On the Origin of Microbodies in Plants

- A New Hypothesis -

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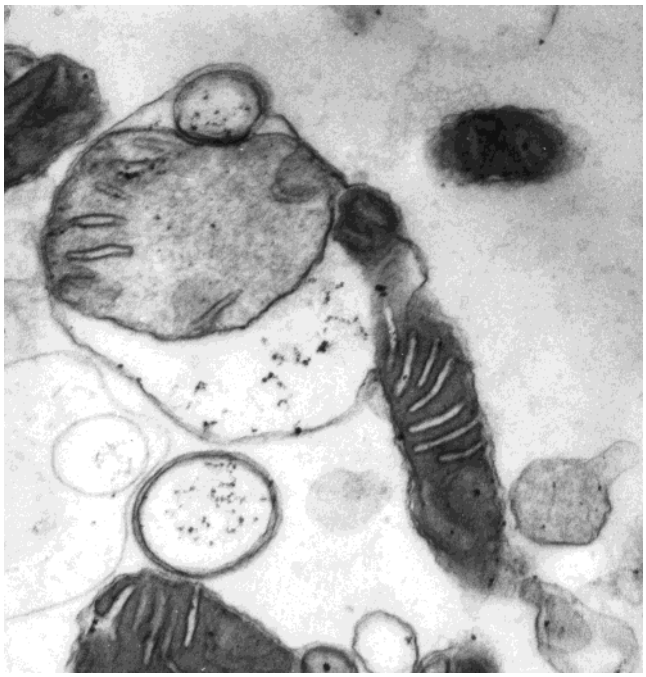

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Abstract. In *Euglena gracilis* grown with acetate in the dark, the glyoxysomal marker isocitrate lyase was found not to be located in microbodies but in vacuolar structures newly formed by disintegrating mitochondria during heterotrophic growth. The mitochondrial vacuoles contained also spherical organelles with diameters in the range of 0.2 to 0.5 µm and characteristic structures of microbodies. After isolation of these organelles they could be shown to possess only enzymes of the ß-oxidation pathway like microbodies of other primitive algae. Mitochondria disintegrated also in seeds of cucumber which were germinated for three days thereby forming ER-like structures which frequently were seen in close contact with developing microbodies. Altogether, we conclude from our data that microbodies in plant organisms originate from disintegrating mitochondria.

Microbodies are organelles which were detected in 1954 and hence demonstrated to be present in eukaryotes of almost all phylogenetic developmental stages but absent from prokaryotes. They commonly show a spheroid structure with an average diameter of 0.5 µm. Their characteristics are a single limiting membrane enclosing a finely granulated matrix which often contains amorphous or crystalline inclusions. After their biochemical characterization more than 30 years ago, the term peroxisome was introduced concomitant with the postulation that all microbodies possess at least one hydrogen peroxide-producing oxidase along with catalase.[10] However, as recently demonstrated, ancestral organisms do not contain catalase and hence no H_2O_2-forming oxidases, either. Enzymes in these organisms which correspond to the oxidases are dehydrogenases, reducing oxygen to water[3]. Altogether, until now more than 40 enzymes have been found to be possible constituents of microbodies, but it depends on the functional type of the organelle which of the enzymes are present[10].

Euglena gracilis is a unicellular alga which is capable of growing in the light with CO_2 as well as in the dark with organic substrates as sources for energy and carbon. It is reported to be one of the phylogenetically oldest and also earliest mitochondrion-containing eukaryotes[1]. Since it was recently reported that microbodies may have been acquired at the same time as mitochondria[2], *Euglena* should be one of the first organisms possessing microbodies as well. Microbodies underwent changes in function during phylogeny (Fig.1). Thus data on the

organelles in *Euglena*, expected to represent a first stage of the organellar evolution, may provide useful information on the origin of microbodies.

When cells of autotrophically grown *Euglena* are transferred to an acetate-containing medium and cultured in the dark, the enzymes isocitrate lyase and malate synthase are induced and their activities increase during heterotrophic growth. Both enzymes are markers of the glyoxylic acid cycle which is activated during growth on acetate[4]. This is a situation very similar to that found in germinating fatty seeds, where these enzymes are located in microbodies of the glyoxysomal type[5]. However, after isolation of organelles from *Euglena* by sucrose gradient centrifugation, we never could find isocitrate lyase and malate synthase at density 1.25 $g \cdot cm^{-3}$ which is characteristic of glyoxysomes. Instead, both enzymes were identified at density 1.19 $g \cdot cm^{-3}$ concomitant with mitochondrial markers. Electron micrographs of this fraction showed mitochondria but also spheroid particles with diameters in the range from 0.2 to 0.5 µm and characteristic structures of microbodies. According to our results, these particles were not trapped but had the same density as mitochondria. Separating the microbodies from mitochondria became possible, because they move slowlier during gradient centrifugation. When the centrifugation time was reduced from 3h to 1h, mitochondria reached their equilibrium density as usual, but microbodies then were found at density 1.17 $g \cdot cm^{-3}$ as demonstrated by electron micrographs (Fig.3). Unexpectedly, isocitrate lyase and malate synthase were not constituents of the microbody fraction, but still were found in fraction 1.19 $g \cdot cm^{-3}$ which suggests their location in mitochondria. The microbody fraction contained only enzymes of the fatty acid β-oxidation pathway which are present in glyoxysomes of higher plants as well (Fig.2).

To get more information on the localization of isocitrate lyase, antibodies were raised against this enzyme. However, as shown in Figs.4C and D, the antibodies did not bind directly to the mitochondria but to vacuolar structures connected with them (Fig.4A). These structures were not seen in autotrophic cells before and therefore must have been formed after their transition to heterotrophic growth conditions. Actually it could be demonstrated that the number of vacuolar structures, which will be named mitochondrial vacuoles in the following, increased concomitant with the activity of isocitrate lyase. While there were about two mitochondrial vacuoles per cross-section of a cell after three days, up to fifteen were seen after six days. On the other hand, mitochondrial vacuoles were dissolved when heterotrophic cells were transferred back to the autotrophic growth conditions. The glyoxylic acid cycle represents a shunt of the TCA cycle in mitochondria. Both cycles possess common enzymes. Therefore, the

formation of a separate compartment for the glyoxylic acid cyle appears to be reasonable to avoid interference of the two pathways.

Within the vacuole of a disintegrating mitochondrion other mitochondria and residues of mitochondria were detected. Thus the mitochondrial vacuoles look like lysosomes (Figs.4A,D) and may be identical with structures which in the literature are described as autolysosomes in *Euglena*[4]. However, lysosomes originate from Golgi complex, while there is strong evidence from numerous pictures that the lysosome-like mitochondrial vacuoles are formed from disintegrating mitochondria (Fig.4A). Furthermore, in cells growing with acetate in the dark the function of mitochondria is much more important than in autotrophic cells. Therefore, digestion of mitochondria after the change to heterotrophic growth would not be reasonable and seems to be unlikely.

