THE FINE LOCALIZATION OF NUCLEOSIDE

TRIPHOSPHATASE ACTIVITY

IN THE RETINA OF THE FROG

DANTE G. SCARPELLI, M.D., and ELSON L. CRAIG

From the Laboratory of Cellular Pathology, Department of Pathology and Department of
Ophthalmology, The Ohio State University, Columbus, Ohio

ABSTRACT

Nucleoside triphosphatase (NTPase) activity was demonstrated at the submicroscopic
level in the frog retina by the Wachstein-Meisel method utilizing various purine and
pyrimidine nucleosides. Under the electron microscope magnesium-activated NTPase was
localized in the outer and inner segments, and in the plexiform layers. NTPase active sites
in the cones were localized diffusely in the 70 to 80 A interspaces between the double
membranes of the stacked lamellae and in the investing cytoplasm. In the rods, on the
other hand, sites of activity were observed at the periphery of the stacked lamellae as discrete
electron opaque deposits measuring 1000 to 1500 A which interdigitated between
the lamellae for short distances. Deposits of reaction product appeared more numerous in
rods of dark-adapted frogs stimulated with monochromatic light with a wave length of 510
mμ. Enzyme activity was also observed in mitochondria of the rod and cone ellipsoids.
In the outer and inner plexiform layers NTPase active sites were present on and between
the membranes of axons and the plasma membranes of some of the neurons.

Studies on the chemistry of visual excitation have
centered primarily on the structure, interactions,
and kinetics of the photosensitive pigments in the
rods and cones. These investigations have yielded
a model of how light energy may be converted into
chemical energy. It has been shown that a single
quantum of light is capable of exciting a retinal
rod or cone (14, 2, 42). In view of the extreme
sensitivity of the photoreceptors to light energy it
has been postulated that additional mechanisms
must be present to amplify weak light stimuli (43,
23). During a systematic histochemical study of
the frog retina (34) adenosine triphosphatase
(ATPase) activity was localized in the photo-
receptors. In the present report, the localization
of nucleoside triphosphatase (NTPase), an enzyme
capable of splitting purine and pyrimidine tri-
phosphate and of supplying additional energy to
the receptors, has been studied with the electron
microscope in the frog retina.

MATERIALS AND METHODS

Both dark- and light-adapted frogs of the species
Rana pipiens were used. Dark adaption was accom-
plished by placing the animals in a totally dark area
for 24 hours. The rods were stimulated by exposure
of dark-adapted frogs to monochromatic light with
a wave length of 510 mμ for 40 seconds. For dark-
adapted animals a dim red light was used to perform
the dissection, and incubation was carried out in the
dark. The frogs were decapitated and the eyeball
dissected in Tris-maleate buffer at pH 7.2. The lens
and vitreous were removed and the exposed retina
was carefully teased loose from the choroid and
pigment epithelium. The retina was divided into
three pieces: one piece was placed directly into the
incubation medium, and the other two were fixed
for 7 minutes in 1 per cent osmium tetroxide con-
taining 0.2 M sucrose and buffer to pH 7.1.

The ATPase medium employed was that of
Wachstein and Meisel (40) which is made up by
8.3×10^{-4} M ATP, 3.6×10^{-3} M Pb(NO$_3$)$_2$ and 1 $\times$

10^{-2} M $MgSO_4$ in Tris-maleate buffer at pH 7.2. Sucrose (0.25 M) was added to afford protection of cellular fine structure during incubation (32).

Additional experiments were performed in which 8.3×10^{-3} M adenosine diphosphate (ADP), inosine triphosphate (ITP), guanosine triphosphate (GTP), uridine triphosphate (UTP), and sodium β-glycerophosphate were substituted for ATP. 3×10^{-3} M of dimercaptopropanol (BAL) was added in several experiments to inhibit non-specific alkaline phosphatase activity and enhance ATPase activity (26). 3×10^{-4} M P-chloromercuribenzoate (PCMB) was added to inhibit sulfhydryl groups. Tissue incubated in a medium devoid of nucleoside triphosphates served as an additional control. Tissue was incubated for 20 minutes at 37°C with constant agitation on a mechanical agitator. Unfixed reacted tissue and one piece of osmium-prefixed tissue were postfixed in cold 1 per cent osmium tetroxide for 45 minutes. The remaining tissue was placed in cold 2 per cent buffered formalin (pH 7.4) and stored in the cold for 24 hours before being embedded as suggested by Essner *et al.* (8). The tissue was placed in 70 per cent ethanol and cut into 1 mm³ pieces for dehydration and embedding. Tissue was embedded in either a 77:23 butyl-methyl methacrylate mixture or EPON 812 (5). Some tissue was dehydrated in ascending concentrations of EPON 812 thus eliminating alcohol dehydration.

