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First ultrastructural demonstration of catalase activity with diaminobenzidine in a liverwort: *Conocephalum conicum* (L.) Dum. (Marchantiales).

The usual presence of microbodies in plant cells has been well known for several years. Tipically they are spheroidal or oblonged bodies delimitated by one unit membrane and containing a finely granular matrix. This has an average electrodensity and contains, in some cases, a paracrystalline core (for reviews see TOLBERT, 1971 and FREDERICK, GRUBER & NEWCOMB, 1975).

According to the enzyme content, two main classes of plant microbodies are distinguished: the glyoxysomes and the peroxisomes. The former lie tipically in the fat-storing tissues of the seeds and are involved in the glyoxylate cycle (RICHARDSON, 1974).

The latter are distinctive of the photosynthetic cells of the Angiosperms and play an important role in the photorespiration as they contain catalase, glycolate oxidase and other enzymes of the glycolate pathway (TOLBERT, OESER, KISAKI, HAGEMAN & YAMAZAKI, 1968; FREDERICK & NEWCOMB, 1969).

The occurrence of microbodies in Bryophytes can be regarded today as fairly documented (HEBANT & MARTY, 1972; GALATIS & APOSTOLAKOS, 1976; IDZIKOWSKA & SWEYKOWSKA, 1978). Moreover a catalase and a glycolate oxidase activity were tested in the cell extracts of some species (FREDERICK, GRUBER & TOLBERT, 1973). Thence the question raises whether also in

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the Bryophytes these key photorespiration enzymes are localized in the microbodies and, therefore, whether such organelles may be regarded as analogous to the leaf peroxisomes of the Angiosperms.

Nevertheless only in *Polytrichum commune* (Polytrichales, Musci) the presence of catalase activity in the microbodies has been demonstrated as yet (HEBANT & MARTY, 1972). Even when the presence of glycolate oxidase and of other leaf peroxisomal key enzymes in the microbodies is not proved, the ultrastructural demonstration of catalase activity alone in such organules leads to consider them as peroxisomes when they are associated with chloroplasts and mitochondria. As a matter of fact, on the ground of such data, HEBANT & MARTY (1972) suggested a peroxisomal nature for the microbodies of the photosynthetic cells of *Polytrichum commune*.

This assumption cannot be extended immediately to all the Bryophytes because of the wideness and the marked anatomical and histological variability of this phylum. To this purpose further ultrastructural and cytochemical data on other species belonging to the most representative taxa are necessary.

In the present paper the results of such an investigation on *Conocephalum conicum* (L.) Dum. (Marchantiales, Hepaticae) are referred and discussed.

MATERIAL AND METHODS

Specimens of Conocephalum conicum (L.) Dum. were gathered in the Botanical Gardens of Naples, where this liverwort grows spontaneously and sporifies. Small pieces (1 mm^3) were cut from the thalli and fixed for 2 h at room temperature with 4% glutaraldehyde in 0.05 M phosphate buffer pH 7.2. The pieces were then washed for 1 h in four changes of the same buffer and post-fixed for 1 h with 2% phosphate-buffered osmium tetroxide. After dehydration with ethanol and propylene oxide the material was embedded in Epon 812.

Ultrathin sections were cut by using a diamond knife and were stained with uranyl acetate and lead citrate.

A 3,3'-diaminobenzidine (DAB) medium was used for the localization of the catalase activity. Very small pieces of thallus were fixed for 1 h at 4° C with 2% glutaraldehyde in 0.05 M cacodylate buffer pH 7.2 and after a rapid washing in several changes of the same buffer were incubated for 1 h in the dark at 37° C in the following freshly prepared medium:

— 3,3'-diaminobenzidine tetrahydrochloride (Baker) : 40 mg

— 2% hydrogen peroxide : 0.2 ml

— 0.05 M 2-amino-2-methyl-1,3-propandiol (AMPD) buffer: 9.8 ml. The final pH was adjusted to 9.4.

Control tests were carried out as follows:

— preincubation for 30' in 0.02 M catalase inhibitor 3-amino-1H-1,2,4-triazole (AT) and then incubation in DAB medium containing the same concentration of inhibitor;

— incubation in the DAB medium without H_2O_2;

— incubation in the complete DAB medium of pieces previously heated for 10' by immersion in boiling water.

The same tests were carried out also at pH 7.2 using a cacodylate buffer instead of AMPD.

After the incubation the pieces were washed, post-fixed with 2% cacodylate-buffered OsO_4 and prepared for the electron microscopy as above described.

