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Microbodies and an Anomalous "Microcylinder" in the Ultrastructure of Plants with Crassulacean Acid Metabolism

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Summary. An ultrastructural study was made of the leaf tissues of four species of plants in three genera with Crassulacean acid metabolism ("CAM" plants): *Kalanchoë daigremontiana* Hamet et Perrier, *K. verticillata* Elliot, *Sedum rubrotinctum* Clausen and *Crassula tetragona* L. Microbodies similar in appearance, with fibrillar or granular nucleoids but no crystalline deposits, were present in the mesophyll of all four species. The microbodies resembled in size and abundance those of C_3 plants more closely than those of C_4 plants, both under long-day and short-day conditions. The reaction for catalase activity employing 3,3′-diaminobenzidine produced a heavy deposit in the microbodies; the reaction was blocked by the catalase inhibitor, aminotriazole.

Some of the plants of the two species of *Kalanchoë* studied contain in the epidermal and mesophyll cells of the leaves and plantlets an organelle-like structure consisting of a hollow cylinder, 90–160 nm in diameter and up to 2 µm or more in length, around which 18–20 or more minute tubules are wound in a steep helix. The tubules are only *ca.* 9 nm in diameter, hence are much smaller than conventional microtubules. The cylinder and surrounding tubules, herein tentatively assigned the term "microcylinder" for convenience, may represent a product of viral infection, or may be an organelle that appears at certain stages of growth or under particular environmental conditions. In any case it may prove to be of considerable importance for investigators of CAM plant physiology.

Introduction

In recent years three groups of higher plants have been distinguished which differ in their mode of photosynthetic carbon assimilation. They are the now widely familiar C_3 and C_4 plants, and the interesting CAM plants, or plants with Crassulacean acid metabolism (see Ting, 1971; Ting *et al.*, 1972). Each of the three types is characterized by a syndrome of properties setting it apart from the others and embracing not only biochemical pathways, but also anatomical and physiological specializations as well as taxonomic affinities and ecological relationships (see Black, 1973).

Leaf ultrastructure of the C_3 and C_4 plants has been explored to some extent, particularly with respect to the chloroplasts and microbodies (leaf peroxisomes), allowing certain generalizations to be made. Thus it is now recognized that in the leaves of C_3 plants the microbodies are relatively large and numerous, and react strongly in the diaminobenzidine (DAB) test for catalase (Frederick and Newcomb, 1969), properties that are correlated with the relatively high levels of photorespiration exhibited by these plants (see Jackson and Volk, 1970; Black, 1973). In C_4 plants, in contrast, the microbodies in the mesophyll are scarce

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and give a relatively weak DAB reaction. Although they are more numerous and more reactive toward DAB in the bundle-sheath cells, even here they are smaller and less abundant than their counterparts in C_3 plants (Frederick and Newcomb, 1971). These observations are correlated impressively with the information on localization of photorespiration in the bundle-sheath cells (Rehfeld *et al.*, 1970; Kanai and Edwards, 1973) and on the relatively low levels of photorespiration observed in C_4 plants (Jackson and Volk, 1970; Rehfeld *et al.*, 1970). The ultrastructural studies have therefore led to a better understanding of the nature of C_3 and C_4 plants by effectively complementing the biochemical and physiological investigations.

The ultrastructure of CAM plants has been less extensively investigated, particularly with regard to the presence and properties of microbodies. The present study attempts to fill this gap in our knowledge by a brief description of microbodies in relation to the general cytoplasmic ultrastructure of the mesophyll in leaves of several species of CAM plants commonly used in physiological experimentation. In the course of the work we have observed an unusual structure consisting of a cylinder surrounded by fine tubules in some of the leaves and plantlets of *Kalanchoë daigremontiana* and *K. verticillata*. In view of its unusual substructure and possible importance for physiologists working with CAM plants, a brief account of the occurrence and ultrastructure of this "microcylinder" is also included.

Materials and Methods

Plants. The present study was conducted with CAM species growing in the greenhouse of the Department of Botany, University of Wisconsin-Madison during the fall and winter months of 1974. The species chosen for study included *Kalanchoë daigremontiana* Hamet et Perrier (*Bryophyllum daigremontianum* Berger), *K. verticillata* Elliot (*Br. verticillatum* Berger), *Sedum rubrotinctum* Clausen and *Crassula tetragona* L. All four species have been maintained in the Botany greenhouse for many years (the two species of *Kalanchoë* vegetatively from plantlets). During the course of the work, daylength in the greenhouses decreased from 13 to 10 h. For organelle counts in plants grown under short-day conditions, the plants were kept in the greenhouse for 7 weeks under 8-h days; during the dark periods they were covered with a canopy of black cloth. For long-day conditions, selected specimens of each species were placed in a controlled environmental chamber for 18 days and maintained under daily light periods of 15 h at 25° and dark periods of 9 h at 20°. All plants received adequate daily watering. Incandescent and Westinghouse Cool White fluorescent lights provided approximately 1200 fc in the chamber at the height of the sampled leaves.

