Protoplasma 125, 124—128 (1985)

PROTOPLASMA

Cytochemical Staining of a Yeast Polyphosphate Fraction, Localized Outside the Plasma Membrane

J. P. F. TIJSSEN and J. VAN STEVENINCK*

Department of Medical Biochemistry, Sylvius Laboratories, Leiden

W. C. DE BRUIJN

Centre for Analytical Electron Microscopy, Laboratory for Electron Microscopy, Leiden

Received June 12, 1984
Accepted August 4, 1984

Summary

Primary aldehyde fixation in the presence of Ca^{2+} and Mg^{2+} followed by alkaline Pb^{2+} staining leads to electron microscopical visualization of lead precipitates in the yeast *Kluyveromyces marxianus*. These lead precipitates are found in vacuoles, cytoplasm, and on the outside of the plasma membrane in the periplasmic and inner cell wall regions.

X-ray microanalysis shows that the precipitates contain high amounts of Pb and P. The amount of precipitated material appeared to correlate with the cellular polyphosphate content. When Ca^{2+} and Mg^{2+} are omitted from the primary fixative no peripheral Pb/P deposits are observed. In a subsequent washing step a small amount of long chain polyphosphate is liberated. It is concluded that this method leads to visualization of cellular polyphosphate, including a fraction localized outside the plasma membrane of *Kluyveromyces marxianus*.

Keywords: Polyphosphate; Volutin; yeast.

1. Introduction

Based on circumstantial evidence it has been postulated that part of the cellular polyphosphate in yeast is localized outside the plasma membrane and may play an essential role in transport-associated phosphorylation of glucose and glucose derivatives (VAN STEVENINCK and BOOIJ 1964). Several studies indicate the existence of a peripherally localized polyphosphate fraction in the yeast *Kluyveromyces marxianus* (TIJSSEN *et al.* 1981, 1982, 1983), as well as in other microorganisms (KULAEV 1975, OSTROVSKII 1980).

Polyphosphates are well documented to occur in (metachromatic) volutin granules in various microorganisms (WIDRA 1959, KUHL 1960, WILKINSON and DUGUID 1960). Electronmicroscopically these granules are observed as electron opaque deposits, containing the elements P, K, Ca and Mg, as determined by X-ray microanalysis (HUTCHINSON *et al.* 1977, BAXTER and JENSEN 1980, CRANG 1980). In eucariotic microorganisms like yeasts, these granules are found in the cytoplasm as well as in the vacuoles (WIDRA 1959, INDGE 1968a, b, c).

Cytochemical staining of polyphosphate has been described by JENSEN (1968) and by WOOL and HELD (1976), both employing 20% $Pb(NO_3)_2$ at pH 3.4. However, no peripheral polyphosphate fraction has been detected by these investigations.

A more promising approach was used by POUX (1963, 1965a, b), who succeeded in visualization of "soluble" plant phytate (a polyphosphorylated inositol) by fixation of cells in the presence of Ca^{2+} and Mg^{2+} at alkaline pH, followed by subsequent exchange of these divalent cations for lead. In the present study this approach was used to examine a possible extracellular localization of polyphosphate in the yeast *Kluyveromyces marxianus*.

* Correspondence and Reprints: Department of Medical Biochemistry, Sylvius Laboratories, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands.

2. Materials and Methods

Kluyveromyces marxianus (*Saccharomyces fragilis*) CBS 397 was cultured during 20 hours with glucose as carbon source, as described before (JASPERS and VAN STEVENINCK 1976). Phosphate starved yeast was obtained by omitting phosphate from the culture medium, inocculating 2 gram yeast per liter. Hypercompensated yeast was prepared essentially according to LISS and LANGEN (1962): 5% phosphate starved yeast was cultured for 90 minutes aerobically in standard medium, which was described before (JASPERS and VAN STEVENINCK 1976). The yeast was thoroughly washed three times in distilled water after harvesting.

