Sodium Accumulation in Decomposing Yolk Platelets during the Development of Xenopus laevis

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ABSTRACT—To cytochemically demonstrate the accumulation of sodium ions in decomposing yolk platelets, we first aimed to develop a method of sodium detection by administrating the magnesium uranyl acetate (double acetate) reagent, which throws down the sodium as triple acetate, to sciatic nerves of *Xenopus laevis*. By observing the cell contour and the volume of the precipitates produced in the intercellular and interlamellar spaces of the myelin sheath around the nerves, we determined that a 15-times dilution of the original double acetate reagent (Caley and Foulk,1929) was the best concentration for biological usage. The validity of the double acetate method was assessed by observing the specific localization of triple acetate precipitates in the skeletal muscle and kidney; In addition to their localization in intercellular spaces, the precipitates were preferentially present in the transverse system of skeletal muscle cells and present in the ground cytoplasm as well as in the organelles other than vacuoles of proximal convoluted tubule cells of the kidney. By applying this method to developing embryos, it was found that the sodium ions are stored in the vesicles during the cleavage stages of development, are apparently transported by the vesicles to decomposing yolk platelets at the early neural plate and later stages, and are accumulated in these platelets. Those results would satisfy the prediction that the sodium concentration must be increased in decomposing yolk platelets, since yolk solubilization by high salt concentrations is prerequisite for amphibian yolk digestion to occur.

INTRODUCTION

Yolk proteins, which constitute a nutrient reserve for developing embryos, must be degraded to their constituent amino acids at some stage of embryonic development in all oviparous animals. In the previous study, we demonstrated that two enzymes, cathepsin D and cysteine proteinase, work cooperatively to digest yolk proteins during the development of *Xenopus laevis* (Yoshizaki and Yonezawa, 1998). However, in that study, we predicted that there must be some mechanism to increase the ionic strength of salts in reservoir yolk platelets, since yolk proteins consisting of lipovitellins and phosvitin in *Xenopus* (Wiley and Wallace, 1981; Wallace, 1985) are stably preserved as a solid form, yolk solubilization is prerequisite to digestion by enzymes (Yoshizaki and Yonezawa, 1996) and yolk proteins can be solubilized with salt concentrations higher than the physiological level (Essner, 1954). In the present study, I focused on the sodium ion because it is a major component of the extracellular body fluid, because the components of the extracellular body fluid are produced by embryonic cells during development, and because the milieu inside the yolk platelet is highly similar to that of extracellular space.

Cytochemical demonstration of sodium was first attempted by Komnick (1962) using potassium antimonate. However, the validity of the antimonate method of sodium precipitation has been questioned by a growing number of papers (Shiina *et al*., 1970; Torack and LaValle, 1970; Yarom and Meiri, 1973; Van Iren *et al*., 1979), such that this method is now used primarily for detecting intracellular calcium localization (Suzuki and Sugi, 1989) and there currently is no appropriate method for detecting sodium cytochemically.

It has been shown by chemical analysis that magnesium uranyl acetate (double acetate) is a potent reagent for precipitating sodium as magnesium sodium uranyl acetate (triple acetate) (Hall, 1952). In the study by Caley and Foulk (1929), use of the double acetate to quantify sodium was unaffected by the presence of other members of the alkali group (K, NH_4, Li) or by that of alkaline earths (Ca, Mg, Sr, Ba) or ferric iron. In this study I applied this precipitation technique to the subcellular localization of sodium. Thus the present study had two aims: to develop a cytochemical method for detecting sodium ions and to apply this method to determining sodium-ion accumulation in decomposing yolk platelets.

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MATERIALS AND METHODS

Samples

Sciatic nerve, thigh muscle and kidney were obtained from adult male frogs of *X. laevis*. Embryos were obtained by artificial fertilization according to the method of Moriya (1976). Embryos were reared in 0.05 De Boer solution (equivalent to tapping water) at 22-24°C. Stages were assigned according to Nieuwkoop and Faber (1967).