Samples of tissue for study were taken from young, developing leaves of *Sedum rubrotinctum* and *Crassula tetragona*. *Kalanchoë daigremontiana* and *K. verticillata* were represented by both leaf tissue and plantlets developing on the leaves. Young, developing leaves and older, fully mature leaves were sampled; the two pairs of leaves of the plantlets were also fixed at several stages of development, as were roots and stems of plantlets.

Preparation of Materials for Electron Microscopy. Large pieces of the tissues to be examined were placed under a solution of the fixative and segmented into small pieces *ca.* 1×1 mm. The fixatives used were either 3% glutaraldehyde or 2% paraformaldehyde-2% glutaraldehyde, buffered in each case with 0.05 M potassium phosphate at pH 6.8. The second-named fixative yielded consistently superior results and was employed exclusively in later embedments. Fixation was for either 1.5 h or 0.5 h at room temperature; in the latter case fixation was continued overnight at 4°. Fixations carried out on the first schedule were begun in the morning, while those conducted according to the second method were begun in the afternoon in order to take advantage of the lower organic-acid contents at that time of day. Neither method was demonstrably superior to the other in the results

obtained. After fixation, the tissues were rinsed for 1.5 h in phosphate buffer and postfixed in 2% osmium tetroxide in 0.5 M phosphate buffer, pH 6.8, for 2 h at room temperature. Postfixation was followed by dehydration in an acetone series and embedment in a mixture of low-viscosity Epoxy resins according to the method of Spurr (1969).

Silver sections were cut on a Sorvall MT-2 Ultramicrotome, mounted on uncoated 75×300 copper grids, post-stained in aqueous 2% uranyl acetate for 10 min followed by lead citrate for 5 min, and viewed in a Hitachi HU-11A microscope operated at 75 kV.

Counting of Organelles. For the counts of organelle profiles, transverse sections of leaves of *K. daigremontiana* taken from plants growing under both long- and short-day condition- were used. The counts were made during observation of thin sections in the electron micros scope, rather than from micrographs. Owing to the large size of the cells, counts were made of all mesophyll cell chloroplasts, microbodies and mitochondria visible within the grid spaces and no attempt was made to make counts on a "per-cell profile" basis. The total counts of organelles for each sample, given in the table under Results, provided a determination of the chloroplast: microbody ratio.

Localization of Catalase. Mature leaves of *Kalanchoë daigremontiana* growing under greenhouse conditions, but receiving artificial illumination to extend the daylength to 16 h, were used in cytochemical experiments to localize catalase. The procedure, adapted from that of Novikoff and Goldfischer (1969), was modified slightly from that of Frederick and Newcomb (1969). The differences were that no attempt was made to remove the epidermis and that a paraformaldehyde-glutaraldehyde fixation was employed instead of glutaraldehyde alone.

All tissues incubated with diaminobenzidine (DAB) were fixed in paraformaldehyde-glutaraldehyde, then rinsed in 0.05 M potassium-phosphate buffer, pH 6.8, and incubated in either the complete reaction mixture or a control mixture. After incubation the tissue was rinsed in phosphate buffer, then subjected to routine postfixation procedures including treatment with osmium tetroxide, dehydration in acetone, and embedment in the low-viscosity Epoxy resin.