Most surprisingly the mitochondrial vacuoles contain also spherical particles very similar in size and structure to the microbodies in fraction 1.17 $g \cdot cm^{-3}$ (Figs.3A; 4B; 5). Since microbodies were not seen in the autotrophic cells under the conditions we used, they must have been formed in the mitochondrial vacuoles. Frequently their membranes are observed in direct contact with the limiting vacuolar membrane (Fig.5). EM pictures show also empty spheroid particles. This is an interesting observation, because there are reports in the literature on peroxisomes which lack their matrix[6]. Mitochondrial vacuoles containing fully developed microbodies sometimes were seen only partly surrounded by a membrane (Fig.5). From these pictures the impression is obtained that the few microbodies seen in the cytosol of three-day-old acetate grown cells (occasionally one organelle per cross-section) were released from dissolving vacuoles.

The spherical particles in the mitochondrial vacuoles occasionally possess inclusions which is an additional criterion for microbodies (Fig.4B). According to the data of gradient centrifugation enzymes of the fatty acid ß-oxidation pathway are the only constituents. Catalase, which is a marker for higher plant peroxisomes, is not present in *Euglena* and other primitive algae[4,7,8]. Thus the microbodies in *Euglena* are of the same type as in *Prasinophyceae*[7] and *Prorocentrum*[8] which are representatives of the phylogenetically oldest organisms in the developmental lines of *Chlorophyta* (Fig.1) and *Chromophyta*, respectively[3]. In the group of *Chlorophyta* higher plants are the evolutionarily most developed organisms and contain glyoxysomes and leaf peroxisomes as highest developed microbodies. The presence of glyoxysomes also in *Euglena* was concluded from some former data[4]. However, the isolation of

organelles from this alga turned out to be problematic and, as recently reported, not all corresponding results may be free of error[9].

Glyoxysomes are formed during germination of fatty seeds[5], and during this phase we have observed disintegration of mitochondria even in higher plant tissue. Mitochondria in three-day-old seedlings of cucumber formed an ER-like structure (Fig. 7A, 8, 9A) which occasionally was seen also in *Euglena* (Fig.6). It is of utmost interest that membranous structures like this „mitochondrial ER" were observed in close contact with microbodies, which after three days of germination should be developing glyoxysomes (Fig. 7B, 8, 9B). In the literature there are many reports indicating a connection of microbodies with ER. Based on these observations the biogenesis of microbodies by budding from the ER was suggested[10]. However, cytochemical studies pointed to a separate identity of ER and microbodies, and as nowadays proposed, microbodies should multiply by growth and division of themselves[11]. Nevertheless, EM-pictures demonstrating the connection between microbodies and ER-like structures still exist. According to our observations there is no contradiction to recent data, if the ER was the „mitochondrial ER" seen in our pictures.

Cavalier-Smith has proposed that microbodies as mitochondria might have originated by adoption of endosymbionts at about the same time[2], though microbodies do not possess DNA or ribosomes and there are no other data supporting this view. On the other hand, the same author suggested that hydrogenosomes evolved from mitochondria. According to our data we would like to add that not only hydrogenosomes but also microbodies have originated and apparently still originate from mitochondria also in higher plants (Figs. 10-13). This would explain why enzymes characteristic of microbodies are present in prokaryotes and also in mitochondria of primitive algae[3,7,8,12,13] which, according to the endosymbiont hypothesis, evolved from adopted prokaryotes. Our hypothesis, which is also supported by the observation that peroxisomes are not present in amitochondrial protists[2], may open a new point of view in microbody research.

Methods

Algal cultures. *Euglena gracilis*, strain Z, was obtained from the algae collection of the University of Göttingen. Autotrophic cultures were aerated with 2% CO_2 in the air. The light intensity was 40 µmol quanta·m^{-2}·sec^{-1}. The growth medium used was as described by Cramer and Myers[14], but all organic carbon sources were omitted. For heterotrophic growth 0.5 % acetate was added to the autotrophic culture medium.

Separation of cell organelles. After homogenization of the cells with glass beads in a Virtis-homogenizer organelles were separated in a gradient of 30-60% sucrose. All steps as well as the enzyme assays used were previously described[16].

Purification of isocitrate lyase. The enzyme of acetate-grown *Euglena* cells was isolated and purified according to Lamb et al.[17]. Antibodies were raised by using a standard protocol.

Electron microscopy. Whole cells or organelles of gradient fractions were fixed with 4% glutaraldehyde and dehydrated in ethanol. After postfixation in 1.5% osmium tetraoxide they were embedded in Spurr resin as decribed before[15]. For immunocytochemical studies material was embedded in LR White resin. Treatment of the sections with antibodies raised against isocitrate lyase from *Euglena* and incubation with anti-rabbit IgG conjugated to 15 nm colloidal gold were performed according to Woijtaszek and Bolwell[18].

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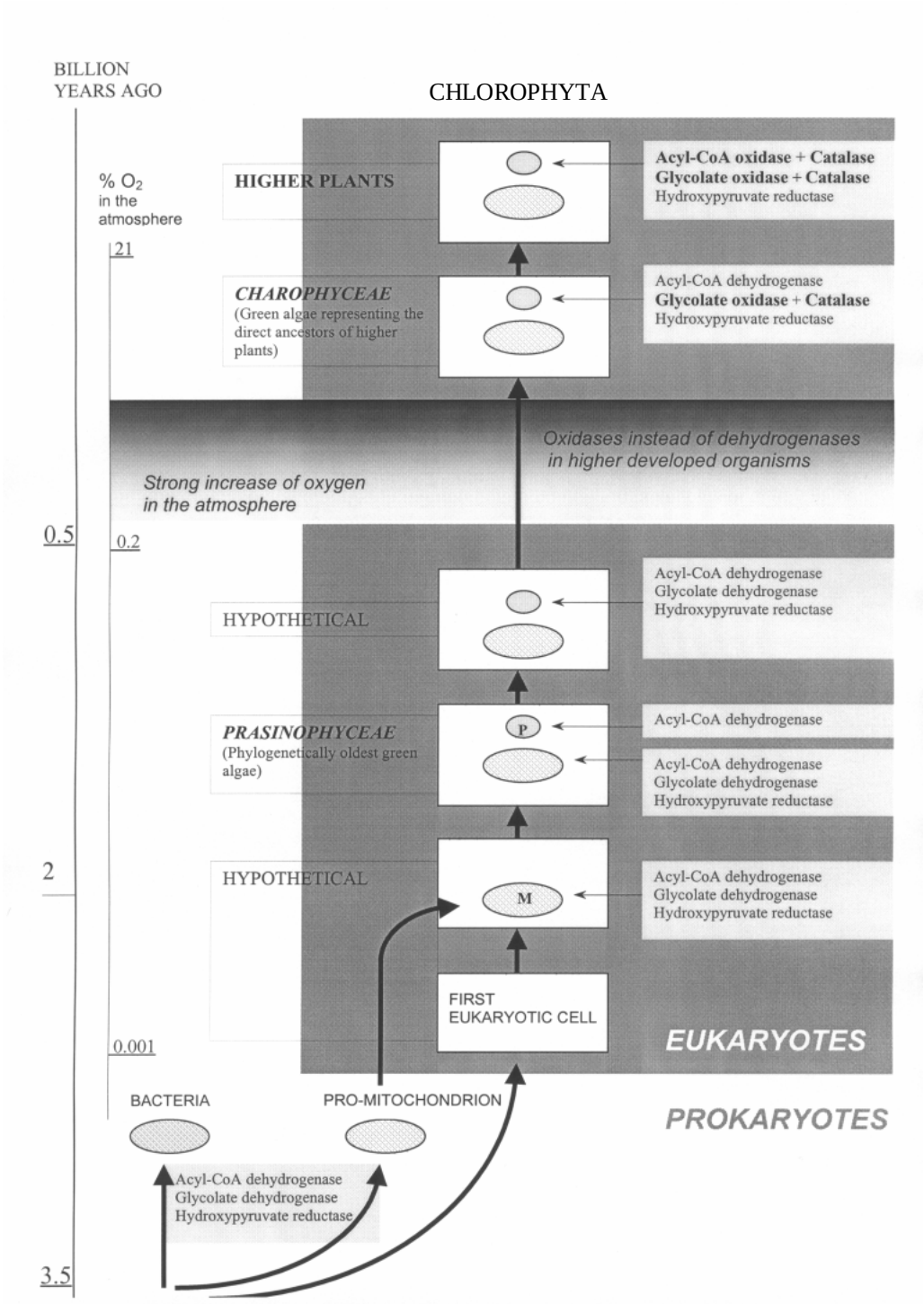

