

THE DEVELOPMENT OF MICROBODIES AND PEROXISOMAL ENZYMES IN GREENING BEAN LEAVES

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ABSTRACT

The ontogeny of leaf microbodies (peroxisomes) has been followed by (a) fixing primary bean leaves at various stages of greening and examining them ultrastructurally, and (b) homogenizing leaves at the same stages and assaying them for three peroxisomal enzymes. A study employing light-grown seedlings showed that when the leaves are still below ground and achlorophyllous, microbodies are present as small organelles (e.g., 0.3 μm in diameter) associated with endoplasmic reticulum, and that after the leaves have turned green and expanded fully, the microbodies occur as much larger organelles (e.g., 1.5 μm in diameter) associated with chloroplasts. Specific activities of the peroxisomal enzymes increase 3- to 10-fold during this period. A second study showed that when etiolated seedlings are transferred to light, the microbodies do not appear to undergo any immediate morphological change, but that by 72 h they have attained approximately the size and enzymatic activity possessed by microbodies in the mature primary leaves of light-grown plants. It is concluded from the ultrastructural observations that leaf microbodies form as small particles and gradually develop into larger ones through contributions from smooth portions of endoplasmic reticulum. In certain aspects, the development of peroxisomes appears analogous to that of chloroplasts. The possibility is examined that microbodies in green leaves may be relatively long-lived organelles.

INTRODUCTION

Microbodies are distinctive organelles which were characterized first in liver and kidney cells of mammals (6) and later in *Tetrahymena* (30). When isolated from cell homogenates, the animal microbodies were found to contain at least one H_2O_2 -generating oxidase and catalase as their characteristic enzymes and thus came also to be called "peroxisomes" in reference to their presumed peroxidative role in cellular metabolism (6). The occurrence of microbodies in plant cells is now known to be extensive (31, 42); moreover, those present in endosperm after seed germination (4,

5, 52) and those occurring in green leaves of angiosperms (14, 16, 47, 48) rank among the most prominent of all microbodies studied. It has become increasingly apparent that microbodies should be regarded as major organelles present in many eucaryotic cells, and that studies of those occurring in plant cells can contribute importantly to our general understanding of their function, development, and regulation.

The microbodies in green leaves are larger than those in most other tissues and often appear to be closely associated with chloroplasts and mito-

chondria (14). These microbodies when isolated and characterized biochemically have also been termed "leaf peroxisomes" (46). They have been shown to contain at least eight enzymes (42), the majority of which participate directly in the metabolism of glycolate, an important product of CO₂ fixation in photosynthesis. Most of the ultrastructural and biochemical work reported on leaf microbodies (peroxisomes) has described their morphology and function in mature photosynthetic leaves; until very recently (11, 12) no work had appeared which reported in any detail their origin, development, or regulation in young leaves. However, it is highly likely that a process involving their genesis and maturation takes place at some stage in the growth of a leaf and gives rise to one or more populations that function as peroxisomes throughout the lifetime of the leaf.

A number of older reports indicate that catalase and glycolate oxidase, two enzymes now considered to be localized exclusively within peroxisomes, undergo significant changes in levels of activity when dark-grown leaves are placed in the light and turn green (1, 9, 44, 45). Considering these reported enzymatic changes, it seemed probable that a marked development of microbodies might occur during leaf greening. A similar conclusion was reached by reasoning that since peroxisomes of mature leaves function in the metabolism of a photosynthetic product, they might well develop significantly during the period in which leaves are acquiring photosynthetic ability. This paper presents the results and discusses the implications of developmental studies conducted (a) on microbodies in greening leaves of light-grown bean seedlings and (b) on microbodies in greening leaves of etiolated bean seedlings placed in the light.

MATERIALS AND METHODS

Bean seeds (*Phaseolus vulgaris* L, cultivar dwarf horticulture) were presoaked for 12 h and then planted in moistened sand. Some seedlings were grown under a 12-12 h light-dark cycle at temperatures varying between 22 and 28°C, with illumination of approximately 800 foot-candles provided by fluorescent and incandescent lamps. Other seedlings were raised in the dark at 24°C for 9 days and then transferred to the 12-12 h light-dark regime. Stages sampled are dated in reference either to germination, which was considered to start upon the first moistening of the seeds, or to initial exposure of the etiolated seedlings to light.

Preparation for Electron Microscopy

Segments of primary bean leaves at specified stages of seedling development were fixed at room temperature in a solution of 3% glutaraldehyde in 0.05 M potassium phosphate buffer (pH 6.8). After this, the leaf segments were rinsed in phosphate buffer for about 1 h, then postfixed in buffered 2% osmium tetroxide for 2 h. They were next dehydrated in an acetone series followed by propylene oxide, and embedded in Araldite-Epon-dodecenyl succinic anhydride (3/3/8). Leaves from the etiolated seedlings never exposed to light as well as those from the 3-day old seedlings were fixed and rinsed under a dim green light and were postfixed in the dark, but all other leaves were processed entirely in the light. The incubation of leaf segments in 3,3'-diaminobenzidine (DAB) to localize catalase cytochemically (32) was performed as described by Frederick and Newcomb (15), except that the epidermis was not peeled off the 5-day old leaves. Silver-gray sections were cut on a Sorvall MT-2 ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.), mounted on copper grids, and stained in aqueous 2% uranyl acetate for 10 min followed by lead citrate for 5 min. The sections were viewed with a Hitachi HU 11-A electron microscope at an accelerating voltage of 75 kV.

Homogenizations and Assays

Primary leaves at the various stages of development were diced with razor blades attached to an electric knife handle (50) in a grinding medium of 0.4 M sucrose and 0.05 M potassium phosphate at pH 7.5. The suspensions were ground in a mortar, then strained through two layers of cheesecloth to yield the homogenates. These steps and the ensuing dilutions were carried out at 4°C. The etiolated and 3-day old leaves were homogenized under a dim green light; all other leaves were homogenized in the light.

The homogenates were assayed at 25°C for three distinctive enzymes of leaf peroxisomes. The assay for catalase (EC 1.11.1.6) was according to Lück (28), with 1 unit of activity corresponding to that amount of enzyme which decomposes 50% of the H₂O₂ present per 100 s in a volume of 3.0 ml. Nicotinamide adenine dinucleotide, reduced form (NADH)-hydroxypyruvate (glyoxylate) reductase (EC 1.1.1.26) was assayed as described by Tolbert et al. (49), with glyoxylate as substrate. Glycolate oxidase activity (EC 1.1.3.1.) was measured anaerobically using 2,6-dichlorophenol-indophenol (DCPIP) as electron acceptor (47). A change of 1 OD per min is equivalent to 121 nmol DCPIP reduced per min; however, since the concentration of dye is not saturating, the results do not represent the maximum activity of the enzyme (43). Protein

was measured by the procedure of Lowry et al. (27), while chlorophyll was determined by the method of Arnon (2). The entire procedure, including the homogenizations, enzyme assays, and protein and chlorophyll determinations, was performed at least twice for each stage examined.

RESULTS

Microbodies in the Greening Leaves of Light-Grown Bean Seedlings

The first of the two studies reported here encompasses a period of 8 days in the development of bean seedlings, a time during which the primary leaves turn green and expand greatly (Table I). At the outset (day 3 after germination), the leaves are still within the seed and below ground; by day 7 they are above ground, freed from the cotyledons which previously enclosed them, and exposed fully to the light; by day 9 they have enlarged into deeply green photosynthetic organs. The outward indications of growth and greening in the leaves between days 3 and 11 are accompanied by several ultrastructural changes that can be used as intracellular markers. One involves the development of proplastids containing some starch grains but few lamellae at day 3 into chloroplasts having grana and other features associated with photosynthesis by day 7. A second indication is in the degree of vacuolation, which is limited at day 5 but is extensive by day 9.

