structural components. These forms have been briefly reviewed by Sitte [1]. One type contains tubules and is found in petals and in fruits, *e. g.* in the rose fruits. The chromoplasts of hips have been described briefly [2–5] but they have never been investigated in detail.

This paper deals with the ultrastructural development of the chromoplasts in the hypanthium (cupula) of *Rosa rugosa* during the ripening process. A chemical characterization of the tubules is given.

2. Material and Methods

2.1. *Plant material*

Hips and leaves of *Rosa rugosa* Thunb. in different developmental stages (corresponding to differences in pigmentation) have been investigated.

Abbreviation: SDS, sodium dodecylsulfate.

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2.2. *Polarization microscopy*

Freehand sections were observed in a Laborlux-Pol-microscope (Leitz, Wetzlar, Germany).

2.3. *Electron microscopy*

Tissues were fixed with 3% glutaraldehyde in 100 mM phosphate buffer pH 7.8 for 3 h. After washing with buffer postfixation was carried out with 2% OsO_4 in 100 mM buffer for 2 h. The samples were then washed with bidistilled water, prestained with 2% aqueous uranyl acetate for 12 h and dehydrated through a graded ethanol series. The above stages were performed at 0 °C. The samples were embedded in Epon 812 [6]. Ultrathin sections, cut on a Reichert ultra-microtome Om U 2, were stained with 2% aqueous uranyl acetate, followed by lead citrate [7]. In certain experiments fixation with 2% $KMnO_4$ was also carried out. The samples were observed in a Siemens electron microscope Elmiscop IA.

Negative staining of tubule fractions was performed with 2% phosphotungstic acid (pH 7.2).

Positive staining: Equal amounts of 2% OsO_4 in 100 mM phosphate buffer and tubule fraction were mixed and left to stand for at least 15 min at 4 °C. One drop of the mixture was then placed onto a Pioloform [8] coated copper grid. After 2 min excess fluid was removed.

2.4. *Isolation of tubules*

Isolation of intact chromoplasts was not successful due to the presence of excessive slime in the homogenate. The tubules were therefore isolated 'directly' from the hip. 400 g tissue were used in each isolation, since yields were small.

Isolation medium: 50 mM phosphate buffer, 400 mM sorbitol, 3% (w/v) dextran (MW 17,700), 1 mM EDTA; adjusted to pH 7.4 with buffer components.

Homogenization: The tissue was chopped into small pieces in ice-cold isolation medium and then homogenized for 15 sec with a rotating knife-homogenizer (Bühler, Tübingen, Germany) set at 25000 rpm. After filtration through two layers of nylon gauze (40 mesh), the homogenate was centrifuged at $200 \times g$ for 5 min. The supernatant was further centrifuged at $3400 \times g$ for 30 min. The resulting supernatant was discarded. The pellet was resuspended in a small quantity of 20 mM phosphate buffer pH 7.4 and homogenized again for 30 sec (35000 rpm). This crude extract was then loaded onto a discontinuous sucrose gradient, consisting of 10%, 25%, 30%, 40% (w/v) sucrose layers. After centrifugation at $70000 \times g$ for 2 h, the resulting four bands (tubule-rich fractions) were removed and concentrated by further centrifugation at $140000 \times g$ for 30 min.

2.5. *Biochemical analyses*

Lipids were extracted according to Folch *et al.*[9]. They were separated and identified by thin-layer chromatography (silica gel 60, Merck 5721) in a two-dimensional system[10]. Analyses of glycolipids[11], phospholipids[12], and acylglycerols (determined as fatty acids[13] after saponification) were obtained from the plates. Carotenoids were estimated directly from the lipid extract[14]. Protein obtained from the interphase of the lipid extraction was analyzed according to Lowry *et al.*[15]. Separation of proteins was carried out directly from the tubule fractions by SDS polyacrylamide gel-electrophoresis[16].

3. Results

3.1. *Early green stage*

The green 'hips' contain normally-developed chloroplasts with thylakoids, grana (consisting of up to 14 stacked thylakoids), and few globules (diameter ca. 90 nm). Difficulties arise in the fixation of the hip as seen by the presence of osmiophilic material within the vacuole, the cytoplasm and the chloroplast matrix (Fig. 1 *).

