LOCALIZATION OF ADENOSINE TRIPHOSPHATASE ACTIVITY IN THE RAT SPERM TAIL AS REVEALED BY ELECTRON MICROSCOPY

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ABSTRACT

The epididymides of rat testis were fixed in glutaraldehyde and cut as frozen sections. The sections were incubated in lead nitrate solution containing as a substrate either ATP, AMP, creatinine phosphate, beta glycerophosphate, or phenyl phosphate. Then they were post-fixed in osmium tetroxide, embedded, sectioned, and examined with the electron microscope. In the sperm tail, when ATP is used as a substrate the reaction product (lead phosphate) is observed both in the tail filament complex and on the surface membrane of the mitochondrial helix of the middle piece. In the tail filament complex, this product is seen near the nine paired peripheral and two central filaments, and in the matrix between the outer coarse fibers. But the product is not observed within these filaments and fibers. In longitudinal sections, no periodicity of the deposits in the complex is observed. When the other phosphate compounds are used as substrates the reaction products appear on the surface membrane of the mitochondrial helix, and are not found in the tail filament complex. No distinctly different localization of the reaction products is observed when substrates other than ATP are used. Possible relationships between the structure and the function of the sperm tail are discussed in the light of these findings.

INTRODUCTION

Since the introduction of aldehyde fixatives for electron microscopy, it has been possible to demonstrate the localization of some enzymatic activities in cells and tissues at the electron microscope level (8, 9, 14, 26, 28).

Nelson, in 1958, published the first report on the electron microscopic localization of adenosine triphosphatase (ATPase) activity in the rat spermatozoon (23). He used a freeze-drying procedure and a calcium method. He has also reported on the localization of other enzymes in the sperm tail (24). Daems and co-workers have noted ATPase activity in the *Drosophila* sperm tail prefixed with osmium tetroxide (6). Recently, Tice and Barrnett have reported on the localization of rat testicular phosphatases, but these enzymes were not observed in the flagellum and surrounding mitochondria of the developing spermatid (33). From biochemical studies it is known that the motile sperm possesses not only ATP and ATPase but also succinic dehydrogenase, cytochrome oxidase, and other enzymes (19, 31). These enzymes are chiefly concentrated in the middle piece of the sperm tail (25). Morphological analyses of sperm tail movement have been performed in considerable detail by electron microscopy (1, 10, 11, 22, 30). It has been suggested that the sperm tail filaments might be contractile,

with ATP-ATPase serving as an energizing mechanism, as in muscle contraction (4, 10).

This paper will describe some electron microscope observations on the rat sperm tail in the epididymis incubated so as to reveal ATP-splitting and other enzymes after glutaraldehyde fixation.

MATERIALS AND METHODS

Young adult male rats of the Wistar strain were used in this study. Under ether anesthesia, both the caput epididymis and the cauda epididymis of the testes were removed and cut into blocks about 3 mm in diameter. The blocks were fixed for 1 to 2 hours in cold 6 per cent glutaraldehyde buffered to pH 7.2 with 0.1 M cacodylate, as recommended by Sabatini *et al.* (28). No sucrose was added to the fixative. After fixation, the blocks were washed, frozen, and sectioned at about 50 μ with a standard freezing microtome. The sections were incubated at room temperature for 5 to 60 minutes in Wachstein and Meisel's mixture (35) at pH 7.2. ATP, adenosine-5'-phosphate (AMP), creatinine phosphate, and beta glycerophosphate were used as substrates. Mölbert's medium (21) containing phenyl phosphate at pH 7.6 for demonstrating alkaline phosphatase was also employed. After incubation, the sections were washed, postfixed for 1 hour in cold 2 per cent osmium tetroxide buffered with *s*-collidine (3), dehydrated with ethanol, and embedded in Epon 812 (18).

Some sections were incubated in substrate-free media as controls. Other sections were immersed in 2 per cent osmium tetroxide for 1 hour before incubation. In some cases, 5×10^{-4} M *p*-chloromercuribenzoate for sulfhydryl group inhibition was added to Wachstein and Meisel's mixture containing ATP. This produced no observable effect in the present study.

Embedded blocks were oriented in a convenient direction and cut with a Porter-Blum microtome. Most of the thin sections were immersed in 2 per cent uranyl acetate (36) or lead solution (20), or in both, for counterstaining. Sections were examined with a Hitachi 11A or JEM 4C electron microscope. Some frozen sections were treated with ammonium sulfide after incubation for light microscope examination.

OBSERVATIONS

The reaction product (lead phosphate) is found as granules about 30 mμ in diameter. The product does not appear in the control preparations (Fig. 1). Although morphological and functional differentiation of sperm cells passing through the epididymis has been reported (5, 12), cytochemical differences in the sperm tails could not be detected in the caput epididymis and cauda epididymis in the present study. For convenience, the nomenclature in this paper is based on Fawcett's review (10).