At the earliest stages considered here, i.e. in 3- and 5-day old leaves, microbodies are already present in the leaf cells (Figs. 1-5). Their matrix is generally granular and slightly flocculent. The microbody profiles are generally small and may measure as little as 0.2 μm in diameter. Many are circular to oval, but a number of them

are quite elongate (Fig. 5). Some of the microbodies in 5-day old leaves appear larger than those in 3-day material, but others in the older leaves, as in Fig. 3, are as small as any microbodies seen at day 3.

At these early stages, various forms of membranous structures are commonly associated with the microbody boundaries (Figs. 2-5). Occasionally, a profile of rough endoplasmic reticulum (ER) can be followed for some distance through the cytoplasm to its abutment on a microbody. The terminal portion of the cisterna adjacent to the microbody appears slightly swollen and free of ribosomes (Fig. 4). In other sections, vesicles can be observed near and in contact with the small microbody profiles (Fig. 2). Such vesicles contain at the most a few faint fibrils and bear no ribosomes on their membrane surface. They often resemble the swollen smooth ends of ER cisternae described above and are probably related to an ER system (see Fig. 3, for example). The possibility that some of these vesicles may be derived from dictyosomes is not excluded, but there are many cases in which this does not appear likely. In yet other sections, the topographical relationship of the external membranes to the microbody profiles is quite confused. Such cases, in which the ER is often viewed obliquely, are instructive, however, when they reveal that ribosomes on the nearby rough cisternae are arranged in polysomal configurations. It must be emphasized that despite the seemingly universal involvement of membranous forms with the outer surfaces of small microbodies, no direct continuities between the membranes of the microbody on the one hand and those of the ER or vesicles on the other have been observed in this study.

The DAB reaction was conducted on 5-day old leaves to determine (a) whether catalase could be localized in these small microbodies, and (b) whether any reaction product could be found in the extraneous membrane systems described above. After incubation in DAB and H_2O_2 , there was a strongly electron-opaque deposit in the microbodies, including their limiting membranes (Fig. 6). However, no such product could be detected in the lumen of the contiguous ER and vesicles, nor apparently on their membranes. The reaction product in the microbodies was eliminated when the tissues were incubated in DAB plus aminotriazole, an inhibitor of catalase activity (Fig. 7).

TABLE I
Postgerminative Development of Light-Grown Bean Seedlings

Stage	Height of seedlings	Length of leaves	Chlorophyll content of leaves
	<i>cm</i>	<i>cm</i>	<i>mg/mg protein</i>
Day 3	Below ground	0.5-1.0	—
Day 5	Ground level	1.2-2.0	0.004
Day 7	9-14	2.5-4.0	0.021
Day 9	13-18	5-10	0.039
Day 11	14-18	7-10	0.051

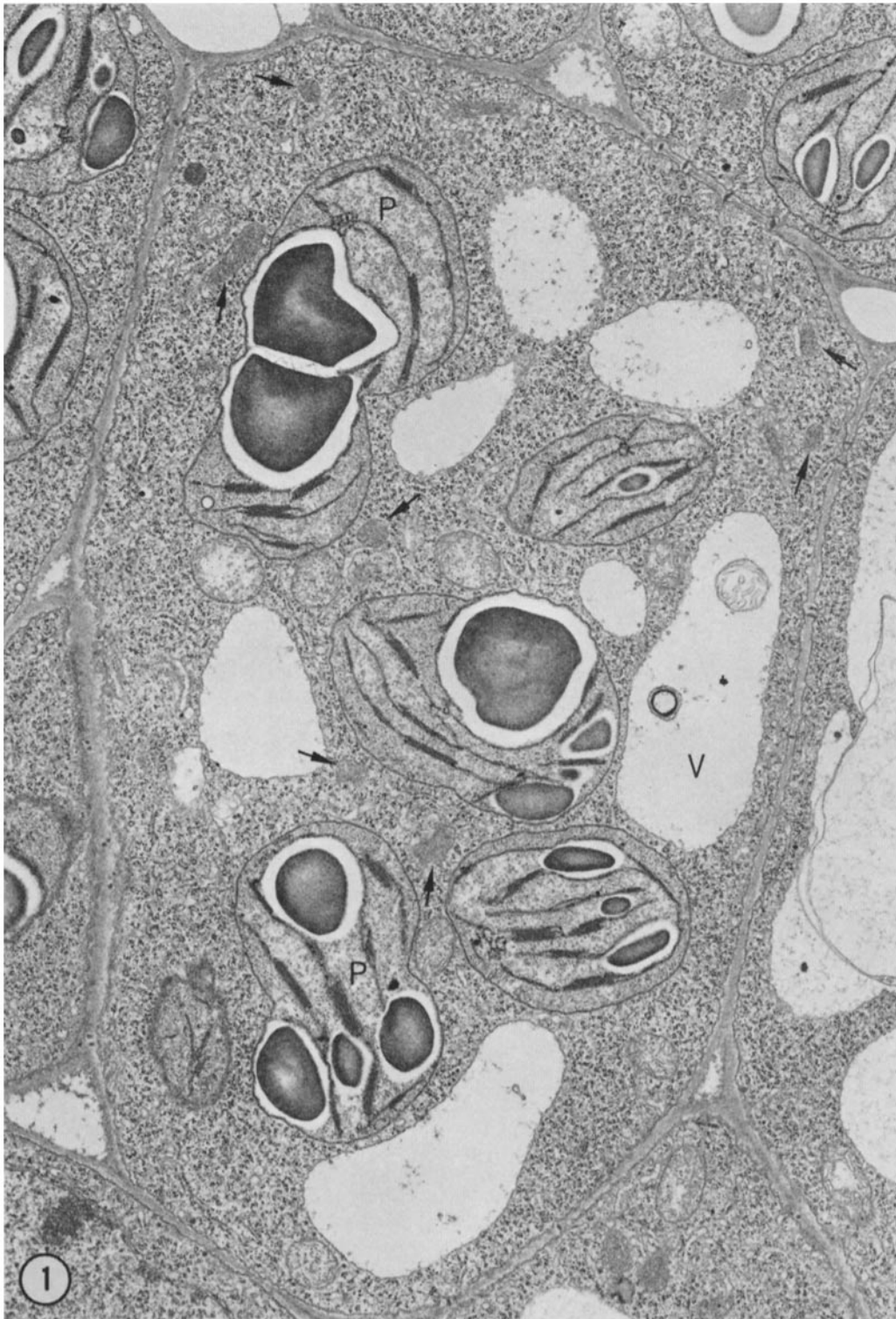


FIGURE 1 Section through a mesophyll cell in a primary leaf from a 5-day old bean seedling. At this stage of development, the leaves are at ground level and already contain low quantities of chlorophyll. Note in this regard that there has been some formation of grana in the plastids (*P*). Seven profiles of relatively small microbodies (arrows) are present in this particular cell section. *V*, vacuole. $\times 13,700$.

At day 7, the bean leaf cells are undergoing enlargement. Vacuolation is occurring in them, although large central regions of cytoplasm are frequently still present (Fig. 8). The microbodies also appear to be enlarging at this stage. While their profiles still do not generally exceed 1 μm in length, profiles less than 0.5 μm in length are rare, and their widths are greater than at earlier stages. Though perhaps not so conspicuous, ER and vesicles still are present around the border of almost every microbody profile examined (Fig. 9).

The cells and their organelles at days 9 and 11 resemble those typically found in mature, photosynthetic leaves (14). The profiles of chloroplasts, mitochondria, and microbodies, occurring in a ratio of roughly 6:3:1, occupy much of the narrow parietal band of cytoplasm surrounding the central vacuole (Fig. 10). The microbodies are large (1.0–1.5 μm in diameter) with an undifferentiated matrix. They tend to be circular at these stages and are irregular or greatly distorted only when associated with or wedged among chloroplasts and mitochondria (Fig. 11). It is again possible to visualize vesicles and ER in the vicinity of microbodies, but these are now dwarfed by the latter and are even less conspicuous than at day 7.