3.2. *Yellow-green stage*

In the large, yellow-green colored hips the elipsoidal chloroplasts already exhibit many symptoms of disorganization of the thylakoid system (Figs 2 and 3). The number of thylakoids per granum decreases. It is presumed that the thylakoids become displaced to yield very elongated double (sometimes triple) thylakoid-complexes. 'Oblique' grana which occasionally show 'incomplete partitions' (Fig. 4, *cf.*[1], l. c., p. 255) may represent a transition stage. The thylakoids are partly swollen (better seen in the yellow stage) or collapsed (Fig. 7). U-shaped thylakoids running parallel to the plastid envelope (*cf.* Figs 2, 3 and 6) are often observed. Other peculiar structures, such as 'thylakoid teeth' (Fig. 5), 'thylakoid noses' (Fig. 7, long arrow), and thylakoid anastomoses (Fig. 7, short arrow) are also sometimes visible.

At this stage, the first tubules are already apparent. They are comparatively short and often connected with membranes such as thylakoids, inner plastid membranes, or the membranes of peripheral vesicles. The tubules occasionally penetrate osmiophilic globules (Fig. 6, globule diameter 30–150 nm).

3.3. *Yellow stage*

At this stage the thylakoid disorganization has advanced. The number of short tubules inside the plastids has increased. The tubules are located mainly in small areas between single or double thylakoids, or between thylakoids and the inner plastid membrane.

3.4. *Orange and red stage*

The red stage differs from the orange one in that the cells now contain anthocyanins in their vacuoles.

The chromoplasts are usually spindle-shaped but sometimes ellipsoidal in outline (*cf.*[2]), especially in the outer layers of the hip. The plastids are filled with bundles of long **, non-ramified chromoplast tubules. These tubules run parallel to each other

* Figs 1–7 see Plate on page 460 a.

** In sections tubules can be followed up to a length of 1 μm. Isolated tubules show lengths of up to 1.5 μm.

and to the longitudinal axis of the chromoplasts (Fig. 12 *). The tubule bundles often reveal a hexagonal pattern in cross-section although no direct contact between the tubules is visible (Fig. 11). Variations in diameter of tubules, and even along the length of a single tubule, occur frequently (12 to 40 nm, mean: 18 nm; Figs 11 and 13). They have an electron-dense core. Individual tubules have irregular polygonal cross-sections (Fig. 11). Osmiophilic globules, through which the tubules usually penetrate, become ellipsoidal.

The thylakoids have become completely disorganized except for irregularly-swollen thylakoid remnants, which resemble irregular cisternae, to which the tubules can be connected (Fig. 14).

In the polarizing microscope, the spindle-shaped chromoplasts exhibit positive dichroism and birefringence, with regard to their longitudinal axes (Figs 8 and 9). The areas of birefringence interrupted by penetrating dark lines most likely represent the areas of parallel bundles of tubules separated by thylakoid remnants (*cf.* Fig. 12).

3.5. *Yellow autumn leaves*

The plastids of the yellow autumn leaves are filled with large globules (Fig. 15, mean diameter 330 nm). Tubules have not been observed.

3.6. *Chemical characterization of the tubules*

The direct isolation procedure (see 2.4) yields 'tubule-rich fractions' which are, however, according to electron microscope observations, not free of contamination (Figs 16 – 18 **). The filamentous structures, as seen in those fractions after negative and positive staining, possess similar diameters to the tubules in sections. Lengths of up to 1.5 μm have been measured. The tubule-associated globules remain intact (Figs 16 and 17) and are more frequent in the lower density fractions (*cf.* Figs 16 and 17 as compared with 18).

The percentual amount of total carotenoid per fraction varies only slightly as can be calculated from Table I. The higher density components (total protein, total phospholipids, but not total glycolipids) increase in amount from the light to the heavier fractions while the amounts of the less dense acylglycerols decrease in the same direction. The ratio of the different phospholipids with respect to one another show a similar pattern in all four fractions. The same relationship is also seen for the glycolipids (Table II). The separation of proteins by SDS polyacrylamide gel electrophoresis shows a main protein band of ca. 31,000 dalton (Fig. 19).