Preparation of the magnesium uranyl acetate

The double acetate (DA) reagent was prepared according to the method of Caley and Foulk (1929) with a slight modification; Solution A consisted of 90g of uranyl acetate (2 H_2O) and 60g of glacial acetic acid in 1000 ml distilled water and solution B consisted of 600g of magnesium acetate (4 H_2O) and 60g of glacial acetic acid in 1000 ml distilled water. The two solutions were separately heated to about 70°C, mixed at this temperature and allowed to cool to 20°C. Finally, the DA reagent was filtered through a dry filter into a dry bottle.

Electron microscopy

Samples were fixed with 2.5% glutaraldehyde in 15-times diluted DA reagent (hereafter referred to as 0.07 DA) for 3 hr at 4°C. They were then washed with 0.2 M sucrose three-times for 30 min each and post-fixed with 1% OsO_4 in 0.2 M sucrose for 3 hr at 4°C. After fixation, samples were dehydrated in an acetone series and embedded in Epon 812 resin.

Controls were fixed with 2.5% glutaraldehyde in the 0.07 reagent with equi-osmolar sucrose substituted for magnesium acetate.

RESULTS AND DISCUSSION

Development and assessment of the methodology

The magnesium uranyl acetate (double acetate; DA) reagent precipitates sodium as triple acetate (Caley and Foulk, 1929). But because the ionic concentration of the DA reagent originally developed for chemical studies is too high for use in biological studies, I first aimed to determine the appropriate concentration of reagent for biological applications. When sciatic nerves were fixed with glutaraldehyde in 0.5 or 0.25 DA reagent, the tissues shrank, probably due to the high osmotic pressure, and no precipitate appeared in any location. With 0.13 or 0.07 DA, normal cell contours seemed to be maintained and the precipitates appeared in intercellular and interlamellar spaces of the myelin sheath of Schwann cells around a nerve axon (Fig. 1). The precipitation was always more pronounced in the interlamellar spaces at the outer region of the myelin lamellar sheath. The volume of precipitates seemed to be higher with 0.07 DA than with 0.13 DA. Based on these results, it is possible that the precipitates are produced by interaction of the DA reagent with the sodium ions in the extracellular body fluid. Then it could be understood from the figure that the sodium ions are precipitated where immobilized by an intervening structure, but in open areas are dislocated after being precipitated. With 0.03 DA, the precipitates also appeared in the spaces mentioned above but the lamellar structure of the myelin sheath was dissociated. The control treatment yielded no precipitate (Fig. 2). Thus I determined that 0.07 DA is the best concentration of this reagent for application to biological materials.

Although the Epon-embedded sections were examined by X-ray microanalysis to detect sodium in the precipitates, no significant amount of sodium was detected. This may have been due to the inherent limitation of X-ray microanalysis for detecting significant amount of light elements (Cliff and Lorimer, 1975).

I therefore assessed the validity of the DA method by applying it to skeletal muscle and kidney in which sodium ions are known to be present in specific locations. The transverse system of skeletal muscles is an indentation of the plasma membrane and the extracellular body fluid follows in the wake of such indentations. The cross sections of the transverse system appear regularly in the vicinity of Z-lines in the sagittal sections of sarcomeres. When the muscle was treated with the 0.07 DA reagent, the precipitates were present in such sections of the transverse system as well as in the intercellular space (Figs. 3 and 4). The precipitates did not appear in any organelle or in the ground cytoplasm. The control treatment did not yield any precipitate in muscle cells (Fig. 5).

One of the functions of the kidney is the reabsorption of sodium ions from the primary urine at the proximal convoluted tubule. When the kidney was submitted to treatment with the 0.07 DA reagent, heavy precipitates appeared within the epithelial cells at the proximal convoluted tubule as well as in the intercellular spaces between them (Figs. 6-8). The precipitates were present in the ground cytoplasm and even in organelles other than the vacuoles (Figs. 7 and 8). In the other portions of the kidney, no significant precipitation was observed in any cell, except for that in intercellular spaces (Fig. 8). The control showed no precipitate (Fig. 9).

As a result of these observations, the locations of the precipitates which were produced with the DA method coincided well with the locations where the sodium ions are supposed to be present, thus showing the validity of this method to localize sodium ions cytochemically.

