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Ultracytochemical study of trimetaphosphatase activity during acrosomal formation in the mouse testis

Q.-S. Jin[1], M. Kamata[2], E. Garcia del Saz[1] and H. Seguchi[1]

[1]Department of Anatomy and Cell Biology and [2]Department of Urology, Kochi Medical School, Kochi, Japan

Summary. The localization of Trimetaphosphatase (TMPase) activity during the acrosomal formation in the mouse testis was enzyme cytochemically investigated by the cerium-salt method. In addition to the lysosomes of the Sertoli cells and the spermatogenic cells in the seminiferous tubules, positive TMPase activity was detected in the Golgi complex and in the acrosomal vesicles of the spermatids, as well as in the acrosomes of both spermatids and spermatozoa. In the Golgi complex of the spermatids, TMPase activity was observed in the first one or two lamellae of the trans-face and in the small vesicles in the vicinity of the Golgi complex. TMPase positive reaction was also detected in the acrosomes of the spermatozoa in the lumina of both the seminiferous tubules and the epididymal duct. The localization of this enzyme activity was compared with that of acid phosphatase (ACPase), as detected by the cerium-based method, using ß-glycerophosphate as substrate: ACPase activity was completely absent from the Golgi complex, small vesicles, acrosomal vesicle and acrosome throughout the entire process of acrosomal formation. TMPase is thought to become one of the acrosomal components, and may be involved in the acrosomal reaction during fertilization.

Key words: Acrososome, Golgi complex, Trimetaphosphatase, Acid phophatase, Cytochemistry

Introduction

Mammalian spermiogenesis is an extraordinarily complex process which involves the formation of the acrosome from the Golgi complex, condensation of the nucleus and formation of the flagellum (Dym, 1983; Fawcett, 1993). This process has been subdivided into 19 steps grouped into four phases and referred to as the Golgi phase (steps 1-3), cap phase (steps 4-7), acrosomal phase (steps 8-14), and maturation phase (steps 15-19).

Offprint requests to: Dr. H. Seguchi, Department of Anatomy and Cell Biology, Kochi Medical School, Kohasu, Okoh-cho, Nankoku, Kochi, 783, Japan

During the early phases of acrosomal formation, glycoprotein-rich acrosomal substances are synthesized in the rough endoplasmic reticulum and transported from the cis-face to the trans-face of the Golgi complex, where they become small vesicles (condensing vacuoles) that fuse together and become a single acrosomal vesicle containing the acrosomal granule. This acrosomal vesicle associates with the anterior pole of the condensing nucleus of the spermatid during the cap phase (Susi and Clermont, 1970; Susi et al., 1971; Clermont and Rambourg, 1978). The acrosome contains a large number of hydrolytic enzymes such as hyaluronidase, acrosin, neuramidase, arylsulphatase, cytidine monophosphatase, thiamine pyrophosphatase and ß-N-acetylglucosaminidase. (Morton, 1976; Clermont et al., 1981; Yanagimachi, 1981; Tang et al., 1982; Eddy, 1988; Thorne-Tjomsland et al., 1988; Fawcett, 1993; Shur, 1993). These enzymes are involved in the acrosomal reaction during fertilization.

Enzyme cytochemistry during spermiogenesis has been hitherto performed to localize various phosphatases present within the distinct regions of the Golgi complex (Clermont et al., 1981; Tang et al., 1982; Thorne-Tjomsland et al., 1988). Nicotinamide adenine dinucleotide phosphatase (NADPase) activity has been localized in the intermediate four or five lamellae, thiamine pyrophosphatase (TPPase) in the last one or two lamellae on the trans-side, and cytidine monophosphatase (CMPase) in the one or two thicker saccules in the trans-face of the Golgi complex (corresponding to the rigid smooth lamellae of Claude (1970), and to the GERL of Novikoff and his colleagues (Novikoff, 1976; Novikoff et al., 1971, 1977)), in addition to the acrosomal vesicle and the acrosome. These findings indicate that there is a distinct relationship between the morphological and the enzymatical aspect of the Golgi complex during spermiogenesis (Clermont et al., 1981; Tang et al., 1982; Thorne-Tjomsland et al., 1988).

Trimetaphosphatase (TMPase) catalyzes the conversion of cyclic TMP in the acidic range. Together with acid phosphatase (ACPase), this enzyme has been cytochemically employed as a lysosomal marker enzyme (Doty et al., 1977). The enzyme activity, however,

differs in its localization from that of the ACPase, being observed in the tubular lysosomes located infranuclearly, and has been reported to be involved in endocytosis (Oliver, 1980, 1983; Petty et al., 1985). In the present study, the localization of TMPase activity during mouse spermiogenesis was investigated by the newly developed cerium-based method of Seguchi et al. (1992) and compared with that of ACPase activity, as detected by the cerium salt method (Robinson and Karnovsky, 1983). Generally speaking, the cerium-based method has been reported to be superior to the other methods currently employed in several points (Robinson and Karnovsky, 1983; Kobayashi et al., 1987; Seguchi et al., 1992). The results obtained indicate a marked difference between the localization of these two enzymes with regard to the acrosomal formation.

Materials and methods

Adult ddy strain mice weighing 45-65 g were employed. The testes were excised under ethyl ether anaesthesia. After removal of the tunica vaginalis, several holes were opened at the equatorial region, through the tunica albuginea, with a tuberculin needle. Subsequently, 1.0 ml of a fixative containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer, pH 7.4, with 2 mM calcium chloride and 5% sucrose, was injected. The testes were immersed in the same fixative, at 4 ºC, for 30 min, and cut into blocks of approx 1.5 mm^3, which were further fixed for 30 min at 4 ºC. The epididymis was excised, cut into slices of 1.5 mm in thickness and immersed in the fixative above for 60 min at 4 ºC. The specimens were then sectioned at 50 µm in thickness with a Microslicer (Dosaka EM. Kyoto, Japan).