Table I. Major components of the various tubule-containing fractions. Carotenoid as reference = 1 (w/w).

Fractions	A	B	C	D
Transition zone % sucrose (w/v)	0/10	10/25	25/30	30/40
Density [g·cm^{-3}]	1.04	1.10	1.11	1.15
Apolar components:				
carotenoid (without ester component)	1	1	1	1
fatty acids (from carotenoid esters and acylglycerols)	1.9	1.1	1.0	0.9
Semipolar components:				
phospholipids	0.63	0.96	1.10	1.26
glycolipids	2.49	1.47	1.50	1.39
protein	1.18	0.96	2.09	2.87
Ratio (w/w) semipolar/apolar	1.48	1.61	2.35	2.91

Table II. Relative amounts of the different phospho- and glycolipids in all four fractions, expressed in percent (w/w) phospholipid (a) and glycolipid (b), respectively.

a. Phospholipids:	
phosphatidylcholine	27–40
phosphatidylinositol	24–28
phosphatidylserine	17–19
phosphatidylethanolamine	12–20
phosphatidylglycerol	6– 9
b. Glycolipids:	
monogalactosylacylglycerol	28–37
digalactosylacylglycerol	25–34
sulfolipid	7–24
sterolglycoside	8–22

4. Discussion

1. *The development of chromoplasts from chloroplasts*

The chromoplasts in *Rosa rugosa* develop from chloroplasts. The disorganization of the thylakoid system is accompanied by the transient appearance of certain characteristic structures. Some of these have been described by other authors, investigating quite different material. For example, 'oblique'

* Figs 8–14 see Plate on page 460 b.
** Figs 16–18 see Plate on page 460 c.

grana have been found in *Cucumis sativus* [17], in *Cucurbita pepo* [18], in *Tropaeolum majus* [19], and in *Chrysosplenium* [1]. Ljubešić [18] reported the presence of 'special triangular connections' which structurally resemble the thylakoid-teeth of *Rosa rugosa*. Structures similar to U-shaped thylakoids have been noted *e. g.* in *Cucurbita pepo* [18] and *Capsicum annuum* [20].

The tubules in the ripe hip of *Rosa rugosa* reveal the same structural features as in *Cucumis sativus*, described by Smith and Butler [17]. These authors proposed the term 'chromoplast tubules' with respect to the cross-sectional appearance where an electron-dense wall and an electron-transparent core are apparant. In negative staining, however, these tubules appear as filaments (see Winkenbach *et al.* [21], and this present paper, Figs. 16 – 18).

2. *Chemical composition of the tubules*

The chromoplast tubules in *Rosa rugosa* possess a similar chemical composition to those in *Tropaeolum majus* (analyzed by Winkenbach *et al.* [21]). These authors set up a model based on their chemical data – the tubules (or filaments) are thought to be made up of two main-components: an outer semipolar wall and an inner apolar core. The *Rosa* chromoplast tubules resemble those of *Tropaeolum* not only with respect to their appearance in the electron and polarized light microscope but also to their chemical composition. It is therefore well possible that the molecular model of Winkenbach *et al.* [21] may also apply to the *Rosa* chromoplast tubules. The protein pattern in *Rosa rugosa* is remarkably comparable to that of *Tropaeolum*. The carotenoid type does not seem to play a crucial role in the formation of tubules, since tubules in chromoplasts have been observed in cases where the main carotenoid component was lycopene (hips of *Rosa canina* [22]), lutein diester (petals of *Tropaeolum* [23]), β-carotene and cryptoxanthin (sepals of *Strelitzia reginae* [24]) and cryptoxanthin (fruits of *Solanum capsicastrum* [22]). Simpson *et al.* [25] found various carotenoids in fruits of several CTPA-treated cultivars of *Capsicum annuum*.