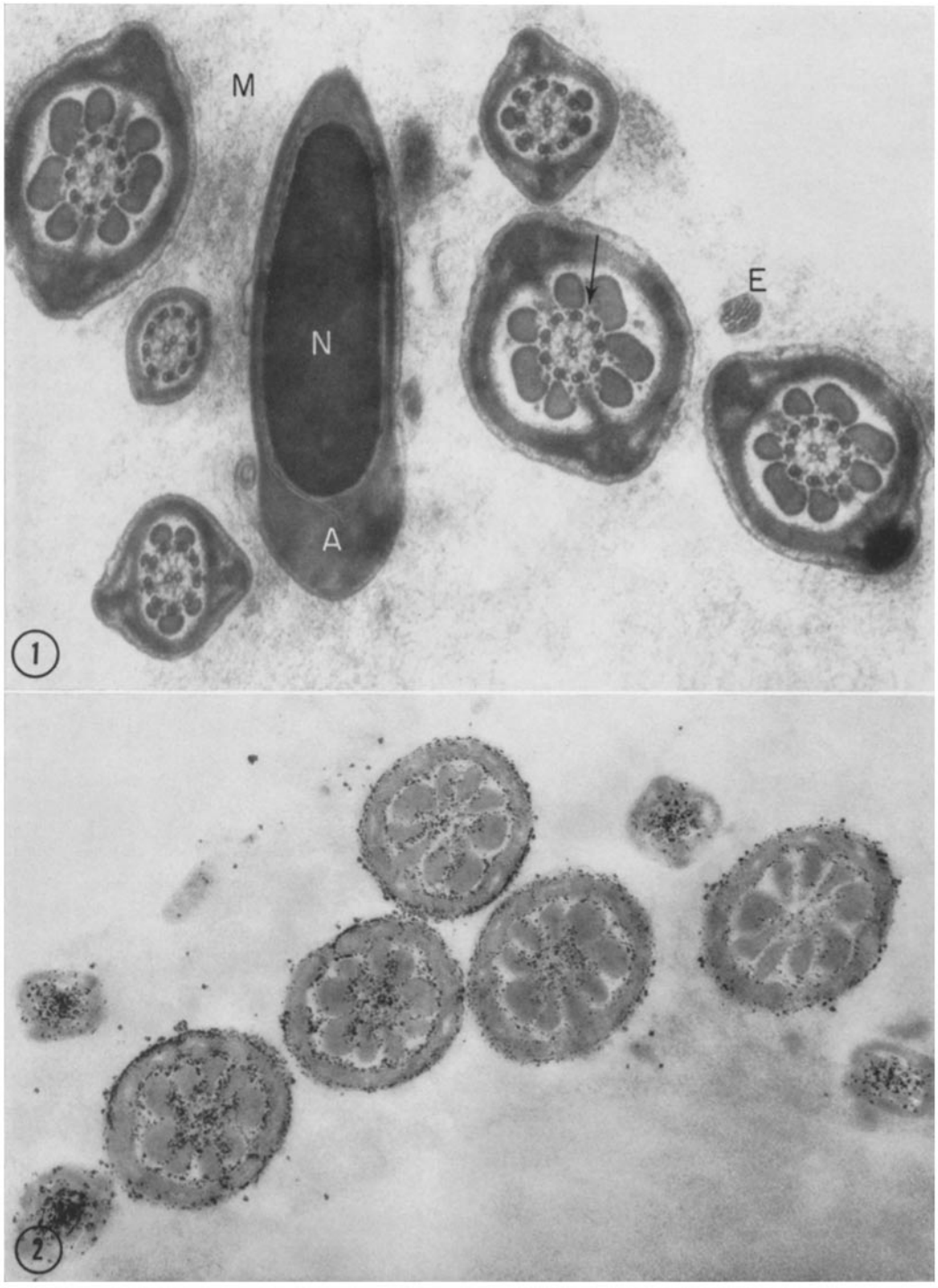
ATP as a Substrate

When ATP is used as a substrate, the reaction product is observed in the following three portions

All electron micrographs are of sections of rat epididymis. The specimens were fixed in glutaraldehyde and frozen-sectioned, and the sections were incubated, postfixed in osmium tetroxide, and embedded in Epon 812. Thin sections were stained with uranyl acetate followed by the lead solution of Millonig (20), or otherwise as indicated.

FIGURE 1 A control section incubated for 30 minutes in Wachstein and Meisel's mixture without a substrate, showing cross-sections of the main pieces of sperm tails and a section through one sperm head. No reaction product is seen in the sperm tail or in the nucleus (*N*) with the acrosome (*A*). The axial filaments, consisting of two central and nine paired peripheral filaments, are illustrated. Each peripheral pair is subdivided into a dense and a light subfiber. Surrounding the axial filaments, the outer coarse fibers and the fibrous sheath are seen. The spokelike structures between the central and peripheral filaments, and a granular component (arrow) between the outer coarse fibers are visible. The tip of an end piece is indicated by *E*. Outside the sperm cells, a fine filamentous component in the lumen of the epididymis is interpreted as representing a mucous substance (*M*). × 40,000.