For the detection of the TMPase activity, the cerium-based method of Seguchi et al. (1992) was employed. The reaction medium contained 20 mM acetate buffer (pH 3.9), 2 mM cerium chloride, 1 mM sodium trimetaphosphate, 5% sucrose and 0.00015% Triton X-100. Incubation was performed at 37 ºC, for 40 to 60 min for both testis and epididymis. ACPase was detected by the method of Robinson and Karnovsky (1983), using cerium as capture agent. The incubation medium contained 0.1M acetate buffer (pH 5.0), 2 mM cerium chloride, 1 mM ß-glycerophosphate, 5% sucrose and 0.00015% Triton X-100. The reaction was carried out at 37 ºC for 40 min. After incubation, the sections were rinsed with the same buffer as that employed in the incubation medium and postfixed with 1% osmium tetroxide for 30 min. Following dehydration in a series of graded ethanols, the specimens were embedded in Spurr's eposy resin (1969). Ultrathin sections were contrasted with uranyl acetate and lead citrate and observed in a JEM-1200 EX (JEOL Co., Ltd., Tokyo, Japan) electron microscope.

TMPase control experiments included the addition of 10 mM sodium fluoride (NaF), an inhibitor of TMPase, to the incubation medium, and omission of the substrate sodium trimetaphosphate. As control experiments for ACPase, addition of 10 mM L-(+)-tartaric acid, an inhibitor of ACPase, and omission of the substrate ß-glycerophosphate were performed.

Results

Throughout the entire process of spermiogenesis, TMPase activity was detected in the Golgi complex,

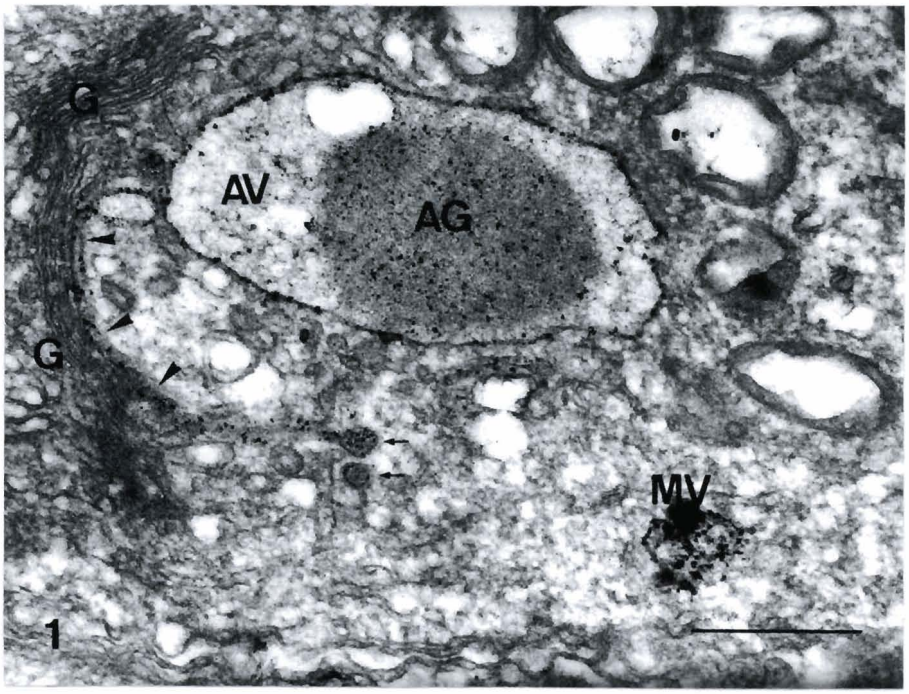


Fig. 1. During the Golgi phase, TMPase activity is detected in the acrosomal vesicle (AV), small vesicles (arrows), the first one or two lamellae (arrowhead) of the trans-face of the Golgi complex (G) and the nearby multivesicular body (MV). In the acrosomal vesicle the reaction product is most densely accumulated in the acrosomal granular area (AG). x 45,000. Bar= 0.5 µm.

multivesicular body near the Golgi complex, acrosomal vesicle and acrosome of the spermatid, in the acrosome of the spermatozoon, and in the lysosomes of Sertoli and spermatogenic cells in the seminiferous tubules (Figs. 1-4). The reaction product was seen as a fine, granular, electron-opaque deposit mostly localized along the inner