3. *The origin of the tubules*

Steffen and Walter [26] found 'fibers' in *Solanum capsicastrum* with associated 'droplet-like structures' or 'local swelling on fibres'. They interpreted that these droplets (*i. e.* the globules) result from the breakdown of the thylakoids and then develop into fibers (*i. e.* tubules) by 'stretching'. This assumption was shared by several authors [5, 27, 28]. However, this mode of chromoplast tubule formation – *i. e.* globules being intermediate stages of developing tubules – seems to be very unlikely in the case of *Rosa rugosa* (and possibly in some other species also) for the following reasons:

1. If tubules are formed from materials of the globules, these tubules should be of comparable and uniform density in cross-sections as compared with the globules. This is, however, not the case. Instead, as compared with the globules, they exhibit an electron-dense wall and an electron-transparent core. A substructure within the globules, as reported by Simpson and Lee [5] (other plant material), could not be detected in the material described here.

2. If the tubules are formed from globular material by direct 'stretching', their chemical composition should be similar. However, our chemical analyses provide evidence that tubules and globules have a different chemical composition. The tubules contain a large amount of semipolar material (see also [21]), whereas the tubule-associated globules are likely to consist mainly of apolar material. Moreover, the fraction with the most frequent tubule-associated globules contains the highest amount of apolar acylglycerols. It is furthermore to be noted that large plastoglobules derived from chromoplasts of various flower petals (*Caltha, Tulipa, Viola*) consist mainly of apolar material [29]; it appears unlikely that the plastoglobules of rose hips differ greatly in their chemical composition from the ones of flower chromoplasts.

3. Globules do not appear at all during the development of tubulous chromoplasts in *Cucumis sativus* [17], *Tropaeolum majus* [19], and *Chelidonium majus* [30]. These examples show clearly that the formation of globules as an intermediate stage is not a necessary prerequisite in the formation of tubules.

Tubule formation is considered to be a consequence of the reorganization of material derived from thylakoid breakdown [17, 19, 31]. However, the origin of tubular lipids and proteins cannot be determined without tracer experiments.

Neither the protein pattern nor the lipid composition of tubules are comparable with data reported for thylakoids (*e. g.* [32, 33]). Simple breakdown of thylakoids by utilization of the same

material without any losses does not fit the data; this suggests that differences occur at least in the degree of breakdown of various lipids and proteins. Furthermore, additional synthesis of lycopene must occur because this carotene is not found in chloroplasts of higher plants.

4. *Autumn leaves of Rosa rugosa*

In yellow autumn leaves of *Rosa rugosa* the predominant structural elements of the plastids are globules. This indicates that, in the same plant (although within different tissues), quite different reorganization processes of the chloroplasts occur, as also shown by Falk [19] in *Tropaeolum* (see also [16]). According to the literature, it seems to be a general rule that senescent plastids (*sensu* Granick [34]) of all autumn leaves, even of those plants with tubulous (or crystalline) chromoplasts in their fruits or petals, contain globules.

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Figs 1–7. Plastids in early stages of hip development in *Rosa rugosa*.

Fig. 1. *Green stage:* Thylakoids partly arranged in grana. Osmiophilic material within the vacuole, the cytoplasm and the chloroplast matrix. 47,000×.

Figs 2–6. *Yellow-green stage:* The thylakoid system with symptoms of disorganisation.

Fig. 2. Oblique grana with a low number of thylakoids per granum. Note the U-shaped thylakoids (arrows). 28,000×.

Fig. 3. Less grana as in Fig. 2. Elongated double thylakoid-complexes. U-shaped thylakoids. 40,000×.

Fig. 4. Incomplete partition (arrow). 80,000×.

Fig. 5. 'Thylakoid teeth'. 80,000×.

Fig. 6. Osmiophilic globules penetrated by tubules in cross-section and longitudinal section. 70,000×.

Fig. 7. *Yellow stage:* The thylakoids are partly swollen or collapsed. 'Thylakoid noses' (long arrow) and thylakoid anastomoses visible (short arrow). 30,000×.

* The line designates 1 μm (fractions of it, when used, are indicated).