FIGURE 2 Incubated for 30 minutes with ATP as substrate. This section was not stained. It shows cross-sections of the middle and main pieces of sperm tails. The reaction product (lead phosphate) is deposited as granules about 30 mμ in diameter in the central area of the sperm tail and along the surface of the mitochondrial sheath of the middle piece. × 30,000.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of the sperm tail: in the axial filament complex, between the outer coarse fibers, and on the surface membrane of mitochondria in the middle piece (Figs. 2 to 9). In cross-sections, the reaction deposits are found near the central and peripheral filaments and in the matrix surrounding the outer coarse fibers (Figs. 5 and 6). No deposit appears within the axial filaments themselves, but one can see the reaction product in the vicinity of the filaments. Relatively little reaction product is found among the outer coarse fibers of the middle and main pieces. It seems that the deposits are fewer in the peripheral area between these fibers than in the central area (Figs. 5 and 6). A granular component between the outer coarse fibers is visible even when specimens are incubated without a substrate (Fig. 1). This has been recognized after OsO_4 fixation (30). In the middle piece, many deposits can be seen on both the outer and inner surfaces of the mitochondrial helix surrounding the axial filament complex and the outer coarse fibers. However, reaction product granules are not found within the mitochondria themselves (Figs. 2 to 7). In the main piece, the deposits are not seen in the fibrous sheath nor on the plasma membrane (Figs. 4 to 6, 8, and 9). In the end piece, a reaction product is not clearly observed. In the head region of the spermatozoon, a few deposits are observed on the acrosomal membrane enclosing the acrosome, but not inside the nucleus (Fig. 5).

When epithelial cells of the epididymis are examined, the deposits are observed within the microvilli. However, the number of deposits is far less than in the sperm tail in the same section (Fig. 4).

In longitudinal sections of sperm tails, the reaction deposits seem to be distributed longitudinally at random in the axial filament complex and around the mitochondria. No periodicity of the deposits can be observed (Figs. 8 and 9). The reaction product is not seen in the segmented fibrous sheath or on the plasma membrane.

When the duration of incubation is short, it seems that the product appears first on the mitochondrial surface, and not in the tail filament complex (Fig. 3).

Other Phosphate Compounds as Substrates

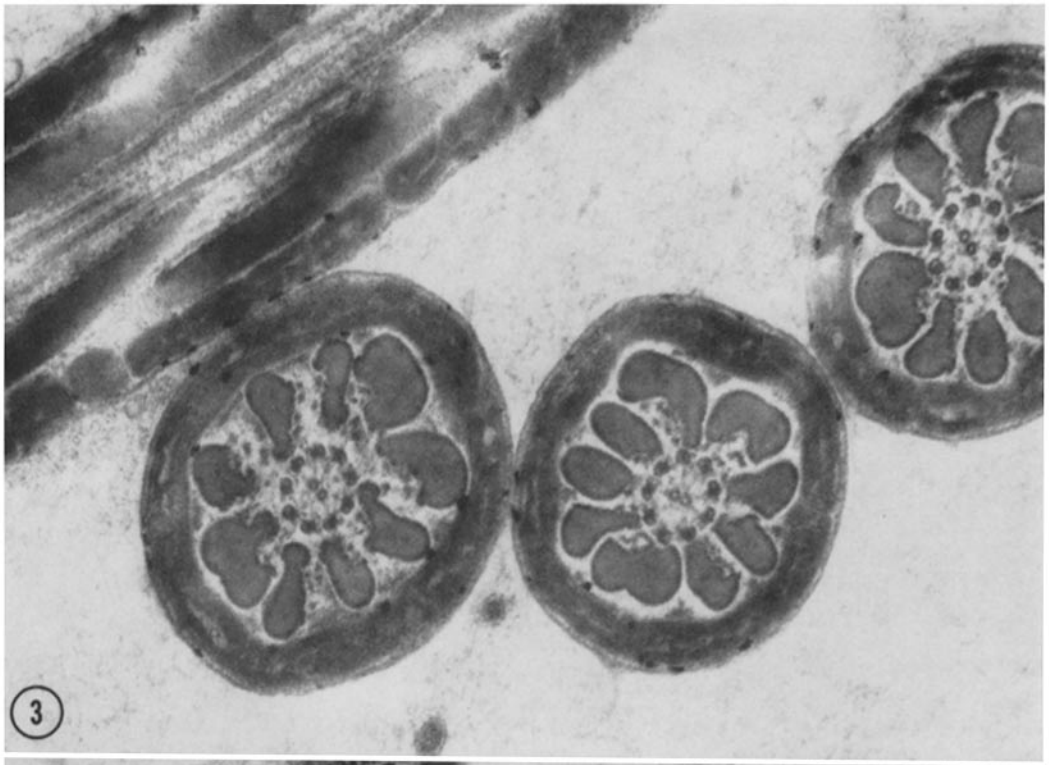
The reaction product appears similarly localized in the sperm tail after AMP, creatinine phosphate, beta glycerophosphate, or phenyl phosphate is used as a substrate. When these substrates are used, however, the reaction deposits are distributed on the mitochondrial surface in the middle piece of the sperm tail, but are seen very sparingly if at all in the tail filament complex. This is the main difference in the localization of deposits when these phosphates are used as substrates instead of ATP. Fig. 10 shows cross-sections of the middle and main pieces and the head after phenyl phosphate has been used as a substrate. In this figure, a few deposits are seen near the axial filaments of the main piece.

