ADENOSINE TRIPHOSPHATASE ACTIVITY IN THE MEMBRANES OF THE SQUID NERVE FIBER

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ABSTRACT

This investigation deals with the localization of sites of ATPase activity, especially of transport ATPase, in nerve fibers of the squid *Doryteuthis plei*, at the subcellular level. Splitting of ATP liberates inorganic phosphate which reacts with lead to form a precipitate in the tissue. The reaction was made on nerve fibers fixed with glutaraldehyde. Frozen slices were incubated in Wachstein-Meisel medium containing ATP and $Pb(NO_3)_2$. Deposits of reaction product were found in the axolemma (towards its axoplasmic side), Schwann cell membranes (mainly at the channels crossing the layer), and mitochondria. Control experiments revealed that no deposits were observed in nerve fibers fixed in osmium tetroxide prior to incubation in the medium containing ATP, or in nerve fibers incubated without substrate or with adenosine monophosphate, adenosine diphosphate, glycerophosphate, or guanosine triphosphate as substrate. For evaluation of transport ATPase activity, these findings were compared with results obtained with nerve fibers treated with G-strophanthin or K-strophanthoside before or after glutaraldehyde fixation. The cardiac glycosides produced a disappearance or diminution of the deposits. The largest inhibitory effect was observed in the axolemma. The findings indicate that the highest ATPase activity is localized in the axolemma and may be due primarily to transport ATPase.

Experiments on squid nerve fibers (1–6) and erythrocytes (7–9) have shown that the energy required for the active sodium-potassium transport comes from adenosine triphosphate (ATP). This active transport depends on Na^+ and K^+ (10–14) and is inhibited by cardiac glycosides (8, 15, 16).

On the basis of those experiments, the requirements of the active sodium-potassium transport system were formulated. An adenosine triphosphatase (ATPase) discovered by Skou (17) in crab nerve was found to satisfy those requirements (18–20). This enzyme has been found in a large number of cells (cf. reference 21). It differs from other ATP-hydrolyzing enzymes in that in addition to Mg^{2+} it requires Na^+ and K^+ in the medium for maximum activation and in that it is inhibited by cardiac glycosides. This cardiac glycoside-sensitive (Mg^{2+} + Na^+ + K^+)-activated ATPase will be referred to as transport ATPase throughout this work.

Caldwell and Keynes (22), and Villegas et al. (23) have shown the inhibitory effect of the cardiac glycosides on the active ion transport in the axon and Schwann cell of the squid nerve fiber.

Bonting and Caravaggio (24) have determined the ATPase activities of the "axon sheath" and axoplasm. They reported that the highest con-

centrations of ATPase, particularly of transport ATPase, were found in the "axon sheath." Recent experiments of Canessa (25) have shown further that this highest ATPase activity is localized in the microsomal fraction of the axon sheath.

Since the axon sheath (or axoplasm-free nerve fiber) used in previous works is a rather complex structure formed by the axolemma, Schwann cells, and some endoneural connective tissue, the present study was carried out for the purpose of localizing by electron microscopy the sites of ATPase activity, especially of the transport ATPase.

The method of localization employed in the present work was similar to that used by Torack and Barrnett (26), and Farquhar and Palade (27). The splitting of ATP by the ATPase liberates inorganic phosphate that reacts with lead, used as trapping agent, and precipitates in the tissue. For study of the transport ATPase, the results obtained in normal nerve fibers were compared with those obtained in nerve fibers treated with G-strophanthin or K-strophanthoside. This approach allowed an estimation of the inhibition of the transport ATPase caused by the cardiac glycosides.

The results of the present work reveal that there are sites of ATPase activity at the axolemma, Schwann cell membrane, and mitochondria, and that the activities at the axolemma and Schwann cell layer are markedly reduced by the cardiac glycosides.

