Some Ultrastructural Observations of Cotyledonary Tissue and Microbodies from *Pisum sativum*

Biochemical and histochemical literature (reviewed by TOLBERT[1]) illustrates that various oxidases and hydrolyases are compartmentalized within particulate components of the plant cell. Biochemical studies[2–5] of plant tissues have shown that single-membrane-bound particles rich in α-hydroxy acid oxidase and catalase, enzymes characteristic of animal peroxisomes, can be isolated from homogenates of plant cells. Recently FREDERICK and NEWCOMB[6,7] have shown an 'equivalence between leaf peroxisomes and leaf microbodies' in photosynthetic tissue by employing an electron cytochemical reagent, 3,3-diamino-benzidine. This compound has also been employed to demonstrate peroxidatic activity of animal microbodies containing catalase[8–11].

In the present study single-membrane microbodies and spherosome-like bodies are demonstrated in pea cotyledon cells.

Materials and method. Seeds of *Pisum sativum* L. var. Homesteader were soaked in distilled water for 4 h and then fully-imbibed seeds were germinated for 92 h at 26°C. The tissue was chopped into 1 mm³ segments on a block of frozen buffered fixative (4% (v/v) Ladd glutaraldehyde in 0.01 *M* potassium phosphate pH 6.8 at 0°C). Fixation was carried out at 0°C for $2^1/_4$ h. After primary fixation the tissue was post-fixed in unbuffered 1% (w/v) OsO_4 for 1/2 h. If the tissue was to be tested cytochemically, incubations were done before post-fixation.

The cytochemical procedure used was that of FREDERICK et al.[6], developed by NOVIKOFF and GOLDFISCHER[11]. Incubations were carried out in covered vials for 1.5 h at 37°C. The standard incubation medium contained 10 mg DAB (Sigma Chemical Co., St. Louis, Mo.) 5 ml of 0.05 *M* 2-amino-2-methyl-1, 3-propanediol buffer pH 10.0 (Sigma Chemical Co.), and 0.1 ml of 3% (v/v) H_2O_2. Before addition of tissue, the pH of the incubation medium was adjusted to 9.0. Five controls in addition to incubation in complete medium were performed. The controls listed were similar to those of FREDERICK et al.[6]: incubation in propanediol buffer; preincubation of tissue segments in propanediol buffer containing 0.01 *M* KCN for 20 min followed by incubation in standard medium containing 0.01 *M* KCN; anaerobic incubation in standard medium; preincubation of tissue segments in 0.02 *M* 3-amino-1, 2, 4-triazole (Aldrich Chemical Co. Inc., Milwaukee, Wis.) for 20 min followed by incubation in standard medium containing 0.02 *M* amino-triazole; and incubation of tissue segments in standard medium minus H_2O_2.

All tissue segments were rinsed with 0.01 *M* potassium phosphate buffer (pH 6.8) prior to post-fixation. After post-fixation, the tissue was dehydrated in an acetone series, stained for 5 h in 70% (v/v) acetone containing 1% (w/v) uranyl nitrate.

After acetone dehydration shrinkage of the plasma membrane was observed. This damage was minimized by using the freeze-substitution method of MEPHAM and LANE[12]. Glutaraldehyde, OsO_4-fixed tissue segments in foil containers were plunged into a mixture of methylcyclohexane: isopentane (2:23, v/v) in a liquid N_2 bath. After freezing, the containers were transferred to vials, containing pre-cooled methanol, in a methanol/dry ice bath. The vials were stoppered and maintained in the bath for 30 days with 10 changes of precooled methanol after which, the tissue was stained for 1 day in 1% (w/v) uranyl nitrate in methanol. The vials were then slowly brought to room temperature, transferred to ethanol/acetone (1:1, v/v), through 2 changes of acetone, and embedded.

Light gold sections were prepared using a Dupont diamond knife on a Reichert Om U2 ultra-microtome, mounted on 200 mesh grids and stained with aqueous lead citrate. The grids were examined with a Phillips EM 200 at 60 KV.