DISCUSSION

The results of electron microscopic observations on the localization of ATPase activity in cells seem to differ among the various investigators. Conflicting results have been obtained even in the same kind of tissue, muscular tissue for example. Tice and Barrnett have described the localization of ATPase in the A band of rat cardiac muscle prefixed or not prefixed with hydroxyadipaldehyde (32). In contrast, de Beyer *et al.* have reported that ATPase localization is in the Z band of mouse heart muscle briefly prefixed in osmium tetroxide (7). Further, Hori and Takahashi (15) have demonstrated ATPase activity in the A band and in the sarcoplasmic reticulum as well as in the

FIGURE 3 Incubated for 5 minutes with ATP as substrate. A few granules of the reaction product are seen on the surface of the mitochondrial sheath in the middle pieces of sperm tails sectioned both transversely and obliquely. No deposits are seen in the axial filament complex or among the outer coarse fibers in this section. × 45,000.

FIGURE 4 Incubation for 30 minutes with ATP as substrate. The section shows sperm tails and the microvilli of the epithelium of the epididymis. The reaction product is seen in the sperm tail and on its mitochondrial surface. The product is also seen within the microvilli, where the number of granules is much less than in the sperm tails. × 35,000.



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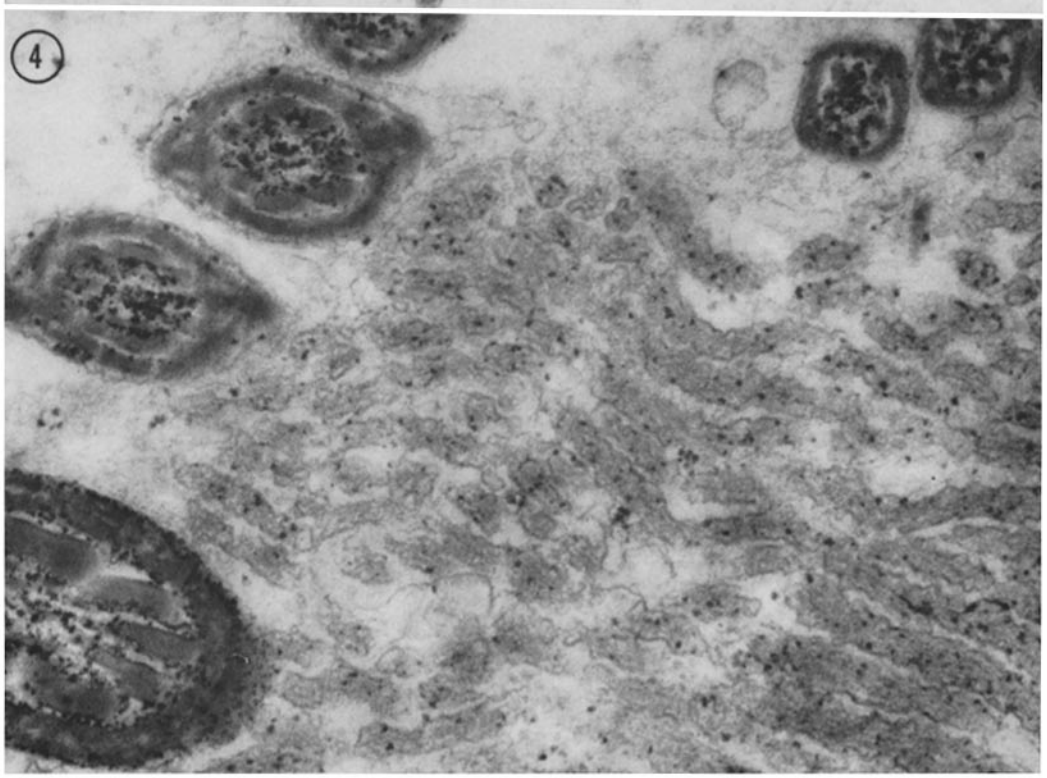

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mitochondria of rat skeletal muscle treated by freezing-substitution. In rat colonic epithelium prefixed in formalin, Otero-Vilardebó *et al.*, using the same technique, have shown that ATPase is localized on the cristae of mitochondria as well as on the microvilli and the cell membrane, although in the distal tubule of rat kidney the mitochondria show no reaction product (27). Lansing and Lamy have reported that in rotifer cilia prefixed in OsO_4 for 6 minutes the reaction granules of ATPase appear in the vicinity of certain peripheral filaments (16). In the *Drosophila* sperm tail briefly prefixed in OsO_4, Daems *et al.* have observed that the ATPase activity is located in the "intersatellite space," outside the peripheral filaments (6). These diverse results may very well reflect differences in tissue treatment and fixation and the use of different tissues from various species of experimental animals.