Preparation for electron microscopy: Primary fixation was performed at 0 °C, during 2 hours. The fixative contained 1% glutaraldehyde, 4% formaldehyde, 0.2 M KCl, 50 mM $CaCl_2$ and 50 mM $MgCl_2$ in 0.1 M tris/maleate pH 6.0. After gently spinning down (± 1,000 × g) the cells were enrobed in a fibrin matrix according to VERMEER *et al.* (1978). Small (1 mm^3) cubes were cut from the fibrin clot, which were quickly washed in ice cold buffer containing 0.2 M KCl and 0.1 M tris/maleate pH 9.0.

Cytochemical staining was performed for 60 minutes at 0 °C in 1% lead acetate, 0.1 M tris/maleate buffer pH 9.0.

After quickly washing in 0.2 M KCl, 0.1 M tris/maleate buffer pH 8.5, postfixation was performed in 1% OsO_4 plus 50 mM $K_4Fe(CN)_6$ in the same buffer overnight, at 4 °C (DE BRUIJN 1973, DE BRUIJN and DEN BREEJEN 1975, 1976, DE BRUIJN and VAN BUITENEN 1980). Specimen were dehydrated in a graded series of aceton and embedded in Spurr. During microtomy the knife reservoir was refilled every 10 minutes with CO_2 free bidistilled water, adjusted to pH 8.0 with NH_4OH.

For acid exposure sections are floated on 0.25 N HCl for 1–5 minutes, only on one side. Subsequently the grid is tilted during drying according to TILLBERG *et al.* (1980).

Ultrathin sections were analysed in a Philips EM 400 analytical microscope, operating at 80 kV. X-ray dispersive microanalysis was performed with a Tracor/Northern 2000 system. The ultrathin sections were loaded in a low-background holder, perpendicular to the X-ray detector and tilted 24°. The precipitates were analysed for 100 seconds life time with a 100 nm ∅ spot size from a 150 nm ∅ condensor-2-aperture.

Extraction of orthophosphate and polyphosphate from intact yeast cells and quantitative analysis of these compounds was performed as described in detail previously (TIJSSEN *et al.* 1983).

3. Results

Primary fixation in the presence of Ca^{2+} and Mg^{2+} of the yeast *Kluyveromyces marxianus*, CBS 387, followed by cytochemical Pb staining and OsO_4 postfixation leads to the electron microscopical visualization of lead precipitates. Analysis of the supernatant after primary fixation revealed that all orthophosphate had leaked out from the fixed cells to the medium, whereas no polyphosphate could be detected in the supernatant. Model experiments, with orthophosphate and polyphosphate in amounts, comparable to those found in yeast, confirmed that orthophosphate remained soluble in the primary fixative, whereas polyphosphate (including pyrophosphate) was precipitated.

On inspection in the electron microscope precipitates were found in cytoplasm and in vacuoles (Figs. 1 *A* and *B*). Some of these intracellular precipitates exhibit a size and shape, typical for the so-called volutin granules, which are well documented to contain large amounts of polyphosphate (WIDRA 1959, KUHL 1960, WILKINSON and DUGUID 1960). Moreover deposits were found on the cell periphery, localized on the outside of the plasma membrane (Figs. 1 *A* and *C–E*).

X-ray analysis of these precipitates showed that they contain both the elements Pb and P in high amounts, compared with other cellular regions. From the obtained spectra the weight ratio Pb/P was calculated by the computer, taking into account the appropriate correction factors. From these calculations the Pb/P ratio appeared to vary between 0.7 and 1.0. In the volutin-like granules the elements Mg, Ca and K were found in addition (Fig. 2).

The cellular orthophosphate and polyphosphate levels in yeast under varying conditions are shown in Tab. 1. Electron microscopical studies of these batches revealed that the amount of lead precipitated material correlates closely to the polyphosphate content of these yeast cells. Acid exposure of ultrathin sections, with subsequent tilting of the grids as described in the method section, leads to dislocation of material from the deposits, in a manner previously described as characteristic for polyphosphate deposits (TILBERG *et al.* 1980). This phenomenon is also observed with peripheral precipitates.