Results

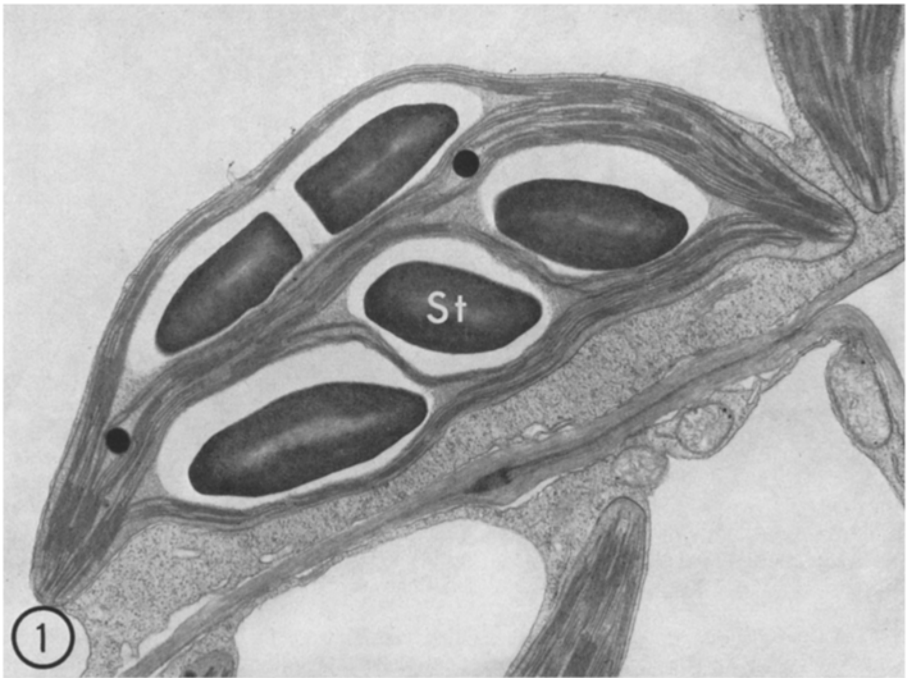
General

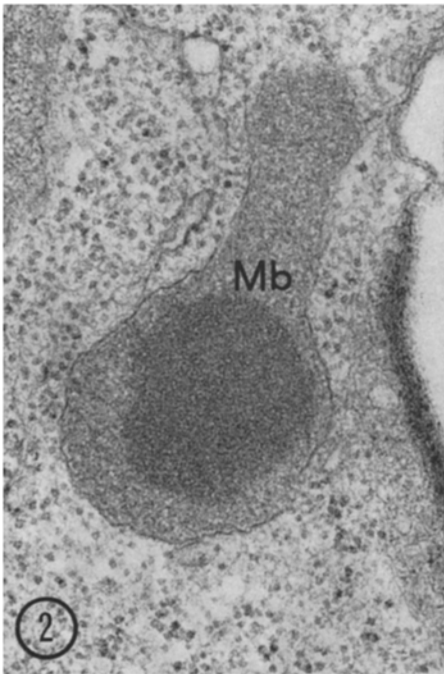
Ultrastructural observations have been made on the succulent leaves of four species of CAM plants representing three genera, with emphasis on *Kalanchoë daigremontiana* and *K. verticillata*. In all four species the mesophyll consists of large, isodiametric, water-storing cells containing a large central vacuole bounded by a thin, peripheral layer of cytoplasm. The presence of chloroplasts in these water-storing cells is characteristic of CAM plants (Ting *et al.*, 1972).

With the exception of the "microcylinders" found in some of the material as described below, the mesophyll cells present no unusual or distinctive features either in the kinds and numbers of organelles they contain, or in the morphology of these organelles. The chloroplasts have well-developed grana; they also commonly possess large starch deposits (Fig. 1). Abundant starch was present in material fixed between 8:00 and 9:00 a.m. as well as in material fixed late in the afternoon. No peripheral reticulum has been noted in the chloroplasts, nor have crystals or other special inclusions been observed in the stroma. Chloroplasts of plants grown under short-day conditions (favoring C_4-like metabolism) are usually thicker than those grown under long-day.

Microbodies

Microbodies are frequently encountered in the mesophyll of all four species. Their abundance relative to chloroplasts and mitochondria was determined for





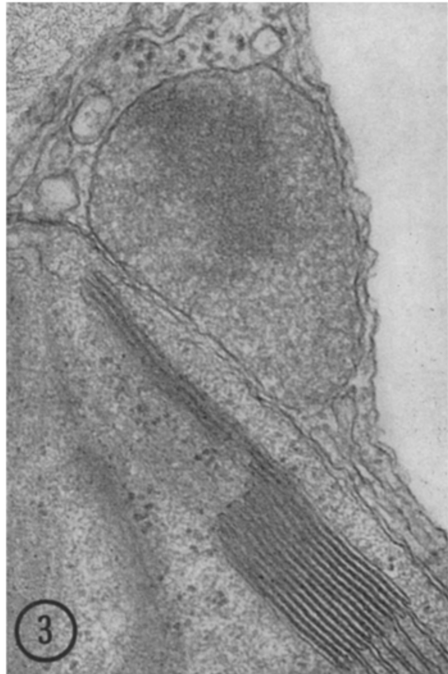


Fig. 1. Portions of two mesophyll cells showing the peripheral layer of cytoplasm in a leaf of a plantlet of *Kalanchoë daigremontiana*. The chloroplasts have well-developed grana and large starch deposits (*St*). ×16000

Fig. 2. An amoeboid microbody (*Mb*) with a prominent nucleoid in a leaf mesophyll cell from a plantlet of *K. daigremontiana*. ×56000

Fig. 3. A microbody appressed to a chloroplast in a leaf mesophyll cell of *Sedum rubrotinctum*. ×64000

Table 1. Counts of organelle profiles in mesophyll cells of *K. daigremontiana*

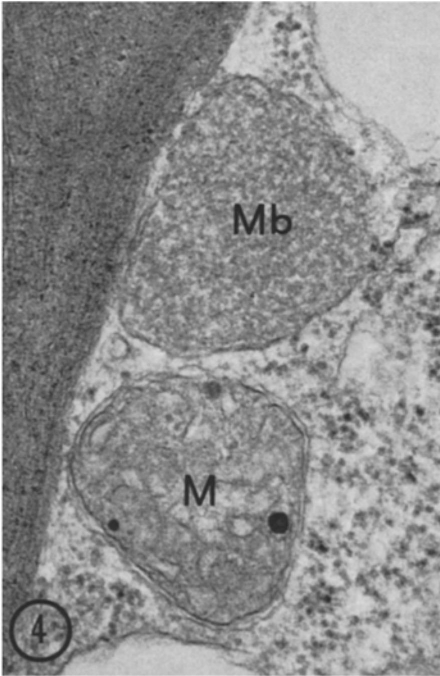
Plant material	Total No. profiles counted			Ratio, chloroplasts: microbodies
	Chloroplasts	Mitochondria	Microbodies	
Mature leaves; long-day (15 h) conditions in growth chamber	892	1455	117	7.6:1
Mature leaves; short-day (8 h) conditions in greenhouse	295	908	66	4.5:1
Very young leaves; short-day (8 h) conditions in greenhouse	393	502	69	5.7:1

K. daigremontiana by making counts of organelle profiles in those portions of the mesophyll cells visible between the grid bars. As shown in Table 1, mitochondria are the most abundant of the three organelles, followed by the chloroplasts. The ratio of chloroplasts to microbodies is similar to the ratio previously obtained for the mesophyll of C_3 grasses and the bundle-sheath cells of C_4 grasses (Frederick and Newcomb, 1971).