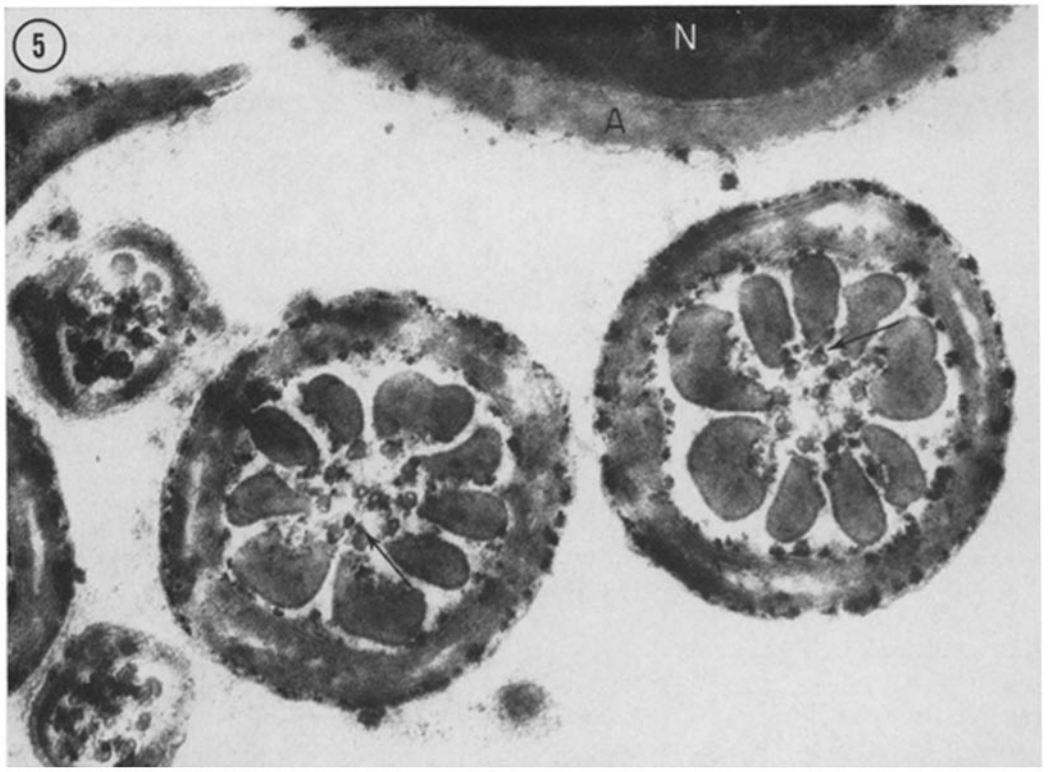
In our results with rat sperm tails, when ATP is used as a substrate significant deposits of lead phosphate appear in the axial filament complex, in the matrix between the outer coarse fibers, and on the surface of the mitochondrial sheath. The surface membrane of the mitochondria also shows the deposits when other phosphates are used as substrates. The axial filament complex and the matrix between the coarse fibers do not show the deposits when phosphates other than ATP are used as substrates.

The deposits are also found within the microvilli of the epididymis epithelium when ATP is used as a substrate, a result similar to that reported in the proximal tubule cells of rat kidney by Wachstein and Besen (34). Since the number of deposits is much greater in the sperm tail than in the epithelial microvilli in the same section (Fig. 4), the activity of the ATPase is considered to be higher in the sperm tail than in the microvilli.

With respect to the rat spermatozoon, Nelson (23) has stated that "ATPase is confined to the nine longitudinal fibers of the outer axial fiber bundle. In none of these is there any darkening of the cortical helix. However, when glycerophosphate is substituted for ATP, regions of the helix do show signs of deposition of Ca-phosphate whereas the fibers do not show increase in density." He suggested that the phosphatase activity is in the mitochondrial helix. It is worth noting that his nine longitudinal fibers probably would not correspond to the nine peripheral pairs of filaments described in the present paper, but rather are to be identified with the outer coarse fibers, to judge from his micrographs (23, 25). Although inhibition tests have not been completed in this study, the observation that the axial filament complex shows reaction product only when ATP is used as a substrate suggests that ATPase is located in this complex, as well as in the matrix between the outer coarse fibers, but probably not within the filaments and fibers themselves. Although ATPase and other phosphatases have not been observed in the spermatid flagellum in the rat testis after glutaraldehyde fixation (33), our results might be explained on the assumption that the sperm tail in the epididymis has greater enzymatic development than the spermatid flagellum in the testis.

Mitochondrial ATPase activity has been demonstrated in some tissues at the fine structural level, whether the tissues have been treated with aldehyde fixatives (2, 17, 27) or prepared without chemical fixatives (15, 17, 29). However, none of these reports has demonstrated that the activity is on the surface membrane of the mitochondria rather than on the cristae. Moreover, when other substrates are substituted for ATP, the reaction product appears also on the surface membrane of the mitochondria. On this surface there may be phosphatase with the capacity to cleave the linkages in several different kinds of phosphates, including ATP. Similar phosphatase activity has been reported in the Golgi complex of the rat spermatid (33). As shown in the sperm tails in Fig. 3, the reaction products appear first on the