### Detection of sodium ions in yolk platelets of embryonic cells

The cytoplasm of ectodermal cells of stage 13 embryos (early neurulae) was occupied mostly by yolk platelets, lipid droplets and mitochondria (Fig. 10). There were a few vesicles which interiorly possessed precipitates produced by the treatment with the 0.07 DA reagent (Fig. 11). Some vesicles were in close apposition to yolk platelets but apparently had not fused with them. Such features were maintained from stage 2 (two-cell embryo) to stage 13. Precipitation of sodium ions in intercellular spaces was observed in embryos at all stages observed.

The number of yolk platelets which have an intimate relationship with precipitate-possessing vesicles increased in the ectodermal cells from embryos of stages 15 (early neural fold stage) to 23 (early tailbud stage) (Fig. 12). Fig. 13 shows apparent fusion of a yolk platelet with such vesicles in the cells of stage 15 embryos. These changes were only observed in small or medium yolk platelets that were distributed in the apical region of the cells.

In the ectodermal cells of stage 29 (tailbud) embryos, yolk
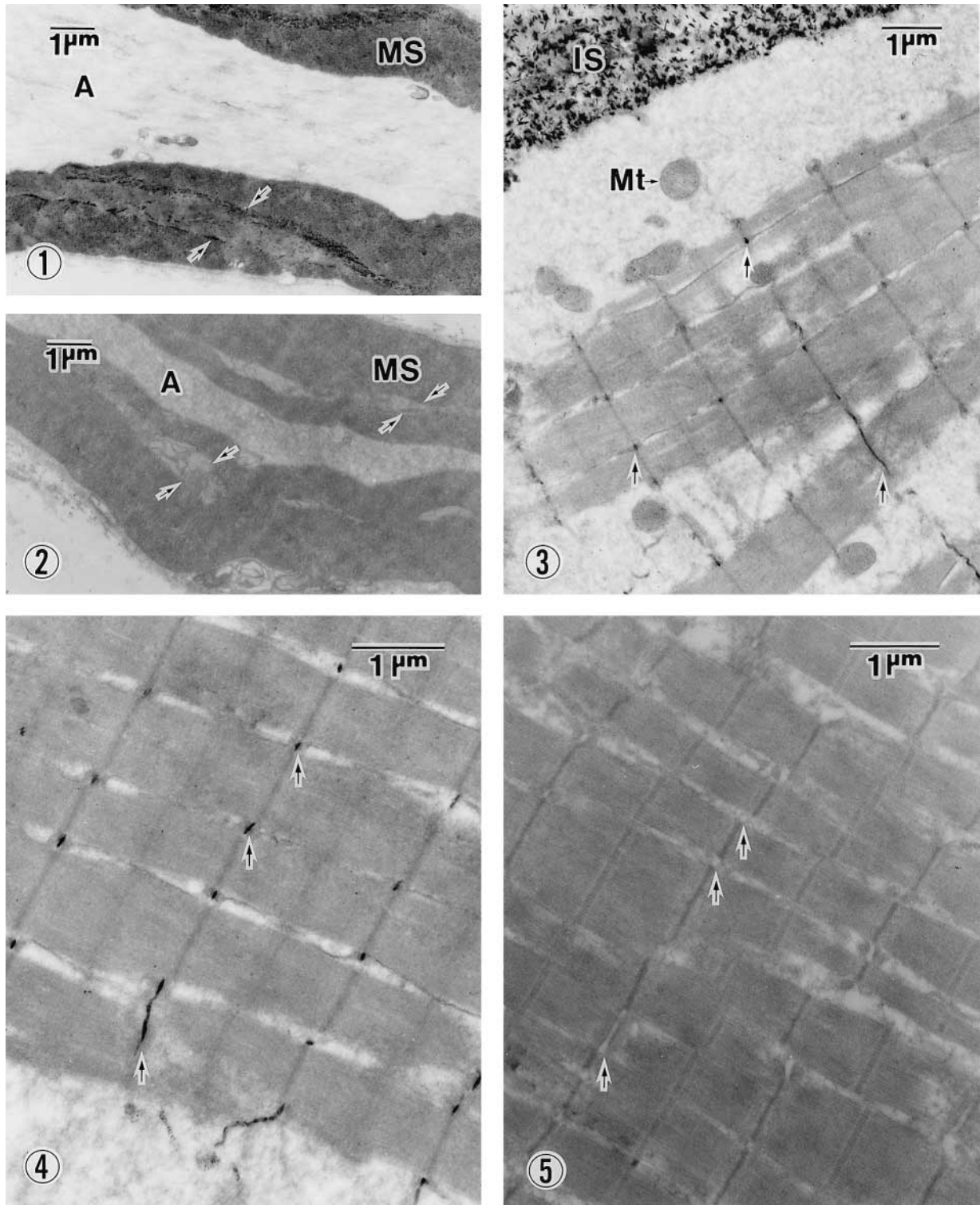
Figs. 1-5. Electron micrographs of sciatic nerves (Figs. 1 and 2) and thigh muscles (Figs. 3-5), showing the extracellular localization of the triple acetate precipitates. The precipitates are present in the intercellular and interlamellar spaces of the myelin sheath (MS) around a nerve axon (A) (between two arrows in Fig. 1). In muscle cells, the precipitates are present in intercellular space (IS) and in the sections of the transverse system (arrows in Figs. 3 and 4). Figs. 2 and 5 are control sections of each tissue treated with uranyl acetate alone, showing no precipitate. Mt, mitochondrion.
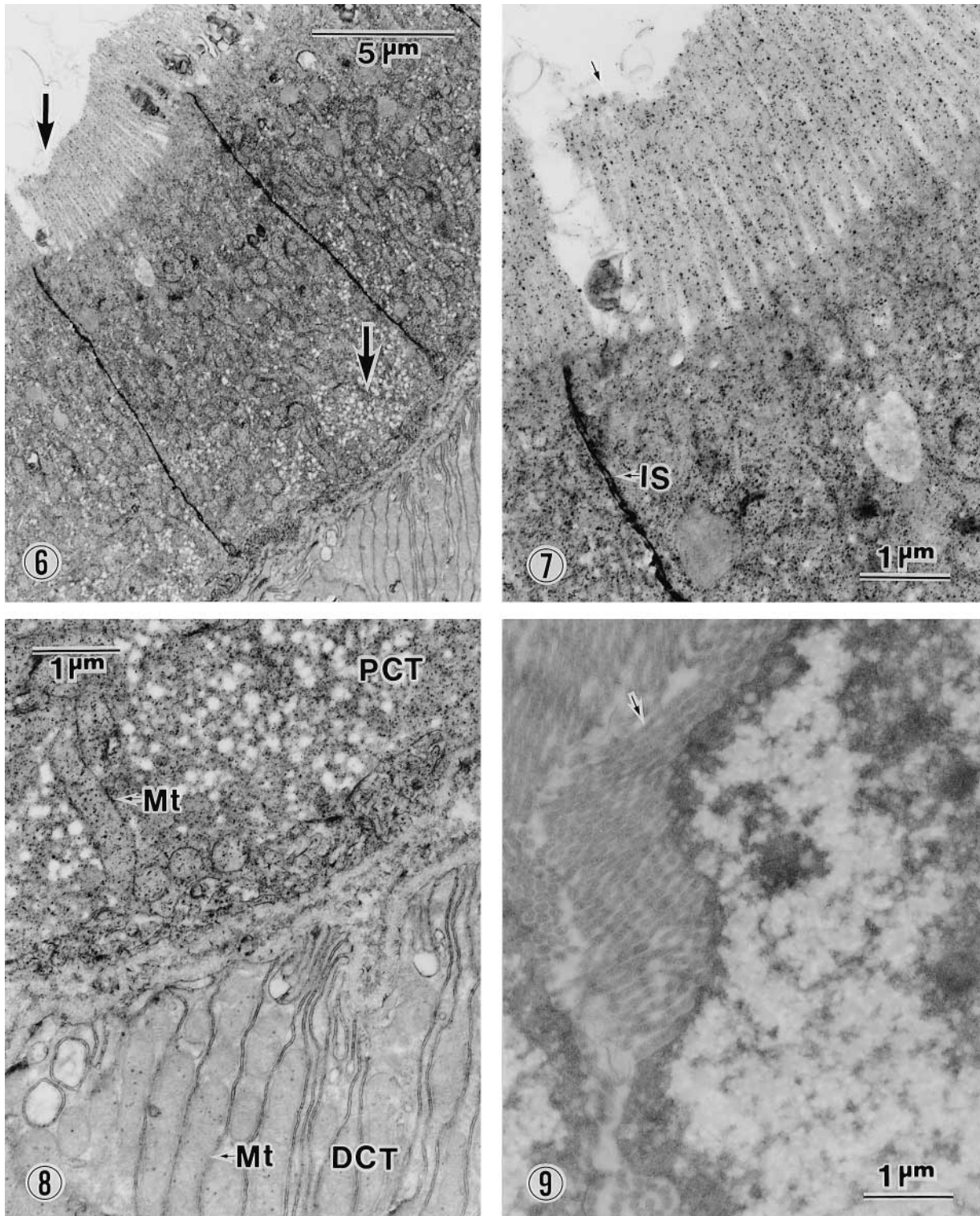
Figs. 6-9. Electron micrographs of kidney, showing the intracellular as well as the intercellular localization of the triple acetate precipitates. Portions indicated by large arrows in Figure 6 are enlarged in Figures 7 and 8. The precipitates are present in the ground cytoplasm and organelles other than