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Redistribution of Phosphate Deposits in the Alga *Scenedesmus quadricauda* Deprived of Exogenous Phosphate—an Ultra-Cytochemical Study

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Abbreviations: ADP = adenosine diphosphate, ATP = adenosine triphosphate, ATPase = adenosine triphosphatase, EDAX = energy dispersive analysis of X-rays, Pi = orthophosphate, PPi = pyrophosphate, PP = polyphosphate, PhAR = photosynthetic active radiation, TCA = trichloroacetic acid.

Summary

The green alga *Scenedesmus quadricauda* (Turp.) Bréb. was cultivated in the presence or absence of orthophosphate and synchronized daughter or mother cells were cytochemically stained. For *in situ* capturing of water soluble phosphates Ca^{2+} and Mg^{2+} ions were added to the ice-cold glutaraldehyde fixative to form a polymeric metal-phosphate complex which was equivalent to the energy-rich condensed polyphosphates in staining by alkaline lead acetate. The X-ray microanalysis of the extensive stained deposits proved the presence of phosphorus. In orthophosphate-supplied daughter cells cytoplasmic vacuoles contained round stained bodies; a layer of phosphate-containing paracrystals encompassing some starch grains and a fine stained layer delineating the chloroplast envelope were also observed. In the equivalent mother cells only the material inside the *loculi* of stacked thylakoids was stained. In orthophosphate starved daughter cells filamentous phosphate-containing paracrystals filled extensive cytoplasmic vacuoles. A stained layer covered the chloroplast envelope and continuous stained layers appeared inside the *loculi* of stacked thylakoids. Mother cells that develop from these daughter cells were filled with starch grains and showed only peripheral stained deposits. The results are compared with the biochemical evidence of phosphate turnover in algal cells.

Keywords: Scenedesmus quadricauda; Phosphate deposits; Electron cytochemistry; EDAX.

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1. Introduction

It is well known that polyphosphates (PP) are stored in green and blue green algae and serve as an osmotically inert intracellular source of phosphorus (see reviews by Harold 1966, Stewart *et al.* 1978). In algae PP accumulate during periods of retarded growth, *e.g.*, in the dark period (Sundberg and Nilshammar-Holmvall 1975) and they were reported to be present in the form of spherical bodies containing also RNA, lipids, proteins and divalent cations (Widra 1959, Roomans 1980), these bodies observed in vacuoles and on the surface of starch grains inside chloroplasts (Atkinson *et al.* 1974, Jensen and Sicko 1974, Sundberg and Nilshammar-Holmvall 1975, Elgavish and Elgavish 1980). However, X-ray microanalysis (EDAX) of *Scenedesmus* cells cultivated under a limited supply of orthophosphate (Pi) showed that the abundace of morphologically observable "polyphosphate" granules can no longer be taken as an index of mobilizable phosphorus reserves (Tillberg *et al.* 1979). Because conventional electronograms and biochemical analyses provide no information on minor phosphate deposits, we have employed the ultra-cytochemical staining developed for plants by Poux (1965). In principle two classes of phosphate containing deposits can be discriminated by the method. One is insoluble during the glutaraldehyde and osmium fixations and the appropriate incubation, washing and dehydration steps (*cf.*, Brierley and Slautterback 1964, Colquhoun and Rieder 1980). This class includes the energy-rich condensed PP (meta-

phosphate, linear polymers and imidophosphate polymers—DAWES and SENIOR 1973) which may be naturally linked by coordination bonds to metal ions (*cf.*, ROOMANS 1980). The second class can be localized only after the addition of Ca^{2+} and Mg^{2+} ions to the alkaline glutaraldehyde fixative. Theoretically, these divalent cations capture *in situ* the water soluble form (sodium or potassium salts) of condensed PP starting from the pyrophosphate (PPi) or can even precipitate free Pi as a stable polymeric metal complex (KULAEV 1975). The stain was lead phosphate formed essentially by the exchange of lead for the divalent cations bound to cellular phosphates. When the procedure was performed at 0 °C, the action of cellular polyphosphatases was eliminated (VOŘÍŠEK and SCHWENCKE 1984). We report the results of the cytochemical staining of synchronized cultures of *Scenedesmus quadricauda* and of EDAX analysis of sufficiently voluminous stained deposits (*cf.*, NILSHAMMAR and WALES 1974).