Duplicate experiments were done to assay NTPase activity. Whole retinas prepared as described above were incubated for 30 minutes in the appropriate medium after which the retina was removed for subsequent protein determinations (11). The inorganic phosphate concentration of the supernatant was determined by the method of Fiske and Subbarow (9).

RESULTS

Although the fine structure of tissue prefixed in osmium tetroxide was well preserved, demonstra- tion of NTPase activity was unsuccessful as a result of complete enzyme inhibition. Incubation of fresh retina in a medium containing 0.25 M sucrose resulted in the preservation of a considerable degree of fine structure, especially of mitochondria, and the fine localization of NTPase active sites in the outer and inner segments of the photoreceptors and both plexiform layers.

In both light- and dark-adapted frogs NTPase activity in the retina was present in the outer segments and ellipsoids of the rods, primary and accessory cones, and in the plexiform layers. Sites of NTPase localization were characterized as intensely electron opaque, granular deposits under the electron microscope. In the cone outer segments NTPase activity was localized in the 70 to 80 A interspaces between the double membranes of the stacked lamellae and in the cytoplasm irrespective of the nucleoside triphosphate used as substrate. The lead phosphate deposits were most intense at the base of the cones (Figs. 1 and 2). NTPase active sites in the cone ellipsoid mitochondria appeared as clusters of lead phosphate granules and were so large that they appeared to be associated with both the membranes of the cristae and the mitochondrial matrix (Figs. 3 and 4). When ITP was used as substrate fewer and smaller lead phosphate deposits were found in the mitochondria of the rod ellipsoids (Fig. 5). In the primary cone ellipsoids the oil droplets were devoid of lead deposits. NTPase activity in the rod outer segments was localized at the periphery of the stacked unit disks as discrete deposits, measuring 1000 to 1500 A, which interdigitated between the disks for short distances (Figs. 6 and 7). These deposits appeared more numerous on the rods of dark-adapted frogs stimulated with 510 mμ light.

FIGURE 1

Accessory cone of the frog retina. Dense deposits of lead indicate sites of NTPase activity. These are most intense in the outer segment, especially at its base. Mitochondrial NTPase active sites appear as irregularly shaped, dense, granular deposits. $\times$ 13,600.