MATERIAL AND METHODS

25 giant nerve fibers from the hindmost stellar nerve of living squids *Doryteuthis plei* were used. They were separated from the rest of the bundle, and both ends were tied with thread and sectioned. About 5 min were spent in the isolation procedure. Immediately after isolation, the nerve fibers were kept for 15 min in artificial seawater (28). Then, each nerve fiber was fixed in ice-cold 2% glutaraldehyde (29) in artificial seawater, buffered with veronal-acetate. This buffer does not appear to modify the fixing properties of glutaraldehyde since the imido groups of veronal do not react with the aldehyde groups. The pH of the solution was 8.0–8.1, which is the pH of natural and artificial seawater in which the isolated nerve fibers are kept (28). The pH of the squid axoplasm is near 7 (30).

After fixation, the nerve fibers were washed for 10 min in isotonic sucrose solution and cut with a freezing microtome into sections about 50 μ thick.

These sections were incubated for 30 min at 22°C in Wachstein-Meisel medium (31) containing 3.6 mM $Pb(NO_3)_2$, and 1 mM ATP as substrate. Sucrose was used for the purpose of making the medium isotonic with seawater (1010 milliosmols/kg of water). The pH of this medium was also 8.0–8.1.

The nerve fiber sections were rinsed in isotonic sucrose solution, fixed for 2 hr with 2% osmium tetroxide in artificial seawater buffered with veronal-acetate, dehydrated in successive changes of ethanol, and embedded in Epon. Thin sections (gray or silver interference color) were mounted on carbon-coated grids and observed in a Siemens Elmiskop IA. Some sections were stained with uranyl acetate and lead citrate.

Control Experiments

Three types of control experiments were made to investigate the specificity of the method: (*a*) nerve fibers were fixed in osmium tetroxide before incubation in the medium containing ATP; (*b*) nerve fibers were incubated in medium lacking ATP; and (*c*) nerve fibers were incubated in medium in which ATP was replaced by one of the following substances: adenosine monophosphate, adenosine diphosphate, Na β-glycerophosphate, or guanosine triphosphate.

Effect of Cardiac Glycosides

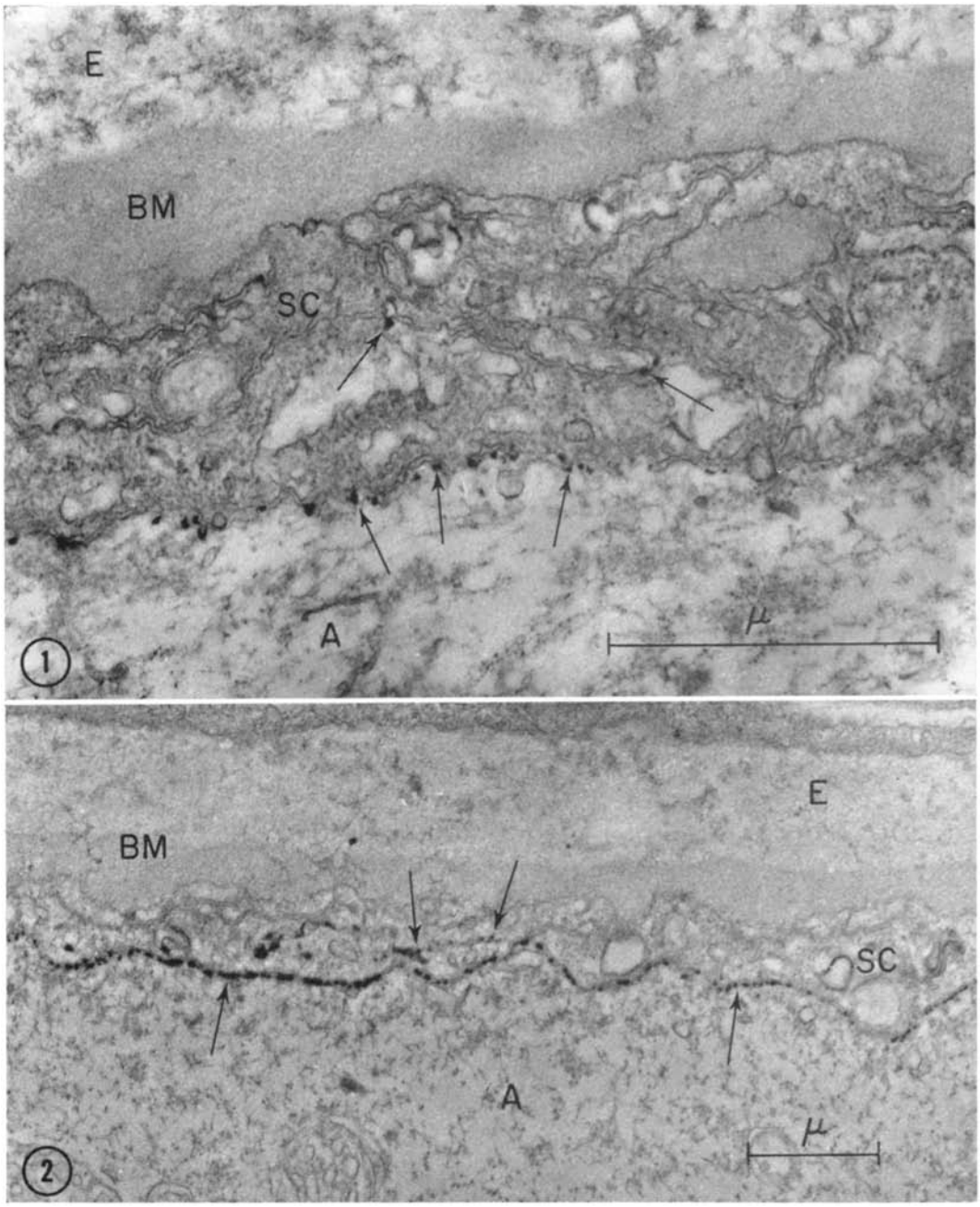
In addition to the experiments carried out for the localization of the sites of ATPase activity and for controlling the specificity of the method used, further experiments were made for the investigation of the major location of the cardiac glycoside-sensitive fraction of the enzyme or transport ATPase. Groups of nerve fibers were immersed for 15 min, immediately after isolation from the animal and before glutaraldehyde fixation, in artificial seawater containing 10^{-3} M G-strophanthin or 10^{-3} M K-strophanthoside; control nerve fibers were incubated in cardiac glycoside-free artificial sea water. Another group was treated with the same concentration of the cardiac glycoside G-strophanthin, added to the Wachstein-Meisel medium containing ATP in which the nerve fibers were incubated after glutaraldehyde fixation.