No clear-cut differences in microbody appearance between the four CAM species have been noticed. Especially in cells of younger leaves, although not exclusively so, many of the microbodies are amoeboid in appearance, suggesting that they may undergo extensive changes in shape. Elongate microbody forms are common, including those with dumbbell shapes or with a head and tail configuration (Fig. 2). Associations of microbodies with cisternae of granular endoplasmic reticulum in young cells are frequent. Microbodies appressed to chloroplasts are also common (Figs. 3–5), although many do not appear to be so closely associated (Fig. 6).

A nucleoid is seen in the microbodies in such a high proportion of the micrographs (Figs. 2, 3, 5, 6) as to suggest that it may invariably be present in this organelle in these CAM plants. Somewhat irregular in outline, the nucleoid is more electron dense than the surrounding matrix, and is fibrillar or granular in appearance and without detectable crystallinity. Crystalline inclusions have not been seen in the microbodies in any of the CAM plants studied.

The equivalence of the microbodies observed in CAM plants to peroxisomes as biochemically defined was examined cytochemically by employing diaminobenzidine (DAB) for the localization of catalase, a marker enzyme for the peroxisome. Segments of mature leaves of *Kalanchoë daigremontiana* grown under long-day conditions were incubated in the complete DAB mixture, resulting in a heavy deposition of an electron-dense reaction product in the microbodies (Fig. 7). Other organelles were not noticeably stained. The darkening of the microbodies caused by DAB was completely prevented when the catalase inhibitor aminotriazole was included in the reaction medium.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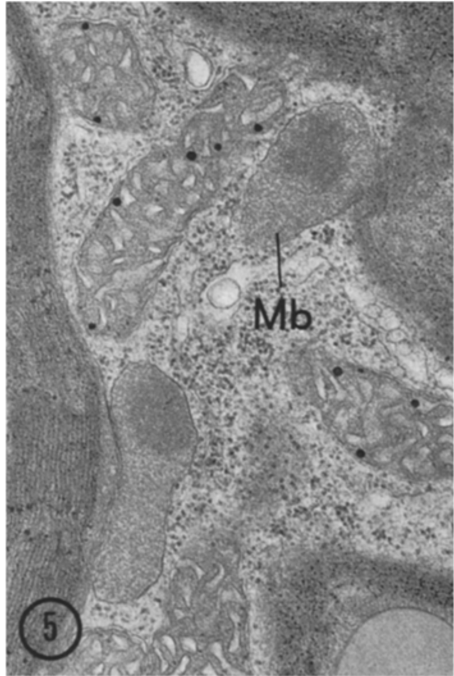
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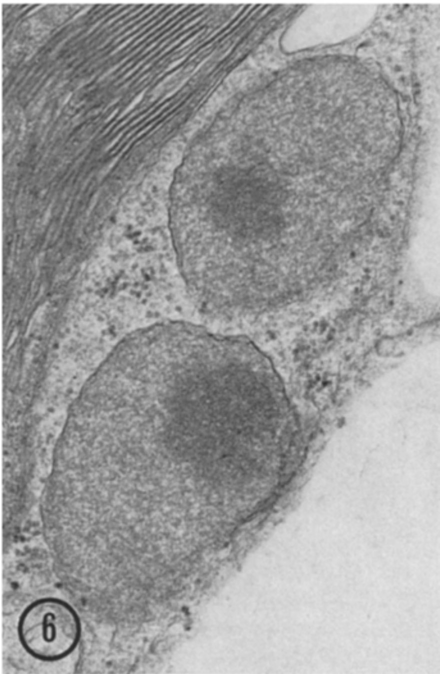
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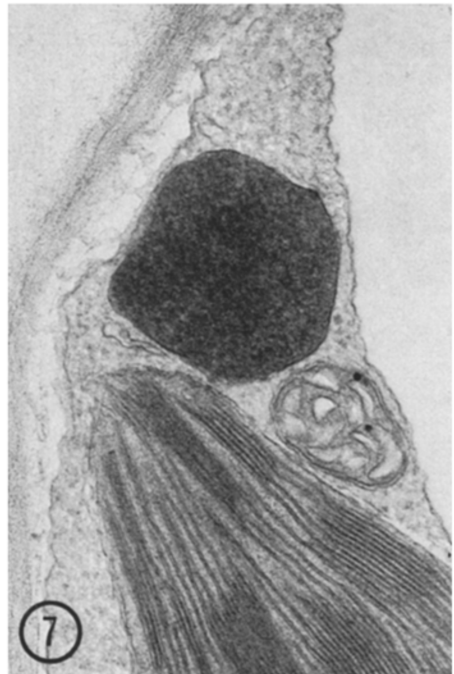
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Microcylinders

We report here the presence in some of the CAM plant material of an unusual organelle-like structure which we are tentatively terming a "microcylinder", with the expectation that a more appropriate designation will be found as additional information about the structure accumulates. The microcylinders have been observed thus far in the mesophyll and epidermis of young leaves in 6 out of 8 mature specimens of *K. daigremontiana* examined, but not in the mature leaves. Plantlets removed from the leaf notches of 5 of the 6 positive plants also contained microcylinders. In an examination of 14 plantlets taken from the same parent plant, microcylinders were found in all cases. In a more limited examination of leaves and plantlets from three mature specimens of *K. verticillata,* microcylinders were seen only in one plantlet. None of the plantlets or parent plants of either species had any visible disease symptoms.