FIGURE 2

Accessory cone of the frog retina. A higher magnification of the cone shown in Fig. 1. Enzyme-active sites appear localized between the lamellae. Dense amorphous deposits of lead reaction product are visible in the cytoplasm of the outer segment. Note the absence of NTPase activity in the membranes adjacent to the foldings of the lamellae (arrow). $\times$ 52,000.
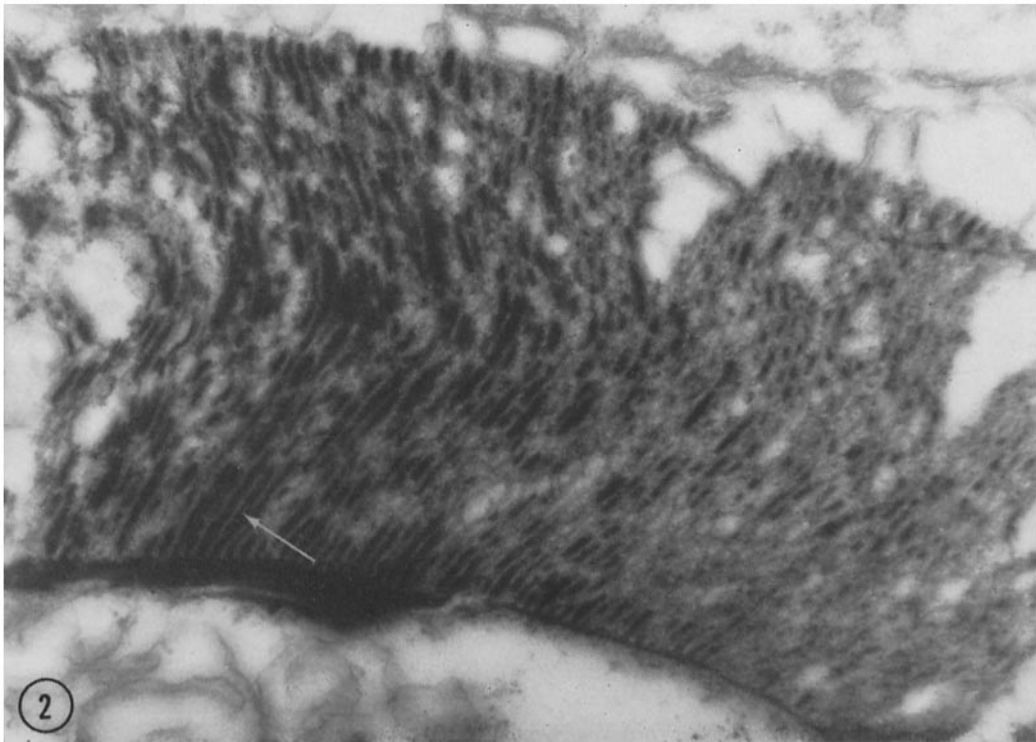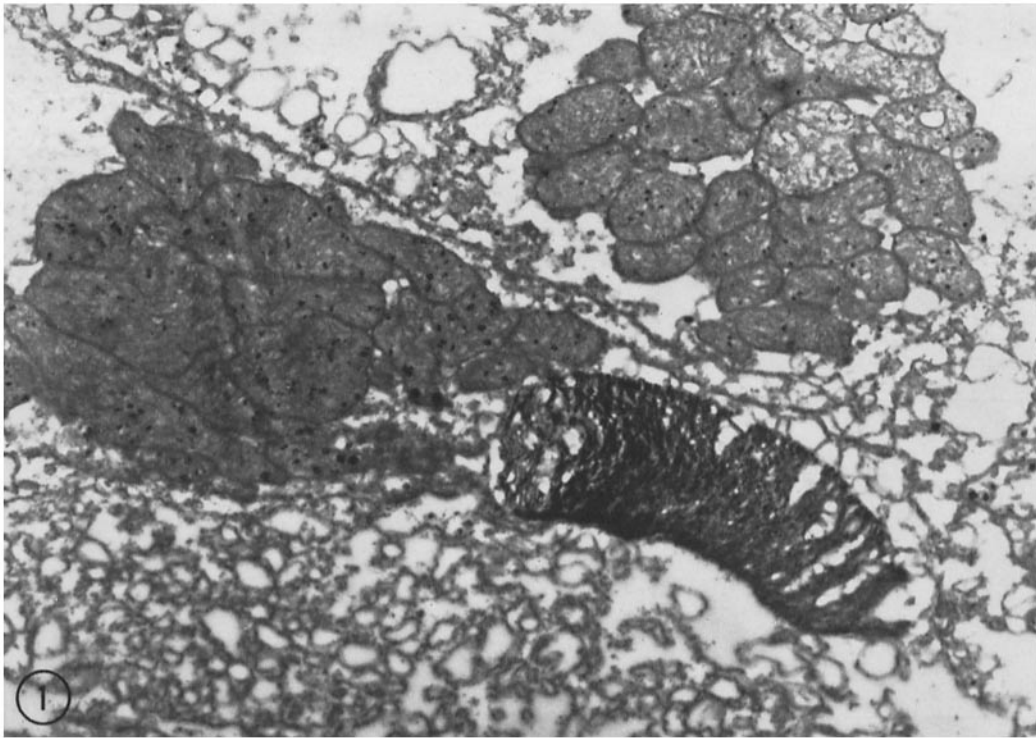
The localization of NTPase in the photoreceptors is summarized in Fig. 8. Enzyme activity in the outer and inner plexiform layers was present on and between the membranes of axons and the plasma membranes of neurons (Figs. 9 to 11).

Enzyme assays of NTPase activity of whole retinas utilizing various nucleoside triphosphates are summarized in Table 1. ATP, GTP, and UTP

TABLE I

Dephosphorylation of Various Nucleoside Triphos-phates by NTPase in the Frog Retina

Activity is expressed in μg P released in 30 minutes per mg of protein nitrogen.