When Ca^{2+} and Mg^{2+} are omitted from the primary fixative no peripheral lead deposits are observed, whereas the intracellular deposits are unaffected. It appeared that in the alkaline washing step subsequent to primary fixation a small amount of polyphosphate is released. About 2.9 µmol polyphosphate per gram yeast was recovered from the supernatant by bariumacetate precipitation. Separation according to chain length of this liberated polyphosphate fraction by Sephadex gelfiltration showed that the released polyphosphate fraction belongs to the high molecular weight polyphosphates contained in yeast (Fig. 3).

4. Discussion

Cytochemical staining of cellular polyphosphates by lead salts has been described before by JENSEN (1960) and by WOOL and HELD (1976). The present studies confirm these results. In the first place X-ray dispersive microanalysis showed that the precipitates contain lead and phosphorus. In spite of the limited spectral resolution of the X-ray analyser the P Kα-peak was

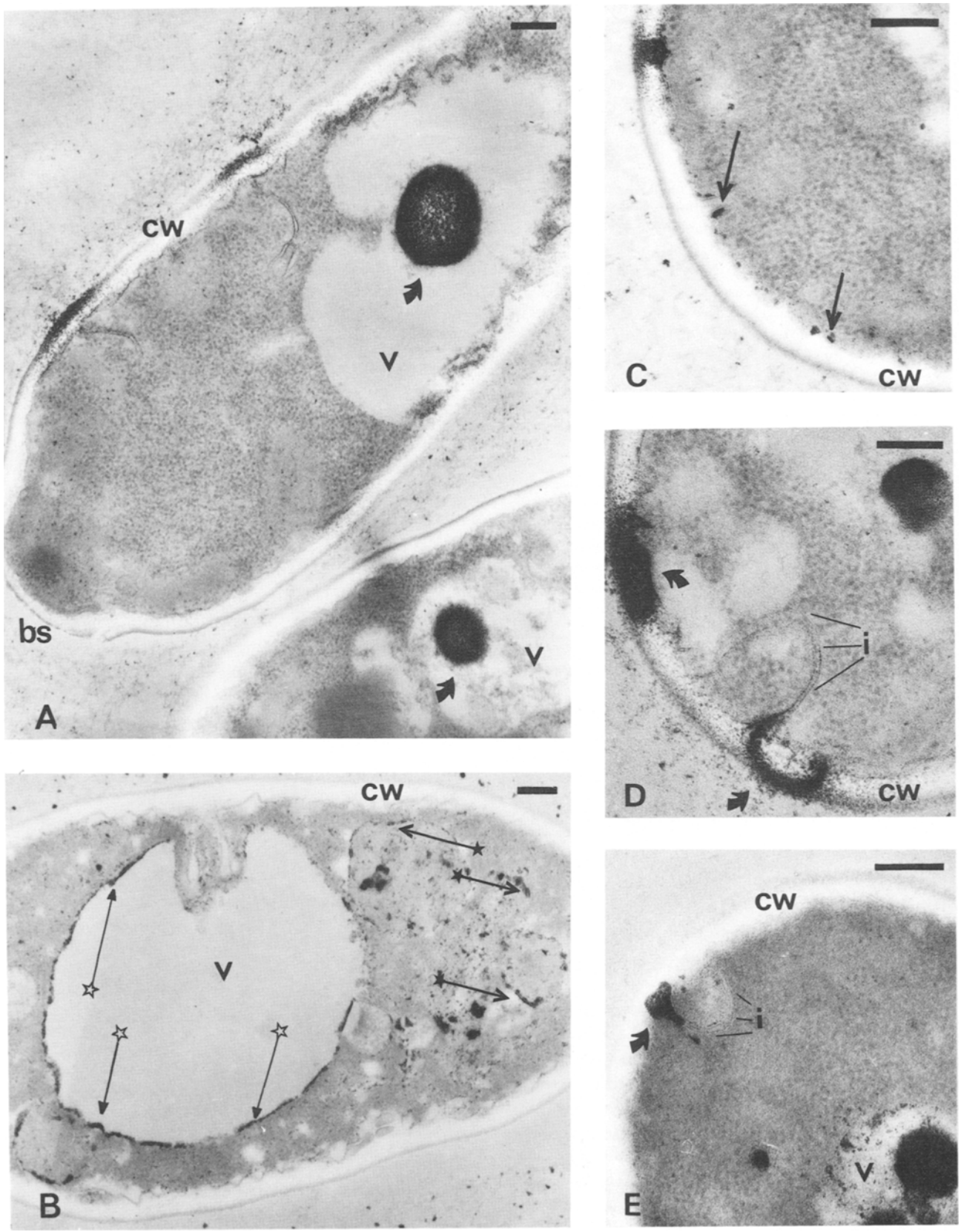