Microbodies not only increase in size, but also appear to undergo development in their associations with other organelles during the period studied. At days 3 and 5, microbody profiles viewed either transversely or longitudinally are not seen in contact with plastids. The primary association at these early stages seems to be rather with the ER and its derivatives. At day 7, a time when well-developed chloroplasts are present yet

expanses of free cytoplasm remain, the degree of association is ambiguous: some profiles are in contact with chloroplasts and others are in their vicinity, but many are quite distant, lying either solitarily in the cytoplasm or near mitochondria. At days 9 and 11 the situation resembles that described for other mature leaves, wherein many profiles of microbodies are in contact with chloroplasts and mitochondria. Clear manifestations of these associations are seen when the outline of the appressed microbody conforms for a considerable distance with the contour of the chloroplast or in cases where the appressed microbody causes an indentation in the chloroplast or mitochondrion.

The enzyme levels in bean leaf homogenates assayed at stages corresponding to those described ultrastructurally undergo significant changes between days 3 and 11 (Fig. 12). During this period catalase, glycolate oxidase, and glyoxylate reductase all increase in specific activity (6-, 10-, and 3-fold, respectively). These three enzymes already are active at day 3, before the appearance of any chlorophyll. After day 5, the activities increase at a rate very roughly parallel to that of chlorophyll, with the rates of increase leveling off between days 9 and 11. Of the three peroxisomal enzymes, catalase exhibits a somewhat independent curve, especially between days 3–5 and 9–11; in the latter interval, it diminishes in specific activity by about 33%, while glycolate oxidase, glyoxylate reductase, and chlorophyll continue to increase.

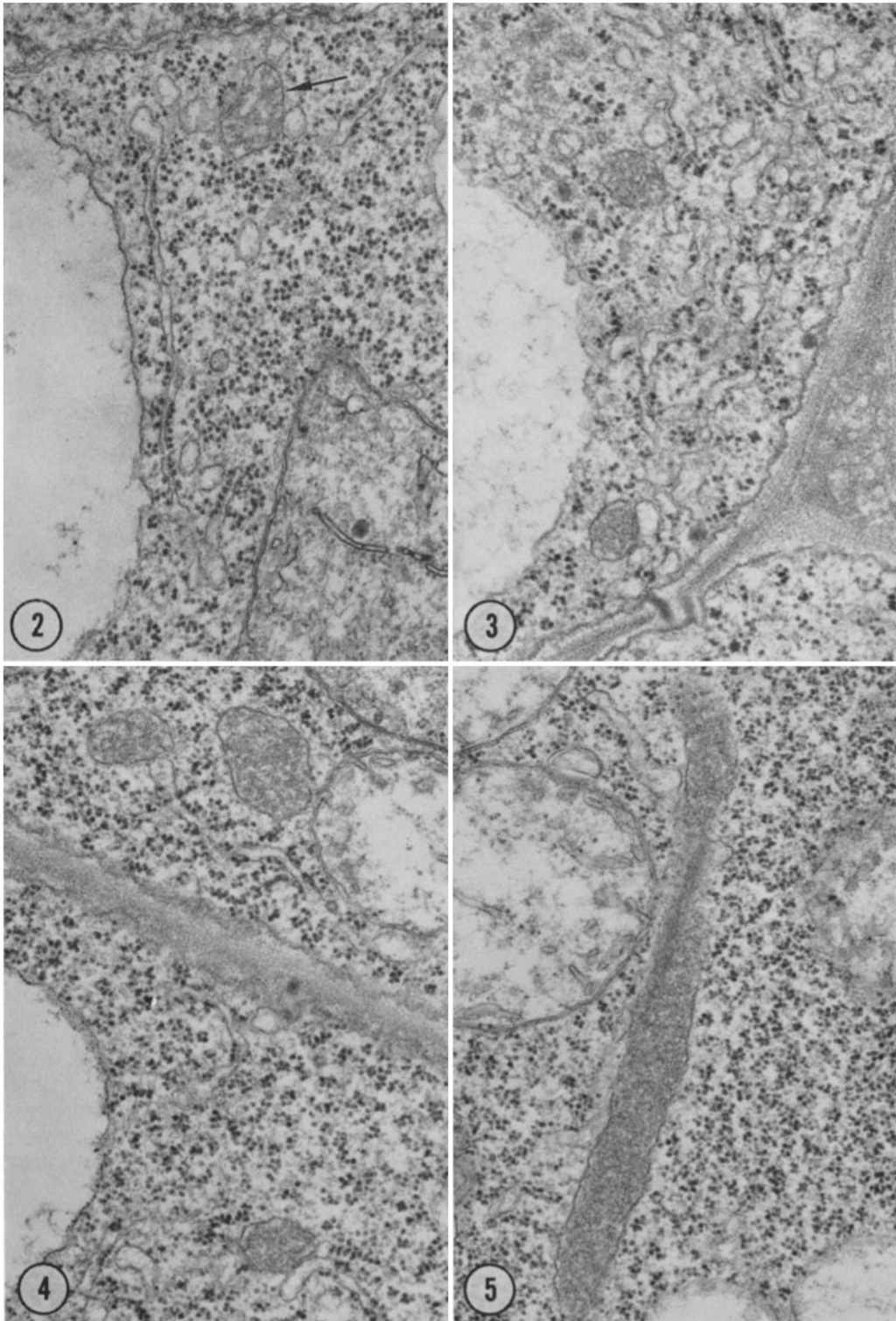
Microbodies in the Greening Leaves of Etiolated Bean Seedlings Placed in the Light

The second developmental study covers the 3 days after the exposure of dark-grown, 9-day old

FIGURE 2 Appearance of microbody (arrow) in mesophyll cell of 3-day old bean leaf. There are electron lucent regions in the somewhat flocculent matrix of the microbody, and electron-lucent vesicles in surface contact with it. The terminal region of the otherwise rough ER cisterna is smooth and slightly swollen, and appears to be budding from the ER. $\times 41,000$.

FIGURES 3 and 4 Small microbodies associated with the ER in 5-day old light-grown bean leaves. In Fig. 3, the ER appears to be undergoing vesiculation, and some of its smooth, swollen regions are in contact with the microbodies. In Fig. 4, the terminal parts of otherwise rough cisternae are smooth and somewhat distended, and abut on the microbodies. The membranes of these and other microbodies are never seen in direct continuity with the membranes of the associated ER. Fig. 3, $\times 46,000$; Fig. 4, $\times 41,000$.

FIGURE 5 An elongate microbody in a 5-day old leaf. The section apparently skims along a portion of the microbody surface, revealing fine parallel fibrils. Such views have been encountered only rarely. $\times 38,000$.