This unusual structure is composed of a hollow cylinder surrounded by a number of very small, more or less evenly spaced tubules; the tubules wind around the body in a steep helix nearly parallel to the cylinder axis. Thus we arbitrarily define the "microcylinder" as the complete structure, and refer to its two major components as a "cylinder" and "tubules", respectively. We refrain from using the term "microtubules" so as to avoid confusing the tubules of the present structure with conventional microtubules, which are considerably larger.

The cylinders are somewhat variable in diameter, ranging in the material thus far examined from *ca.* 90 to 160 nm (excluding the ring of tubules). Their lengths are difficult to determine, since most of those observed do not lie entirely within the plane of section. At least some of them, and perhaps the majority, extend for 2 μm or more, such as the example in Fig. 8, which is 2.7 μm in length. Frequently cylinders have been observed with one end closed by a vesicle (Fig. 9). Since they come to an abrupt end in these cases, it seems unlikely that they are passing out of the plane of section; we conclude, therefore, that their true ends are being seen.

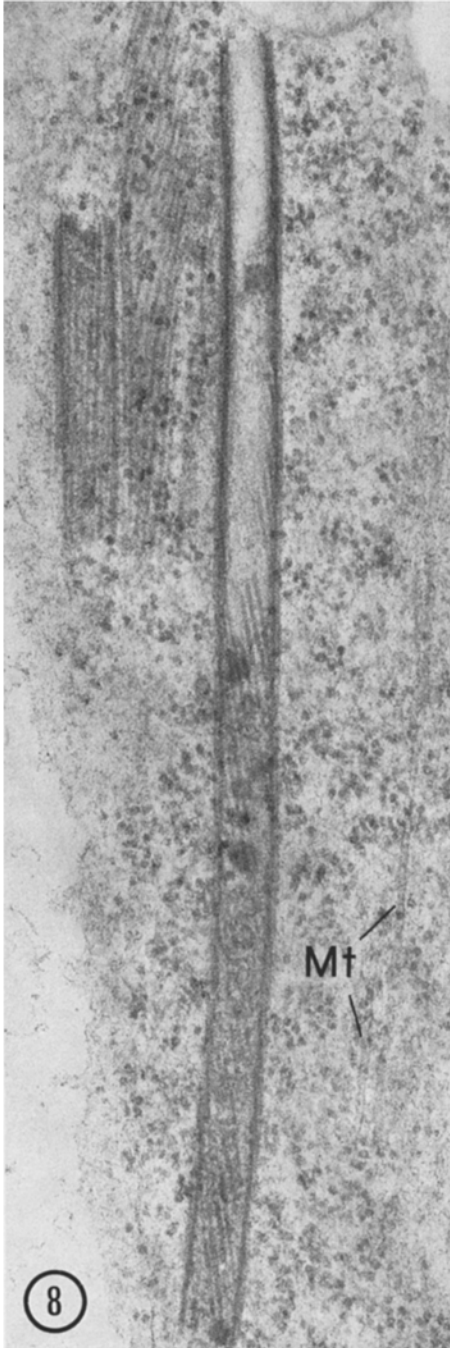
The lumina of the cylinders contain a number of unevenly distributed masses of amorphous or faintly fibrillar material (Figs. 8, 10). The masses are variable in size and occasionally appear to be membrane-bounded. In cross section the cylinder wall consists of an electron-dense layer with the approximate dimensions of a unit membrane, though it is without detectable unit-membrane structure. To the inside of this is less electron-dense material of varying thickness (Fig. 11).

Fig. 4. A microbody (*Mb*) and a mitochondrion (*M*) in a leaf mesophyll cell of *Crassula tetragona*. ×60000

Fig. 5. Microbodies (*Mb*) and mitochondria among chloroplasts in the mesophyll of a plantlet of *Kalanchoë verticillata*. ×32000

Fig. 6. Microbodies with nucleoids in a mature leaf of *K. daigremontiana* maintained under long-day conditions in the growth chamber. ×50000