Substrate	μg P/mg protein nitrogen
ATP	1.20
ATP with 3 $\times$ 10^{-3} M BAL	1.30
ATP with 3 $\times$ 10^{-4} M PCMB	0
ADP	0.10
ITP	0.43
GTP	0.91
UTP	1.10
Sodium β-glycerophosphate	0

were dephosphorylated with equal ease. ITP was dephosphorylated more slowly. ADP was dephosphorylated only slightly and β-glycerophosphate not at all. The addition of BAL did not significantly alter the dephosphorylation of ATP while PCMB completely inhibited it. These results indicate that NTPase activity is not due to non-specific alkaline phosphatase and that the enzyme is a sulfhydryl-dependent one.

DISCUSSION

Localization of enzyme-active sites in cells at the electron microscope level depends on the degree of tissue preservation and the amount of enzyme activity that survives the rigors of tissue preparation and histochemical incubation (33). In the case of the Wachstein-Meisel ATPase method lead ions markedly inhibit ATPase activity (25). Wachstein and Lange (41) also observed the complete inhibition of mitochondrial ATPase by prefixation in cold neutral formalin. More recently, however, Persijn *et al.* (27) were able to localize ATPase in the mitochondria of liver cells which had been prefixed in cold osmium tetroxide for 3 minutes. The successful demonstration of this enzyme by Essner *et al.* (8) in liver after brief osmium tetroxide fixation is at variance with our results. This variation may be explained by one or more of the following possibilities. 1) Since tissue blocks were used in their experiments and osmium tetroxide penetrates tissues very slowly, fixative may not have penetrated sufficiently into the tissue to inhibit all of the ATPase present. 2) Inhibition of all the NTPase activity in the retina by prefixation in osmium tetroxide in our experiments may be due to the fact that the dissection of retina free from the choroid and pigment epithelium prior to fixation as a thin film of tissue decreased the problem of penetration of fixative to negligible significance. 3) Another possibility may be that the ATPases present in the liver are indeed resistant to brief osmium fixation while those of the retina are not. 4) The amounts of ATPase in the liver may be of sufficient magnitude that, despite inhibition by osmium, enough enzyme activity


FIGURE 3

Ellipsoid of the principal cone of the frog retina. Sites of NTPase activity in the mitochondria appear as electron opaque granules of varying size and shape. Note the absence of reaction product in the oil droplet. $\times$ 18,500.

FIGURE 4

A high magnification of a portion of a princiapl cone ellipsoid. The reaction product granules do not appear to be membrane associated. $\times$ 52,000.