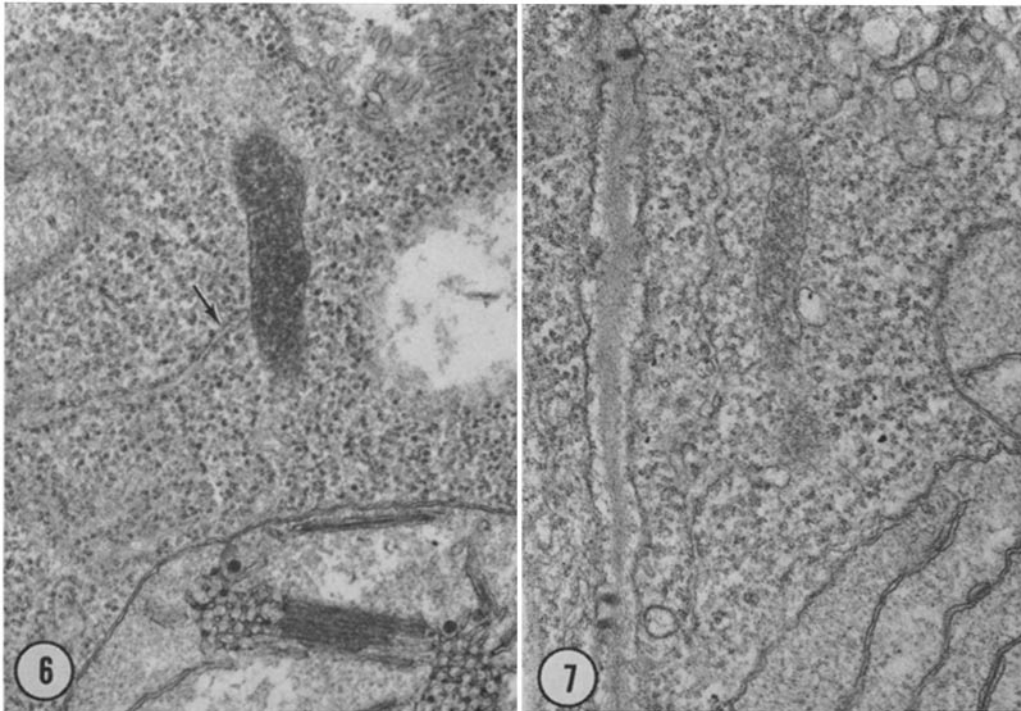


FIGURE 6 Reaction product in microbody of 5-day old bean leaf after incubation in DAB medium. The stain seems to be quite localized in the microbody proper, and does not appear in the ER cisterna nearby (arrow). $\times 46,000$.

FIGURE 7 Appearance of microbody in 5-day old bean leaf treated with aminotriazole in addition to DAB and hydrogen peroxide. The electron density of the microbody matrix resembles that of microbodies in untreated tissues. The inhibition of deposit by aminotriazole indicates that the reaction product observable in Fig. 6 is due to the activity of catalase. $\times 36,000$.

bean seedlings to light. At the beginning of this period the primary leaves, though more developed than the etiolated leaves of some species, are yellow in color and quite small. 72 h later the leaves have doubled or tripled in size although the plants have gained little in height. Moreover, the leaves have acquired an amount of chlorophyll comparable with that in the 9-day old light-grown seedlings (Table II). Ultrastructurally, at least two major changes, again involving the plastids and vacuoles, occur in the cells during this 72 h period. First, the etioplasts are transformed into chloroplasts, with the disaggregation of prolamellar bodies and the formation of grana. Under the conditions used here, this process starts during the initial 12 h and is largely complete after 36 h. Second, a large vacuole forms within the cells. This appears to occur more slowly than the rearrangements in the etioplasts, but after 48 h vacuoles occupy the centers of a majority of the cells.

The microbodies in 9-day old etiolated bean leaves are illustrated in Fig. 13 and have already been described elsewhere (19): many profiles are small, but there are indications that at least some of the organelles may be attenuated, with the shorter axis measuring between 0.2 and 0.4 μm . When the etiolated leaves are placed in light, it does not appear that their microbodies undergo any major or immediate changes. At least, after 6 and 12 h in the light many profiles are still elongate and generally resemble those in dark-grown leaves. Occasionally even smaller profiles associated with ER and/or vesicles are observed; these resemble profiles seen in early stages of light-grown leaves.

Further changes in the microbodies are difficult to describe on a 12 h basis, because the transformation appears to be gradual and the sampling performed at each stage was limited. Fig. 14 has been chosen to illustrate microbodies in mesophyll cells

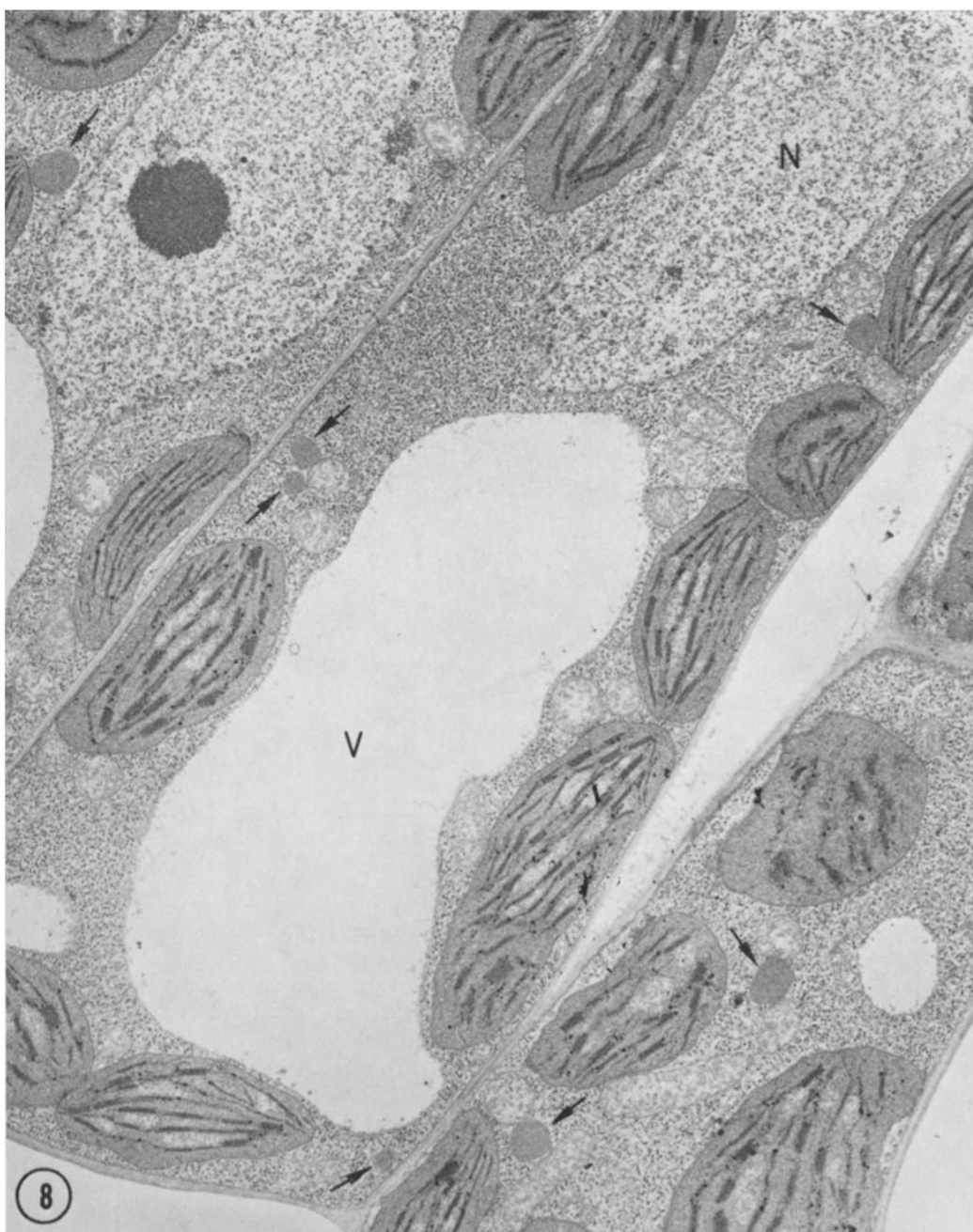


FIGURE 8 Longitudinal section through palisade cells of a 7-day old bean leaf. At this stage, the chloroplasts are quite fully developed, but the vacuoles (V) are only partially consolidated into a large central region. Microbodies (arrows) are associated with chloroplasts or mitochondria or lie free in the cytoplasm. N, nucleus. $\times 8,200$.