Fig. 1. Occurrence of Pb/P precipitates in the yeast *Kluyveromyces marxianus*. *A*: granular precipitate in vacuole; *B*: precipitates in cytosol, associated with membrane structures (★→) and aligning the vacuolar membrane (☆→); *C*: precipitates on the inner side of the plasma membrane; *D*: precipitates outside the plasma membrane, partially in the cell wall; *E*: precipitate outside the plasma membrane, partially in plasma membrane invagination. Abbreviations used: *bs* bud scar; *cw* cell wall; *i* plasma membrane invagination; *v* vacuole. Bar represents 0.2 μm

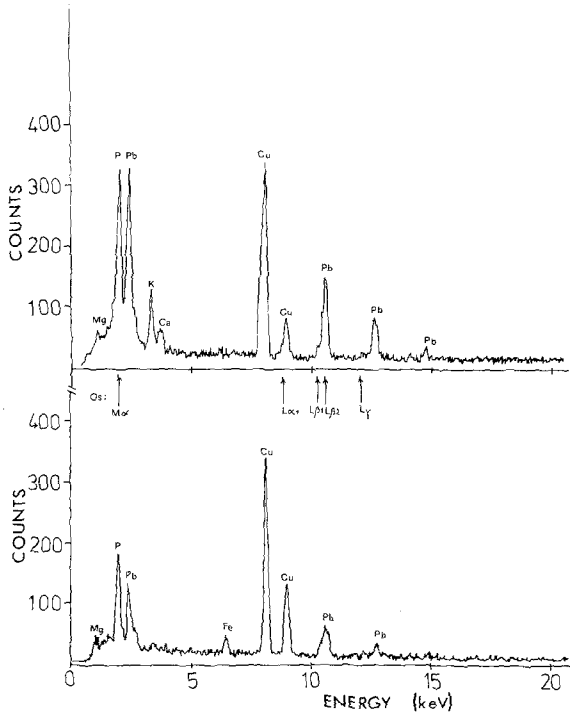

Fig. 2. X-ray dispersive micro-analysis of a granular-shaped intracellular lead deposit (A) and of a lead deposit, localized outside the plasma membrane (B) (80 kV, ΦC_2 aperture 150 µm, spot size 100 nm, life time 100 seconds). Arrows indicate the positions where Os peaks should be expected, if Os would have been present in detectable amounts

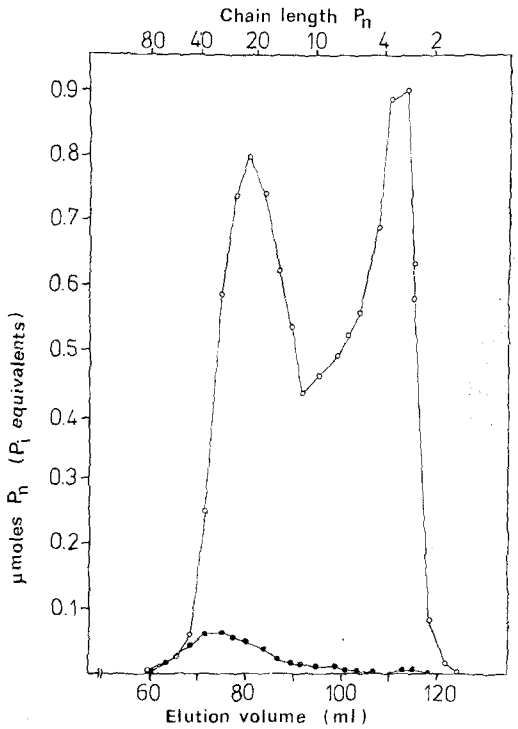

Fig. 3. Sephadex elution pattern of polyphosphates isolated from yeast by boiling (○—○) and liberated from yeast when Ca^{2+} and Mg^{2+} are omitted from the primary fixative (●—●). When Ca^{2+} and Mg^{2+} are present in the primary fixative, no liberation of polyphosphates was observed