FIGURE 5

NTPase active sites in a rod and cone when ITP was utilized as substrate. Deposits of reaction product in mitochondria of the rod ellipsoid in the upper left portion of the micrograph are smaller and fewer than those in the cone ellipsoid below it. $\times$ 13,000.
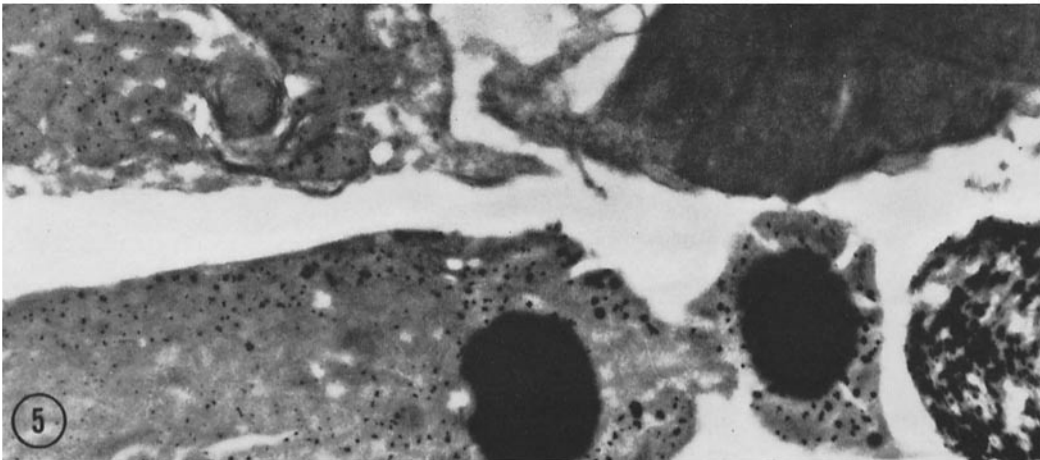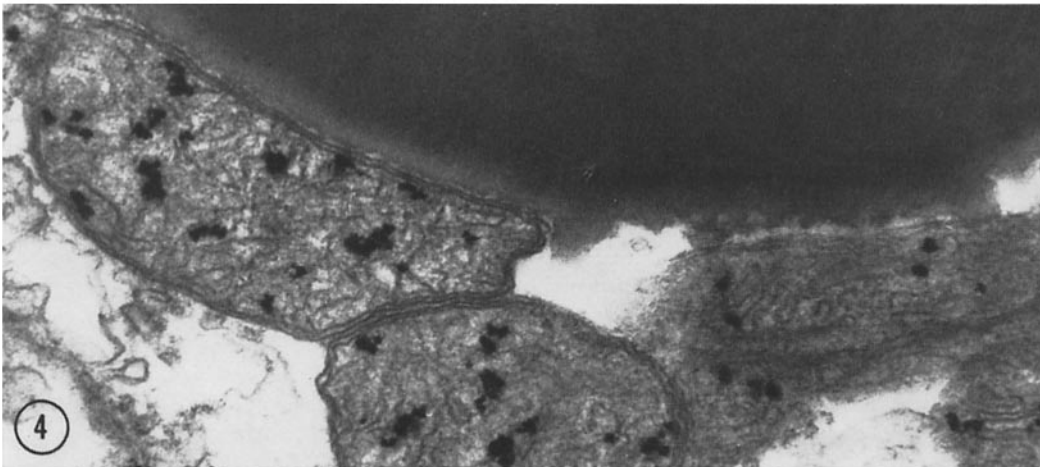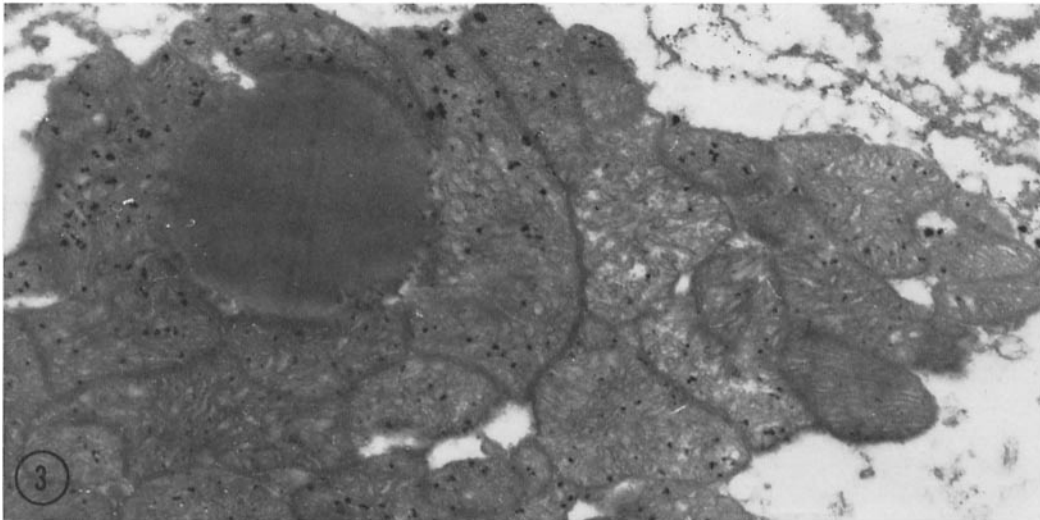
remains to be demonstrable histochemically. Dephosphorylation of a variety of nucleoside triphosphates in addition to ATP by the enzyme-active sites in the frog retina is also a property of ATPases in other tissues (3, 10, 17, 19). Use of the term ATPase for the nomenclature of these enzymes is retained solely for reasons of convention (18). In the frog retina the substrate specificity of enzyme active sites is sufficiently wide and hence they are better referred to as NTPase.

The demonstration of NTPase activity in the frog retina was first reported by de Berardinis and Aurrichio (6) utilizing ATP as substrate and magnesium ion as the activating cation. In their experiments they also found significant dephosphorylation of ADP. Under the experimental conditions employed in the present study ADP was also dephosphorylated, but only very slowly. Although this might represent nucleoside diphosphatase activity (28) it is most probably due to spontaneous phosphorolysis. More recent studies by Sekoguti (36) on the NTPase activity of the retina have shown that differences exist between the ATPase activity of whole retina homogenates and suspensions of rod outer segments with respect to the effect of activators and inhibitors. These results indicate that different NTPases are present in the retina.