2. Material and Methods

2.1. Strain and Cultivation Conditions

Synchronous populations of *Scenedesmus quadricauda* (Turp.) Bréb. strain Greifswald/15 were grown in a chemostat in flat cultivation vessels with plane-parallel glass walls (volume 1,200 ml, distance of the glass walls 18 mm) submerged in a water bath at 30 °C and illuminated from one side by incandescent lamps (500 W, Tesla). The irradiance at the surface of the cultivation cuvettes was 140–180 $W \cdot m^{-2}$ PhAR (photosynthetic active radiation). Different values of the mean irradiance of one cell were achieved by varying the culture density. The latter was controlled by adjusting an appropriate dilution rate; the dilution rate was 0.10 $hour^{-1}$ and the culture density expressed as O.D. 750 = 0.4 (Co = 1.5×10^6 cells per ml). The cultures were synchronized by alternating light and dark periods (14 : 10 hours).

2.2. Medium and Sampling

The components of the mineral medium were (in mg per l) KNO_3—4,042, KH_2PO_4—340, $MgSO_4$—988, EDTA Fe/Na—18, $CaCl_2 \cdot 6H_2O$—10.96, H_3BO_3—3.09, $MnSO_4 \cdot 4H_2O$—1.18, $CoSO_4 \cdot 7H_2O$—1.4, $CuSO_4 \cdot 5H_2O$—1.24, $ZnSO_4 \cdot 7H_2O$—1.43, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$—0.88. The first samples from phosphate starved and control cultures were harvested after the releasing of autospores, *i.e.*, after 14 hours in the light plus 10 hours in the dark (daughter cells). The next sampling followed after 14 hours in the light (mother cells).

2.3. Phosphate Analysis

Free phosphates were quantified in trichlor acetic acid axtracts (5%) of algal cells and the values were compared with those obtained for total polyphosphates in hot 0.5 N $HClO_4$ extracts (nucleotides adsorbed on charcoal). The amount of phosphates was measured by a direct colorimetric method (phosphomolybdate-tartarate) in the modification of SIGLER and KOTYK (1976).

2.4. Cytochemistry

The procedure was described in detail previously (POUX 1965, VOŘÍŠEK *et al.* 1982). Rapidly chilled algal culture was harvested by centrifugation (100 × g) and fixed in ice-cold glutaraldehyde (MERCK) buffered by tris-HCl to pH 7.9 and supplied alternatively with 100 mM $MgCl_2$ and 100 mM $CaCl_2$. The fixed cells were stained by lead acetate buffered to pH 8.9. After staining the cells were postfixed overnight in ice-cold 1% OsO_4 in 50 mM cacodylate, pH 7.2, dehydrated in the alcohol series and embedded in Vestopal W. Ultrathin sections were viewed under a JEOL JEM 100 B microscope (60 kV) with or without poststaining (REYNOLDS 1963).

2.5. EDAX Analysis

Energy dispersive analysis of X-rays was performed with Tesla 613 transmission EM combined with Si(Li) detector from ÚJV Řež (Prague) (take-off angle 45°, resolving capacity 35 a J, Be window 25 nm thick) and an NTA 1024 multichannel analyzer from EMG (Hungary). Calibration was on Al foil, the primary energy was 77.8 fJ; selected areas on ultrathin sections (100 nm thick) were irradiated for 100–500 s; diameter of the analyzed area was 0.5–5 μm.

3. Results

3.1. Phosphorus Content in Algal Cells

The values of free phosphate and polyphosphate content in daughter and mother cells cultivated in the control or phosphate-free medium are summarized in Table 1. It is obvious that both free phosphate and polyphosphate levels were higher in control cells. In phosphate starved cells the levels of total phosphorus decreased during the first cell cycle to a residual constant value. In both cultures the decrease after the cell division reflects the dilution of phosphate into daughter cells.

3.2. Cytochemical Staining of Control Culture Supplied with Orthophosphate

The cells under study were harvested at the beginning of the cell cycle, *i.e.*, at the end of the dark period (daughter cells). In the cytoplasmic compartment small round stained bodies with a diameter up to 300 nm were found inside vacuoles adhering to the tonoplast (Fig. 1). They varied in number and size. The most conspicuous substructures inside some of vacuoles were aggregates of stained paracrystals (Fig. 2), which were sometimes present simultaneously with the above described bodies. Paracrystals were composed of stained subunits arranged in three-dimensional filaments. One or both membranes of the chloroplast envelope were delineated by a fine discontinuous layer of stained material (Fig. 1). Small round stained bodies adhered to this boundary in the cytoplasm. The stained paracrystals appeared also inside chloroplasts. They