Figure 1 Hypothetical evolution of plant microbodies according to Stabenau[3]. In the scheme the phylogenetic change of compartmentation of only three enzymes is indicated. Generally, enzymes characteristic of leaf peroxisomes and glyoxysomes (e.g. glycolate oxidoreductase, hydroxypyruvate reductase, isocitrate lyase, malate synthase, and enzymes of the ß-oxidation pathway) are constituents already of prokaryotes. Therefore, their location in the mitochondria of primitive eukaryotes can be explained by the endosymbiont hypothesis. Apparently these enzymes were transferred to microbodies during the evolutionary development of organisms. In the microbodies of the phylogenetically oldest eukaryotes only enzymes of the fatty acid ß-oxidation pathway could be demonstrated, though corresponding enzymes were found in the mitochondria as well. However, in higher developed organisms the ß-oxidation pathway is present in the microbodies, exclusively. Moreover, in these organisms glycolate and acyl-CoA are oxidized not by dehydrogenases as in bacteria and primitive algae but by H_2O_2-forming oxidases. Thus the presence of catalase became also necessary in the microbodies. The appearance of oxidases seems to correlate with a dramatic increase of oxygen in the atmosphere. M = mitochondrion; P = microbody/peroxisome.

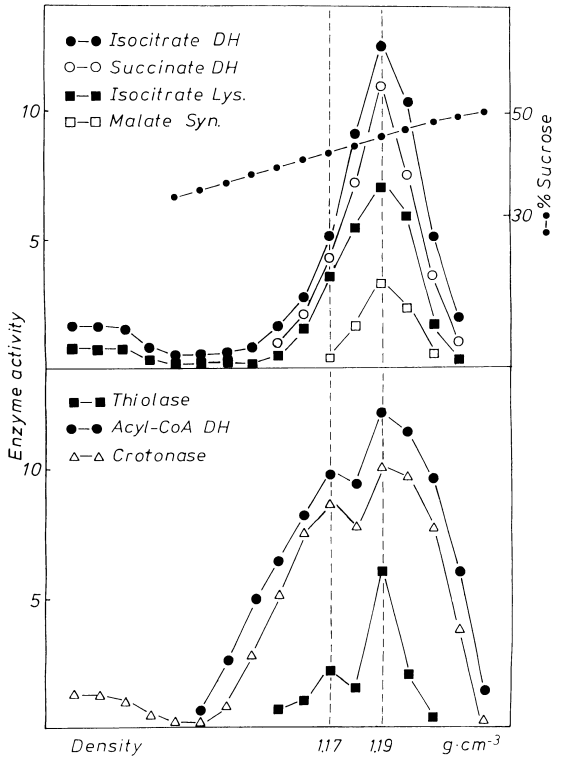

Figure 2 Distribution of enzymes after separation of organelles from *Euglena gracilis* by short-time centrifugation in a sucrose gradient. Cells were grown on acetate in the dark for three days. Peaks of succinate dehydrogenase and isocitrate dehydrogenase indicate the presence of mitochondria at density 1.19 g · cm^{-3}. Microbodies moved to density 1.17 g · cm^{-3} as indicated by peaks of the ß-oxidation enzymes. Ten units of enzyme activity on the ordinate correspond to the following activities in pkat · ml^{-1} fraction: isocitrate dehydrogenase *(Isocitrate DH)*, 200; succinate dehydrogenase *(Succinate DH)*, 20; isocitrate lyase *(Isocitrate Lys.*), 250; malate synthase *(Malate Syn.)*, 200; acyl-CoA dehydrogenase (*Acyl-CoA DH)*, 200*;* crotonase, 6,000; thiolase, 20.

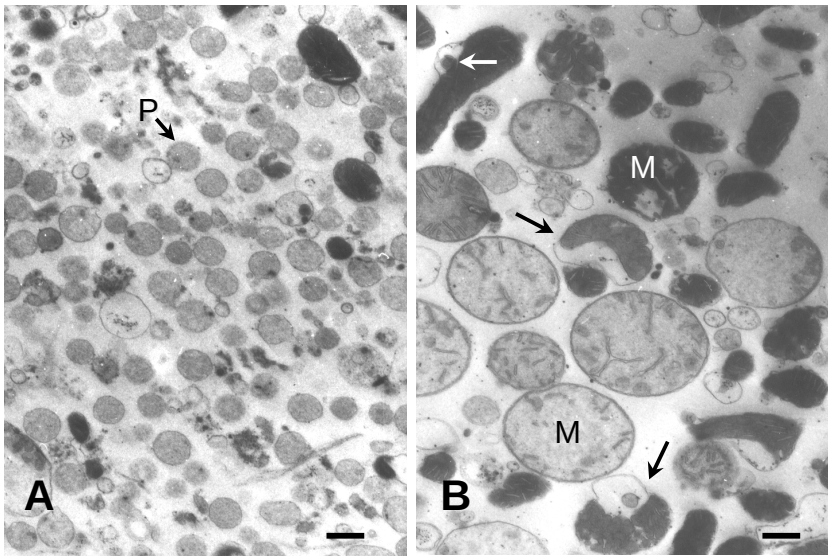

Figure 3 Organelles from acetate-grown *Euglena gracilis* isolated by short-time gradient centrifugation. **A**, Microbodies (P) in the fraction of density 1.17 g · cm^{-3}. The arrow indicates an organelle with an inclusion (compare with figure 4B). **B**, Mitochondria (M) at density 1.19 g · cm^{-3}. Disintegrating organelles possess a dense matrix. Several of them have formed a vacuolar structure (arrows). A small microbody can be seen in the mitochondrial vacuole at the bottom of the picture. Bars = 0.4 µm.