unequivocally determined by the computer program, without apparently any interference of a Os Mα-peak in the vicinity of the P Kα-peak. Moreover the absence of other Os peaks (see Fig. 2) contra-indicates the presence of interfering amounts of Os. Secondly there is a close parallel between the cellular polyphosphate content and the amount of lead precipitated material. Finally an acid induced dislocation of the precipitated material was observed, characteristic for polyphosphate deposits (TILBERG *et al.* 1980).

Lead precipitated material was also observed outside the plasma membrane, extending in the cell wall and in plasma membrane invaginations. Despite the fact that the appearance of these deposits is more dense and spotty, as compared to the intracellular deposits, it seems highly probable that they represent polyphosphates. First, all deposits outside the plasma membrane had the same composition, as judged from X-ray dispersive microanalysis (Fig. 2*B*), which is in accordance with the proposed origin of these deposits. Further, these deposits exhibited the typical acid-induced dislocation, characteristic for polyphosphates. Finally, deposits outside the plasma membrane were not observed in phosphorus-starved yeast (containing only trace amounts of polyphosphates) but were abundant in hypercompensated yeast, containing very high concentrations of polyphosphate.

In previous studies no polyphosphate, located outside the plasma membrane of yeast cells has been described, despite the fact that such a polyphosphate localization is suggested by several studies. It could be rationalized that a polyphosphate fraction, localized outside the plasma membrane, could easily be dislocated during fixation. Therefore we employed in the present studies the fixative described by POUX (1963, 1965a, b), containing Ca^{2+} and Mg^{2+}. As shown in the results section

Table 1. *Cellular orthophosphate and polyphosphate levels in µmol phosphate per gram yeast, for yeast cultured under various conditions*

	Ortho-phosphate	Poly-phosphate
Phosphate starved	2	2
Normal 20-hour batch culture	32	18
Hypercompensated	28	68

the presence of Ca^{2+} and Mg^{2+} in the fixative is essential for the visualization of polyphosphate, localized outside the plasma membrane. Omitting these ions from the fixative results in the absence of peripheral polyphosphate deposits, whereas a high molecular weight polyphosphate fraction is liberated into the supernatant.

Acknowledgement

We wish to thank J. VORÍSEK and A. KOTYK of the Czechoslovac Academy of Sciences for initiating this work.
We are also grateful to J. N. VAN 'T NOORDENDE for his excellent technical assistance and to J. J. BEENTJES and L. D. C. VERSCHRAGEN for photographic assistance.

References

BAXTER, M., JENSEN, T., 1980: A study of methods for *in situ* X-ray energy dispersive analysis of polyphosphate bodies in *Plectonema boryanum*. Arch. Microbiol. **126**, 213—215.

CRANG, R. E., 1980: Polyphosphate bodies in *Aureobasidium pullulans*. Micron **11**, 3—4.

DE BRUIJN, W. C., 1973: Glycogen, its chemistry and morphologic appearance in the electron microscope. I. A modified OsO_4 fixative which selectively contrasts glycogen. J. Ultrastruct. Res. **42**, 29—50.

— DEN BREEJEN, P., 1975: Glycogen, its chemistry and morphological appearance in the electron microscope. II. The complex formed in the selective contrast staining of glycogen. Histochem. J. **7**, 205—229.

— — 1976: Glycogen, its chemistry and morphological appearance in the electron microscope. III. Identification of the tissue ligands involved in the glycogen contrast staining reaction with osmium(VI)—iron(II) complex. Histochem. J. **8**, 121—142.

— VAN BUITENEN, J. M. H., 1980: X-ray microanalysis of aldehyde-fixed glycogen contrast stained by $Os^{VI}Fe^{II}$ and $Os^{VI}Ru^{IV}$ complexes. J. Histochem. Cytochem. **28**, 1242—1250.

HUTCHINSON, T. A., CANTINO, M. E., CANTINO, E. C., 1977: Calcium is a prominent constituent of the gamma particle in the zoospore of *Blastocladiella emersonii* as revealed by X-ray microanalysis. Biochem. biophys. Res. Commun. **74**, 336—342.