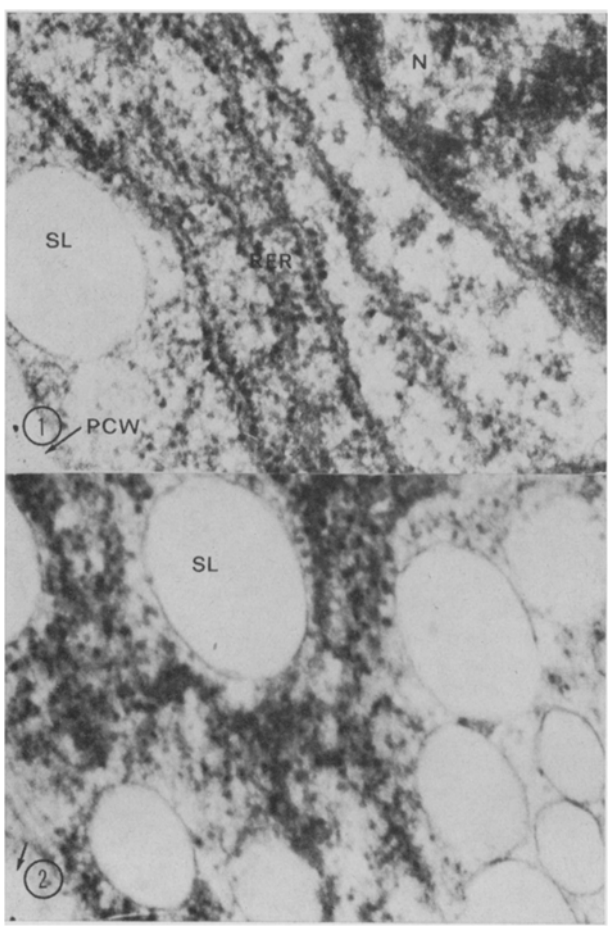

Fig. 1. Cotyledon tissue not incubated with DAB. Dehydration of tissues by freeze-substitution give excellent preservation of rough endoplasmic reticulum (RER), nucleus (N) and primary cell wall (PCW→). Note spherosome-like bodies (SL). ×51,840.

Fig. 2. Spherosomes of cotyledon tissue not incubated with DAB. Note abundance of spherosome-like bodies (SL) in the peripheral area of the cell. ×51,840.

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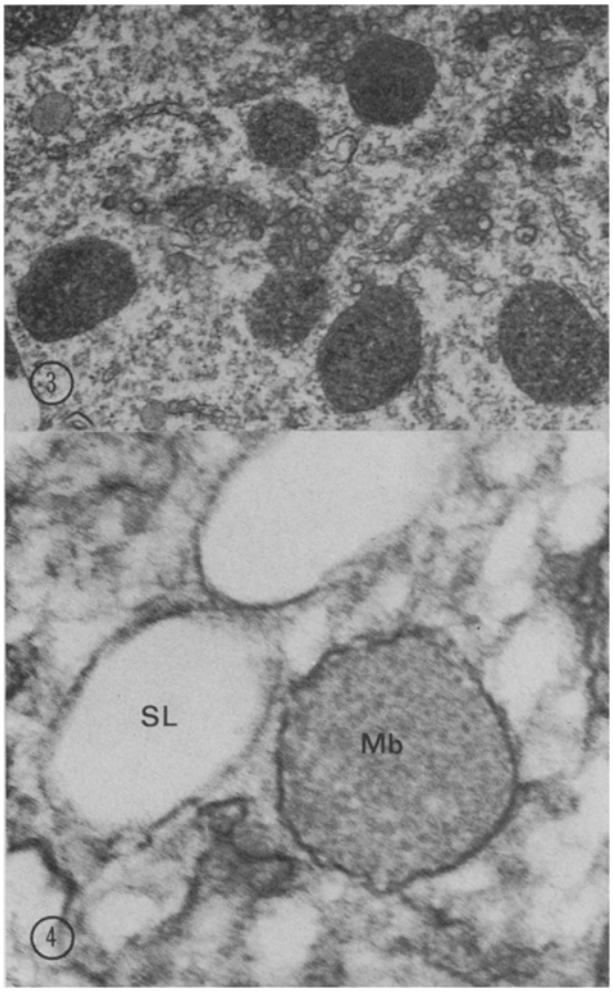

Fig. 3. Incubation of the cotyledon tissue in the DAB staining system illustrates that deposition of reaction product is intense within single membrane microbodies (Mb). ×23,045.