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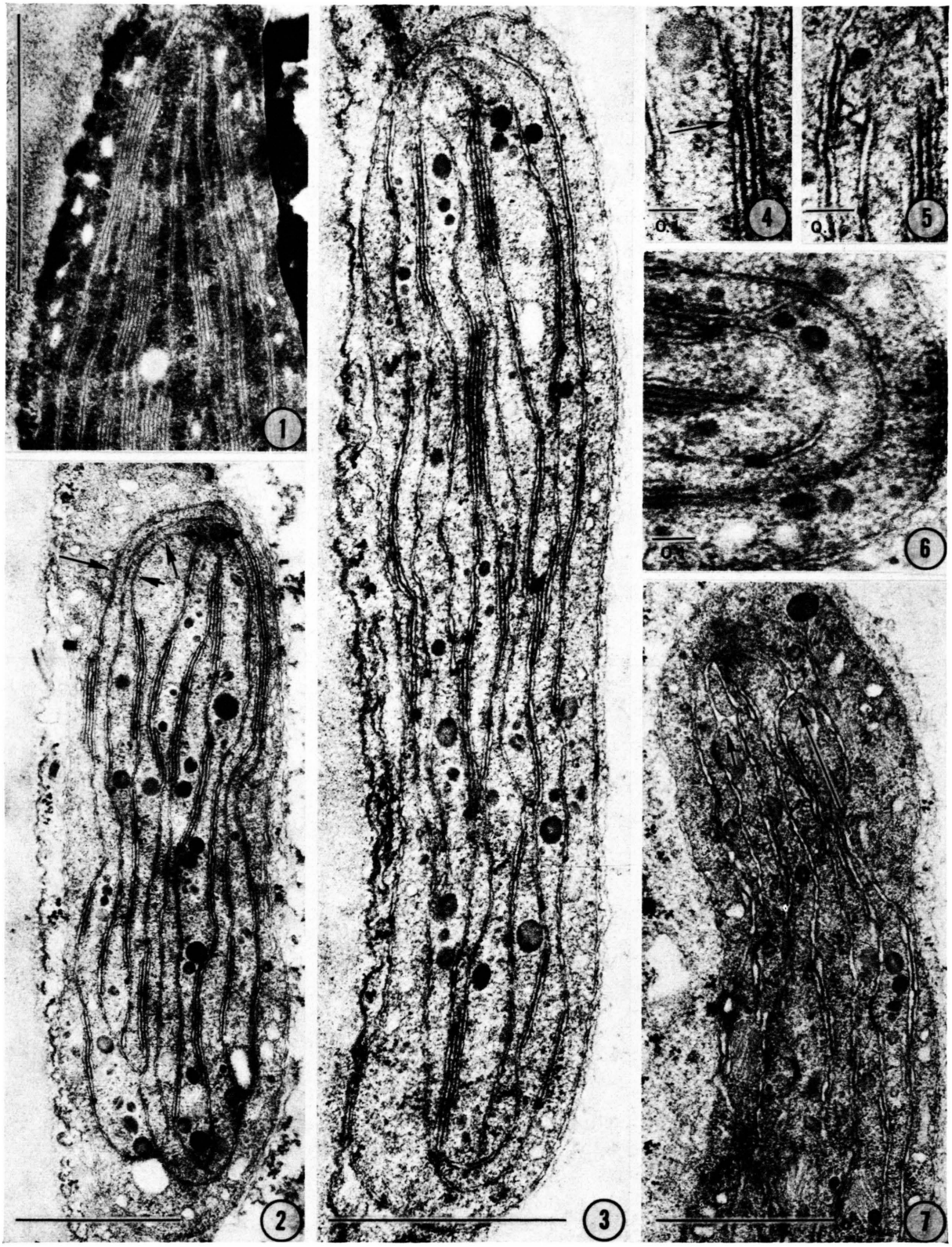