Table 1. *Values of free phosphate and polyphosphate content in daughter and mother cell from two subsequent cells cycles in the control or phosphate-free medium*

	Free inorganic phosphate [1]		Total polyphosphate [2]		Total phosphate	
	—P	Control	—P	Control	—P	Control
Cultivation medium						
the 1st cell cycle						
Daughter cells	1.36 [3]	1.36	1.70 [3]	1.70	3.06 [3]	3.06
Mother cells	0.49	1.90	1.56	6.80	2.05	8.70
Synchronous cell division in the dark [4]						
the 2nd cell cycle						
Daughter cells [5]	0.12	0.29	0.44	1.46	0.56	1.75
Mother cells [5]	0.08	1.03	0.43	3.28	0.51	4.31

[1] Pellets extracted by cold 5% TCA.
[2] Residual pellets extracted by hot 0.5 N $HClO_4$ (nucleotides from extracts adsorbed on charcoal).
[3] PO_4^{3-} pg. cell^{-1}.
[4] Mother cells divided in 8 autospores in control medium and in 4 autospores in phosphorus deficient medium.
[5] Principal sampling for cytochemical studies.

were clustered on the rim of some starch grains or in the chloroplast matrix (Figs. 1 and 3). Photosynthetic membranes of thylakoids were neither stained nor poststained by the described procedure and were hardly visible.