Fig. 4. This illustration of an unstained microbody (Mb) is typical of tissue incubated in propanedial buffer only, for the duration of the experimental period. Note spherosome-like bodies (SL). ×36,175.

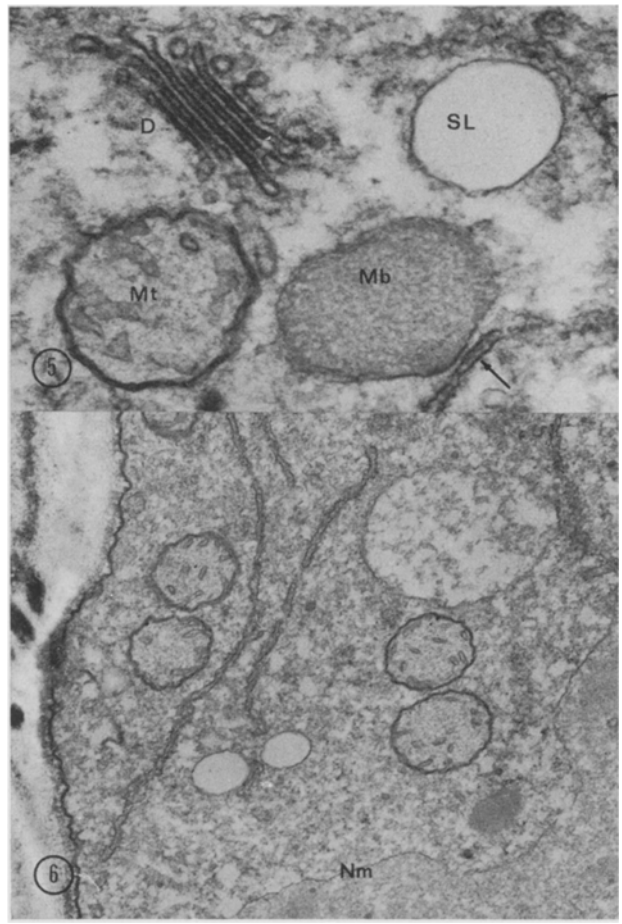

Fig. 5. Section of cotyledonary tissue treated with 0.02*M* amino-triazole as a specific inhibitor for catalase. The 4 structures shown are: a microbody in which staining was completely inhibited by 0.02*M* amino-triazole (Mb), spherosome-like body (SL), a mitochondrion (Mt), and a dictyosome (D). Note well defined limiting membrane of the microbody (Mb). Extension of endoplasmic reticulum can also be seen (→). ×28,672.

Fig. 6. Dehydration by freeze-substitution yields clear images of membranes as shown by mitochondrial and nuclear membranes (Nm) of pea cotyledon tissue not incubated in the experimental DAB medium. ×13,824.

Results and discussion. The cotyledon tissue examined was found to contain single-membrane microbodies similar to those reported by FREDERICK et al.[6,13] and numerous spherosome-like bodies. The latter were distributed throughout the cell, though frequently more numerous in peripheral areas as illustrated in Figures 1 and 2.

The intracellular localization of catalase within single-membrane microbodies of the cotyledon is substantiated by observations presented in Figures 4–6. As illustrated in Figure 5 prior incubation with 0.02*M* amino-triazole inhibits catalase activity, thrrdeby inhibiting DAB staining of the microbody. The observed browning reaction of the tissue was inhibited by cyanide but not by amino-triazole. Crystalling inclusions were absent from the microbody matrix. The location of catalase-containing microbodies appeared to be primarily peripheral and frequently in close proximity to the RER.

Ultrastructural studies of senescing cotyledons have led BAIN and MERCER[14] to correlate ultrastructural degeneration with the senescence of the cotyledon. Biochemically, it has been shown that reduced pteroylglutamate derivatives in pea cotyledons reach a peak value 3–4 days after germination[15,16] and consequently metabolism of C-1 fragments is maximal during this period. By 6–7 days, cotyledon senescence, exemplified by fungal and bacterial decomposition of cotyledon cells, is well established. In lieu of complementary biochemical and ultrastructural information and with the ubiquitous occurrence of microbodies, it would seem feasible to predict an age functional difference within microbodies of the cotyledon that could parallel the general observations on the senescence cycle in pea cotyledons. If catalase alone is accepted

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as the histochemical marker distinguishing this organelle or microbody such differences should be interpreted with a cautionary note[17].