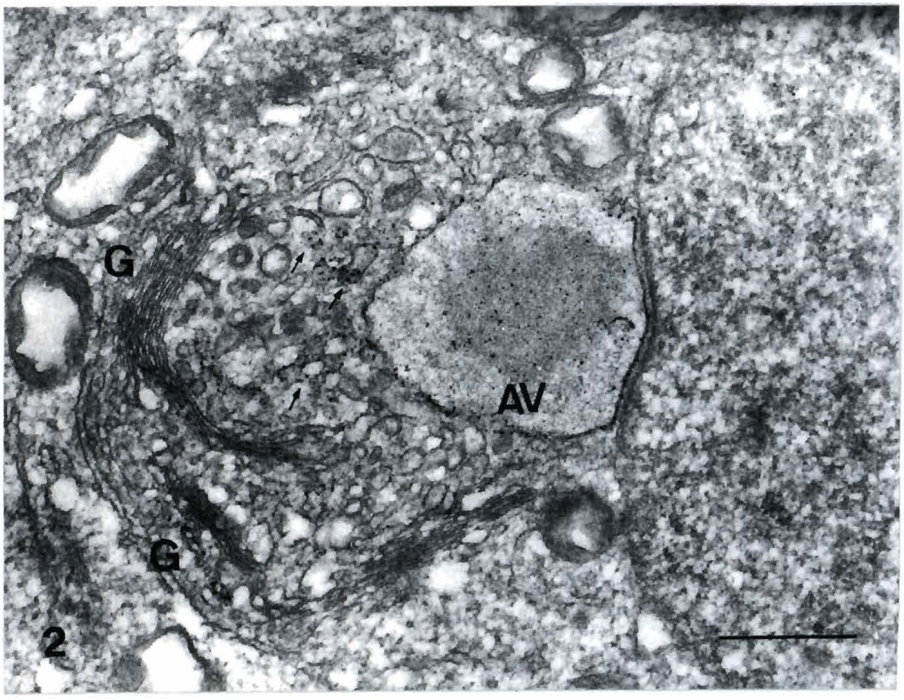
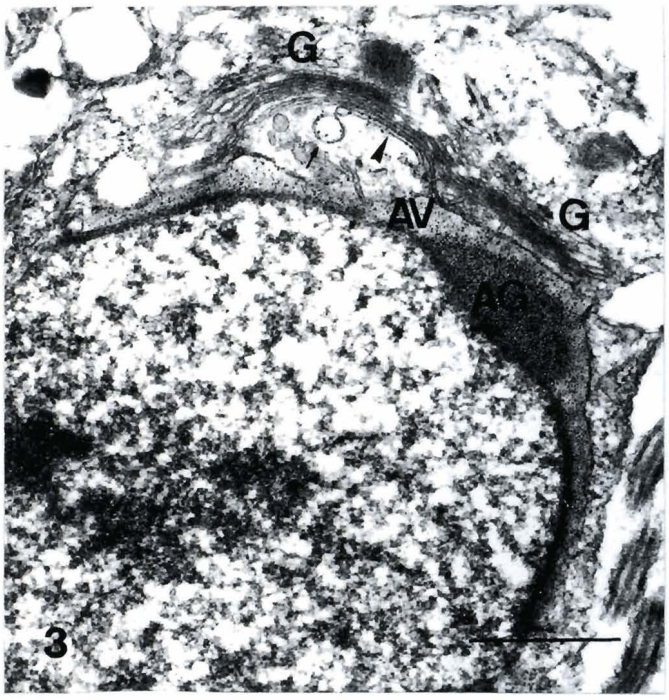


Fig. 2. In the early cap phase, TMPase activity is detected in the acrosomal vesicle (AV) bound to the nuclear envelope and in small vesicles (arrows). Golgi complex (G). x 37,000. Bar= 0.5 µm.



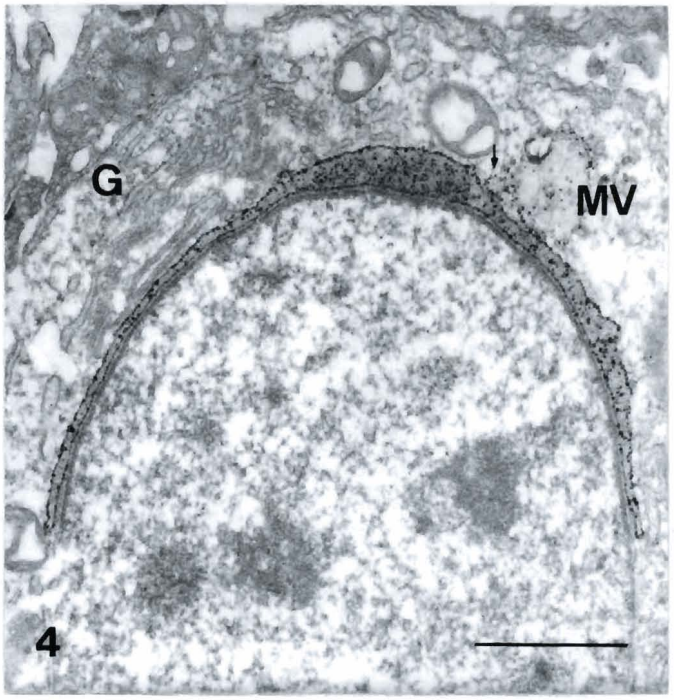


Fig. 3. During the cap phase, TMPase activity is observed in the acrosomal vesicle (AV) which has begun to wrap the nucleus, in the innermost lamella (arrowhead) of the trans-face of the Golgi complex (G) and in small vesicles (arrow). The reaction product is more densely precipitated in the acrosomal granule (AG). x 22,000. Bar= 1 µm.

Fig. 4. In the acrosome phase, the TMPase activity is detected in the acrosomal vesicle wrapping the nucleus, in small vesicles (arrows) and in the adjacent multivesicular body (MV). Golgi complex (G). x 20,000. Bar= 1 µm.

surface of the limiting membrane of the Golgi lamellae, acrosomal vesicle, acrosome and multivesicular body, and sometimes in the lumen of these structures (Figs. 1-4). In semithin (0.5-1.0 µm) sections, the reaction product was evenly distributed (except for the acrosomal granule) throughout the lumen of both the acrosomal vesicle and the acrosome (Figs. 5, 6). In the innermost trans-face lamella of the Golgi complex, the reaction product appeared as an anastomosing reticular tubular network (Fig. 7), which corresponds to the rigid smooth lamellae of Claude (1970) and to the GERL of Novikoff et al. (1971, 1977).