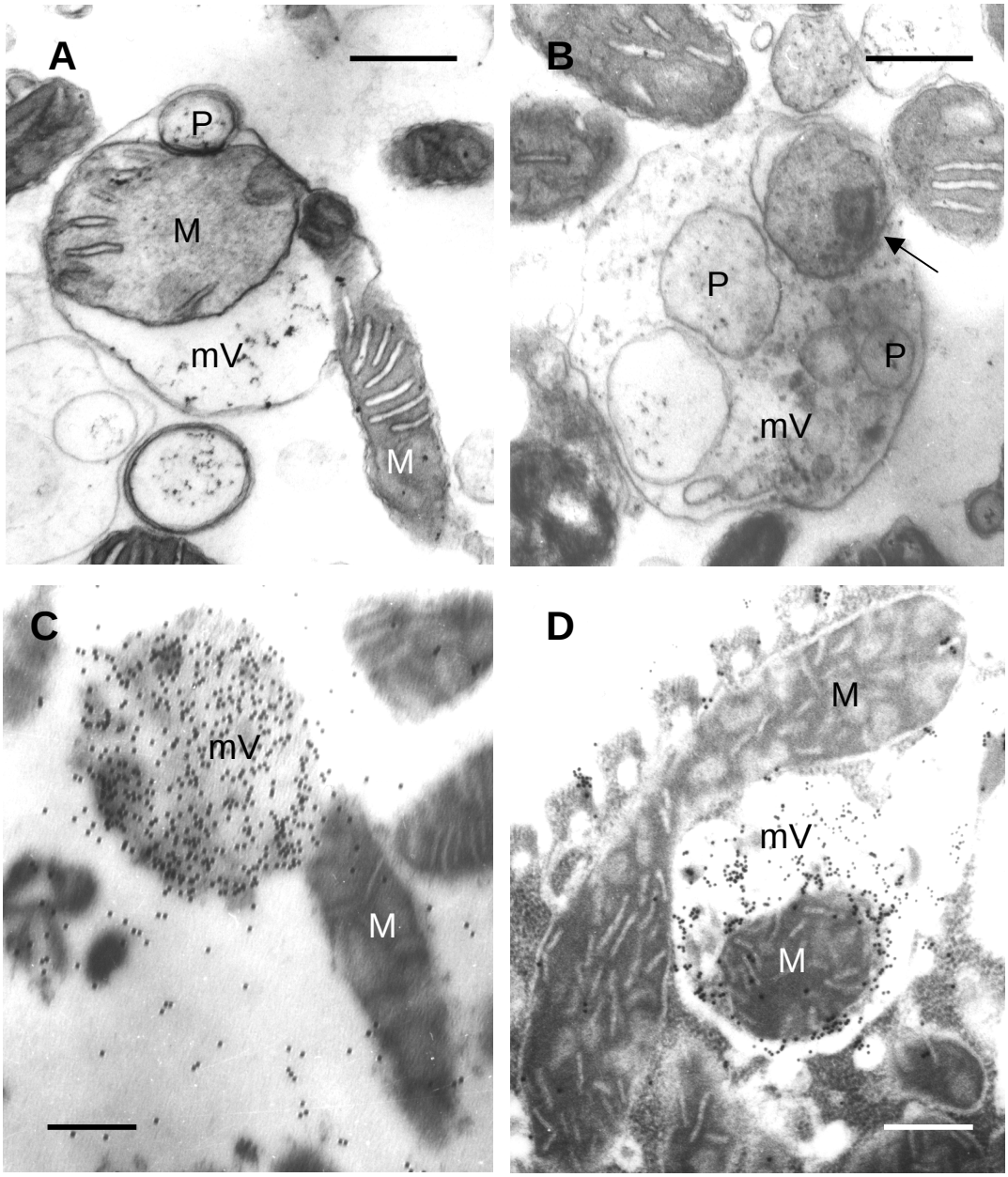

Figure 4 Organelles in acetate-grown *Euglena gracilis*. **A**, Isolated mitochondrion which has formed a vacuolar structure. The mitochondrial vacuole contains a disintegrating mitochondrion and a microbody. **B**, Mitochondrial vacuole with microbodies in the mitochondria fraction of a gradient. The arrow indicates an inclusion characteristic of microbodies (compare with figure 3A). **C**, Antibodies raised against isocitrate lyase bind to the mitochondrial vacuole of an isolated organelle. **D**, Cross-section of a whole cell. Antibodies against isocitrate lyase bind only to the matrix in the mitochondrial vacuole. M = mitochondrion, mV = mitochondrial vacuole, P = microbody. Bars: 0.4 µm.

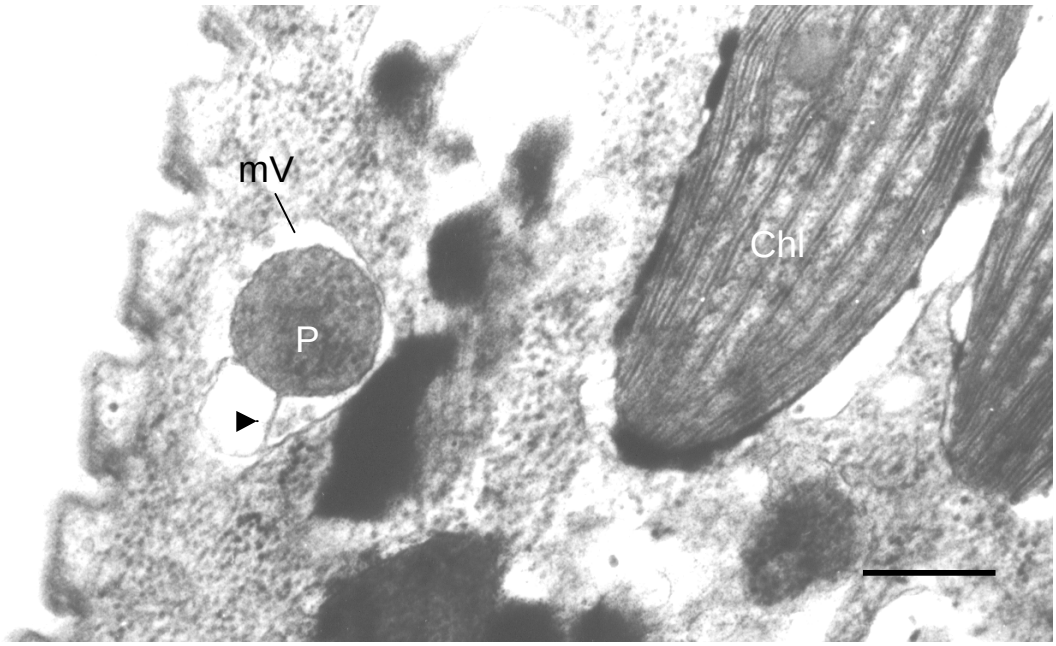

Figure 5 *Euglena gracilis*. Cross-section of an acetate-grown cell demonstrating a microbody (P) in a mitochondrial vacuole (mV) which partly lost its limiting membrane. Note the membranous connection between the microbody and the vacuolar membrane (wedge). Chl = chloroplast. Bar: 0.4 µm.