RESULTS AND DISCUSSION

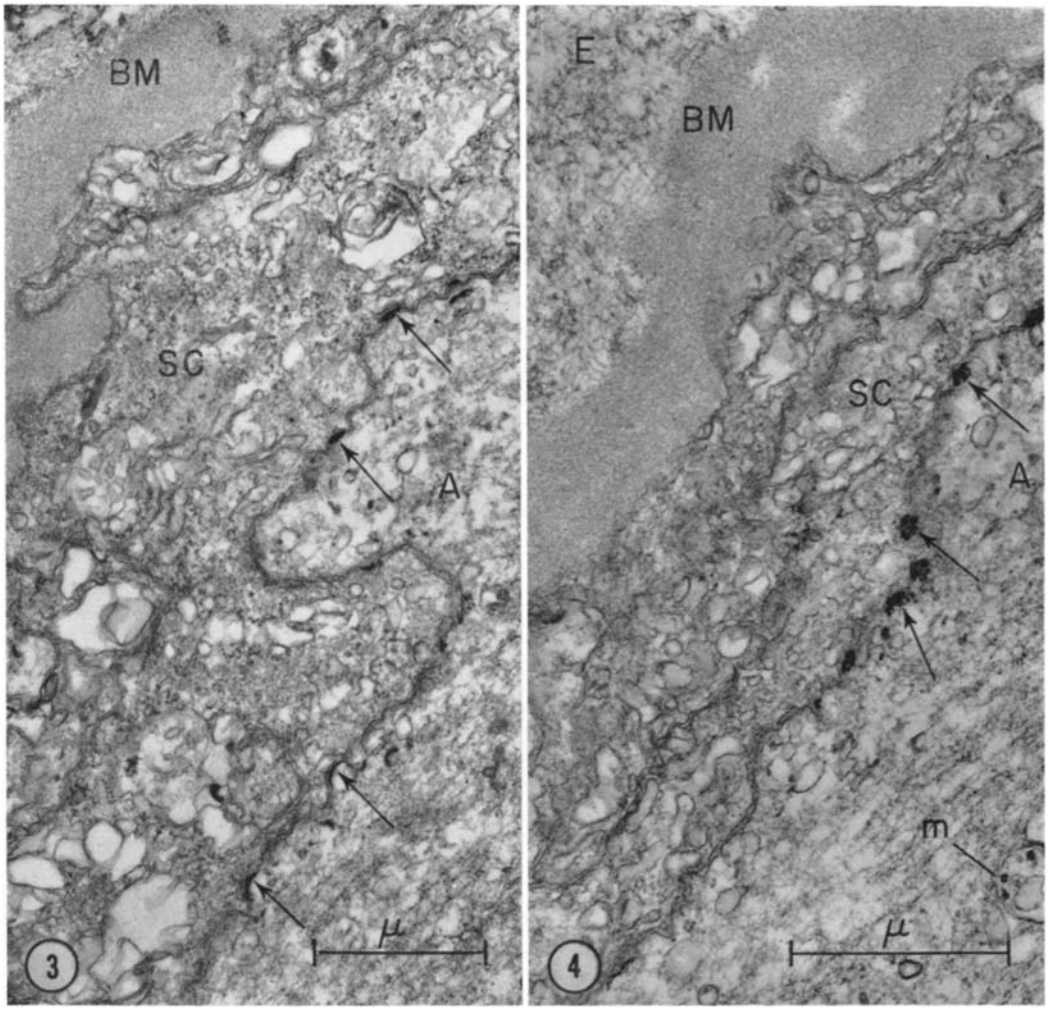
The electron microscopic examination of the nerve fibers revealed a good preservation of the fine structure of the tissue. The axon, Schwann cell layer, basement membrane, and endoneurium showed the same ultrastructural features and relationships described previously (32).