vacuoles in the epithelial cells at the proximal convoluted tubule (PCT in Fig. 7). No precipitate is observed in the cells at the distal convoluted tubule (DCT). The small arrows in Figs. 7 and 9 indicate microvilli of the cells at PCT. There is no precipitate in the control section (Fig. 9). IS, intercellular space; Mt, mitochondrion.
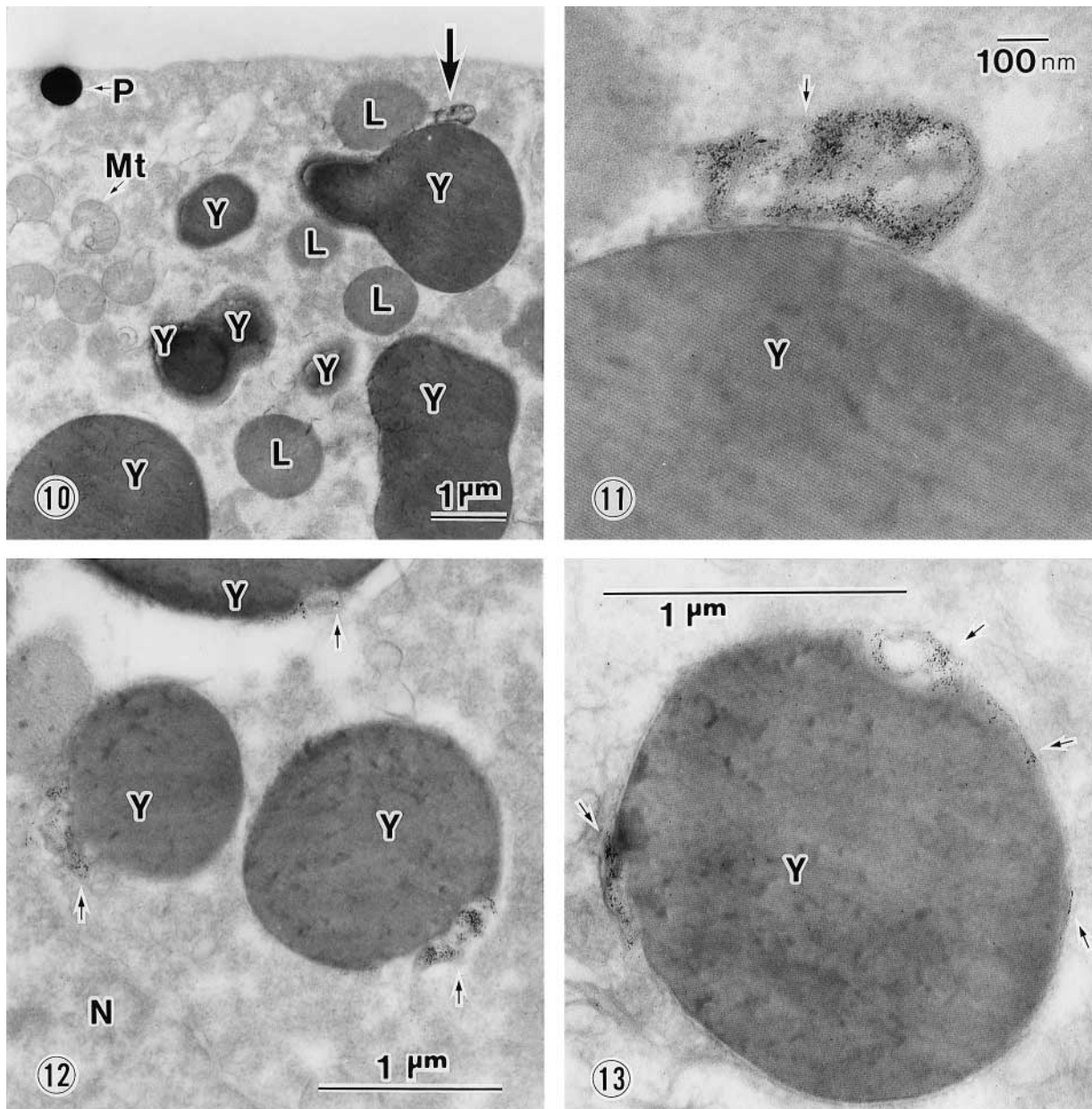
Figs. 10-13. Ectodermal cells of embryos treated with the double acetate reagent. The cytoplasm of the stage 13 embryo is occupied by yolk platelets (Y), lipid droplets (L) and mitochondria (Mt) (Fig. 10). The portion indicated by large arrow is enlarged in Fig. 11. The small arrow in Fig. 11 indicates a vesicle possessing triple acetate precipitates. Many yolk platelets in the ectoderm of the stage 15 embryo have a close association with the vesicles (arrows in Fig. 12). Some yolk platelets possess the precipitates as well as the vesicle at its superficial layer (arrows in Fig. 13), suggesting a fusion of the platelet with precipitate-possessing vesicles. P, pigment granule.