When divalent cations were omitted in the fixative, the stained paracrystals were still found on the rim of starch grains. In vacuoles extensive deposits of stained amorphous material were found instead of paracrystals.

The control cells harvested after another 14 hours in the light (mother cells) contained numerous large starch grains in extensive chloroplasts and no cytoplasmic vacuoles. The above-mentioned stained deposits were not present but more or less continuous layers of stained material appeared in chloroplasts, obviously delineating membranes of stacked thylakoids (Fig. 4). From their average distance (14 nm) and thickness (4 nm) we concluded that the stained material filled the loculi of thylakoid sacs. The chilling of the cells throughout the whole cytochemical procedure was essential for the staining which was otherwise not influenced by the absence of divalent cations in the fixative.

3.3. Cytochemical Staining of Orthophosphate-Starved Culture

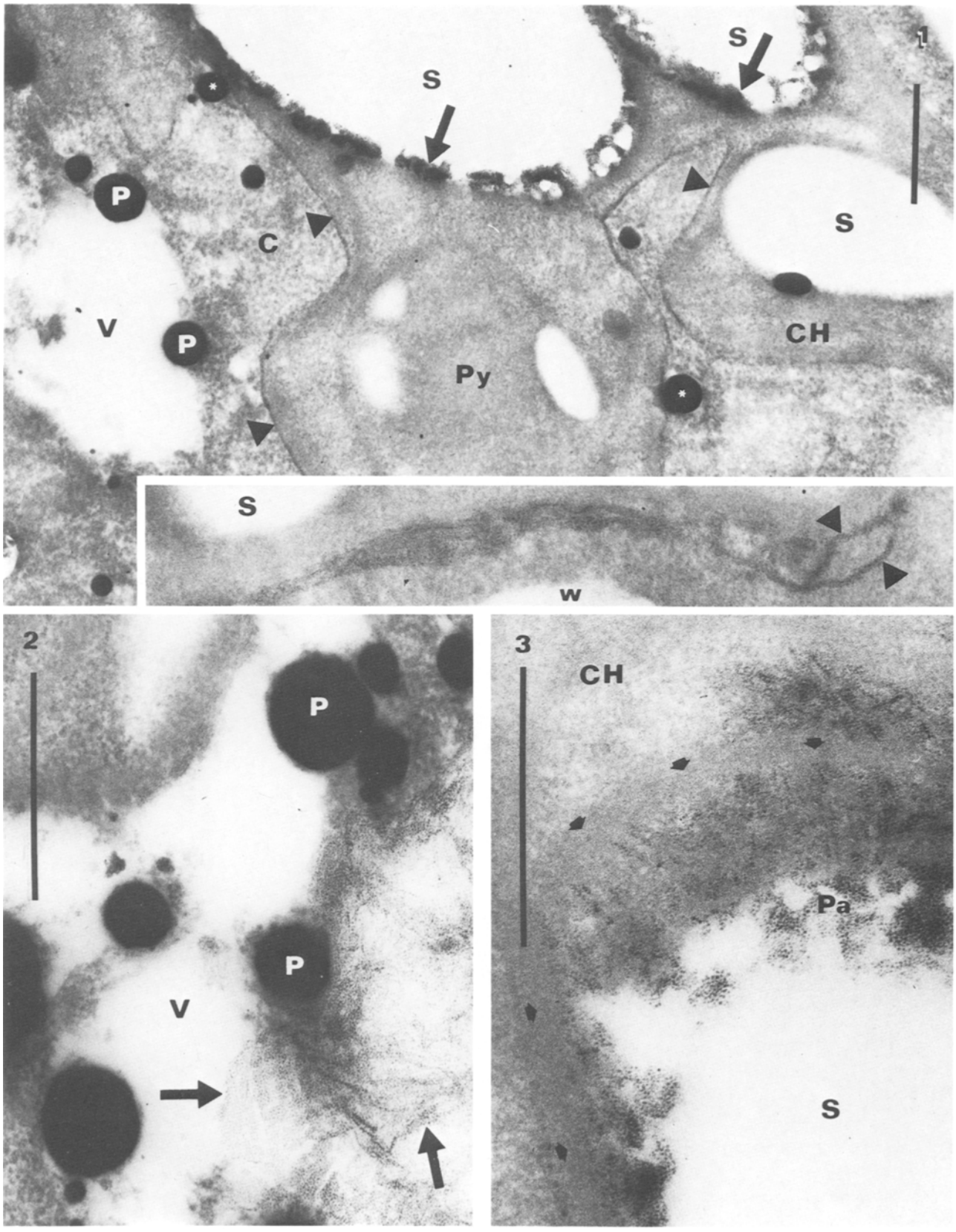
The synchronized cells were cultivated for 24 hours in the medium without orthophosphate and were also harvested at the end of the dark period (daughter cells). When compared with the control culture extensive vacuoles more or less filled with filamentous paracrystals of stained material appeared in the cells