Results and Discussion

Microbodies are very common in the photosynthetic cells of the subepidermal layer, whilst generally they are absent in the white parenchymatic cells below (fig. 1).

The microbodies of C. conicum have a spheroidal or, more rarely, an oblonged shape with sizes ranging from 0.5 to 1.5 μm. Their matrix is finely granular, laks any paracrystalline inclusions and shows the same electrondensity as the chloroplast stroma.

As a rule a more or less intimate association of the microbodies with chloroplasts and mitochondria was observed (figg. 2 and 5).

The incubation in the standard DAB medium at pH 9.4 resulted in an evident staining of the matrix, that became more electrodense than in the untreated samples (fig. 2).

Both the addition of AT and the absence of hydrogen peroxide in the DAB medium, as well as the exposition of the samples to boiling water before the incubation in the complete DAB medium, prevented the staining (figg. 3, 4 and 5).

No staining was ever observed in the samples incubated at neutral pH.

The 3,3'-diaminobenzidine has been largely used as a substrate for the ultrastructural localization of several enzymes (Graham & Karnowsky, 1966; Seligman, Karnowsky, Wasserkrug & Harker, 1968; Novikoff & Goldfisher, 1969). For the action of these enzymes the DAB is oxidized and forms a water- and lipid-insoluble polymer that gives rise to a well localized osmiophilic deposit.

The specifity depends upon the experimental conditions used and upon a selective inhibition of the enzymatic reactions. As for the catalase it has been proved that the DAB cytochemical test is suitable when glutaraldehyde fixative, high temperature, alkaline pH, high hydrogen peroxide concentrations, relatively long incubation time and selective inhibitor AT are employed (Sexton & Hall, 1978).

The marked and well localized staining of the microbodies in the samples incubated in the complete DAB medium is cer-

tainly attributable to an enzymatic oxidation of the DAB since it is suppressed by too high temperatures. Moreover the reaction appears to be catalase-dependent as it is highly sensitive to the presence of AT and of exogenous hydrogen peroxide.. This is confirmed by the absence of staining in the samples incubated at pH 7.2.

These results prove that the microbodies house the catalase activity which was previously tested, togheter with glycolate oxidase and hydroxypyruvate reductase activities, in the cell extracts of *C. conicum* (GAMBARDELLA, CASTALDO & LIGRONE, *in press*).

Such data allow to believe that the microbodies of this liverwort are quite analogous to the leaf peroxisomes of the Angiosperms (TOLBERT, 1971; FREDERICK *et al.*, 1975), as also their usual association with chloroplasts and mitochondria demonstrates.

Conocephalum conicum is the first liverwort investigated in this respect. The results of this study therefore constitute a further confirmation of the « high degree of physiological similarity that exists among the different phyla of Archegoniatae », that was already stressed by HEBANT & MARTY (1972) as a conclusion of their researches on the microbodies of *P. commune.*

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SUMMARY

The occurrence of catalase activity in the microbodies of the photosynthetic cells of *Conocephalum conicum* (Marchantiales, Hepaticae) is proved by means of the DAB cytochemical test.

The AA. suggest that such organelles are analogous to the leaf peroxisomes of the Angiosperms.

RIASSUNTO

Viene dimostrata citochimicamente, attraverso l'uso del DAB test, la presenza di attività catalasica nei « microbodies » delle cellule fotosintetiche di *Conocephalum conicum* (Marchantiales, Hepaticae).

Gli AA. suggeriscono che tali organuli siano analoghi ai perossisomi delle foglie delle Angiosperme.