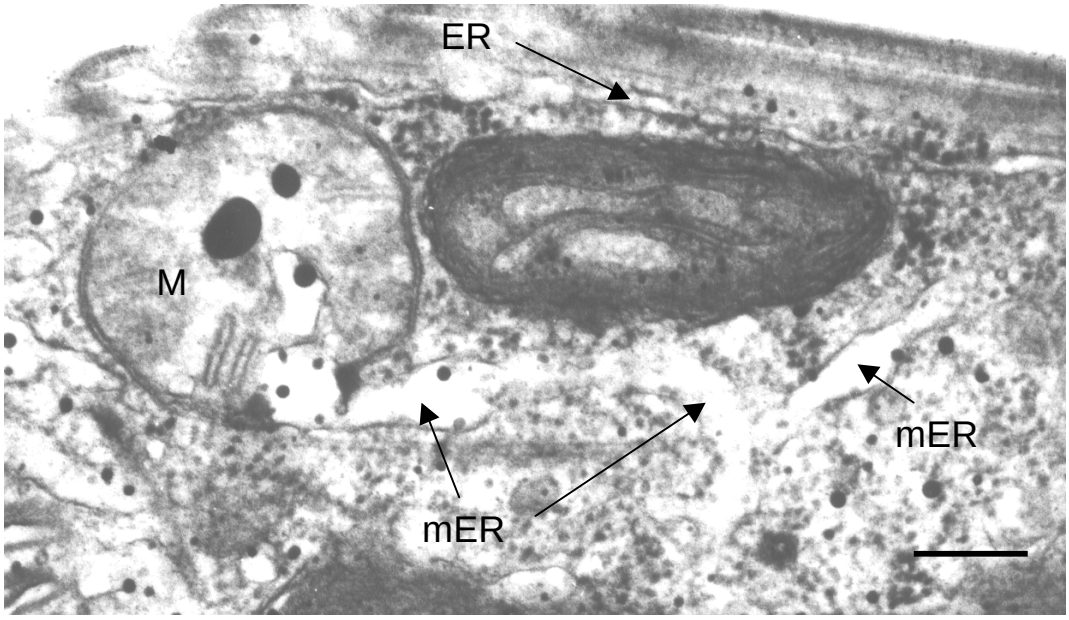

Figure 6 *Euglena gracilis*. Disintegrating mitochondrion which has formed an ER-like structure (mER). Mitochondrial ER and mitochondrial vacuoles can be formed by the same organelle. Since the mitochondrial ER occasionally was seen in contact with larger vacuoles it may provide membrane material for growth of the mitochondrial vacuoles. ER = endoplasmic reticulum. Bar: 0.4 µm.

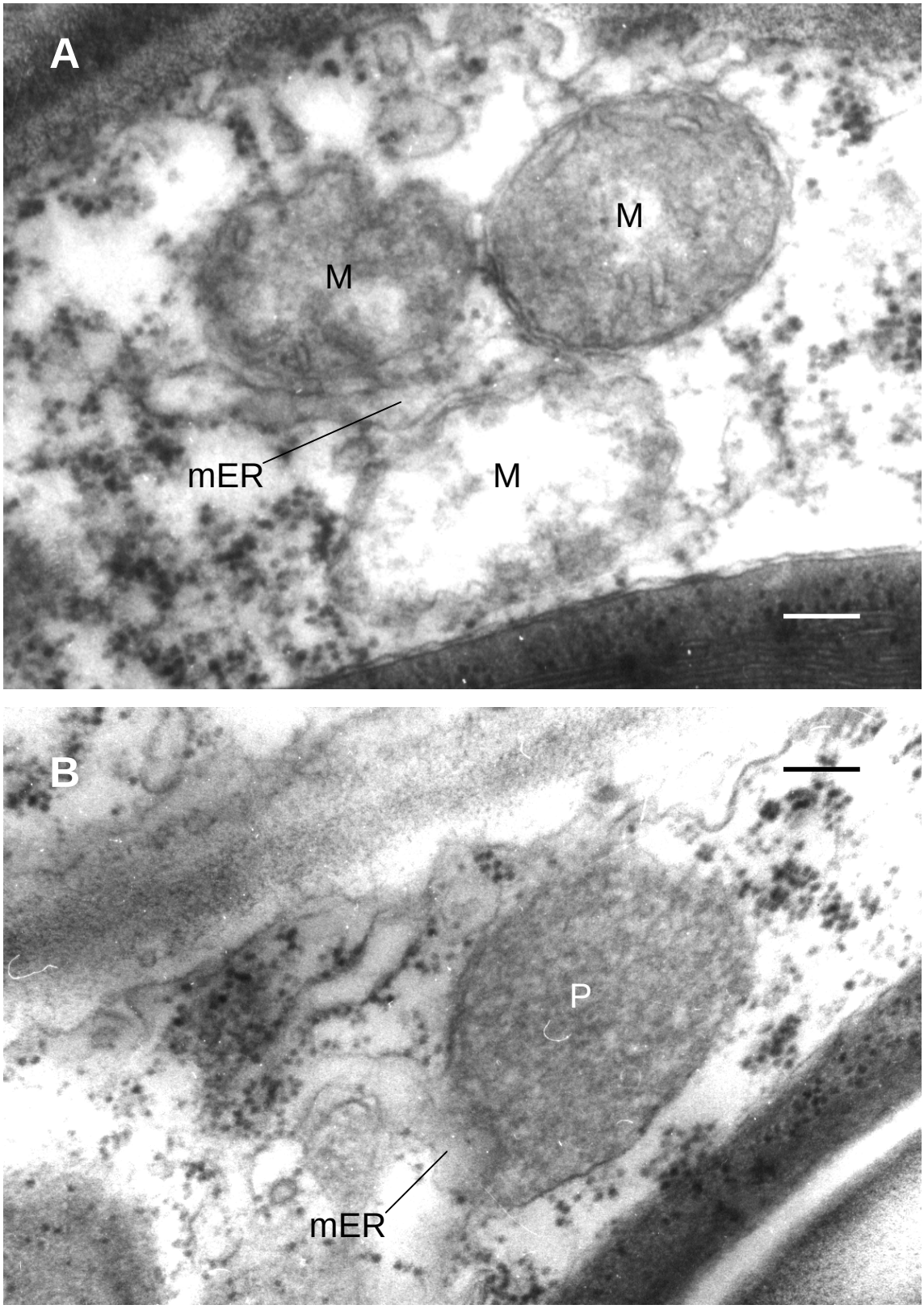

Figure 7 *Cucumis sativus* (cucumber). Seed-tissue after three days of germination. **A**, Disintegrating mitochondria forming an ER-like structure. **B**, Microbody in close contact with a membranous structure which is very similar to the mitochondrial ER. P = microbody, M = mitochondrion, mER = mitochondrial ER. Bars: 0.2 µm.