INDGE, K. J., 1968a: Metabolic lysis of yeast protoplasts: J. gen. Microbiol. **51**, 433—441.

— 1968b: The isolation and properties of the yeast cell vacuole. J. gen. Microbiol. **51**, 441—447.

— 1968c: Polyphosphate of the yeast cell vacuole. J. gen. Microbiol. **51**, 447—455.

JASPERS, H. T. A., VAN STEVENINCK, J., 1976: Transport of 2-deoxy-D-galactose in *Saccharomyces fragilis*. Biochim. biophys. Acta **443**, 243—253.

JENSEN, T. E., 1968: Electron microscopy of polyphosphate bodies in a Blue-Green Alga. Nostoc pruniforme. Arch. Mikrobiol. **62**, 144—152.

KUHL, A., 1960: Die Biologie der kondensierten anorganischen Phosphate. Ergeb. Biol. **23**, 144—183.

KULAEV, I. S., 1975: Biochemistry of inorganic polyphosphates. Rev. Physiol. Biochem. Pharmacol. **73**, 131—158.

LISS, E., LANGEN, P., 1962: Versuche zur Polyphosphat-Überkompensation in Hefezellen nach Polyphosphatverarmung. Arch. Mikrobiol. **41**, 383—392.

OSTROVSKII, D. N., SEPETOV, N. F., RESTRETNYAK, V. I., SILBERL'DINA, L. A., 1980: Investigations of the localization of polyphosphates in cells of microorganismes by the method of high-resolution ^{31}P-NMR-145.8 MHz. Biokhimiya **45**, 517—525.

POUX, N., 1963: Localisation des phosphates et de la phosphatase acide dans les cellules des embryons de Blé (*Triticum vulgare* VILL.) lors de la germination. J. Microsc. (Paris) **2**, 557—568.

— 1965a: Localisation de l'activité phosphatasique acide et des phosphates dans les grains d'aleurone I. Grains d'aleurone renfermant à la fois globoides et cristalloides. J. Microsc. (Paris) **4**, 771—782.

— 1965b: Mise en évidence des phosphates, en microscopie électronique, dans les cellules embryonnaires de quelques Dicotylédones. C. R. Acad. Sci. Paris **261**, 1064—1066.

TIJSSEN, J. P. F., BEEKES, H. W., and VAN STEVENINCK, J., 1981: Localization of polyphosphates at the outside of the yeast cell plasmamembrane. Biochim. biophys. Acta **649**, 529—532.

— — — 1982: Localization of polyphosphates in *Saccharomyces fragilis*, as revealed by 4',6-diamidino-2-phenylindole fluorescence. Biochim. biophys. Acta **721**, 394—398.

— DUBBELMAN, T. M. A. R., VAN STEVENINCK, J., 1983: Isolation and characterization of polyphosphates from the yeast cell surface. Biochim. biophys. Acta **760**, 143—148.

TILBERG, J. E., ROWLEY, J. R., BARNARD, T., 1980: X-ray microanalysis of leakage from polyphosphate granules in *Scenedesmus*. J. Ultrastruct. Res. **72**, 316—324.

VAN STEVENINCK, J., BOOIJ, H. L., 1964: The role of polyphosphate in the transport mechanism of glucose in yeast cells. J. gen. Physiol. **48**, 43—60.

VERMEER, B. J., DE BRUIJN, W. C., VAN GENT, C. M., DE WINTER, C. P. M., 1978: Ultrastructural findings on lipoproteins *in vitro* and in xanthomatous tissue. Histochem. J. **10**, 299—307.

WIDRA, A., 1959: Metachromatic granules of microorganisms. J. Bacteriol. **78**, 664—670.

WILKINSON, J. P., DUGUID, J. P., 1960: The influences of cultural conditions on bacterial cytology. Int. Rev. Cytol. **9**, 1—76.

WOOL, S. H., HELD, A. A., 1976: Polyphosphate granules in encysted zoospores of *Rozella allomycis*. Arch. Microbiol. **108**, 145—148.