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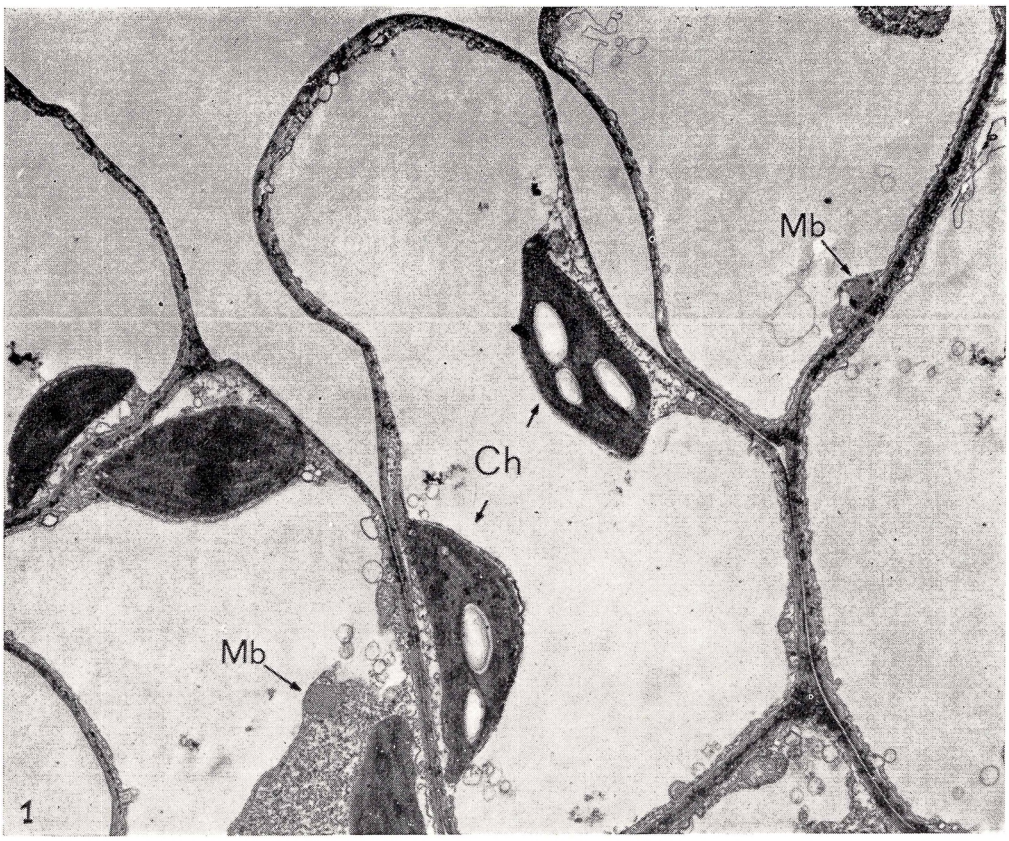
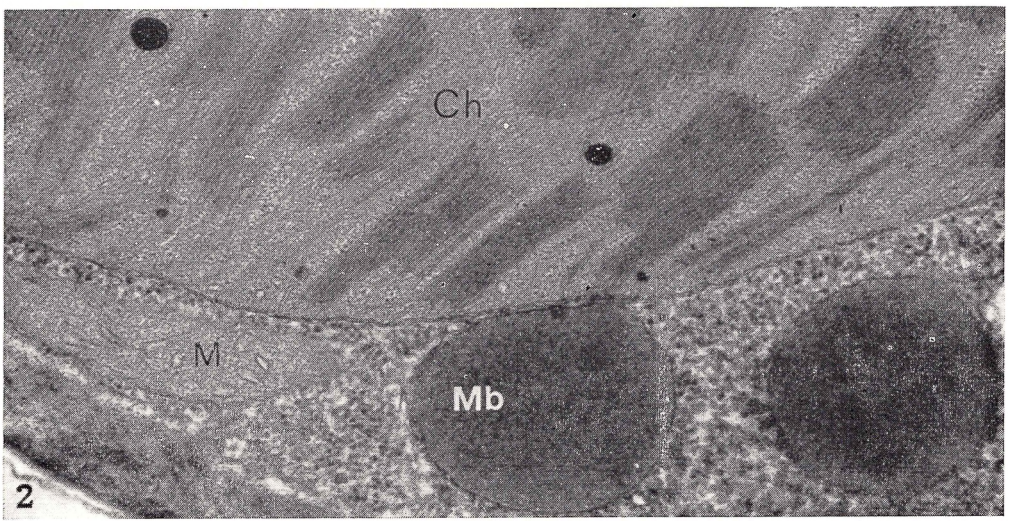
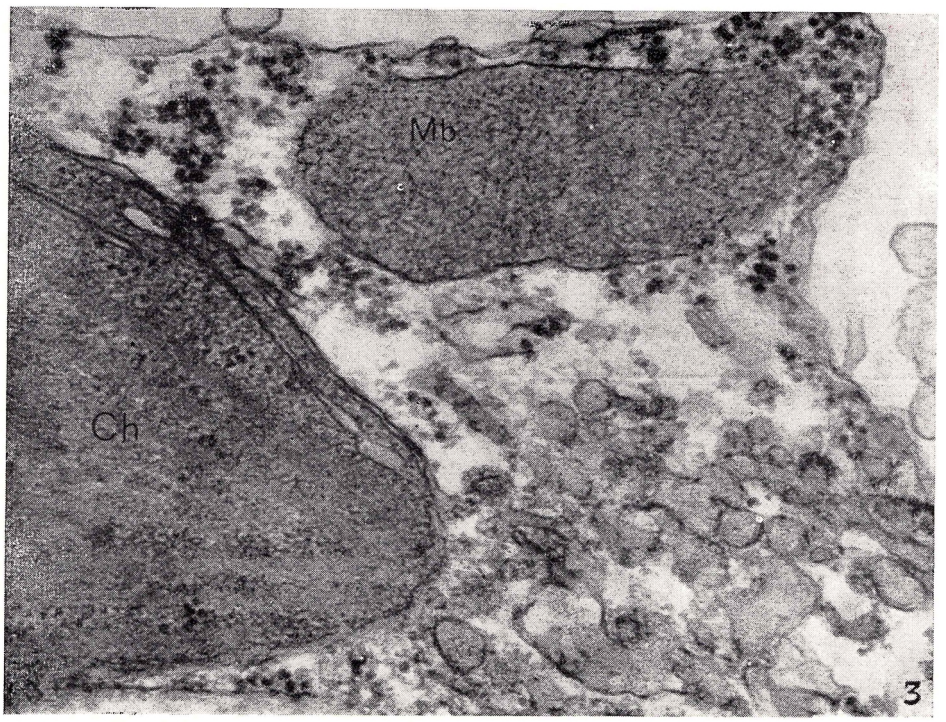
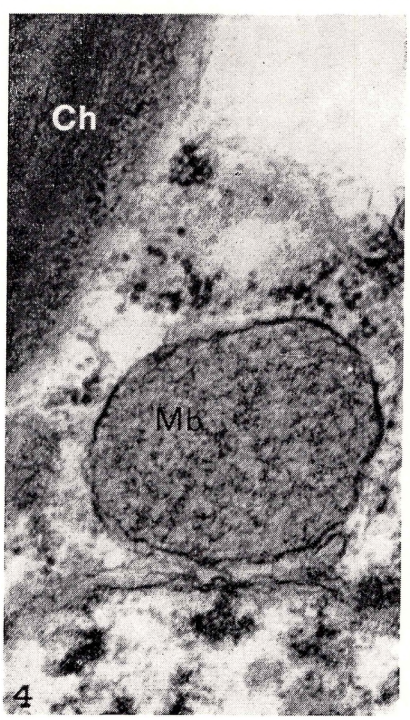
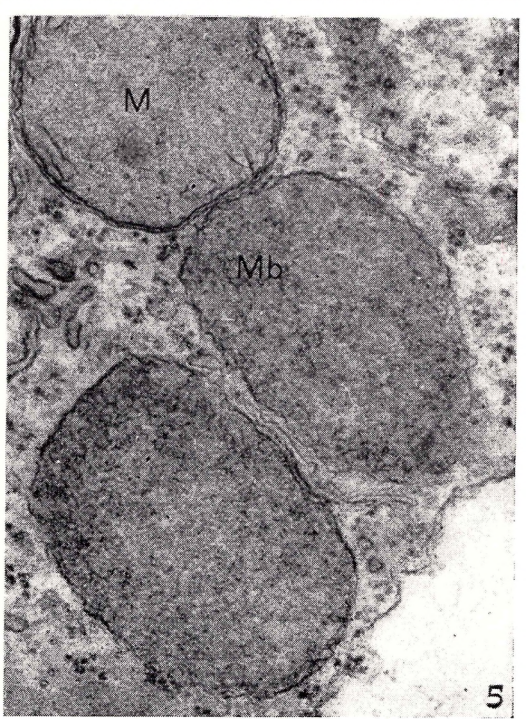




1) Cells of the photosynthetic subepidermal tissue, containing microbodies (Mb) togheter with large and well developed chloroplasts (Ch) (x 7,000).
2) Detail of a photosynthetic cell of a sample incubated in the complete DAB medium pH 9.4. Two intensely stained microbodies are visible. Note their association with a chloroplast and a mitochondrion (M) (x 36.000).







3) Incubation in the complete DAB medium pH 9.4 with addition of AT. No reaction occurred in the microbodies (x 84,000).
4) Incubation in the DAB medium without hydrogen peroxide. The microbody matrix shows no increase in its electrondensity (x 75,000).
5) Sample scalded before the incubation in the complete DAB medium pH 9.4. Note the unreacted microbodies in close proximity to a mitochondrion (x 50,000).