24 h after exposure to light. It appears that the microbodies gradually increase in size during the 3 day period of illumination, since after 72 h their profiles are clearly larger than they were in

etiolated leaves (Fig. 15). They now average about $1.2 \mu\text{m}$ in their greatest dimension and thus are comparable in size with those in 9- and 11-day old light-grown leaves. They also tend to be more

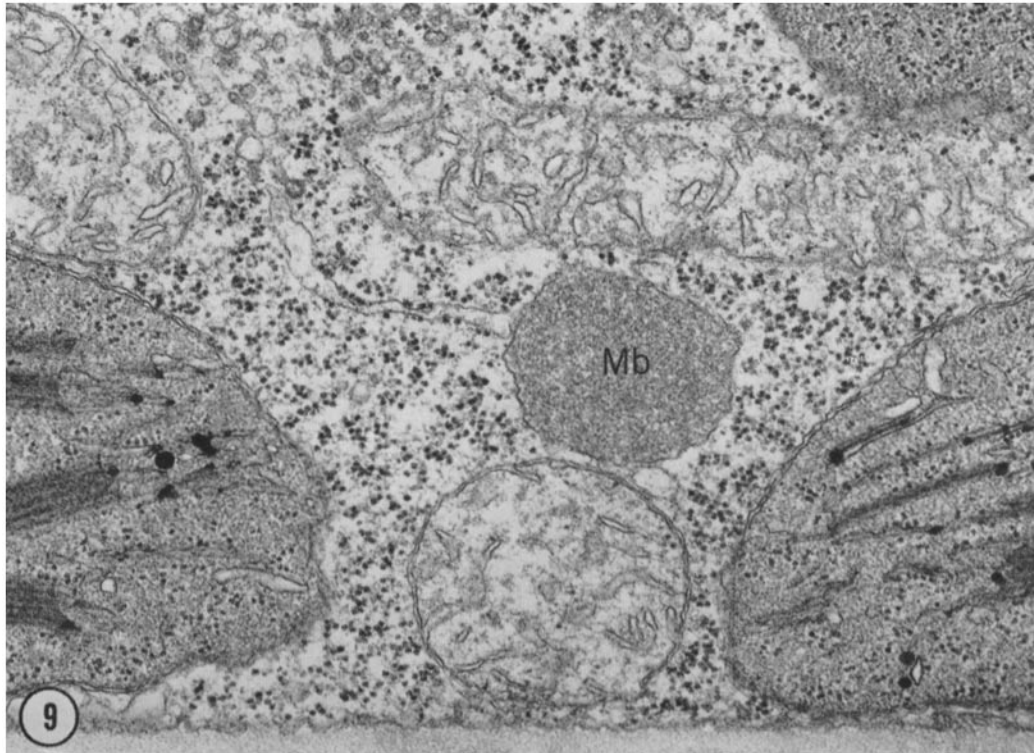


FIGURE 9 Microbody (*Mb*) with a diameter of $0.7 \mu\text{m}$ in a mesophyll cell from a 7-day old light-grown leaf. Microbodies at this stage are generally intermediate in size, larger than at day 3 or 5 but smaller than at day 9. Note that this microbody still has ER associated with it. $\times 46,000$.

circular in outline than previously, again unless distorted by their associations with other organelles. It would be difficult to distinguish these microbodies from their counterparts in mature green leaves.

The status of microbody-plastid associations in this 72 h period of greening is hard to assess. In the first place, even in etiolated leaves some of the microbodies are already associated with the etioplasts (19). During the first 48 h of light, there is certainly no great increase in the number of microbodies apposed to plastids, and in fact, those smaller profiles involved with ER and vesicles are clearly not in contact with the chloroplasts. By 72 h, however, the extent of microbody-mitochondrion-chloroplast associations (Fig. 15) is as great as in the cells of 9- or 11-day old light-grown plants. At this stage, great lengths of the microbody surfaces appear in contact with the chloroplast envelopes.

The three peroxisomal enzymes, catalase, gly-

colate oxidase, and glyoxylate reductase, are already active in homogenates of 9-day old etiolated leaves. Upon exposure of the leaves to light, the specific activities increase by two- to threefold within the 72 h period (Fig. 16), attaining levels equivalent to those in 9-day old green leaves. The changes again parallel roughly those of chlorophyll, glyoxylate reductase activity resembling chlorophyll content even in its diurnal fluctuations, i.e., in the dip in its activity during each 12 h period of darkness. Catalase again exhibits a certain independence in its behavior, since it is unlike the other two enzymes in that its initial response to light is a slight decrease in activity, whereas that of glycolate oxidase and glyoxylate reductase is an increase in activity. If the values are plotted in 24-h intervals, the fluctuations of glyoxylate reductase and chlorophyll disappear; in slope and absolute value, the increases occurring during these 72 h then approximate those observed

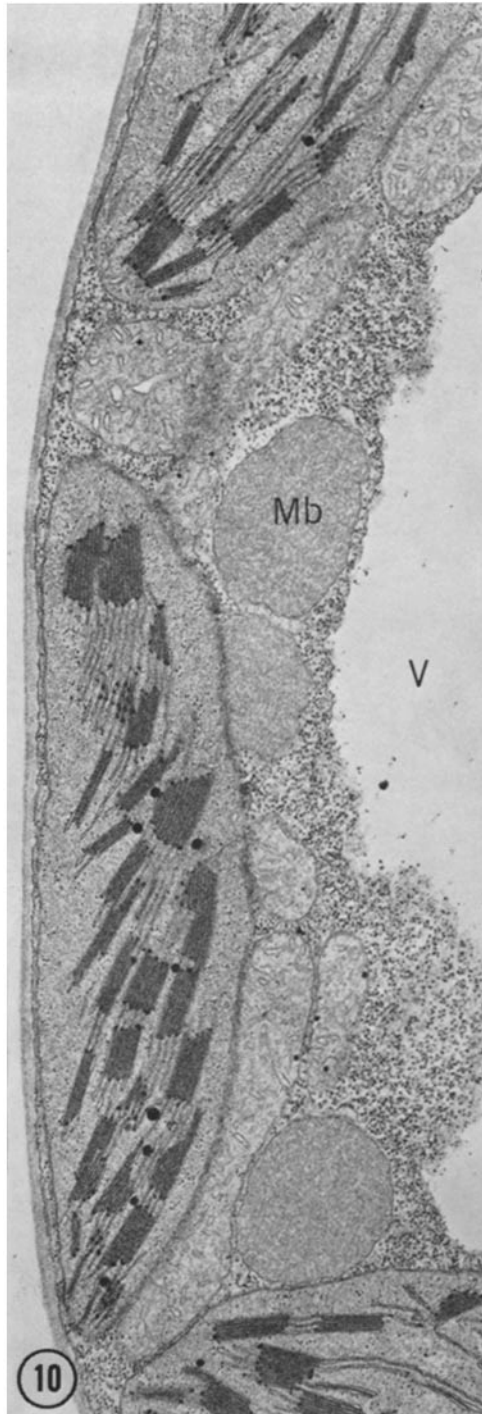


FIGURE 10 Portion of a mesophyll cell in a 9-day old light-grown bean leaf. At this stage of development, the leaf is deep green and fully expanded. A large vacuole (*V*) occupies the center of the cell. In the

in the light-grown leaves between days 6 or 7 and day 9.

DISCUSSION

Origin and Growth of Leaf Microbodies

How animal and plant microbodies arise has proved to be quite difficult to demonstrate. The best supported view has been that these organelles are derived from dilated regions of ER (8, 17, 21, 26, 33, 38, 51, 52). One possibly disturbing feature of this hypothesis, however, has been the considerable difficulty experienced in illustrating it convincingly. Many of the published micrographs fail to demonstrate a direct continuity between the bounding membrane of the nascent microbodies and the membranes of the presumed parent ER cisterna, although establishing this relationship would seem to be a prerequisite to proving that microbodies form by budding from the ER.

In the present work it was considered likely that microbodies would be undergoing formation on days 3 and 5, because during this period of leaf development these organelles are characteristically very small and their marker enzymes are entering the period when they increase substantially in activity. However, it did not prove possible to observe particles clearly recognizable as microbodies in *direct* continuity with the ER. At the same time, it was apparent that the regions of cytoplasm surrounding small microbodies are populated by even smaller smooth vesicles whose interiors lack a discernible matrix, and also by ER cisternae containing smooth and often swollen terminal regions. Both the specialized areas of ER and the smooth vesicles (which may actually still be attached to ER in a plane outside of the section) are often in intimate association with the similarly smooth-surfaced microbodies. These relationships of the ER and derived vesicles with microbodies are most obvious at days 3 and 5, but as described in the Results, they can also be seen at later stages of leaf development when the microbodies are larger.