platelets of mostly large size were located in the basal region of the cells. The precipitates of triple acetate were observed in the superficial layer of these yolk platelets at this stage (Figs. 14 and 16). The precipitates were also observed at the periphery of lipid droplets, which tended to coalesce with each other during the development (Figs. 15 and 16); Some sodium ions in the ground cytoplasm might have been trapped by lipid droplets and concentrated in their hydrophobic environment. Observation of the control again showed no precipitate in the embryonic cells (Fig. 17).

We previously reported that the yolk proteins of *X. laevis* are effectively digested by the cooperative action of two enzymes, that is, cathepsin D, which is preserved in yolk platelets from the beginning of development (Yoshizaki and Yonezawa, 1996), and cysteine proteinase, which is produced in embryos at stage 13 or later (Yoshizaki and Yonezawa, 1998). However, since amphibian yolk proteins are preserved in solid form, yolk solubilization is prerequisite to digestion by enzymes, and thus these particular enzymes would not be able to digest yolk proteins under a physiological salt concentration, but would be able to do so under the high sodium concentrations (Yoshizaki and Yonezawa, 1996; Yoshizaki *et*
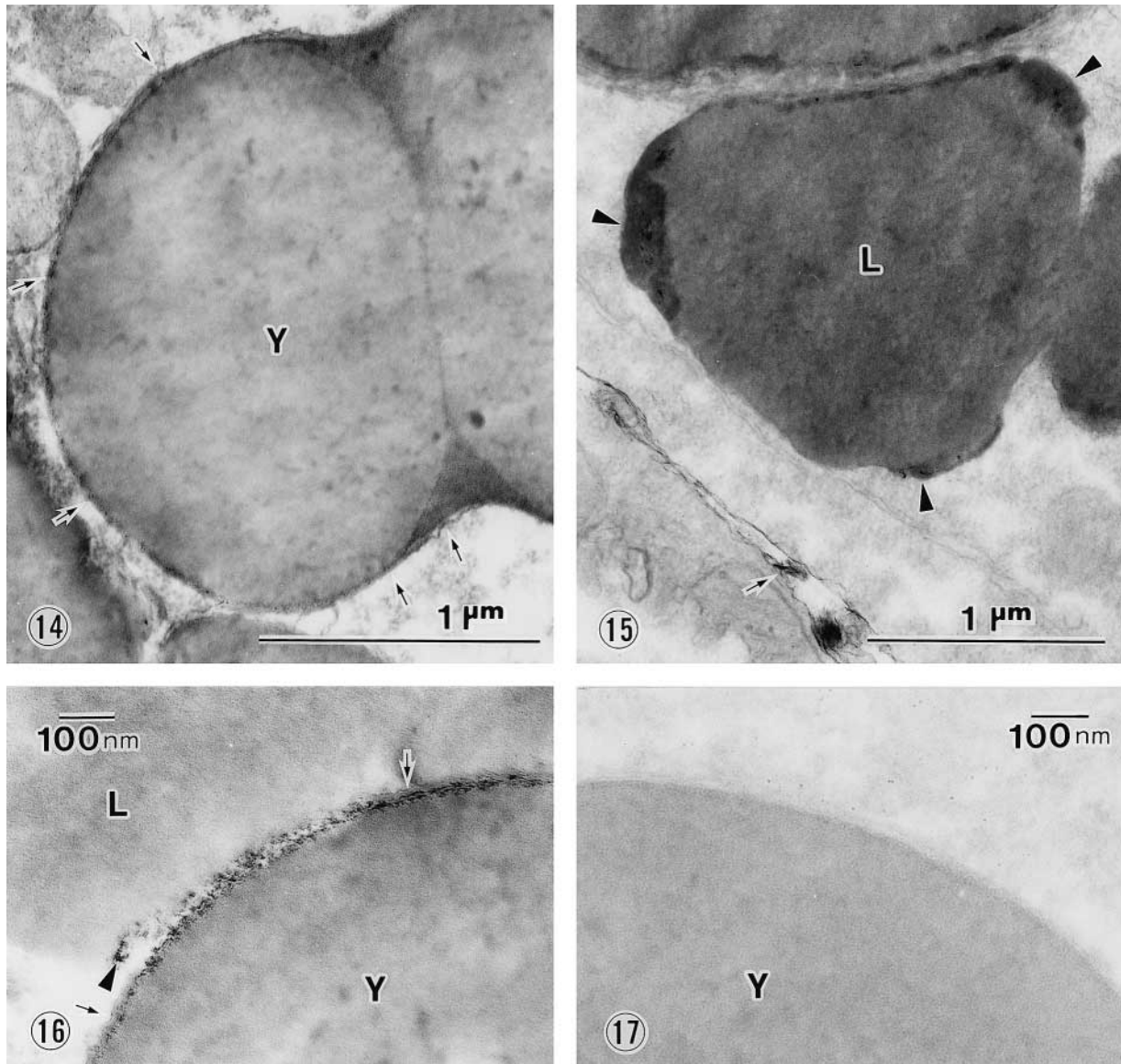
Figs. 14-17. Electron micrographs of ectoderm of the stage 29 embryo. Large yolk platelets (Y) at the basal cytoplasm of the cells possess triple acetate precipitates at the superficial layer (arrows in Fig. 14). Lipid droplets (L) also possess the precipitates at their periphery (arrowheads in Figs. 15 and 16). The small arrow in Fig. 15 indicates the precipitates in intercellular space. The control section does not show any precipitate (Fig. 17).