Fig. 1. Stained deposits in daughter cell from control culture (autospore from the end of the dark period). Beside to stained granules in vacuoles the phosphate deposits are layered on the rim of starch grains (arrows). Chloroplast envelope is delineated by a fine stained boundary (arrowheads) with adhering phosphate granules (*). × 42,000. Insert shows staining of both membranes of chloroplast envelope. × 120,000

Abbreviations Figs. 1–9: *c* cytoplasm, *CH* chloroplast matrix, *L* liposomes, *M* mitochondria, *N* nucleus, *P* phosphate granule, *Pa* phosphate paracrystals, *Py* pyrenoid, *S* starch, *T* thylakoids, *V* vacuoles, *w* cell wall. The bar marker indicates 0.5 μm if not indicated otherwise

Fig. 2. Stained deposits in vacuoles of daughter cell from control culture. Both phosphate granules and phosphate paracrystals (arrows) are present. × 81,000

Fig. 3. Stained paracrystals in chloroplasts matrix and on the rim of starch grain of daughter cell from control culture; arrows indicate the thylakoid. No poststaining. The bar marker indicates 0.25 μm. × 200,000



Figs. 1–3

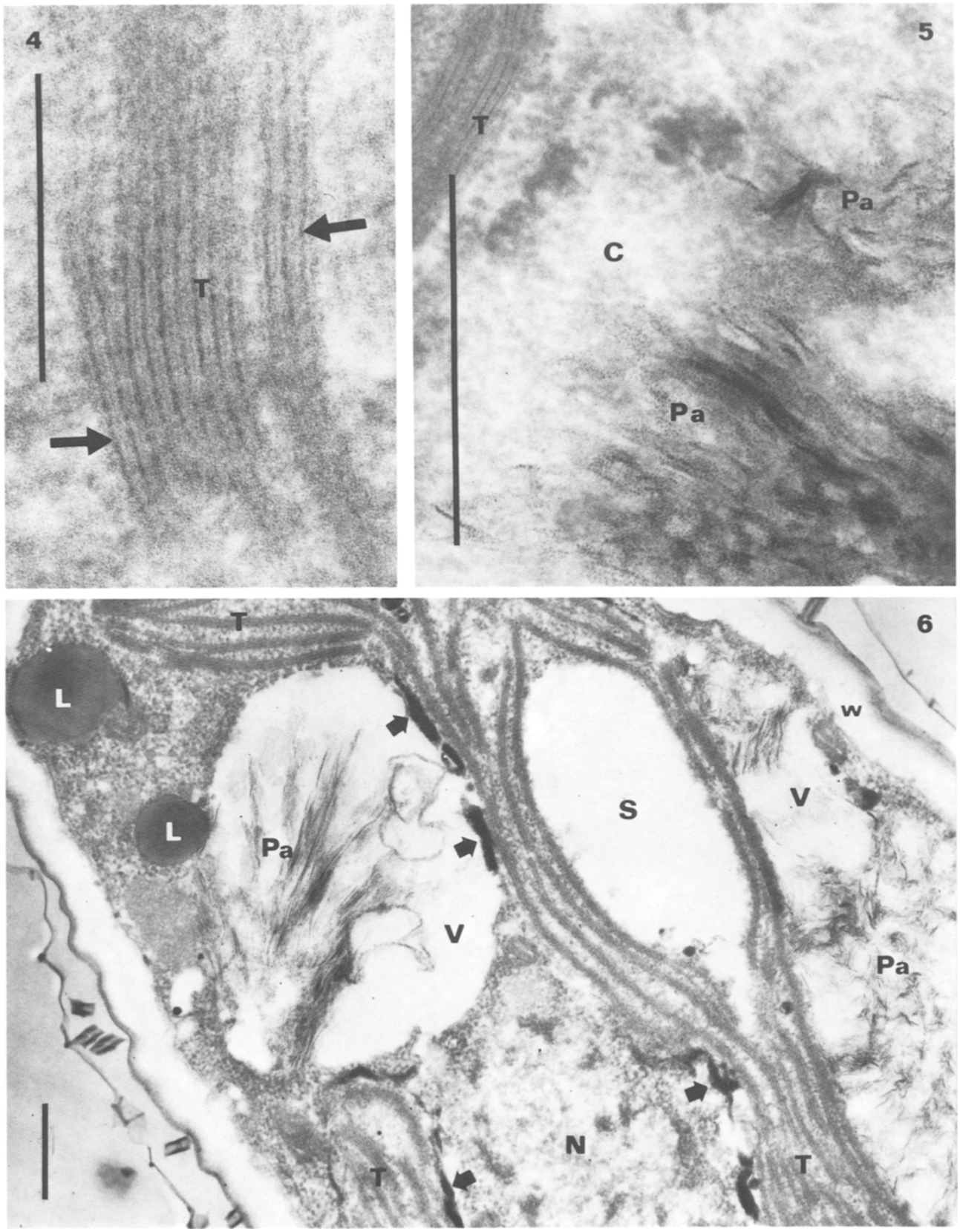


Fig. 4. Stained deposits (arrows) in the *loculi* of stacked thylakoids in mother cell from control culture. No poststaining. The bar marker indicates 0.25 μm. × 220,000

Fig. 5. Phosphate paracrystals in the cytoplasm of phosphate starved daughter cell. Notice the stained deposits in thylakoids. No poststaining. × 132,000

Fig. 6. Stained deposits in daughter cell from phosphate starved culture containing synchronized autospores from the end of dark period. Arrows indicate the stained deposits on chloroplast envelope. Stained deposits are distinct also in thylakoids prominent here. Phosphate paracrystals fill the extensive vacuoles. × 32,000