During the Golgi phase, the reaction product was detected in the acrosomal vesicle formed from the Golgi complex, which was located in close vicinity of the spermatid nucleus (Fig. 1). Moreover, the enzyme

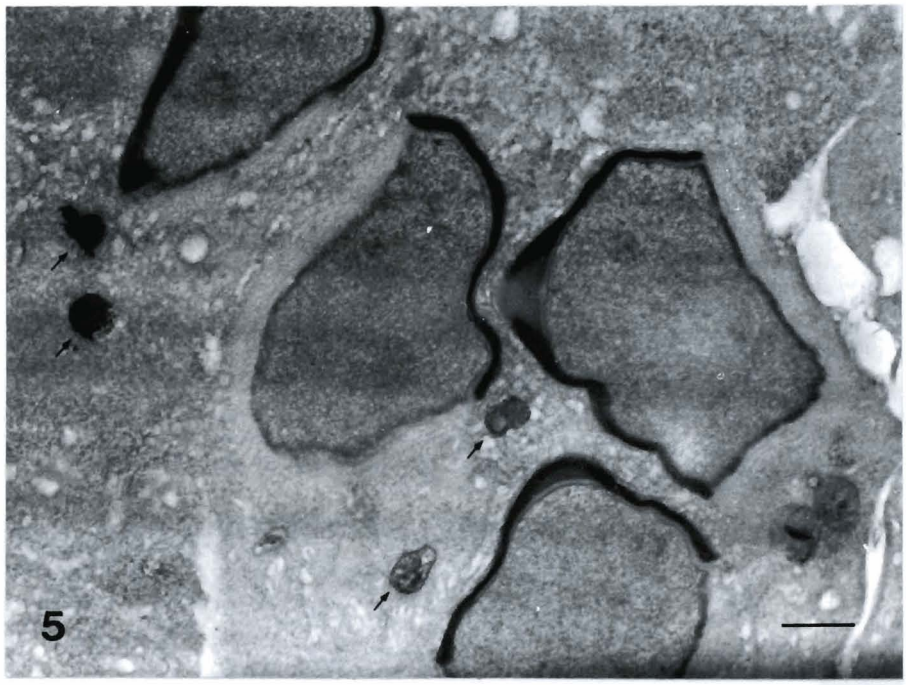


Fig. 5. TMPase activity in a semithin section. in addition to the lysosomes (arrows) in the Sertoli cell, positive reaction is observed in the acrosomal vesicle of the spermatid in the acrosomal phase. x 12,000. Bar= 1 µm.

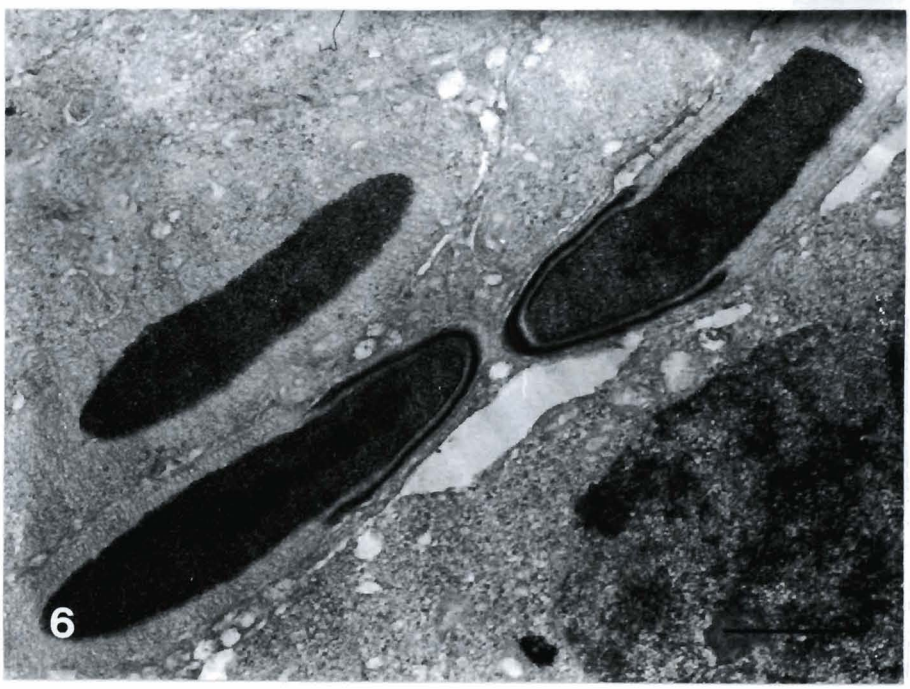


Fig. 6. TMPase activity in a semithin section. At the maturation phase, the reaction product is detected in the acrosome wrapping the condensed nucleus. x 18,000. Bar= 1 µm.

activity was also detected in the small vesicles and in the first one or two lamellae of the trans-face of the Golgi complex, and in a multivesicular body near the Golgi complex (Fig. 1).

At more advanced stages (cap-phase), when the acrosomal vesicle begins to distend and to envelop the anterior portion of the spermatid nucleus, the reaction product of the TMPase activity was observed in the acrosomal vesicle, small vesicles and the Golgi complex (Fig. 3). During the acrosome phase, when the Golgi stacks begin to separate from the acrosome and displace toward the tail portion, the reaction product was observed in the small vesicles derived from the Golgi complex, in the acrosome, and in the multivesicular body (Fig. 4).