From our observations it seems most likely that leaf microbodies do in fact originate by budding from smooth regions of the ER, but that at the time of their release into the cytoplasm they exist

narrow band of cytoplasm along the wall, there are microbodies (*Mb*), mitochondria, and chloroplasts with full complements of grana. $\times 21,000$.

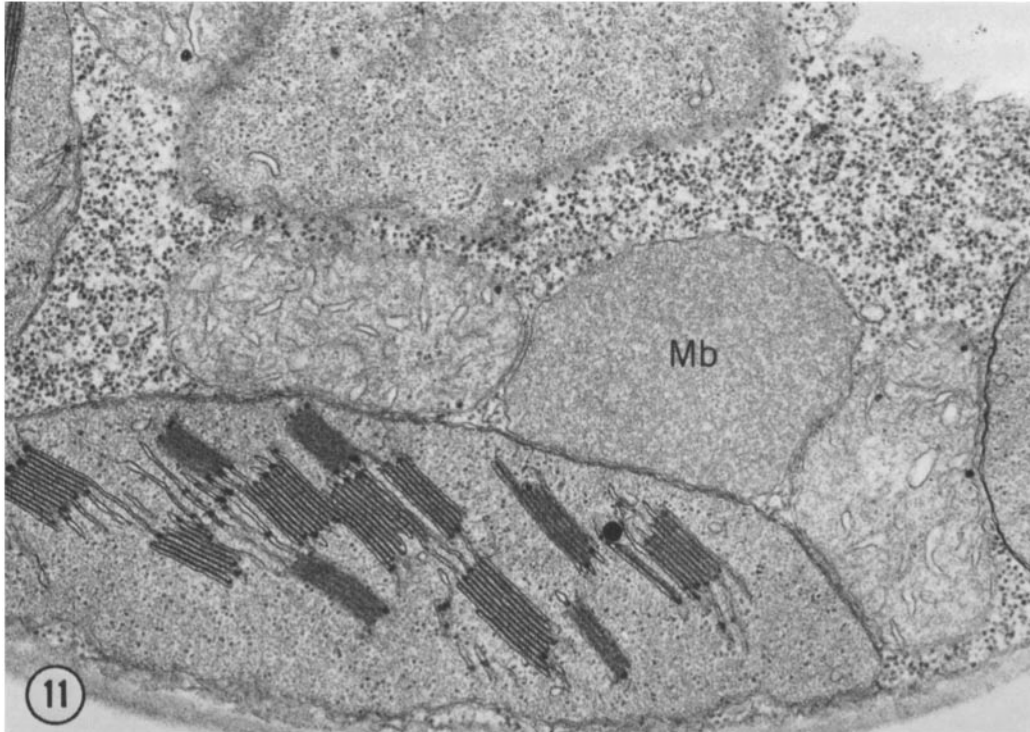


FIGURE 11 Appearance and disposition of a microbody (*Mb*) in a bean leaf from a 9-day old seedling. The microbody measures almost $1.5 \mu\text{m}$ in its greatest dimension. Its surface is in extensive association with the envelopes of a chloroplast and two mitochondria. $\times 34,000$.

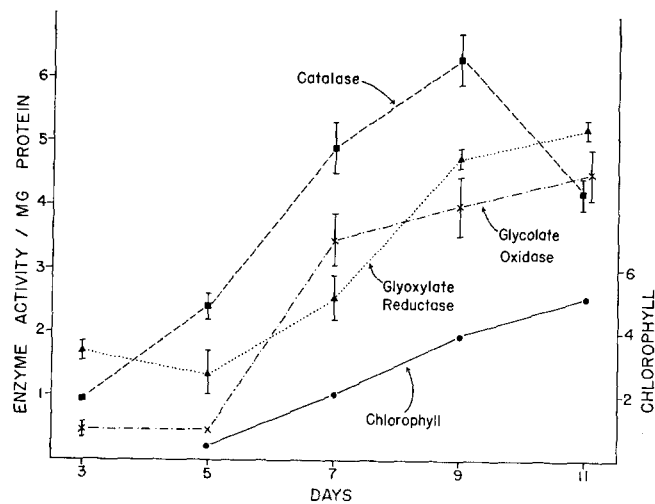


FIGURE 12 Levels of peroxisomal enzyme activity in the homogenates of light-grown bean leaves at five stages of seedling development after germination. Catalase specific activity is plotted as units per milligrams of protein, glycolate oxidase as $20 \times \Delta\text{OD}/\text{min}$ per mg protein, and glyoxylate reductase as $0.05 \times \text{nmol substrate consumed}/\text{min}$ per mg protein. Chlorophyll content is plotted as $100 \times \text{mg}/\text{mg protein}$. Vertical lines represent ranges from determinations on two independent sets of seedlings.

TABLE II
Development of Etiolated Bean Seedlings Exposed to Light

Time in light	Height of seedlings	Length of leaves	Chlorophyll content of leaves
<i>h</i>	<i>cm</i>	<i>cm</i>	<i>mg/mg protein</i>
0	25-30	2-3	—
12	27-35	2-4	0.018
24	22-31	3-5	0.011
36	28-35	3-6	0.037
48	28-32	4-7	0.027
60	27-40	5-7	0.047
72	28-35	5-9	0.036

as electron-lucent vesicles whose identity is obscured. It is further suggested that once such vesicles are formed, they can either persist as discrete particles and develop into recognizable microbodies, or, along with the appressed ER, contribute material to previously formed microbodies, thereby allowing the latter to grow. If fusion were occurring, then the membrane of the smooth vesicle would be incorporated into that of the microbody. At the same time, the matrix of the vesicle would be added to the matrix of the microbody. The value of such a contribution is open to question, since there seems to be little content in the lumen of most vesicles. However, some of the smooth vesicles contain delicate fibers, evident especially near the inner surface of the bounding membrane. It is also possible, of course, that there is additional material present *in vivo* in the vesicles that is washed out during fixation or is not visualized after the standard preparative procedures.

It is clear that between days 3 and 9, while the specific activities of three peroxisomal enzymes are increasing 3- to 10-fold, the microbodies change from particles as small as 0.2 μm to ones over 1.0 μm in diameter. The differences in organelle volumes are great: for example, a 5-fold increase in diameter would mean a 125-fold increase in volume. Since there is no evidence whatsoever for a sudden appearance of already fully enlarged microbodies, it can only be that the large microbodies arise by growth of the small ones. The concept that microbodies grow and that this growth is achieved by a transfer of materials from the smooth ER and/or derived vesicles that are so characteristically appressed to them receives additional support from other

studies conducted with the electron microscope, both developmental (26, 51) and cytochemical (10, 39, 53). While the biochemical results of Poole et al. (34) on liver peroxisomes do not support a model involving a gradual growth of smaller microbodies into larger ones, those of Feierabend and Beevers (12) on peroxisomes in greening leaves do indicate that mature leaf microbodies develop from "precursor particles of lower buoyant density."

When the DAB reaction was tried on 5-day old bean leaves, neither the ER appressed to the small microbodies nor the vesicles in contact with them appeared to be stained. The lack of reaction product in the spaces within the membrane systems is consistent with the results from rat liver (53), but the apparent lack of deposit on the membranes or adjoining ribosomes is not. It is questionable, of course, whether even with a positive reaction, the faint fibrils and the membranes would stain strongly enough to contrast with unreactive regions of the cytoplasm. It is also possible that, as appears to be true of catalase in liver peroxisomes (25), the catalase molecule in leaf cells is not fully assembled before its incorporation into the microbody, and thus cannot react with DAB. In summary, a negative DAB reaction within or on the surface of vesicles and ER does not exclude the possibility that these structures are contributors to the growing microbodies.