al., 1998) known to solubilize yolk proteins (Essner, 1954). Although we have not yet confirmed the coexistence of such two enzymes and sodium in a single yolk platelet, the fact that all these substances were located at the superficial layer of the yolk platelets (present paper; Yoshizaki and Yonezawa, 1994, 1996, 1998) may suggest that yolk crystals are gradually solubilized and digested at their periphery during embryonic development.

According to Slack *et al.* (1973), the intracellular sodium concentration falls steadily from 90 mM in eggs to 30 mM in embryos at the beginning of gastrulation in *Xenopus*, with sodium activity held to a relatively constant 14 mM during this period. This implies that the lost sodium has moved to the intercellular spaces and comprised the sodium in the extra-

cellular body fluid. A similar movement of sodium might be possible into vesicles and/or yolk platelets by the mechanism of Na^+/H^+ exchange as proposed by Fagotto and Maxfield (1994). The present study showed an apparent increase of sodium ions in yolk platelets as a result of ion transportation through vesicles, thus showing that the occurrence of yolk digestion by these enzymes is possible in decomposing yolk platelets. However, it remains to be shown how high concentrations of sodium ions are maintained in decomposing yolk platelets. Future studies using confocal microscopy and fluorescent probes sensitive to sodium ions could quantitatively measure the concentration of these ions.

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