In the late stage of spermiogenesis (maturation phase), the TMPase activity was observed in the acrosome (Figs. 8, 9). The reaction product was more densely distributed on the outer than on the inner acrosomal membrane in the anterior portion of the acrosome of the spermatid (Fig. 8). The acrosomes of spermatozoa supposedly just separated from the Sertoli cells (Fig. 9), observed in both the lumina of seminiferous tubules and epididymal duct, were positive for TMPase (Fig. 10). No difference in the enzyme activity in the acrosome of the spermatozoa was recognized among the head, body and tail of the epididymal duct. In control experiments using a substrate-free medium, or a medium supplemented with 10 mM sodium fluoride, no reaction products were detected (Fig. 11).

The reaction product of the ACPase activity was particularly strong in the innermost lamella on the trans-face of the Golgi complex in spermatogonia, and in primary and secondary spermatocytes (Fig. 12). The enzyme activity was also detected in the lysosomes of spermatogenic cells and Sertoli cells (Fig. 13), but it was never observed in either the acrosomal vesicle, the acrosome or the multivesicular body (Fig. 14). In the controls, the reaction product of the ACPase activity was neither observed when a substrate-free medium was used nor when the inhibitor was added to the medium (Fig. 15).

Discussion

Partially purified TMPase has been biochemically demonstrated to be a 130KD protein with pI 6.1 which electrophoretically appears in a different band from that of ACPase (Seguchi et al., 1992). TMPase catalyzes the conversion of cyclic trimetaphosphate to tripoly-phosphate in the acidic pH range (Oliver, 1983; Petty et

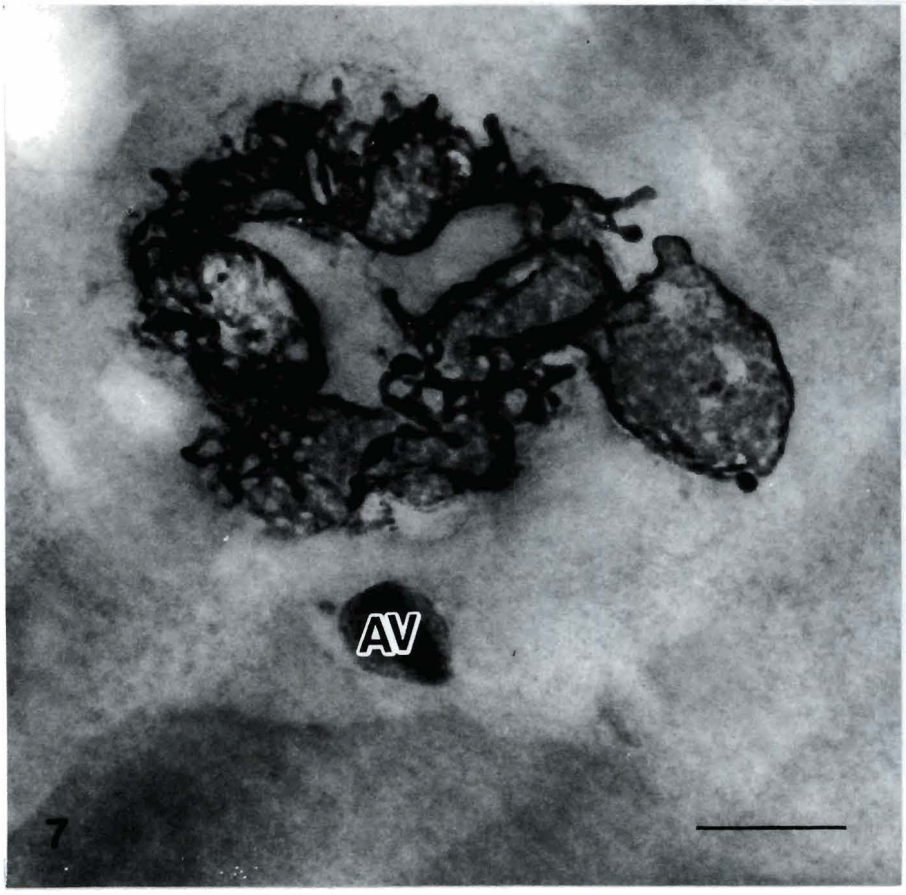


Fig. 7. TMPase activity in a semithin section. The reaction product is detected in an anastomosing reticular structure which corresponds to the innermost lamella of the trans-face of the Golgi complex and in the acrosomal vesicle (AV) during the Golgi phase. x 40,000. Bar= 0.5 µm.

al., 1985). Together with ACPase, this enzyme is currently widely employed by many researchers as a cytochemical marker for the lysosomes (Doty et al., 1977; Oliver, 1980, 1982, 1983; Livne and Oliver, 1986; Sasaki et al., 1988; Bainton et al., 1989). According to tracer experiments using cationized ferritin, horseradish peroxidase (HRP), latex and yeast, the lysosomes possessing TMPase activity do actually take up these substances, and have been observed to be involved in endocytosis (Oliver, 1982; Petty et al., 1985; Livne and Oliver, 1986). Moreover, some researchers demonstrated the existence of TMPase activity in the Golgi complex (Doty et al., 1977; Sasaki et al., 1988; Bainton et al., 1989). However, differences in the localization of these two enzymes have been reported (Oliver, 1980, 1983; Petty et al., 1985; Livne and Oliver, 1986; Sasaki and Grant, 1986; Zhang et al., 1991). The results of the present study support these previous findings and, in addition, demonstrate that TMPase activity is also present in the acrosomal vesicle and acrosome of the mouse spermatid (Figs. 1-4) and spermatozoa (Figs. 8-10). The Golgi complex has been demonstrated to play a