Regulation of Microbody Growth

Microbodies (peroxisomes) and their characteristic enzymes are present in very young leaves which are still completely below ground, as well as in etiolated leaves which have never been exposed to light (19). Thus, it is apparent that they form in leaf cells without induction by light or photosynthetic products such as glycolate. The presence of light, however, does appear to have a stimulatory effect on the microbodies, causing their further growth and a further increase in their enzymatic activities. In the case of the etiolated bean leaves placed in the light, the initial levels of enzyme activity are relatively high, but exposure to light increases this activity even more (see reference 13 also). On the ultrastructural side, microbodies are relatively small in etiolated leaves and undergo considerable growth upon exposure to light. What appears to happen qualitatively in the case of dark-grown leaves is that microbodies and their enzymes develop to a

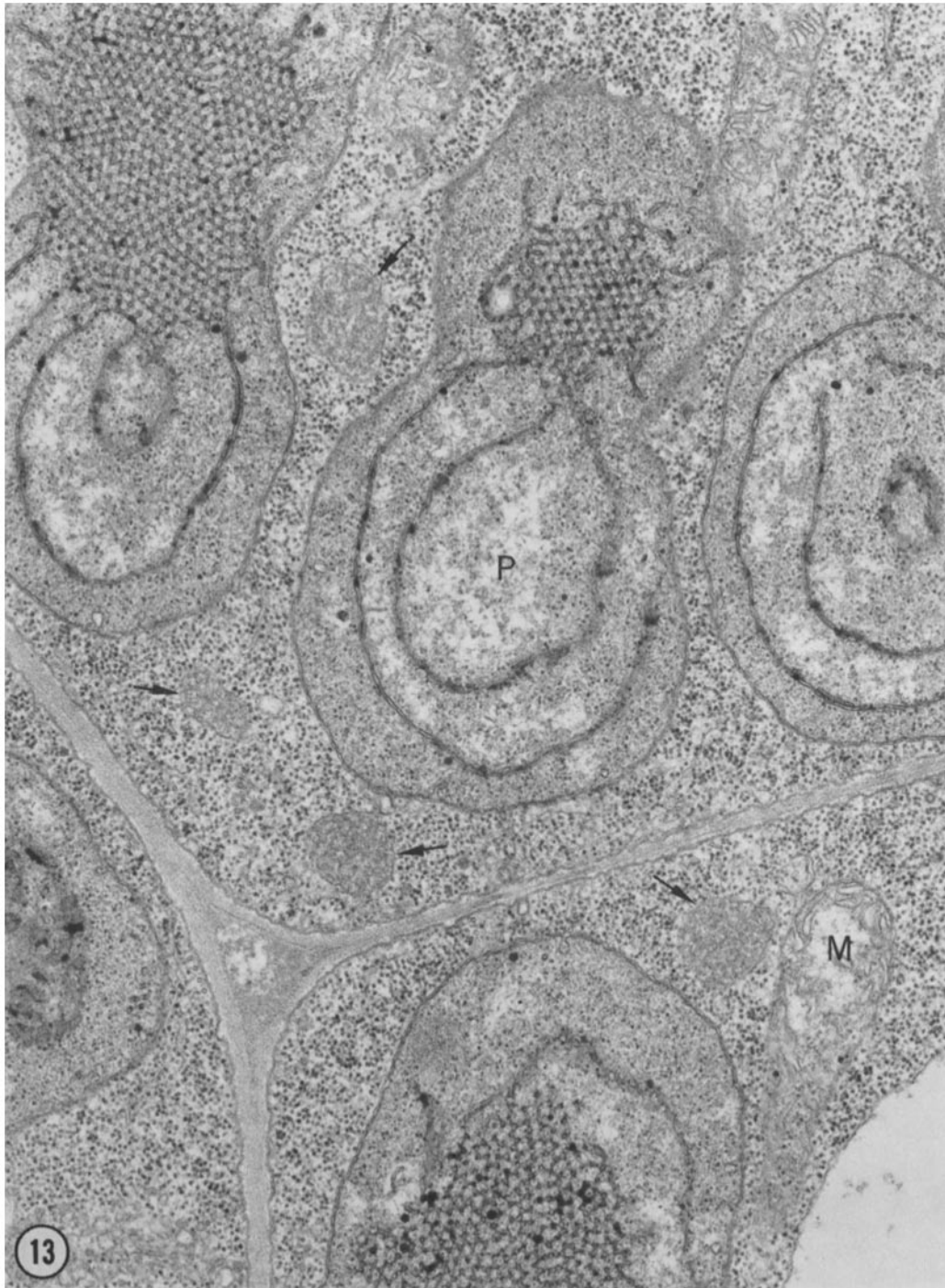


FIGURE 13 Section through portions of several mesophyll cells of a 9-day old etiolated bean leaf. Microbodies (arrows) are present in the cytoplasm along with mitochondria (*M*) and several etioplasts (*P*) with prolamellar bodies. In the second developmental study described in Results, leaves at this stage were exposed to light. $\times 31,000$.

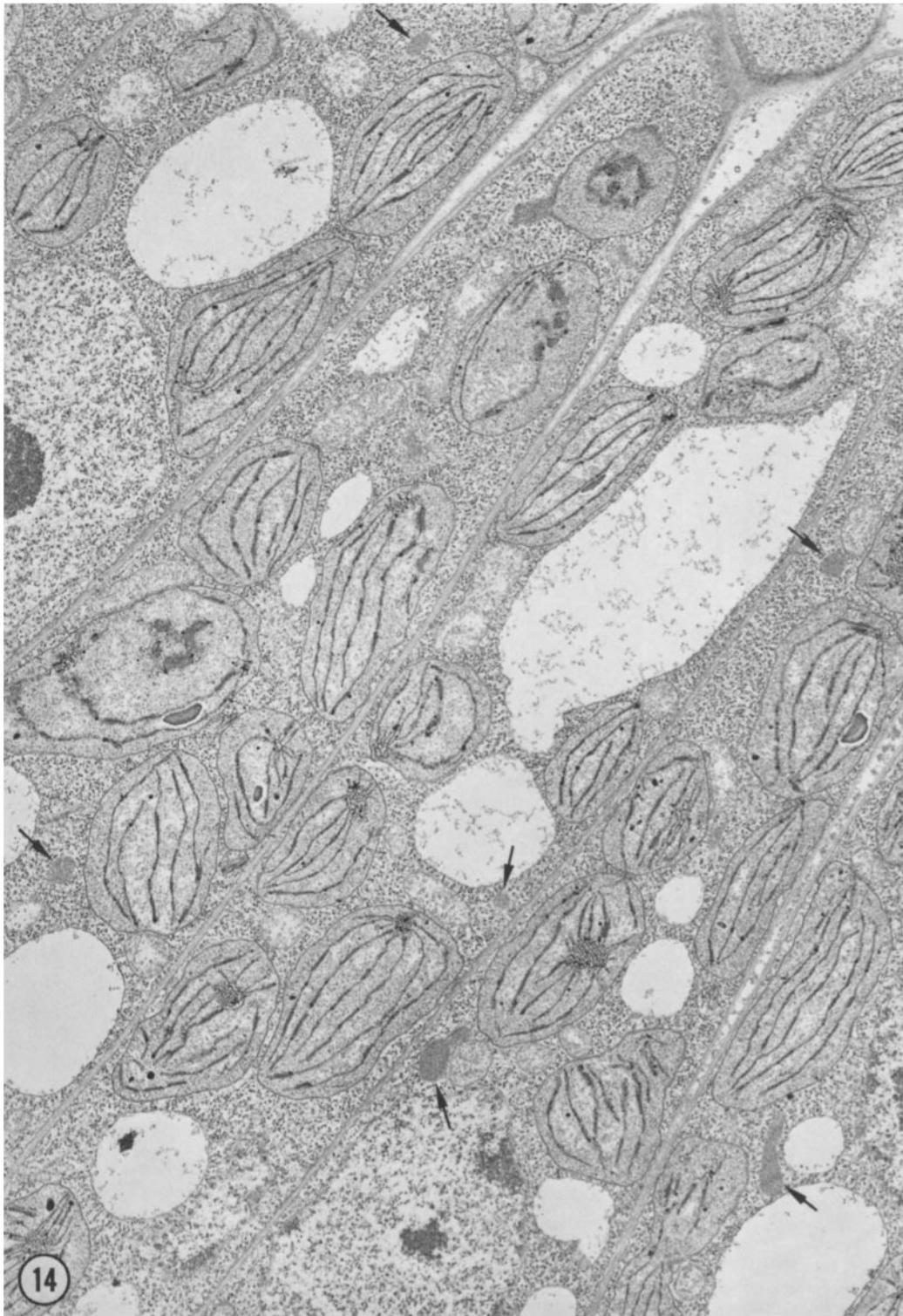


FIGURE 14 Palisade cells of a bean leaf 24 h after initial exposure of an etiolated plant to light. Some grana have formed in the chloroplasts, but disaggregation of the prolamellar bodies is not yet complete. The cells have undergone a certain amount of vacuolation by this stage. Microbodies are designated with arrows. $\times 9,100$.