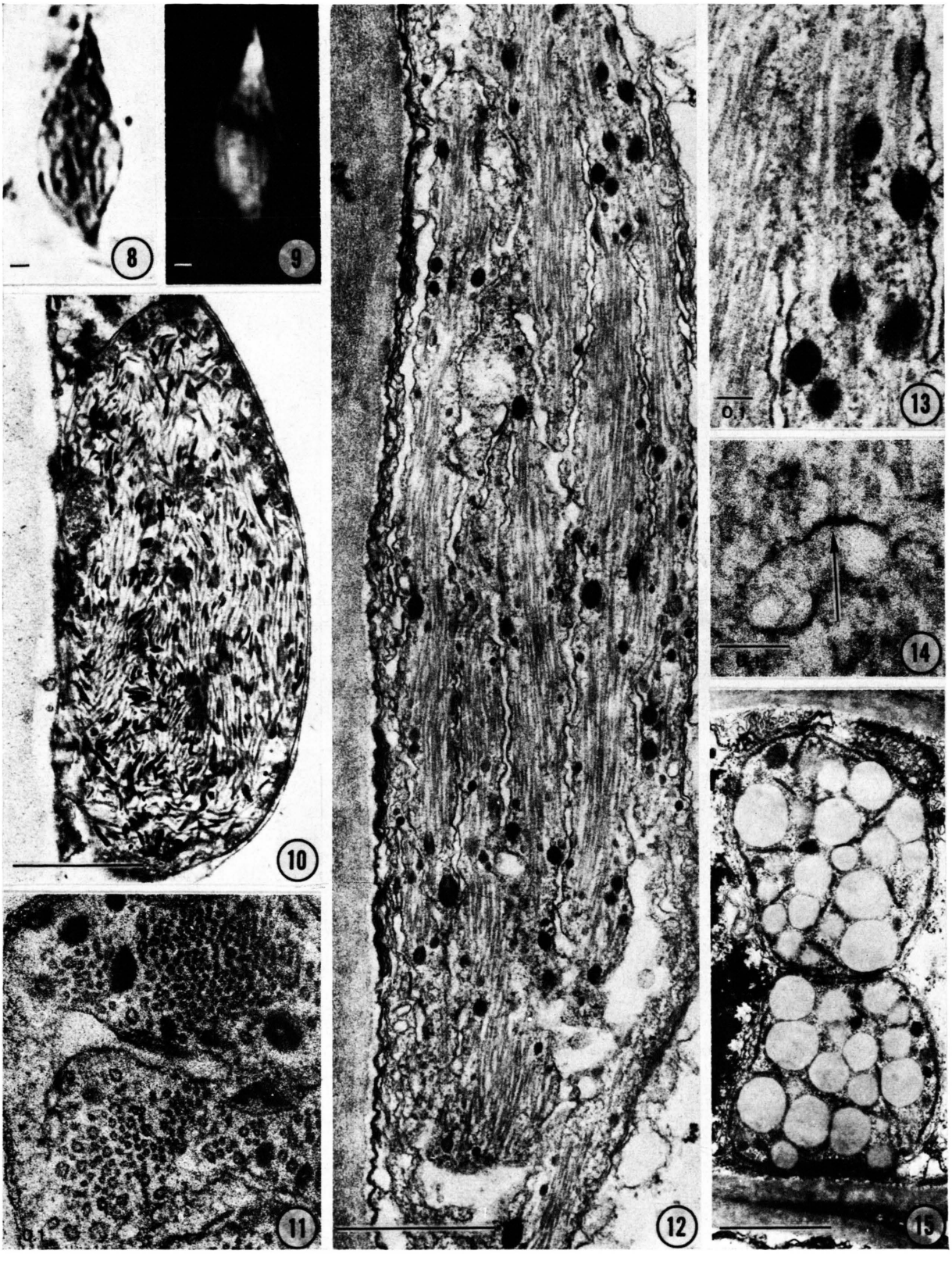
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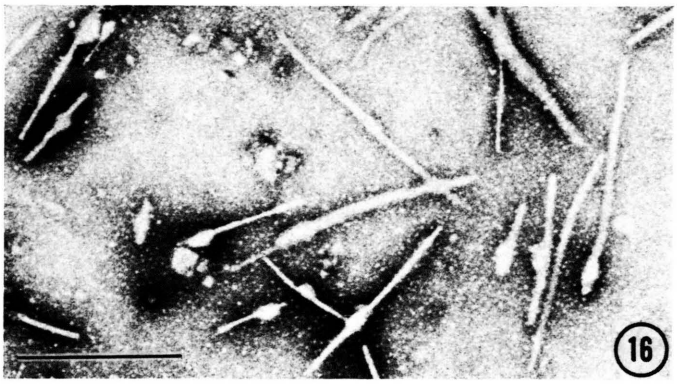