FIGURES 5 AND 6 Incubated for 30 minutes with ATP as substrate. Many granules of reaction product can be seen in the axial filament complex and near the outer coarse fibers of sperm tails. The deposits are also exhibited on both the inner and outer surfaces of the mitochondrial helix, but not inside the mitochondria. Some deposits located between the subfibers of the peripheral pairs are visible in Fig. 5 (arrows). Others are seen near the central pair or spokes in Fig. 6 (arrow). A few deposits are found on the surface of the acrosome (*A*) but not within the nucleus (*N*). × 60,000.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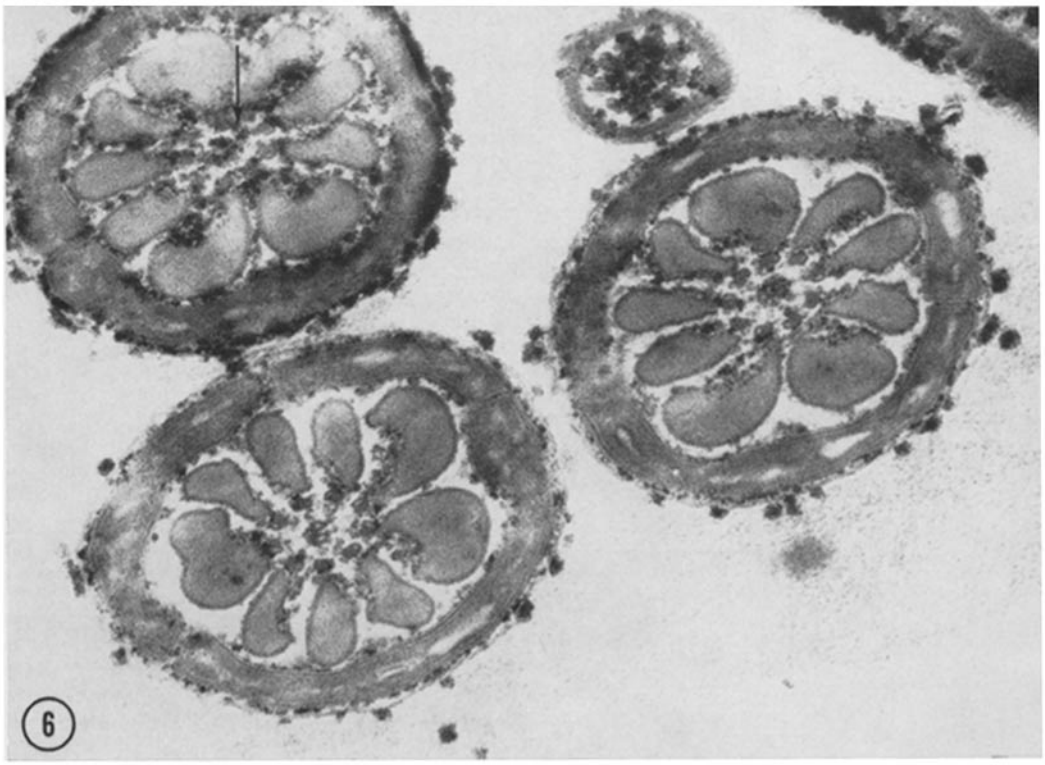
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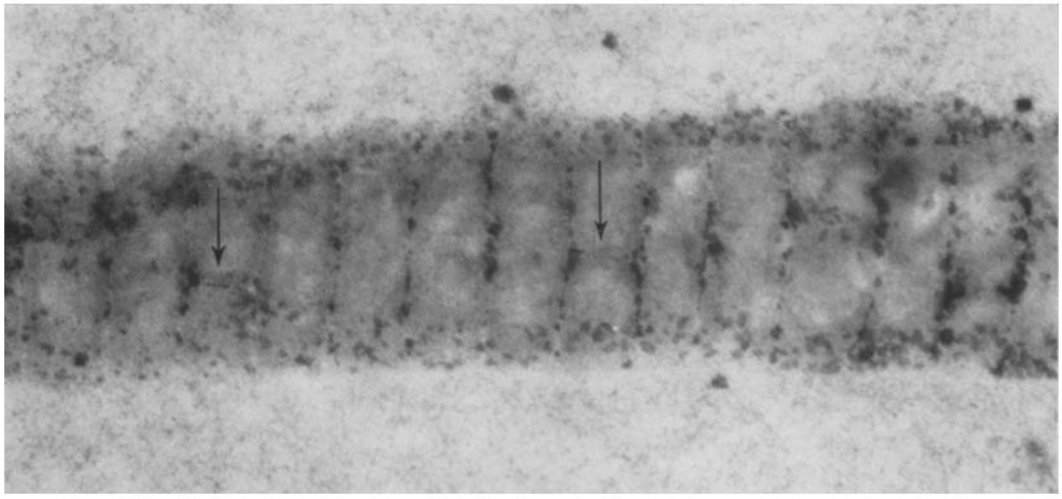

FIGURE 7 Incubated for 20 minutes with ATP as substrate. A slightly oblique section of the middle piece of a sperm tail. The reaction product is seen on the surface of each mitochondrial turn. The junctions between mitochondria are indicated by the arrows. Stained with uranyl acetate. × 70,000.

surface of the mitochondria after brief incubation using ATP as a substrate. With ATP as a substrate, the deposits do not appear in the mitochondria of the lining epithelium of the epididymis but are seen within the microvilli to a small extent, whereas in the sperm tail many deposits are found on the surface of the mitochondria as well as in the axial filament complex. Thus it seems that the enzymes of the mitochondria may be more chemically resistant than the others.