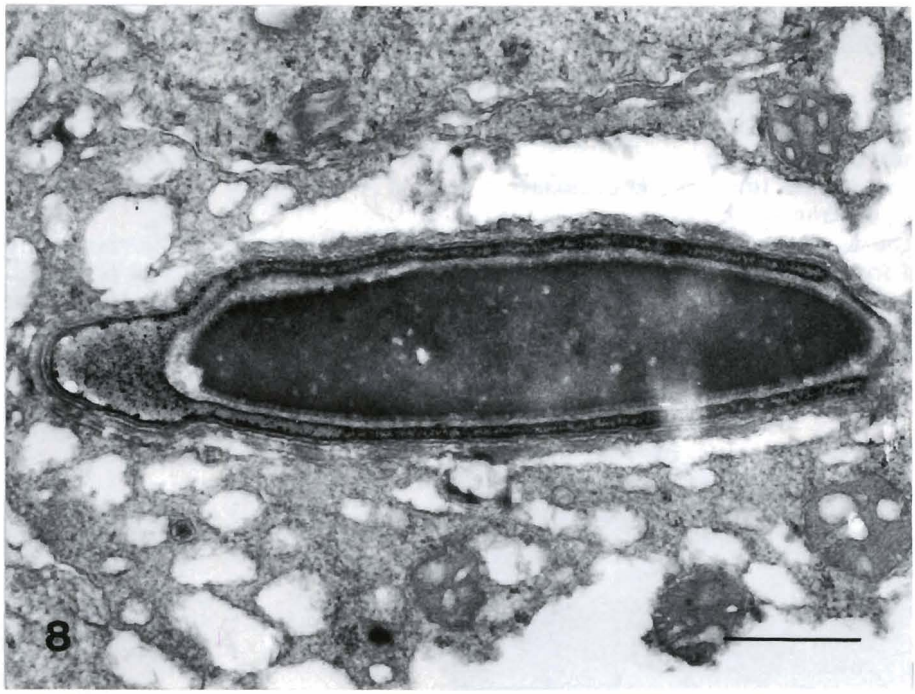
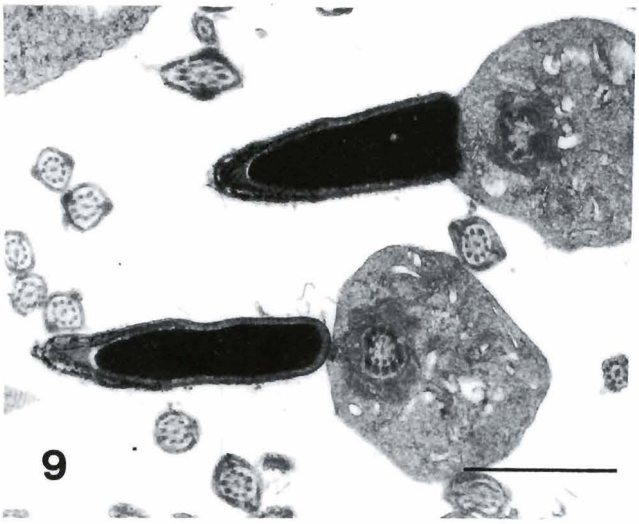


Fig. 8. In the late stage of spermiogenesis. TMPase activity is detected in the acrosome of the spermatid. x 35,000. Bar= 0.5 μm.



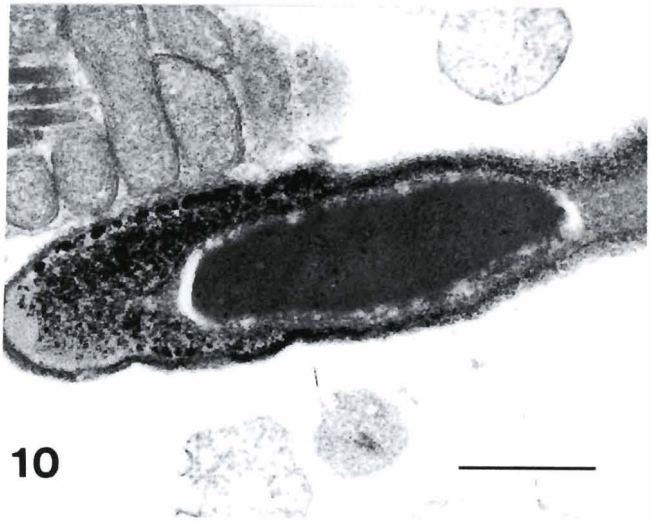


Fig. 9. The acrosomes of spermatozoa in the lumen of the seminifeorus tubule are positive for TMPase activity. x 20,000. Bar= 1 μm.

Fig. 10. TMPase activity is detected in the acrosomes of spermatozoa obtained from the head portion of the epididymis. x 36,000. Bar= 0.5 μm.

key role in the formation of the acrosomal system (Clermont et al., 1981; Shur, 1993). The innermost lamella in the Golgi complex of the spermatid becomes positive for TMPase activity as soon as the acrosomal vesicle appears. Positive TMPase activity is detected here until the acrosomal vesicle comes into contact with the nuclear envelope, expanding along its surface and wrapping the anterior portion of the spermatid nucleus to form the acrosomal cap. However, as soon as the acrosome has developed sufficiently and the Golgi complex detaches itself from the anterior pole moving down to the tail, the enzyme activity is no longer detected in the Golgi complex, although reaction deposits are still observed in the small vesicles and in the acrosome. These findings are in good agreement with those of Tang et al. (1982) and suggest that the production and transportation of TMPase from the Golgi complex is carried out by these small vesicles until the acrosomal formation is almost completed. The conjecture of Tang et al. (1982) regarding the involvement of each of the Golgi components in the acrosomal system formation thus seems to be well founded.