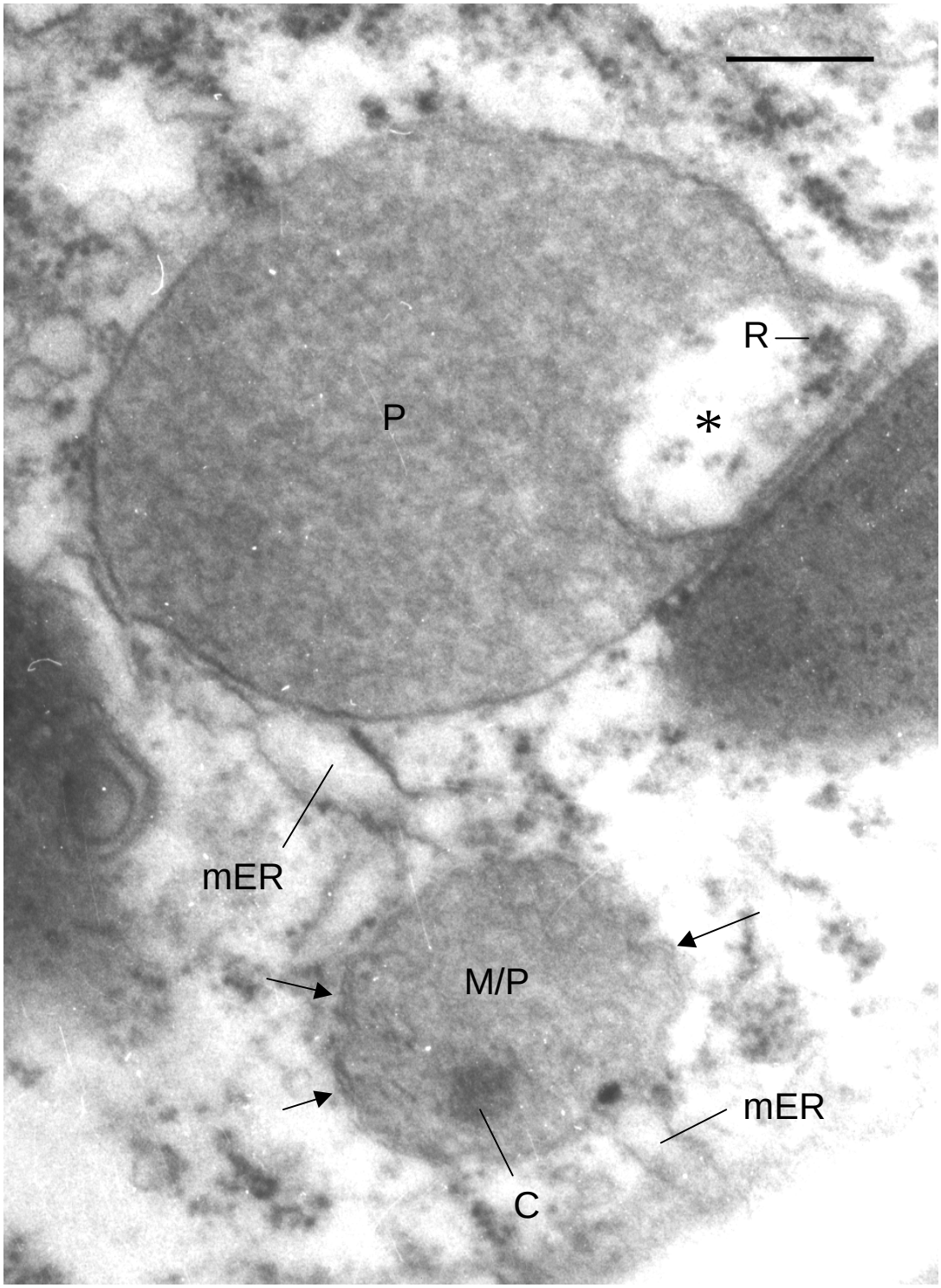

Figure 8 *Cucumis sativus* (cucumber). Cross-section of tissue. Seeds were germinated for three days. Microbody and disintegrated mitochondrion are connected by mitochondrial ER. Remnants of the mitochondrial two limiting membranes are marked by arrows. The structure of the matrix of the mitochondrial residue is very similar to that of the microbody. It already contains an inclusion (core) which is characteristic of microbodies. The microbody possesses a cytosolic inclusion (asterix) which is not completely enclosed by a membrane. Such a picture should not be possible if microbodies originated by division. C = core, M = mitochondrial residue, mER = mitochondrial ER, P = microbody, R = ribosomes. Bar: 0.4 µm.