The present findings appear to confirm and support to some extent previous studies of the structure and function of sperm tails. ATPase in the axial filament complex might serve to mediate contraction of the filaments as ATPase in muscle mediates contraction of myofilaments. ATPase activity has been reported in the cross-bridges between the thick and thin myofilaments in rat heart muscle (32). If the axial filaments contract in the same way that myofilaments do, and if the spokes of the filament complex correspond to the cross-bridges between myofilaments, the spokes might show signs of ATPase activity. The results of this study do not show very clear direct evidence of similarities between axial filaments and myofilaments, though Gibbons and Grimstone have postulated morphological similarities between them (13). Fawcett has suggested from his comparative studies of sperm tails (11) that the outer coarse fibers may be contractile and the fibrous sheath non-contractile. In the present study the interfibrillar matrix shows signs of ATPase activity and the fibrous sheath does not. The localization of ATPase activity is very close to the granular component of the interfibrillar matrix (30).

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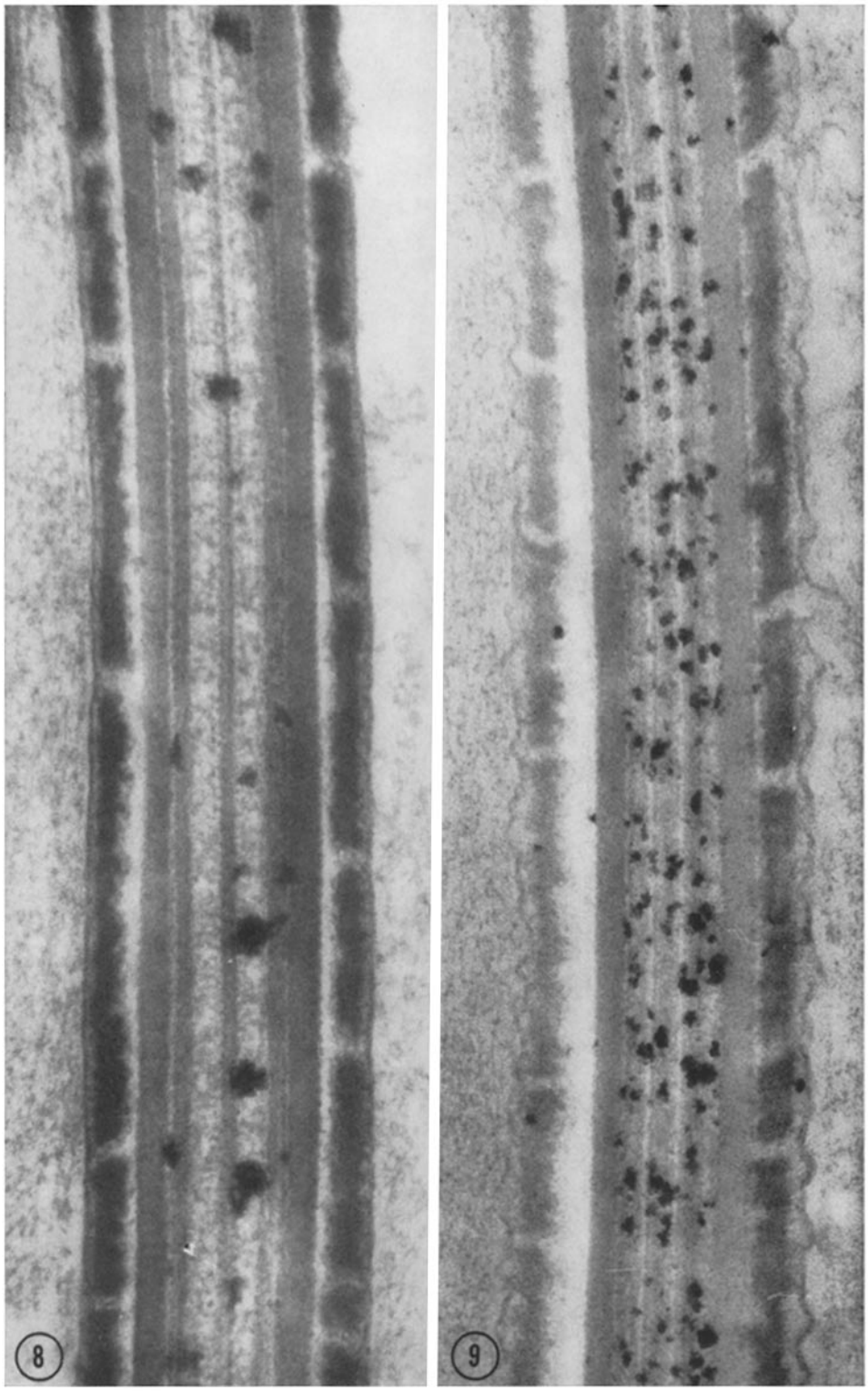
Some of the results reported in this paper were

FIGURES 8 AND 9 Incubated with ATP as substrate. Longitudinal sections through the main pieces of sperm tails. Several deposits are seen in the axial filament complex in Fig. 8 (incubated for 10 minutes), whereas many more deposits are demonstrated in the complex in Fig. 9 (incubated for 30 minutes). No periodicity of the deposit is evident. No deposit is seen on the outer coarse fibers, segmented fibrous sheath, or plasma membrane. Fig. 8, × 100,000; Fig. 9, × 85,000.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presented at the 10th Symposium of the Society of Electron Microscopy, Japan, 1963, and at the 14th Symposium of the Society for Cellular Chemistry, Japan, 1963.