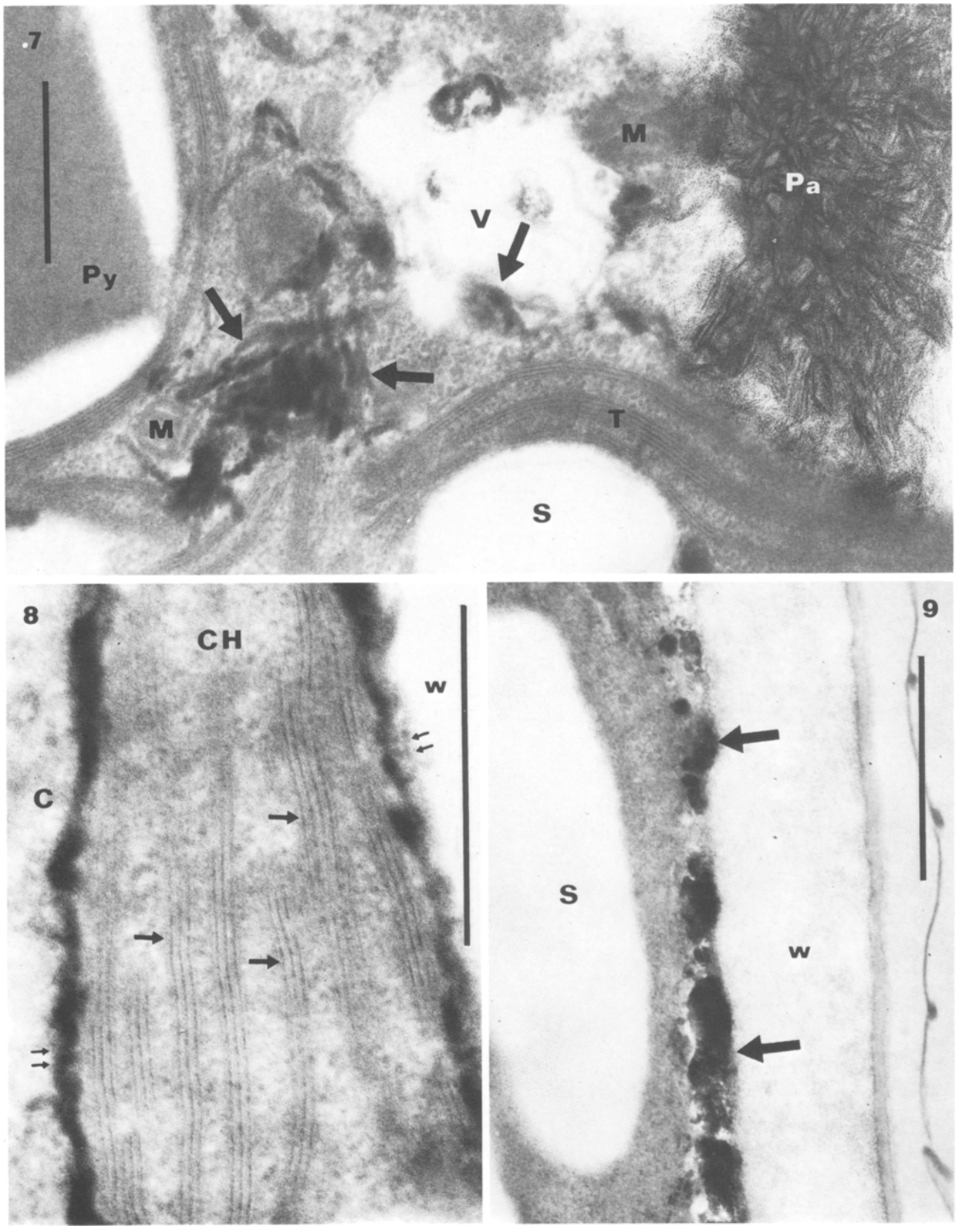


Fig. 7. Ribbon-like stained deposits (arrows) in daughter cell from phosphate starved culture. Notice the stained deposits in thylakoids. Extensive vacuole is filled with phosphate paracrystals. No poststaining. × 64,000

Fig. 8. Transversal section of stained deposits in thylakoid *loculi* (arrows) in daughter cell from phosphate starved culture. Average periodicity was 14 nm. Chloroplast envelope is delineated by a stained layer up to 40 nm thick (double-arrows). No poststaining. × 122,000

Fig. 9. Stained deposits (arrows) on the periphery of phosphate-starved daughter cell cultivated for another 14 hours without phosphate in the light. × 78,000

(Fig. 6). As a rule such vacuoles adhered to the chloroplast envelope and often substituted the cytoplasmic layer between the chloroplast and the cell wall. The stained filamentous paracrystals appeared also in the cytoplasmic matrix (Fig. 5). The vacuoles or the cytoplasm contained also ribbon-like stained deposits equivalent to those delineating the chloroplast envelope (Fig. 7). The chloroplasts themselves were delineated by a discontinuous layer of stained material up to 50 nm thick (Figs. 6 and 8). The stained paracrystals on the surface of starch grains were not observed. A distinct layer of stained material appeared in the loculi of stacked thylakoids (Figs. 5–8).

When phosphate-starved cells were further cultivated without phosphate for 14 hours in the light (mother cells), stained granules were observed on the cell periphery (Fig. 9). The cytoplasm became homogeneous and the stained paracrystals disappeared from vacuoles. The stained deposits in thylakoids were still present. Generally, the cells contained so many starch granules that these represented about one half of the cell volume. Such cells were further cultivated for another 10 hours in the dark, in the presence or absence of orthophosphate; the stained paracrystals in some vacuoles reappeared and the deposits in thylakoids were distinct in phosphate starved cells. The peripheral stained layer was observed in phosphate supplied cells.