Fig. 7. Portion of a mesophyll cell from a segment of leaf tissue of *K. daigremontiana* incubated in a medium containing diaminobenzidine (*DAB*) and hydrogen peroxide. Dense reaction product is confined to the microbody. ×47000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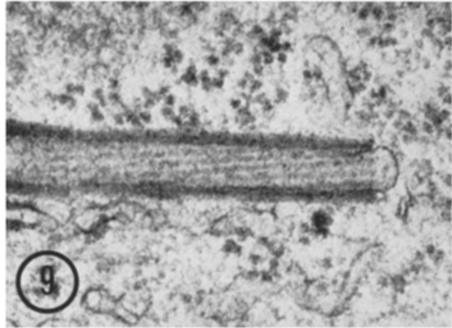
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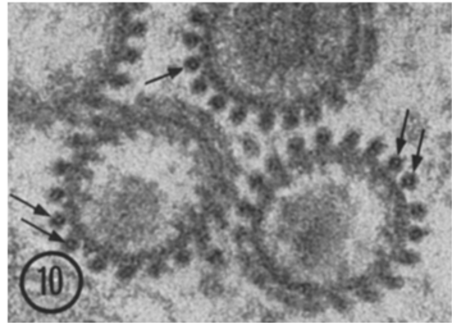
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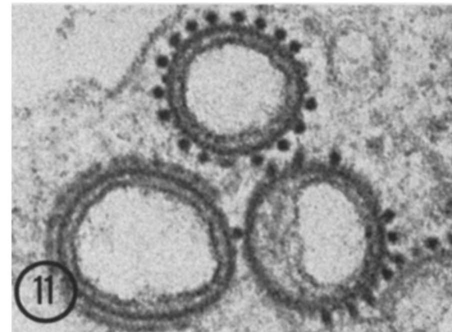
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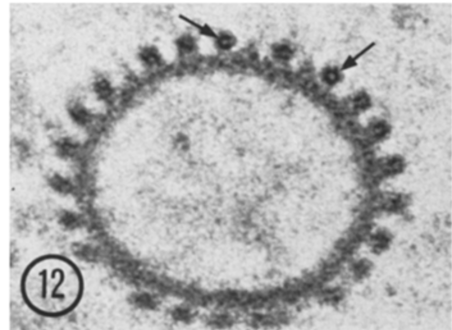
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Bounding the inside of the latter material there is frequently a second electron-dense layer (Fig. 11; inset, Fig. 13) which is thinner and less rigid in appearance than the wall layer. We interpret the vesicle that closes the cylinder in Fig. 9 as an extension of this inner layer.

The tubules surrounding the cylinder are *ca.* 9 nm in diameter, their exact limits being difficult to establish owing to the presence of an irregular coating of amorphous material (Fig. 12). They are clearly tubular in nature, as is seen when they are cut normally, showing the electron-transparent core (arrows, Figs. 10 and 12). They are positioned about 4.5 nm distant from the outer surface of the cylinder wall, and laterally are more or less equidistantly located from one another with a center-to-center spacing of approximately 23 nm between adjacent members. Whether there are arms or bridges anchoring the tubules to the cylinder surface has not yet been determined, although some of the micrographs are suggestive of this (Fig. 12).

The number of tubules around the cylinders can only be approximated in most cases because, owing to their helical inclination, when the tubule profiles are clearly seen on one side of a cylinder, they appear blurred on the other (Figs.10–12). In one particularly clear cross section (inset, Fig. 13), there are 24 tubules associated with the cylinder; in other cross sections this number appears to be at least 18–20.

In describing a steep helix around the cylinder, the tubules in most cases are inclined approximately 7–9° from the cylinder axis (Figs. 8, 9). In some cases, however, the inclination appears to be somewhat greater or less (Fig. 8, upper left). It is not certain whether this represents an actual variation in the pitch of the helix or results merely from the orientation of the microcylinder with respect to the plane of section. Occasionally a reversal in inclination of the tubules from right to left with respect to the axis can be seen along the length of a cylinder (Fig. 14, top center). This results when the microcylinder passes obliquely through the section so that first one surface and then the other is seen clearly.

The microcylinders lie predominantly parallel to the cell surface (Fig. 14), but otherwise show no preferential orientation; sometimes they are oriented at right angles to one another in close proximity in the same cell. While they may occur singly in a particular cell, more commonly they appear in masses (Figs. 13,

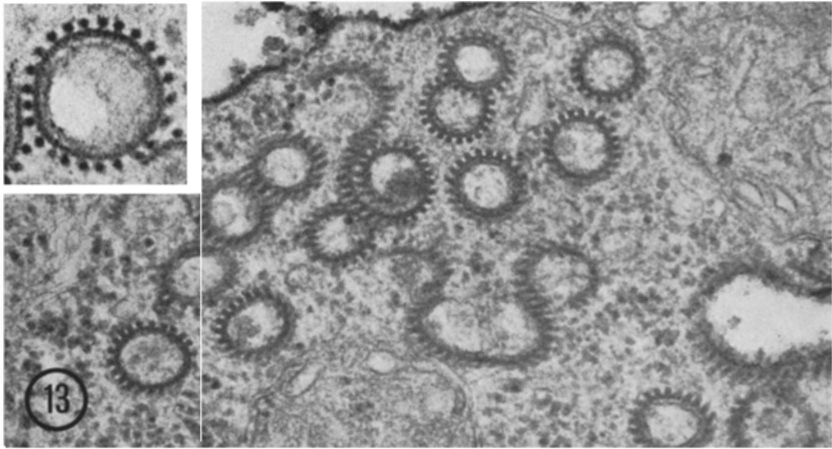
Fig. 8. A microcylinder seen in longitudinal view in a leaf mesophyll cell from a plantlet of *K. daigremontiana*. Portions of other microcylinders are visible at upper left; conventional microtubules (*Mt*) are present on the right. ×63000