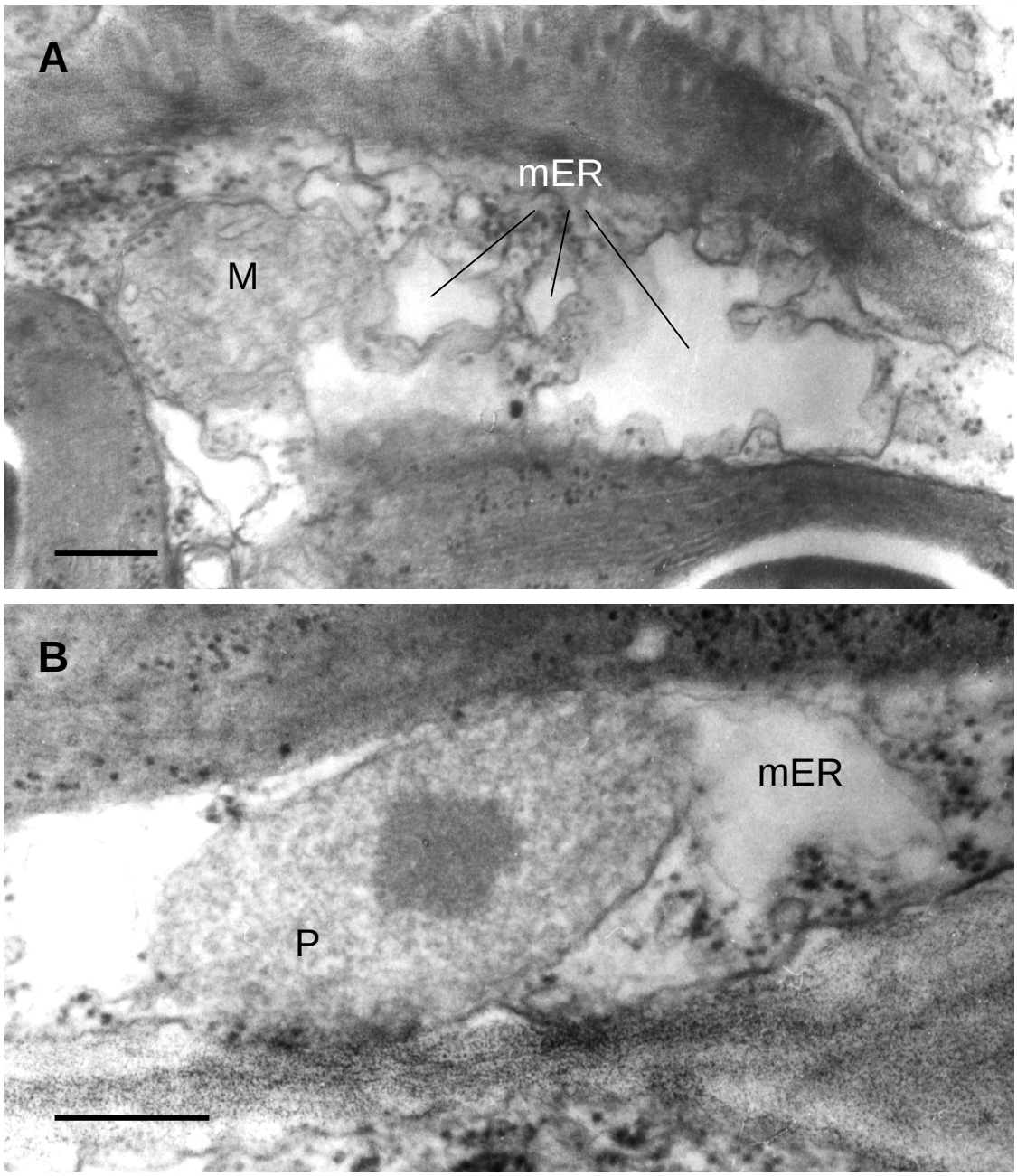

Figure 9 *Cucumis sativus* (cucumber). Cross-section of tissue. Seeds were germinated for three days. **A**, Disintegrating mitochondrion forming a membranous structure which looks like inflated ER (mitochondrial ER). **B**, Microbody in close contact with a membranous structure very similar to the mitochondrial ER in picture A. M = mitochondrion, P = microbody, mER = mitochondrial ER. Bars: 0.4 µm.

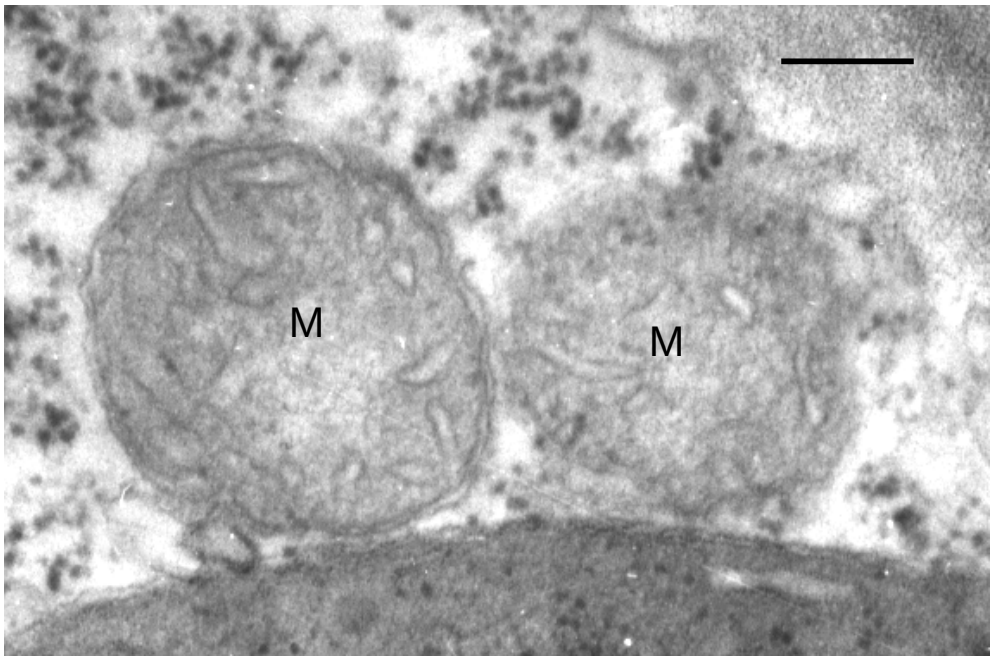

Figure 10 *Cucumis sativus* (cucumber). Disintegration of mitochondria in seeds which were germinated for three days. The organelle on the right side has completely lost its limiting membranes. M = mitochondrion. Bar: 0.2 µm.

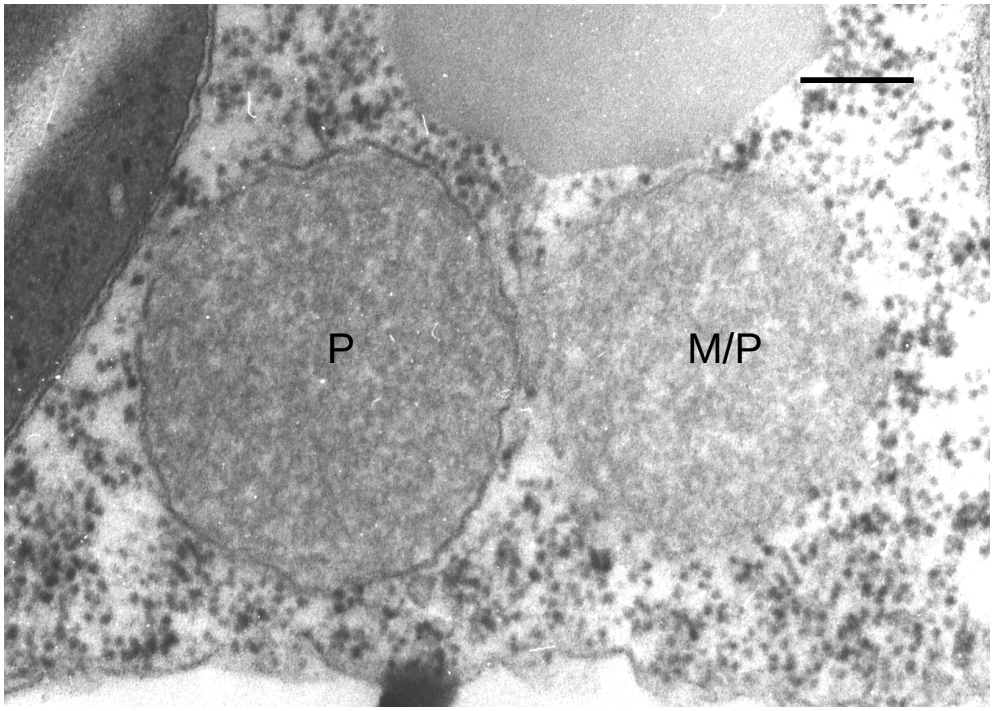

Figure 11 *Cucumis sativus* (cucumber). At a final stage of disintegration the mitochondrial residue shows a structure very similar to the matrix of the microbody. M = residue of mitochondrion, P = developing microbody. Bar: 0.2 µm.