3.4. X-Ray Energy Dispersive Analysis

The packed paracrystals in the vacuoles and cytoplasm of phosphate-starved cells and the paracrystals encompassing starch grains in a control culture were analysed. Sections of whole cells without extensive stained deposits were used as a reference. Fig. 10 shows the typical emission spectrum of the paracrystalline mass inside a vacuole of a phosphate-starved cell. The peaks indicating the presence of P, Pb and Cu in the sample are specified. While Cu was present in the material of the support grid, the electron-opacity of analysed parts of sections was obviously due to precipitated lead. The volume of stained deposits on the cell periphery, inside thylakoids or on chloroplast envelopes was below the resolution power of the apparatus. The spectrum of stained paracrystals delineating starch grains in control cells was identical with the spectrum of vacuolar deposits.

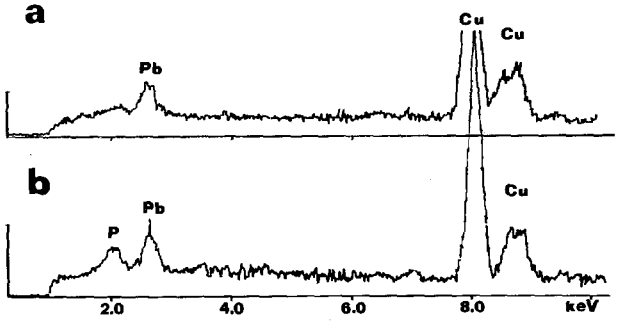


Fig. 10. X-ray microanalysis emission spectra. *a* Phosphate starved daughter cell without extensive stained deposits. Ultrathin section (Vestopal W) was 100 nm thick and the selected area covering the whole cell (diameter 5 μm) was irradiated for 200 seconds. Vertical scale 100 counts. *b* Stained paracrystals in the vacuole of phosphate starved daughter cell. Diameter of the selected area was 0.5 μm

4. Discussion

A recent study in this Institute (ZACHLEDER, unpublished) has revealed that *S. quadricauda* cells contained sufficient endogeneous pool of phosphorus for at least one cell cycle in the absence of external Pi. During this cell cycle both protein and starch synthesis were unchanged. In the second half of the cell cycle the RNA synthesis was stopped and, consequently, also the following number of DNA replications and the number of daughter cells were restricted. During a second cell cycle under phosphate limitation no RNA or DNA synthesis was found but the synthesis of starch (calculated per cell) continued at nearly the rate found in the control culture. The rate of protein synthesis was reduced. This disturbance of the metabolism in *S. quadricauda,* which was finally manifested in the lethal hypertrophy of starch deposits, was soon reflected in the described changes of distribution of phosphorus-containing deposits.

For an evaluation of our cytochemical findings it is necessary to point out that only an extremely high rate of synthesis of an intermediate or an impairment of its turnover, *e.g.*, in aged cells, facilitate its accumulation over a threshold concentration which is necessary for a cytochemical staining (VOŘÍŠEK and POKORNÝ 1975). In the control culture the first event may occur in photosynthesizing mother cells. The layers of stained material, obviously featuring a phosphorylated compound, appeared inside stacked thylakoids and can be plausibly compared with the material observed by NICOLSON (1971) in *loculi* of chloroplasts fixed by cold glutaraldehyde and embedded in protein (*cf.*, TOKUYASHU 1974). This material is certainly dissolved during the classical processing of cells for electron microscopy either in fixation by pure glutaraldehyde (BRIERLY and SLAUTTERBACK 1964) and/or in the subsequent dehydration by ethanol series (COLQUHOUN and RIEDER 1980). Therefore it was not considered until

now (*cf.*, HAGGARD and REGAN 1977) and we would like to assume that it represents a phosphorylated intermediate(s) of photosynthesis (discussed below).
In control cultures the changes of phosphate turnover and subsequent redistribution of phosphate deposits can be expected at the end of the dark period (daughter cells) when starch is not synthesized (for review see WALKER 1976). Thus we assumed that the layer of stained paracrystals around some starch granules does not reflect the enhanced synthesis of PPi from ATP by the ADP-glucose pyrophosphorylase (EC 2.7.7.27) in the first reaction of starch synthesis, but rather a low PPi turnover in non-photosynthesizing control chloroplasts (*cf.*, SUNDBERG and NILSHAMMAR-HOLMVALL 1975). The clouds of paracrystals without any starch core in the matrix of chloroplasts may represent the onset of starch deposition between thylakoids and perhaps also illustrate the primary sites of hexose polymerization.