Fig. 9. One end of a microcylinder closed by a vesicle. ×65000

Fig. 10. Several microcylinders seen in cross-section. The arrows point to several tubules the electron-lucent cores of which are easily seen. ×174000

Fig. 11. Microcylinders sectioned transversely. Note the presence of electron dense material between the outer wall and inner bounding layer. ×134000

Fig. 12. Transverse view of a microcylinder greatly enlarged, showing the ring of helically wound tubules outside the cylinder wall. Arrows point to tubules the electron-lucent cores of which are easily seen. ×223000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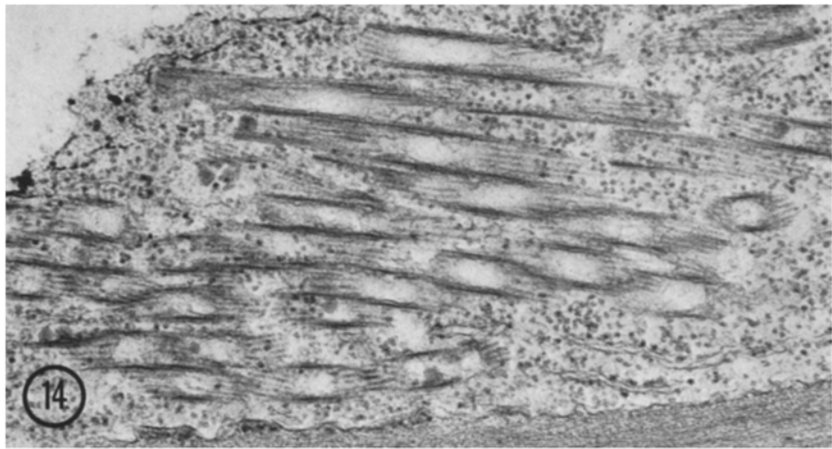
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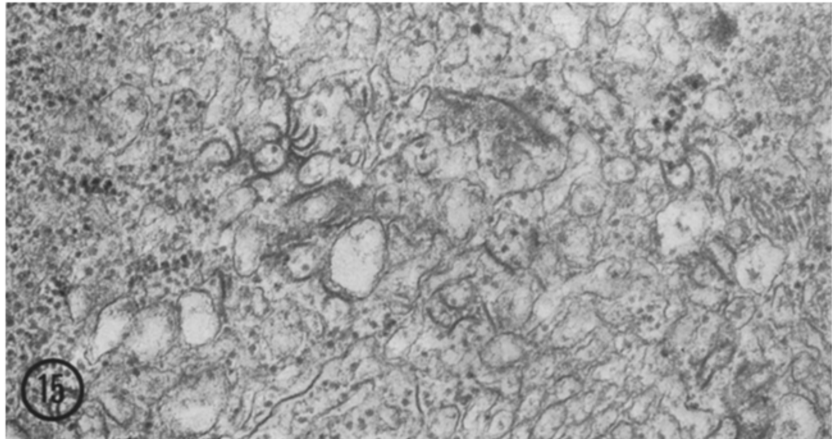
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14). They have never been found in the nucleus, nor within any of the cytoplasmic organelles. No pattern to their distribution within the leaf tissues has been detected. The number of profiles per cell section is highly variable and ranges from zero to as many as 30. In a limited examination of cells in the aerial roots of the plantlets, no microcylinders have yet been seen.

In some cells of the leaf, regions of intense vesiculation of the endoplasmic reticulum are encountered which may contain a few small pinwheel-like structures and a number of short, curved membrane-like segments that are thicker and more intensely stained than the other membranes (Fig. 15). We believe these structures represent stages in the ontogeny of the microcylinders. Results of a more detailed investigation of the origin and development of the microcylinders will be reported in a later paper.

Discussion

General

In the present investigation, the ultrastructure of mesophyll cells has been examined in leaves of four species of CAM plants in three genera, including one of the CAM species (*Kalanchoë daigremontiana*) most frequently used in physiological experimentation. No unusual features have been noticed in the kinds, numbers, disposition or morphology of the organelles. The chloroplasts possess well-developed grana, but no peripheral reticulum. Laetsch (1971) has remarked that the lack of a peripheral reticulum is characteristic also of a number of other CAM plants, including pineapple, although he has noted that a species of *Opuntia* and species of *Ferocactus* have a system of tubules in the peripheral chloroplast stroma. Thomson and Platt (1973) have observed that in the chloroplasts of the barrel cactus *Echinocactus acanthodes*, also a CAM plant, there is no evidence of a peripheral reticulum, although there is a slight vesiculation of the chloroplast inner membrane. Gifford and Stewart (1968) have described inclusions in the proplastids of shoot apices of *Kalanchoë* and *Bryophyllum*, while Lee and Thompson (1973) have reported the presence of an inclusion identified as a stromacentre in leaf plastids of *Kalanchoë pinnata*.