ATPase Distribution

Six nerve fibers were used. The sites of ATPase activity are revealed as black deposits of lead phosphate. The reaction product appeared as



FIGURES 1 and 2 Cross-sections of two squid nerve fibers showing the axon (*A*), Schwann cell (*SC*), basement membrane (*BM*), and endoneurium (*E*). The arrows indicate some of the sites of ATPase activity (deposits of lead phosphate) at the axolemma and Schwann cell channels. These nerve fibers were fixed in glutaraldehyde. Frozen slices were incubated in Wachstein-Meisel medium containing ATP and $Pb(NO_3)_2$, postfixed in OsO_4, and embedded in Epon. Sections stained with uranyl acetate.



FIGURES 3 and 4 Cross-sections of two squid nerve fibers showing the axon (*A*), Schwann cell (*SC*), basement membrane (*BM*), and endoneurium (*E*). Some of the sites of ATPase activity in the axolemma are indicated by arrows. The dense particles of lead phosphate aggregate to form elongated (Fig. 3) or round deposits (Fig. 4). Note also the deposits in a mitochondrion (*m*, Fig. 4). The nerve fibers were treated as stated in the legend of Figs. 1 and 2. These sections were doubly stained with uranyl acetate and lead citrate.

small dense particles aggregated to form round (maximum diameter 50–60 mμ) or elongated deposits (maximum length 160–180 mμ). These deposits were observed, as shown in Figs. 1–4 and 6, mainly in the axolemma. The smallest deposits, which because of their size permitted a better localization, were observed predominantly towards the axoplasmic side of the axolemma.

Deposits were observed also in the Schwann cell membrane, in the channels crossing the Schwann cell layer, and inside the mitochondria of the axon and Schwann cells. Most of the deposits found in the Schwann cell layer were located in the membrane and lumen of the channels as shown in Figs. 2 and 5. The channels are formed by the spaces between neighboring cells and by the spaces left between the finger-like processes of the Schwann cell surface (33).