Under phosphate limitation a second type of metabolic disturbance can be deduced from the known biochemical findings. It was postulated previously (CHEN-SHE *et al.* 1975) that Pi sequestration on the exterior of isolated chloroplasts suppressed the phosphate translocator in the chloroplast inner membrane which counter-exchanges cytoplasmic Pi for triose phosphates synthesized in the Benson-Calvin cycle in the chloroplast matrix. Because chloroplasts *in vivo* are also Pi consuming organelles (for review see HEBER 1974, WALKER 1976) we assumed that in *S. quadricauda* cultivated for one cell cycle without external Pi the phosphate pool was preferentially and rapidly transformed into metabolically inert PPi and PP and deposited in vacuoles. The lack of free Pi in the cytoplasm suppressed the function of the phosphate translocator (this could give rise to the thick layer of stained material on chloroplast envelope) and the triose-phosphate produced in chloroplasts was retained in the organelle. This might result in retention of stained material in thylakoids and also in a subsequent accumulation of enormous starch deposits in phosphate-starved and illuminated (mother) cells during which also the phosphorus deposited in the vacuoles gradually disappeared (*cf.*, Table 1).

In algae fixed and dehydrated routinely at room temperature only the extensive stained bodies are being currently found. This was certainly due to the above-mentioned leakage of phosphates caused by fixation and dehydration. Which of the deposits of condensed phosphates are attacked by polyphosphatases remains to be investigated (*cf.*, VOŘÍŠEK and SCHWENCKE 1984).

Our variations of the cytochemical procedure showed that the presence of divalent cations in the fixative was not essential for the staining of paracrystals around starch grains; these were most probably composed of insoluble condensed phosphates. In vacuoles the stained paracrystals were observed only in the presence of divalent cations in the cold fixative, alternatively with clouds of fine stained grains formed in pure cold glutaraldehyde. However, the X-ray microanalysis did not show a substantial content of Ca^{2+} in paracrystals (*cf.*, DAVIS *et al.* 1982). Because the precipitation of proteins by PP at acid pH is a well known phenomenon (VANDEGRIFT and EVANS 1981) and because the cold glutaraldehyde fixation is generally more favorable for conserving proteinaceous cellular entitites (LANGENBERG 1979) we assumed that paracrystals resulted from steric interactions of PP, proteins and divalent cations. The described presence of paracrystalline mass containing phosphates inside vacuoles of phosphate limited cells versus the preferential presence of round PP bodies or both ultrastructural forms in vacuoles of control cells may imply that under normal Pi supply the paracrystals or their amorphous equivalents were transformed into large bodies by as yet unknown steric interactions (*cf.*, TILLBERG *et al.* 1979).

The stained paracrystals observed in the cytoplasmic matrix of daughter phosphate-starved cells probably indicate the deposits of Pi and PPi formed during sucrose synthesis (*cf.*, WALKER 1976) and trapped by divalent cations.
The high resolution capacity of the described staining procedure and negligible diffusion artefacts (VOŘÍŠEK *et al.* 1982) indicate a low incidence of artefactual staining. The EDAX analysis confirmed that the stained paracrystals in vacuoles and in the rim of starch grains contained phosphate and lead (*cf.*, VAN STEVENICK 1979).

As concerns the non-voluminous stained deposits which appeared inside the stacked thylakoids we can only estimate their composition. A manifestation of reaction product of thylakoid ATPase, which may also produce Pi (FIRTH 1978) can be excluded in our experiments because our staining at 0 °C shows only the lead-philic compounds present at the moment of cell chilling and the subsequent rapid penetration of the fixative. The staining of ATP itself in conditions of unimpaired Benson-Calvin cycle is also hardly probable and it is necessary to emphasize that no free Pi was formed in the light reaction catalysed in photosynthetic thylakoid membranes (MILLER 1979).

Consequently, we concluded that the subtle stain deposits in thylakoid *loculi* represent most probably the phosphorylated precursors of starch; the translocation of such compounds through channel system of thylakoids to the relatively few sites of starch synthesis is quite probable.

The importance of Ca^{2+} for stacking of negatively charged thylakoids was also described (NILSHAMMAR-HOLMVAL 1976). Therefore the exchange of lead for the Ca^{2+} bound naturally to the surface of stacked thylakoids cannot be excluded as a reason for the faint staining there (Fig. 4). Our results might also illustrate the previously described light-driven phosphorylation of thylakoid proteins (BÉLIVEAU and BELLAMARE 1979a, b). However, the stain is distinctly located outside the thylakoid membrane leaflets. The importance of thylakoid stacks in the photosynthetic process was recently demonstrated by VAUGHN and DUKE (1981). They localized the photosystem II donor sites on the lumen of stacked thylakoids by ultra-cytochemical staining.

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