The localization of NTPase activity in the retina is of considerable interest since these enzymes mediate the energy necessary for the various synthetic and functional activities of a wide variety of cells (22). The localization of NTPase active sites in mitochondria is consonant with biochemical data on its distribution. Biochemical assays of ATPase activity of various cellular constituents isolated by differential centrifugation indicate that the majority of ATPase resides in the mitochondrial fraction (Schneider et al., 35, and Novikoff et al., 24), and is associated with the membrane and particle fraction obtained by comminution of mitochondria (Novikoff et al., 24).

Although these results suggest that mitochondrial ATPase may be membrane associated, such a relation was not clearly evident at the electron microscope level in our experiments.

Membrane localization of ATPase was established by biochemical techniques by Rothstein and Meier (31) in yeast, and by Libet (20) in the giant axon of the squid. Since then, a number of investigators have corroborated this finding by diverse experimental techniques in a variety of cells (21, 12, 8, 38). ATPase appears to mediate the transport of inorganic phosphate (21, 12, 30) and sodium and potassium across cell membranes (37, 16, 29); thus the histochemical localization of NTPase fits well with this function. The membrane localization in some of the cell membranes in the plexiform layer of the retina is morphologically similar to that observed by Essner et al. for ATPase (8) in the plasma membranes of liver cells. Localization of enzyme activity between membranes does not necessarily preclude membrane localization of the enzyme. Enzyme-active sites situated on membranes may precipitate sufficient reaction product to fill interspaces between membranes, thus giving an impression of intermembranous localization.

The presence of NTPase in the photoreceptors is noteworthy, in view of their proximity to the densely packed mitochondria which constitute the ellipsoid. Since mitochondria are the major source of ATP (15, 13, 7), the close anatomical relation

Figure 6

Localization of NTPase activity in the membranes of the rod outer segment. In some instances the reaction product seems to be between lamellae. × 101,000.

Figure 7

Sites of NTPase activity in the lamellae of the rod outer segment. The deposit of reaction product (A) has been cut in cross-section, the deposit (B) appears in longitudinal profile and appears to be localized between the lamellae. × 109,000.

Figure 8

A diagram indicating the sites of enzyme activity in the outer segments of rods and cones (OS). The deposits on the rods are restricted to the periphery of the segments, while in the cones the deposits are densely distributed between the lamellae at the base of the segments. Both rod and cone ellipsoid mitochondria contain deposits (IS). The oil droplet (OD) is devoid of enzyme-active sites.
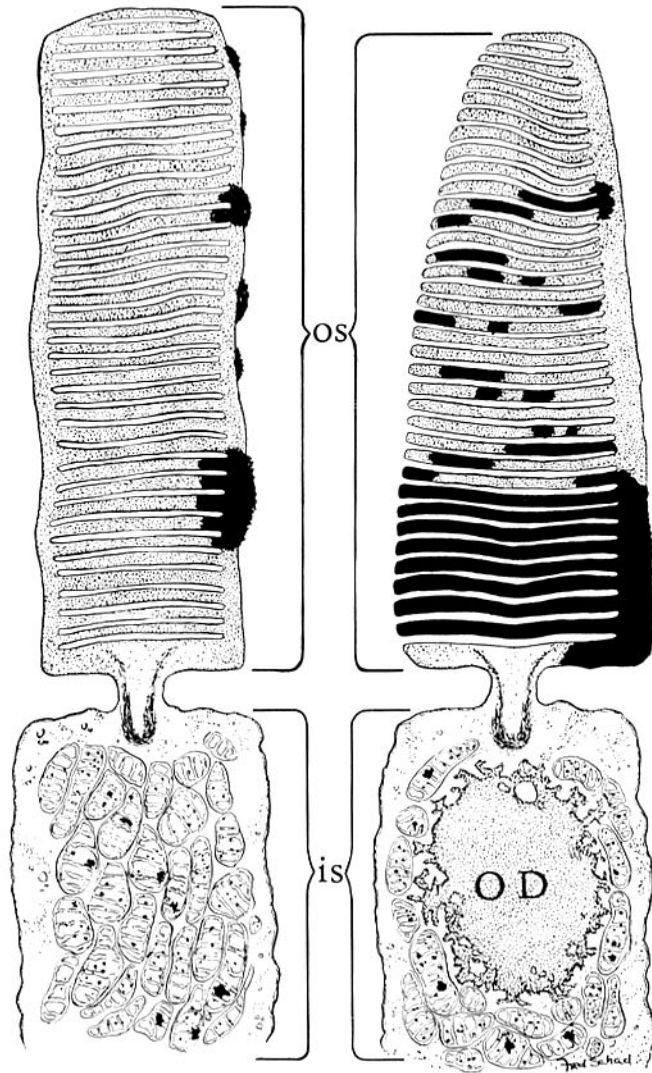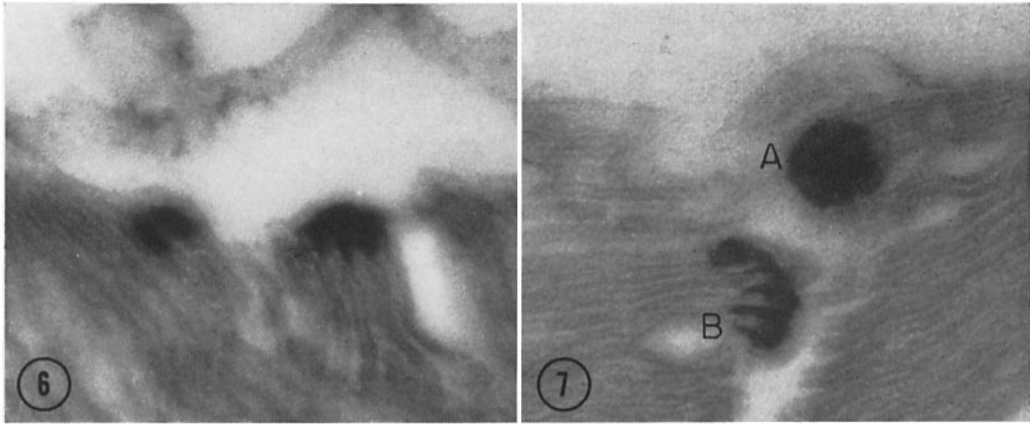
FIGURE 8