FIGURE 15 Pocket of cytoplasm in a spongy cell 72 h after transfer of etiolated seedling to light. The microbodies (arrows) are now as large as those in 9- or 11-day old light-grown leaves. All three microbody profiles present in this section are appressed to chloroplasts. *N*, nucleus; *V*, vacuole; *I*, intercellular space. $\times 10,000$.

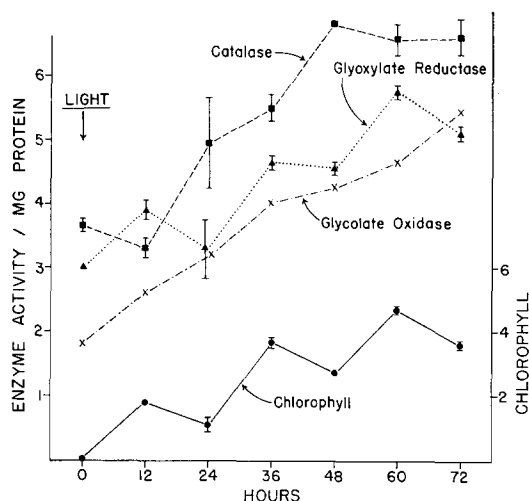


FIGURE 16 Peroxisomal enzyme activity in the homogenates of etiolated bean leaves placed in the light. At the beginning of the 72 h period, dark-grown plants were transferred to a growth room having a 12-12 hr light-dark cycle. Catalase specific activity is plotted as units per milligrams of protein, glycolate oxidase as $20 \times \Delta OD/\text{min}$ per mg protein, and glyoxylate reductase as $0.05 \times \text{nmol substrate consumed}/\text{min}$ per mg protein. Chlorophyll content is plotted as $100 \times \text{mg}/\text{mg}$ protein. Vertical lines represent ranges from determinations on two independent sets of seedlings.

degree perhaps equivalent to that of 6- to 7-day old light-grown peroxisomes, but complete their growth only after exposure to the light, recapitulating in this latter period what occurs in the light-grown plants between days 6-7 and day 9.

Of special interest here is that the development of the photosynthetic apparatus appears to follow a similar growth pattern, i.e. many of the photosynthetic components already accumulate to certain levels in etiolated leaves, then undergo added growth or final elaboration upon exposure to light. This appears to be the case with the carboxylating enzyme ribulose-1,5-diphosphate carboxylase (3, 13, 24, 29), with protochlorophyllide (22, 41), and with plastid ribosomal RNA (40). It may also be noted that the etioplast, in which the above components are stored, is a differentiated form of plastid whose complexity is certainly greater than that of a proplastid (22). It too develops to a point, then is arrested in growth until exposure to light permits its final transformation into a chloroplast. It appears, then, that plastids in etiolated leaves are already equipped to a

certain extent for photosynthesis even before the factor required for the process, i.e. light, is present. It seems probable that the peroxisomes likewise play a preparative role in the dark, in that they become specialized for the metabolism of glycolate before the actual onset of photosynthesis.

What appears to initiate the renewed or final formation of the various photosynthetic components is not light regardless of spectral quality, but more specifically, red light. It is thought that renewed ribulose-1,5-diphosphate carboxylase synthesis, renewed protochlorophyllide formation, increased plastid ribosomal RNA production and the differentiation of etioplasts into chloroplasts are all directly or indirectly under control of the phytochrome system (13, 22, 40). As regards the peroxisome, the results from several laboratories now indicate that the activities of some of its distinctive enzymes, e.g. glycolate oxidase and glyoxylate reductase, respond to red, far-red treatments in ways analogous to other processes affected by phytochrome (23, 36, 37). Thus, it seems attractive to propose that peroxisomes develop under controls similar to those operating on the photosynthetic plastids, at least to the extent that they reach a certain plateau of development in the dark, then are boosted to the final levels found in mature green leaves by red light operating through the phytochrome system.

A final observation on the regulation of peroxisomes is that apparently not all of their enzymes undergo identical patterns of change. This has been demonstrated to some extent in the Results by the increases and decreases in catalase activity which, in their timing and response to light, for example, differ somewhat from the changes in activity undergone by glycolate oxidase and glyoxylate reductase. This independence of catalase has been detected also in postgerminative sunflower cotyledons (20) and in wheat leaves (11). It has been found that catalase activity is not related to a phytochrome system (7), an observation contributing to the conclusion of Poucke and Barthe (36) that there does not exist a common underlying mechanism which regulates the activity of all enzymes contained within the microbody compartment. If it is conceded that microbodies grow at the expense of small discrete contributions derived from the ER, and that enzymes already present in them may be differentially sensitive to stimuli such as light, then it is not conceptually difficult to visualize different controls and responses for the various microbody enzymes.

Longevity of Leaf Microbodies

The results from the two developmental studies allow some suggestions to be made regarding the longevity of leaf microbodies. The ultrastructural observations on young bean leaves indicate strongly that microbodies are initially small particles and then develop into larger ones. It follows that the lifetime of such growing particles could be no less than the time required for them to develop from their young into their mature states. Both developmental studies have indicated that a minimum of 2 days and possibly as many as 4 days are required for the microbodies to attain their full size. Those in the 9-day old etiolated bean leaves are already somewhat enlarged, so that the entire time involved in their maturation, spanning the period in the dark plus that in the light, may well exceed 72 h. It is not excluded that the smaller microbodies observed after 12 h in the light may have been formed *de novo* after removal from the dark; but even so, this suggests a lower limit of 48 h as the time required for their enlargement.

Furthermore, no electron microscope evidence was obtained from 9- and 11-day old leaves suggesting that the microbodies undergo a rapid breakdown once they reach maturity. This impression is supplemented by ultrastructural observations on fully developed leaves that provide little if any indication of a turnover of microbodies (14, 16). In such leaves, microbodies generally appear medium to large sized, being neither very small nor in an obvious state of dissolution. Moreover, the small microbodies described in young leaves in this paper and those illustrated in the process of breakdown by autophagy (26, 52) or atrophy (26) should serve as prototypes which would allow manifestations of microbody formation and degradation to be recognized if they were occurring. It has been concluded partially on the basis of similar evidence that peroxisomes in greening fatty cotyledons are formed from pre-existing glyoxysomes and thus represent a relatively long-lived population of microbodies (50). It is perhaps valid to suggest at this stage of investigation that once higher plant microbodies do attain a large size, whether as glyoxysomes in fatty cotyledons or as peroxisomes in leaves exposed to the light for several days, they seem capable of persisting for considerable periods of time, unless of course, the entire tissue degenerates as in the case of castor bean endosperm (18, 52).

The data presented in this paper, however, relate primarily to the synthesis of leaf peroxisomes and bear only secondarily on their longevity as mature organelles. Studies on the turnover of leaf microbodies, analogous to those on the turnover of rat liver microbodies (35), remain to be performed.

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