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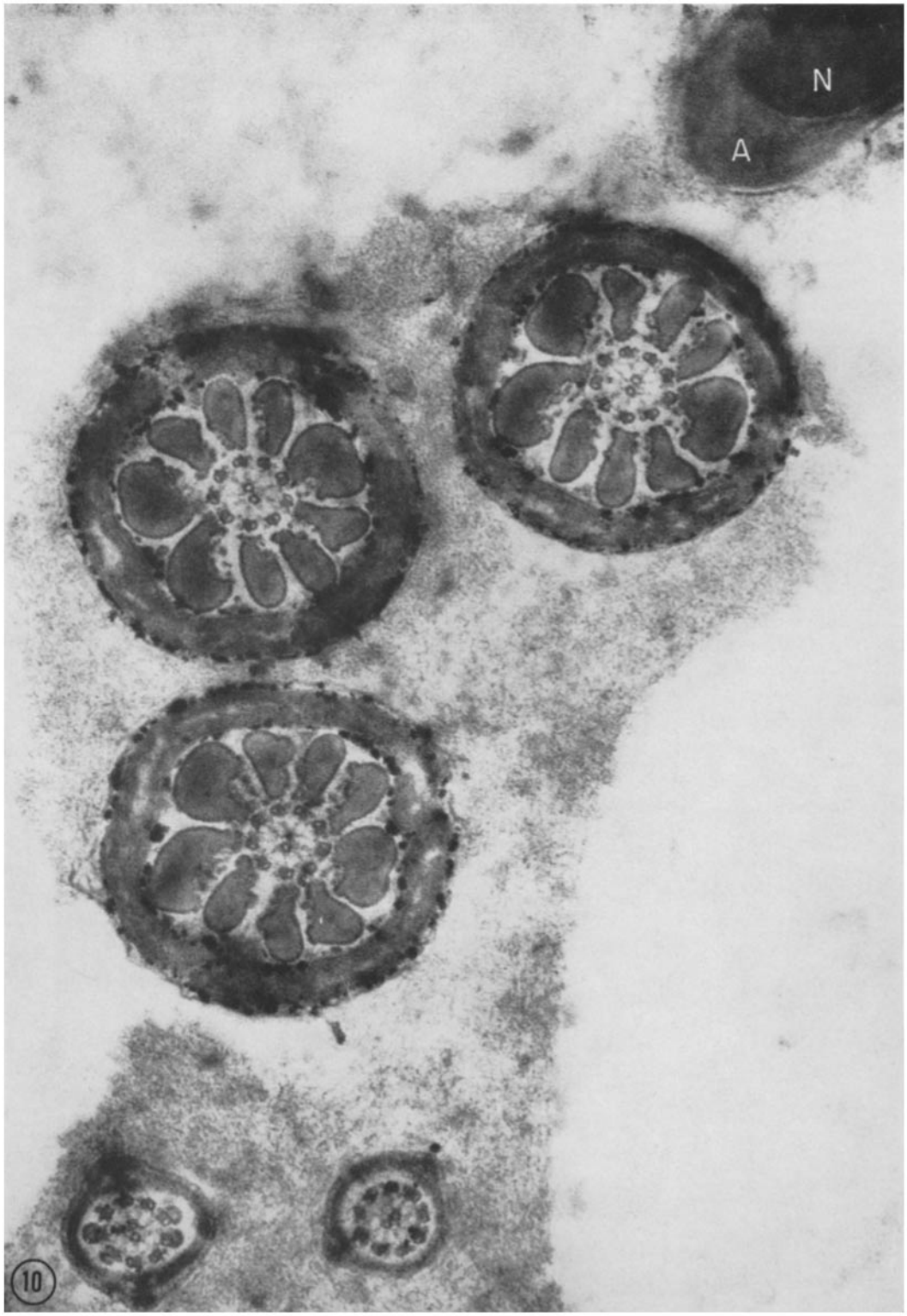
FIGURE 10 Incubated in Mölbert's mixture for 30 minutes with phenyl phosphate as substrate. The section shows cross-sections of the middle and main pieces of sperm tails and a section through one sperm head with an acrosome (*A*). The reaction product is found on the surface of the mitochondrial helix. No deposits are observable in the axial filament complex, between the outer coarse fibers, or in the nucleus (*N*). However, a few deposits appear in the axial filament complex of the main piece. × 48,000.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