of NTPase active sites to them is an ideal arrangement for rapid energy production. Sjöstrand (39) postulated such a relation a number of years ago on the basis of electron microscopic studies of the retina.

Intense NTPase activity throughout the cytoplasm of the outer segment of the cones which morphologically consist of numerous small vesicles and amorphous cytoplasm (4) suggests that this enzyme may also be associated with cytoplasm ground substance. It is of interest to note that ATPase has been localized by biochemical means in the microsomal fraction of rat nerve and the submembranous cytoplasm of squid axons by Abood and Gerard (1).

Furthermore, since visual pigments constitute the principal structural components of the rods and to a lesser degree of the cones (43), the intimate contact of the lamellae of the photoreceptors with NTPase active sites suggests a possible interaction between them. How this enzyme functions in the conversion of electromagnetic energy to chemical energy in the phenomenon of visual excitation remains to be elucidated.

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FIGURE 9

The inner plexiform layer of the frog retina. A complex of electron opaque membranes indicates sites of NTPase activity. × 10,000.

FIGURE 10

A higher magnification of the area shown in Fig. 7. Sites of NTPase activity appear localized on and between double membranes of bipolar cell processes. × 52,000.

FIGURE 11

The inner nuclear cell layer of the frog retina. The nuclei of two bipolar cells are visible on either side of a Müller cell cytoplasmic process containing fibrillar material. Sites of NTPase activity appear as electron opaque deposits localized between the plasma membranes of the various cells. × 60,500.
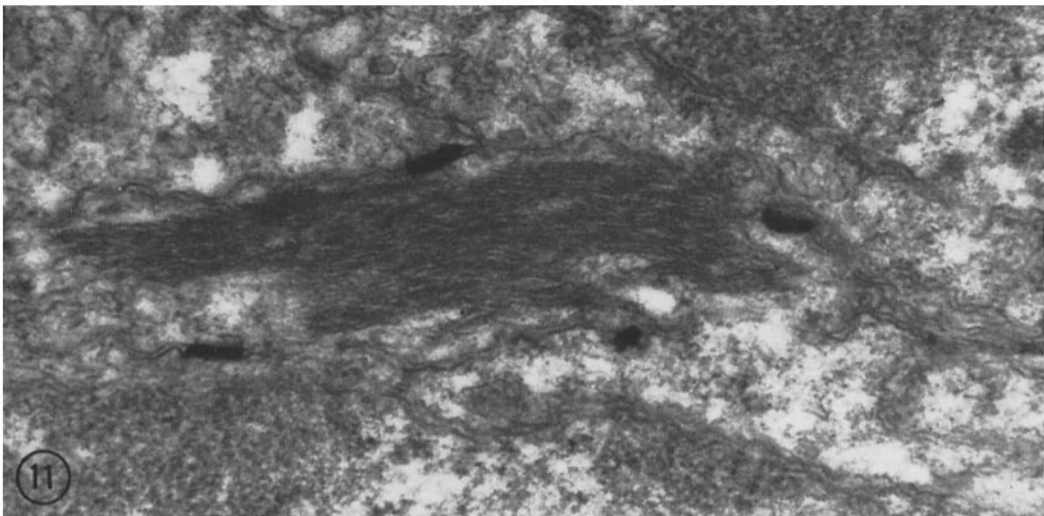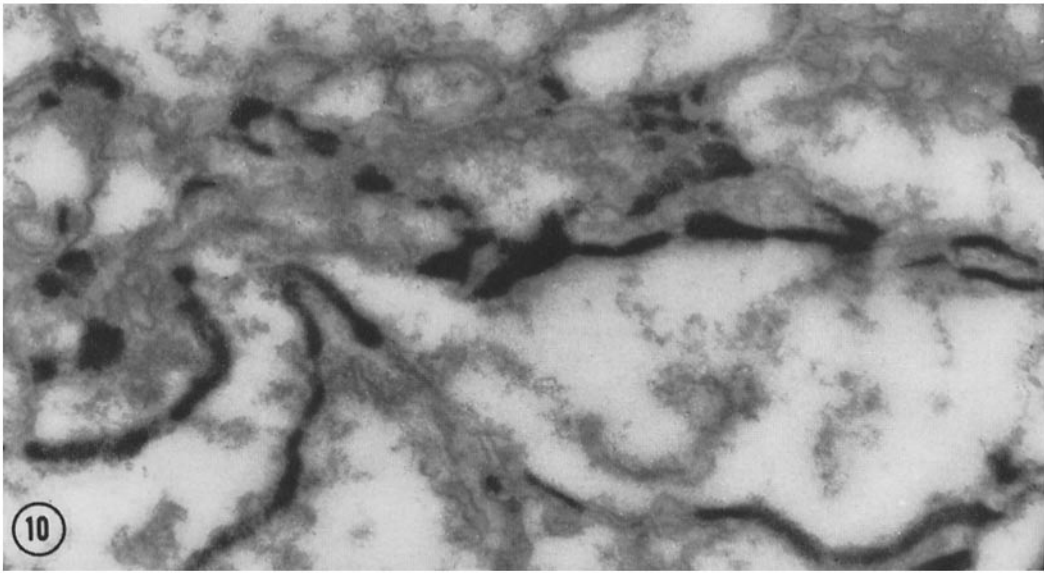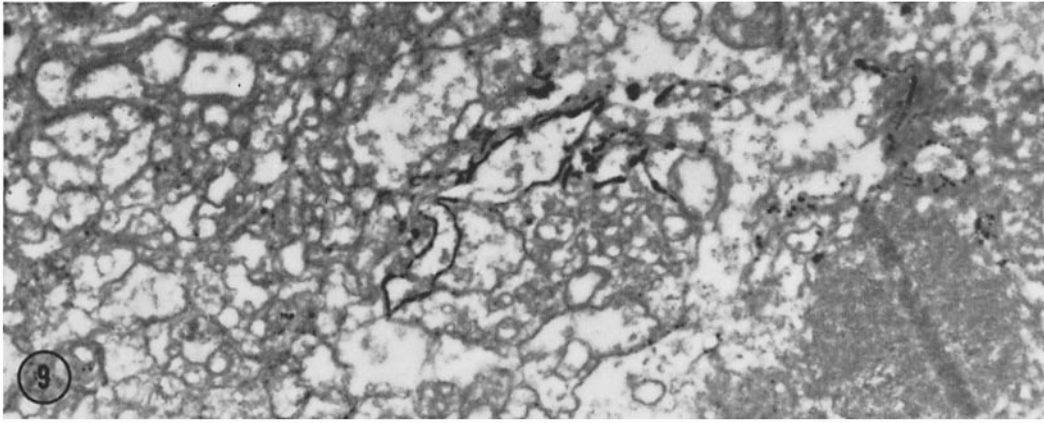
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