Microbodies

The microbodies of the leaves of CAM plants are of particular interest in view of the differences between C_3 and C_4 plants both in the numbers and morphology of this organelle (Frederick and Newcomb, 1971) and in the magnitude of the photorespiratory process in which it participates (see Jackson and Volk, 1970;

Fig. 13. Transverse views of numerous microcylinders present in the cytoplasm of a mesophyll cell of *K. daigremontiana*. ×68000. *Inset*: Cross-section of a microcylinder showing 24 tubules in a ring outside the cylinder wall. ×113000

Fig. 14. A region of cytoplasm containing numerous, similarly oriented microcylinders that pass obliquely through the plane of section. ×42000

Fig. 15. Intense vesiculation of cytoplasm in a leaf-mesophyll cell from a plantlet of *K. daigremontiana*. Especially electron-dense membrane-like segments include a structure resembling a pinwheel. ×52000

Black, 1973). However, the only previous ultrastructural report of the presence of microbodies in CAM plants of which we are aware is that by Thomson and Platt (1973); these authors observed the organelle in association with chloroplasts in the stem of the barrel cactus.

It is now well established that CAM plants have the option of following either the C_3 or the C_4 mode of photosynthetic metabolism, and that a variety of environmental conditions regulate which mode is followed (Bender *et al.*, 1973; Osmond *et al.*, 1973; Lerman and Queiroz, 1974; and others). For example, a CAM plant subjected to short hot days and long cool nights will fix CO_2 almost entirely in the dark, the primary carboxylation being that of phosphoenolypyruvate, as in C_4 plants. On the other hand, if the CAM plant is grown under conditions of long cool days and short warm nights, CO_2 fixation is largely restricted to the light period, the primary carboxylation being that of ribulose diphosphate, as in C_3 plants (Osmond *et al.*, 1973).

When CAM plants were grown under the two conditions in the present study, in both cases the microbodies resembled their counterparts in C_3 plants much more closely than those in C_4 plants both in size and relative abundance. Also, in the diaminobenzidine procedure, *Kalanchoë daigremontiana* kept under long-day conditions gave an intense reaction for catalase activity. These results are not surprising in view of the high value for the CO_2 compensation point (one indication of the level of photorespiratory activity of microbodies) reported for CAM plants (Osmond *et al.*, 1973). They are also consistent with the fact that CAM plants lack the spatial differentiation of structure and function that in C_4 leaves has resulted in a reduced number of microbodies and their concentration in the bundle-sheath cells.

The relative abundance of chloroplasts and microbodies has been correlated previously with other characteristics of C_3 and C_4 plants (Frederick and Newcomb, 1971). Generally the mesophyll cells of C_3 plants have 4–5 chloroplasts per microbody while those of C_4 plants have 10 or more. Thus the ratios obtained for *K. daigremontiana* grown under the two conditions conform to the values expected for C_3 plants. A caveat must be mentioned, however, since it is not clear whether the organelle ratios would be expected to reflect principally the influence of relatively short-term environmental conditions, or the conditions which were present in the very young leaves when the endoplasmic reticulum was presumably much more active in forming new microbodies. We have not investigated this problem.

Microcylinders

Some of our observations suggest that the microcylinders described herein may represent products of viral infection, particularly infection by the potato virus Y-group (PVY). These observations include the intense ER vesiculation with the appearance of structures which we interpret as early stages in the elaboration of the mature cylinders, and the presence of occasional "pinwheels" among the ER vesicles. All of these structures resemble, to some degree, cytoplasmic modifications observed in PVY infections (Edwardson, 1974). We emphasize, however, that the microcylinders we have encountered are themselves

unlike the structures previously reported for PVY infection. None of the latter bears any close relationship in appearance to the microcylinders in *Kalanchoë* leaves, and none consists of, or includes, minute tubular structures such as the tubules we find closely associated with the cylinder walls. It should also be noted that infection by PVY has apparently not been previously reported for any member of the Crassulaceae (Edwardson, 1974).

An alternative possibility is that the microcylinders and associated tubules are not related to viral infection, but rather are organelles that develop in *Kalanchoë* at certain stages of growth or under particular environmental conditions. That this is a reasonable assumption is suggested by the work of Wilson *et al.* (1974), who have found that in cultured carrot cells, the occurrence of abundant inclusions similar to those thought to be induced by PVY infection is related to the particular culture medium on which the cells are grown. Furthermore, attempts by these workers to demonstrate that the inclusions represent active viruses in the carrot cells have given negative results. It appears, then, that formation of inclusions such as are found in their material may not be dependent exclusively upon viral infection.

We believe that whether induced by a virus or not, the presence of these remarkable components in the fine structure of *Kalanchoë* leaf cells will be of considerable interest to workers studying Crassulacean acid metabolism. If viral in origin, it is important that this fact be established and the ramifications explored, so that the problem posed by possible viral infection of experimental material can be taken into account. If, on the other hand, the structures in question reflect a response of the *Kalanchoë* cytoplasm to particular environmental conditions in the absence of an inciting agent, they are if anything of even greater intrinsic interest in view of their uniqueness and the fundamental problems posed concerning their origin, development and function. The tubules associated with the microcylinders are of special interest in view of the importance of conventional microtubules and the current interest in their substructure and mode of action.

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Note added in proof: Recent observations on thin sections and on negatively stained leaf-dip preparations of *K. daigremontiana* reveal long flexuous rods resembling viruses of the PVY-group.