In spermatids, a multivesicular body is always associated with the Golgi complex during the Golgi and cap phases (Susi and Clermont, 1970; Tang et al., 1982; Eddy, 1988). According to Tang et al. (1982), the close topographical relationship of the multivesicular body and the Golgi complex, its positive reaction for NADPase and CMPase, as well as its positive ^{3}H-fucose labelling, clearly indicate its Golgi origin. Zhang and his colleagues, on the other hand, reported the presence of TMPase-positive multivesicular bodies in epithelial cells and suggested that these structures may be a kind of endosome, because of the fact that they originated from the surface plasma membrane marked with tracers (Zhang et al., 1991, 1992; Zhang and Seguchi, 1994). However, as already pointed out by Tang et al. (1982), this apparent discrepancy may be explained by the existence of two possible pathways for their formation;

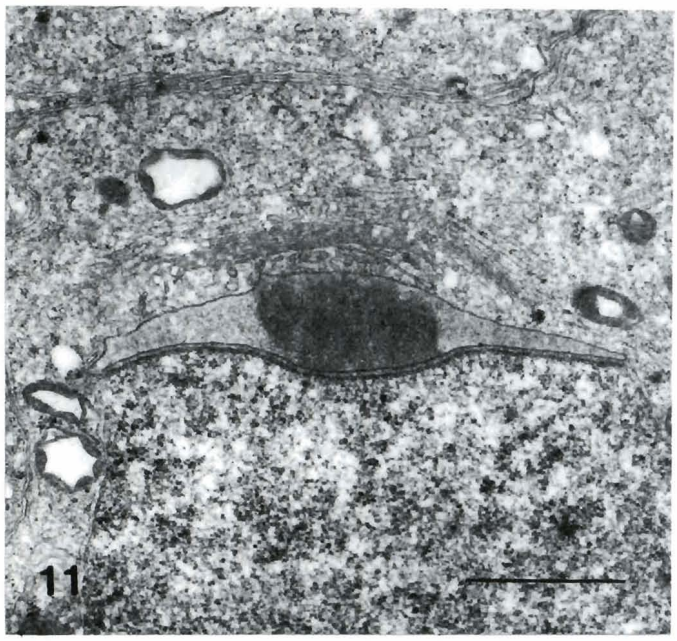
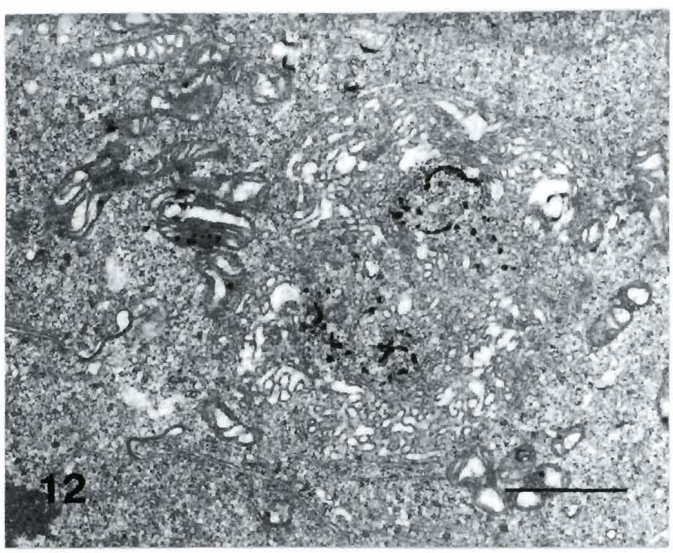


Fig. 11. No TMPase activity is observed in the specimens incubated in a substrate-free medium. x 21,000. Bar= 1 µm.



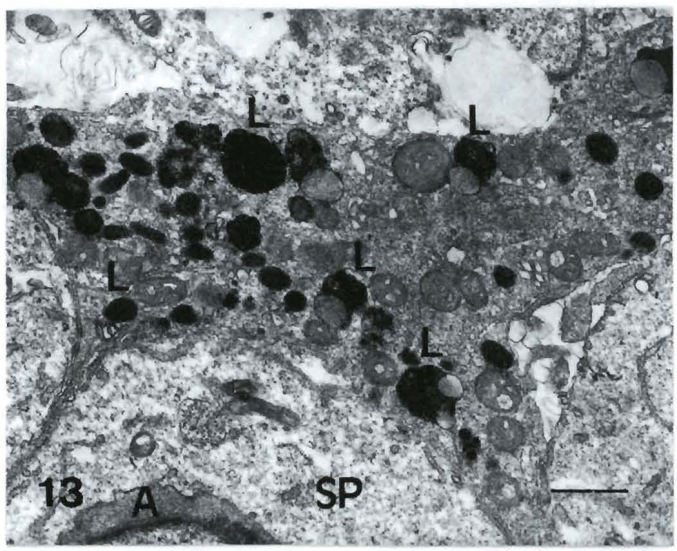


Fig. 12. The ACPase activity in the trans-face of the Golgi complex of a primary spermatocyte. x 16,000. Bar= 1 µm.

Fig. 13. ACPase activity is observed in the lysosomes (L) of Sertoli cells. The acrosome (A) of the spermatid (SP) in the cap phase is negative for the enzyme activity. x 10,000. Bar= 1 µm.

one that involves the direct participation of the Golgi complex, and another related to endocytosis. These two pathways may differ according to the cell type, or even according to the function within the same cell. In the present experiment, the multivesicular body was positive for TMPase as were the innermost lamellae of the Golgi complex, but since it is difficult to discern whether the matrix or the inclusion vesicles are stained (Tang et al., 1982), it is hard to say from which of these two pathways it may have arisen.