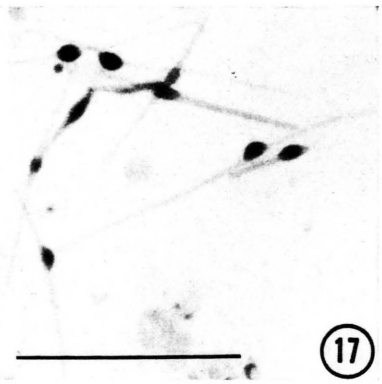

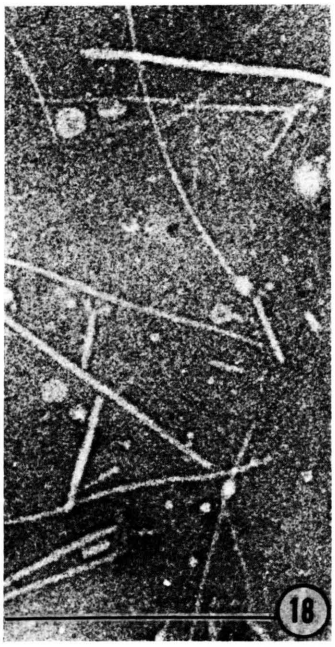

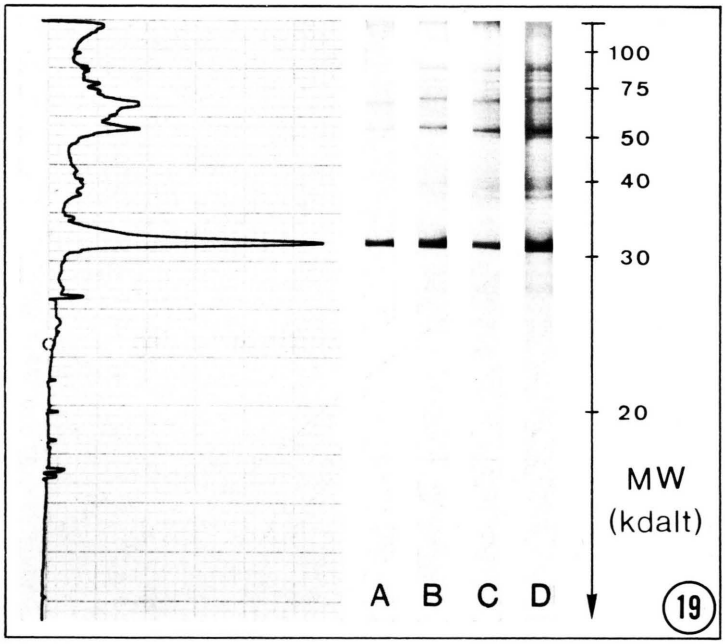

Figs 8—14. *Orange (and red) stage.*

Fig. 8. Spindle-shaped chromoplasts under the light microscope. 3,000×.

Fig. 9. The same chromoplast as in Fig. 8 under polarized light. Note areas of birefringence interrupted by dark lines. 3,000×.

Fig. 10. Tubule containing chromoplast, $KMnO_4$ fixation. 22,000×.

Fig. 11. Irregular polygonal cross-sections of the tubules. 65,000×.

Fig. 12. A spindle-shaped chromoplast with areas of parallel bundles of tubules separated by thylakoid remnants. 26,500×.

Fig. 13. The tubulus penetrate osmiophilic globules. An individual tubule varies in diameter along its length. 60,000×.

Fig. 14. Tubules connected with swollen thylakoid remnants (arrow). 120,000×.

Fig. 15. Globules in plastids of yellow autumn leaves of *Rosa rugosa*. 19,000×.

Figs 16 and 17. A lower-density tubule fraction (0/10% sucrose (w/v), $\varrho = 1.04$) after negative and positive staining. Note the large amount of globule-associated tubules. 22,000× and 30,000×.

Fig. 18. A high-density tubule fraction (25/30% sucrose (w/w), $\varrho = 1.11$). Note the low amount of globule-associated tubules. 36,000×.

Fig. 19. Densitogram of gel A)low density tubule fraction, as represented in Figs 16 and 17). Gel B, C and D represent the fractions 10/25%, 25/30%, 30/40% (w/v) sucrose, respectively.