Zusammenfassung. Mikrokörperchen mit Einfachmembranen wurden bei 4 Tage alten Kotyledonen von *Pisum sativum* studiert und für einen cytochemischen Katalase-Test elektronenmikroskopisch verwendet. Das granuläre Reaktionsprodukt aus dem 3, 3-diamino-benzidin Test für Katalase wurde ausschliesslich in den Einfachmembranen der Mikrokörperchen gefunden.

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Possibility of the Use of Some Physical-Chemical Agents for the Differentiation of Phages *Pseudomonas aeruginosa*

Some phages with a wide spectrum of effectiveness cannot be differentiated sufficiently by means of known criteria (spectrum of phages effectiveness, electron microscopy, serological group etc.) used at present[1–5]. In our former experiments we utilized the inactivation effect of UV-and X-ray radiation, acridine orange (AO) in darkness, photodynamic inactivation of AO, and hydroxylamine for the differentiation of a group of polyvalent staphylococcal phages, and we succeeded in the differentiation of some polyvalent phages by means of various inactivation effects of these physical-chemical agents[6–8]. In this study we tried to use these new tests for the differentiation of phages *Pseudomonas aeruginosa*.

Material and methods. Bacteriophages *Pseudomonas aeruginosa* P57[3], Pb and 7S were multiplicated on host bacteria PS 1 (phages P57[3]) and PS 2 (phages Pb and 7S). Bacteria were cultivated on the tryptone-yeast extract medium (15 g tryptone Oxoid, 7 g NaCl, 2 g yeast extract Oxoid; for solid media 15 g of agar per 1000 ml of liquid medium were used). When plating the phages, the method of the double-layer agar was used[4].

Irradiation of phages with UV-light was carried out in phosphate buffer. For irradiation a 15 W germicide fluorescent lamp Philips was used. The dose-rate was 20 ergs/sec/mm^2. At selected time intervals, irradiated phages were plated on Petri dishes together with the corresponding bacterial host. For X-ray irradiation the Chaul apparatus, Machlett's tube with the exposition of 8,200 rads/min was used. After irradiation the samples were plated together with bacteria on Petri dishes.

Photodynamic inactivation. The phage lysate was diluted 10 times with phosphate buffer (PH 7.0) and

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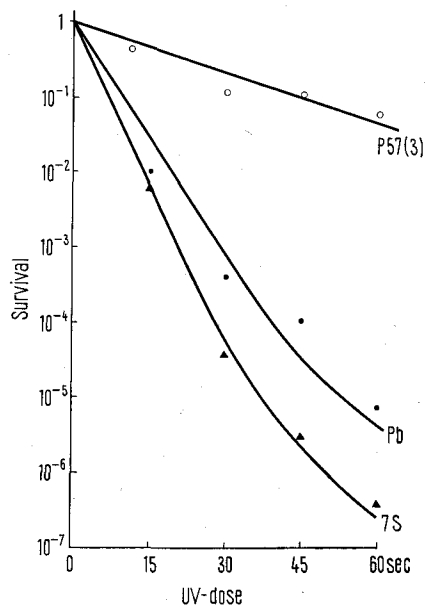

Fig. 1. Inactivation effect of UV-radiation on phages *Pseudomonas aeruginosa* P57[3], Pb and 7S.

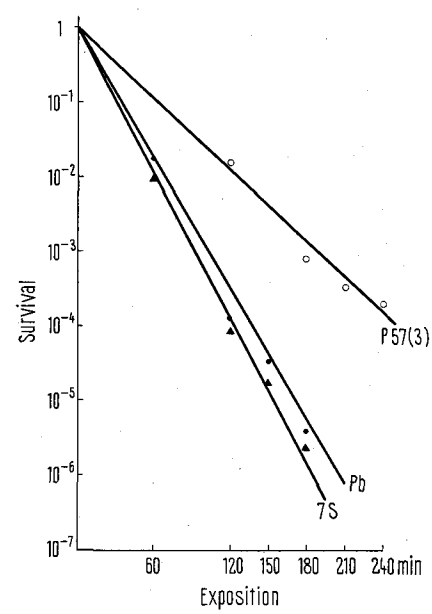

Fig. 2. Inactivation effect of X-ray radiation on phages *Pseudomonas aeruginosa* P57[3], Pb and 7S.