In vitro fertilization experiments conducted with mice revealed that the spermatozoon passes intact through the follicular cell layer surrounding the oocyte and that only when it reaches the zona pellucida is the acrosomal reaction first elicited (Wasserman, 1987). The acrosomal reaction, which enables the spermatozoon to penetrate the zona pellucida, is mediated by species-specific receptors in the zona pellucida (Wasserman, 1987; Shur, 1993). Together with other hydrolytic enzymes, the presence of TMPase in the acrosome may play an important role in the acrosomal reaction.

Allison and Hartree (1970) have biochemically and histochemically investigated the existence of the ACPase, in addition to several other lysosomal enzymes, such as arylsulphatase, ß-N-acetyl-glucosaminidase, phospholipase A and proteases, in the acrosome of ram spermatozoa using Gomori and Burstone methods. However, no morphological evidence was provided to support the reported data (Allison and Hartree, 1970). Clermont and his colleagues have clearly demonstrated the existence of CMPase activity, an ACPase isozyme, both in the acrosomal system and in the Golgi complex of the rat spermatids (Clermont et al., 1981; Tang et al., 1982; Thorne-Tjomsland et al., 1988). The localization pattern of this enzyme completely coincides with that of the TMPase activity reported in this paper. However, this was not the case with ACPase activity, as detected by the method of Robinson and Karnovsky (1983) using ß-glycerophosphate as substrate and cerium chloride as capture agent, as no reaction product was detected at all in the acrosomal system and Golgi complex of the spermatid, in spite of the fact that other structures, such as the lysosomes in the constituent cells of the seminiferous tubule, and the innermost lamella of the Golgi complex of spermatogonia, and of primary and secondary spermatocytes, were positively stained within the same section. The discrepancy between the present results and those of other researchers may be due to the different substrates employed. The present results seem to indicate that the Golgi complex stops producing ACPase during acrosomal formation, contributing instead TMPase to the acrosome. This latter structure lacks, therefore, ACPase (as detected with ß-glycerophosphate as substrate) but exhibits TMPase activity, being thus considered to belong to a type of lysosome different to that staining positively for ACPase as demonstrated by the present enzyme cytochemical method.

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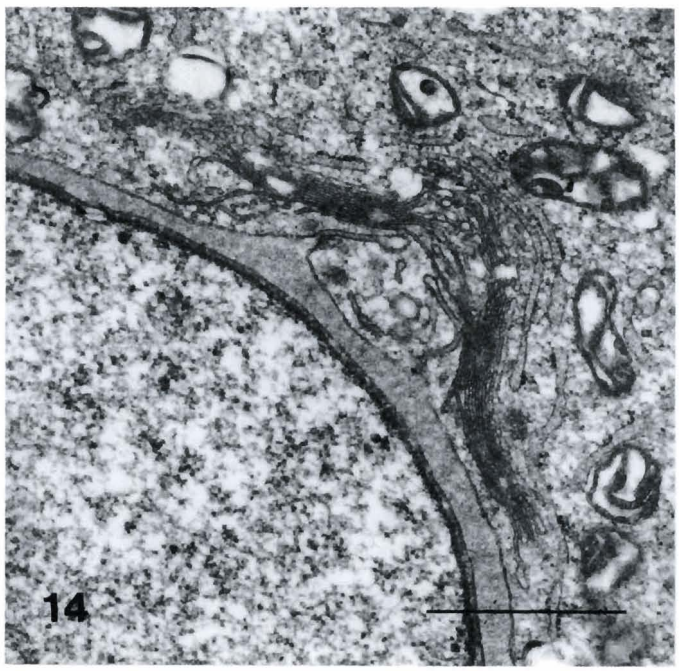

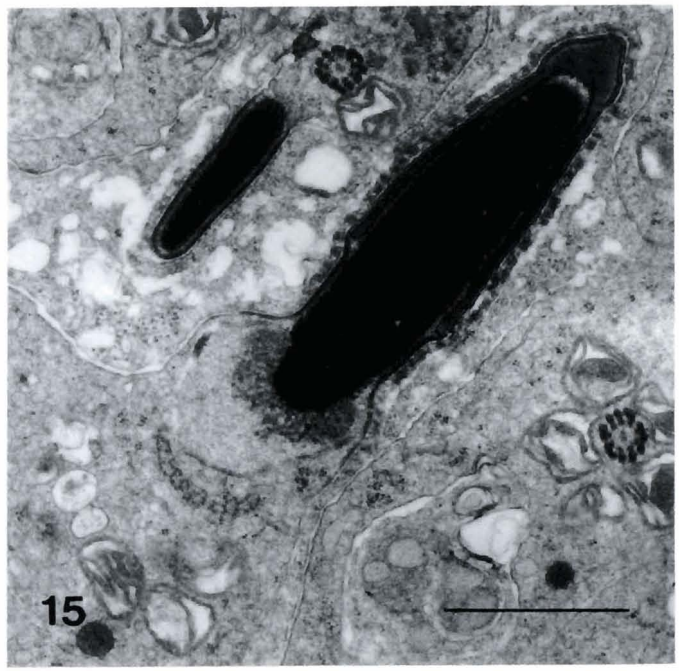

Fig. 14. Cap phase spermatid. Both the acrosomal system and the Golgi complex are negative for ACPase activity. x 26,000. Bar= 1 µm.

Fig. 15. No ACPase activity is observed upon addition of 10 mM tartaric acid to the incubation medium. x 25,000. Bar= 1 µm.

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