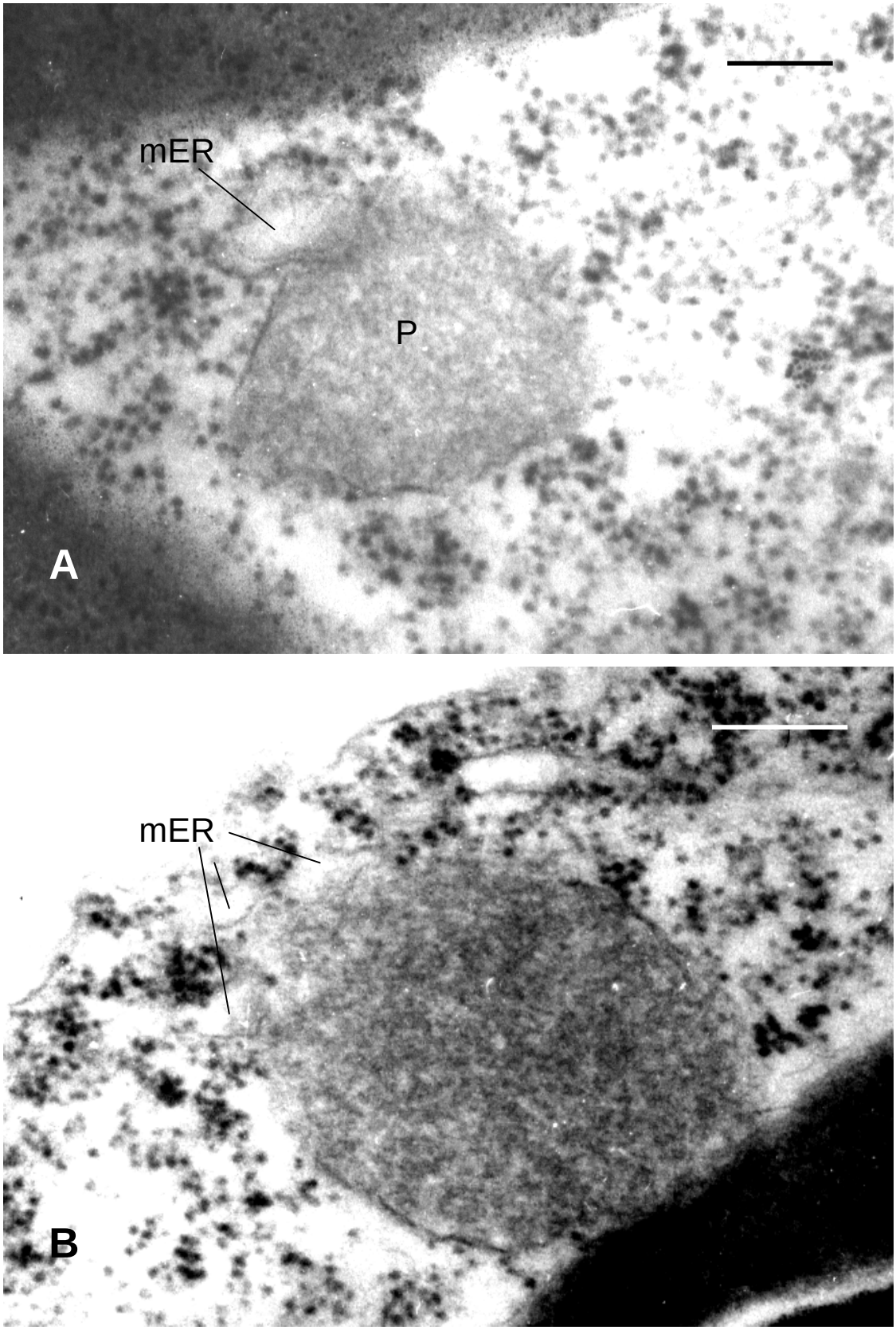

Figure 12 *Cucumis sativus* (cucumber). Developing microbodies. P = microbody, mER = mitochondrial ER. Bars: 0.2 µm.

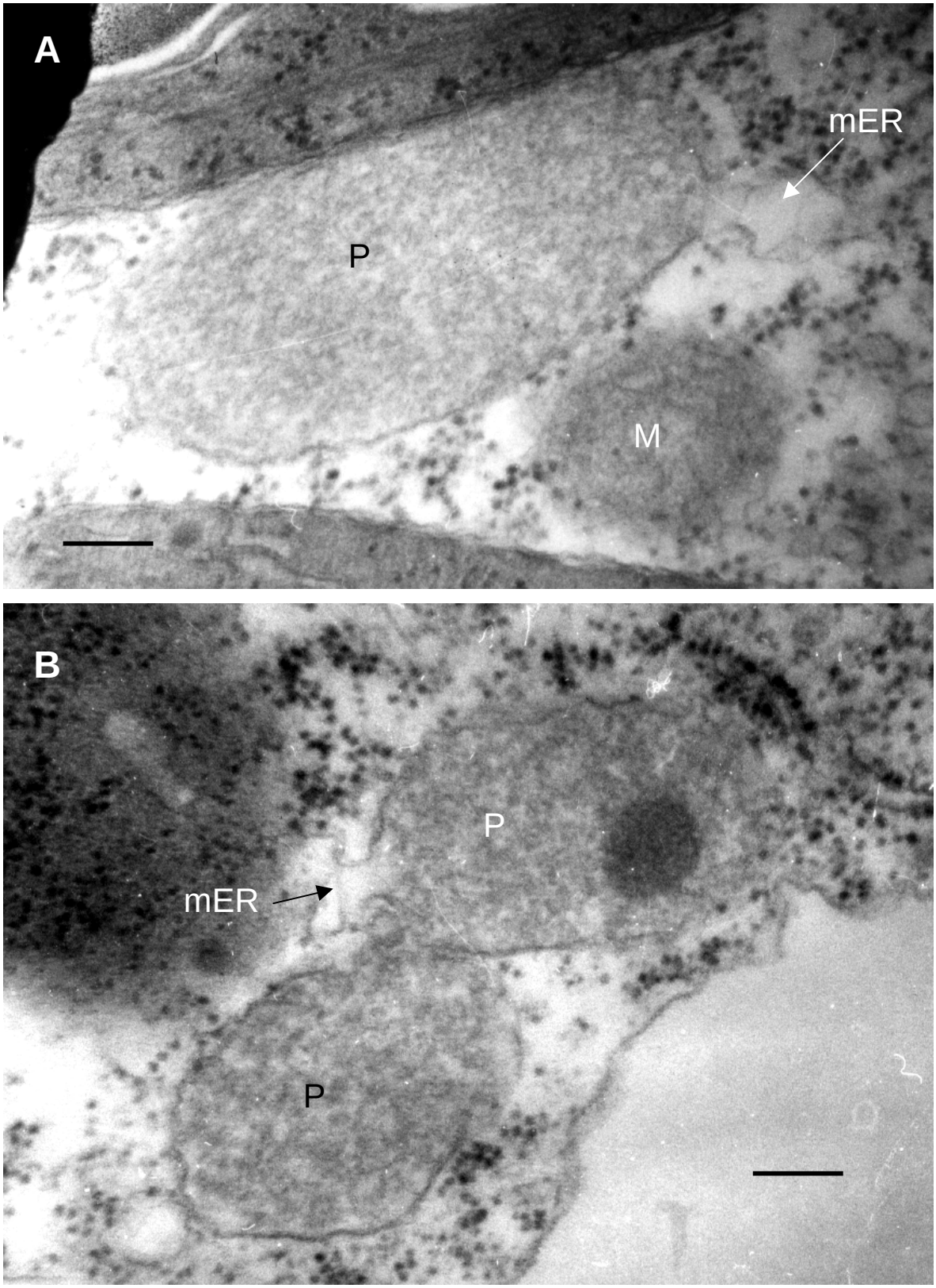

Figure 13 *Cucumis sativus* (cucumber). Microbodies in close contact with membranous structures very similar to mitochondrial ER. P = microbody, mER = mitochondrial ER, M = residue of a disintegrated mitochondrion. Bars: 0.4 µm.