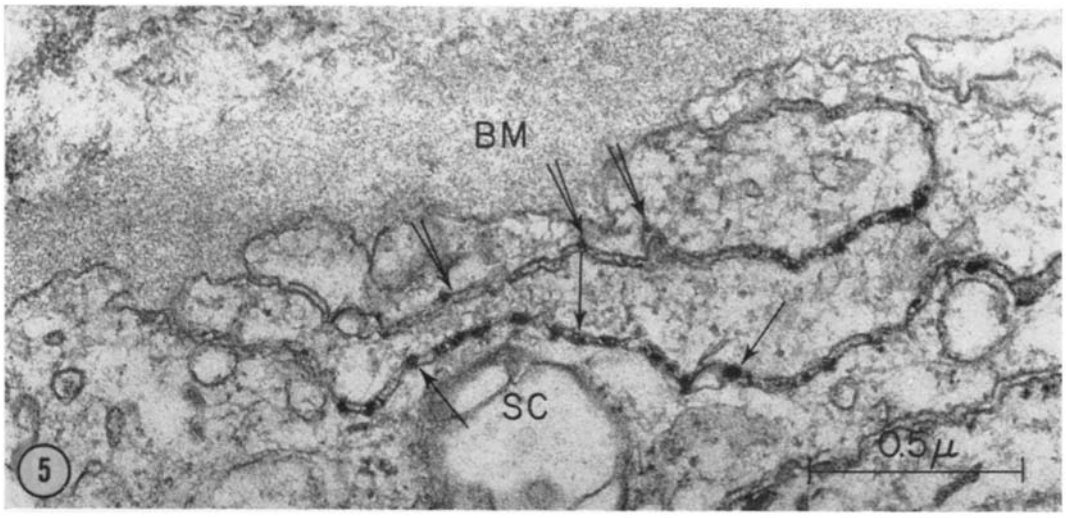


FIGURE 5 Cross-section of a squid nerve fiber showing the Schwann cell layer (*SC*), covered by the basement membrane (*BM*). Notice the sites of ATPase activity in the channel walls (double-stemmed arrows) but mostly in the channel lumina (single arrows). The nerve fiber was treated as stated for the preceding figures. Section doubly stained with uranyl acetate and lead citrate.

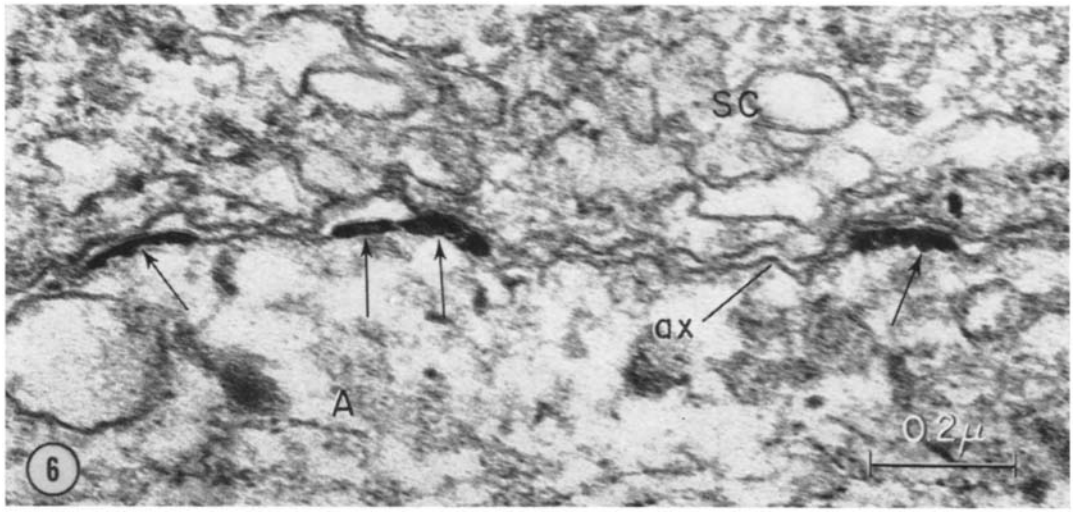


FIGURE 6 A detail of the axon–Schwann cell boundary of a nerve fiber, showing the sites of ATPase activity (deposits of lead phosphate) at the level of the axolemma (*ax*). The deposits are indicated by arrows. Notice that the deposits are situated predominantly towards the axoplasmic side. The nerve fiber was treated as stated in the legend of previous figures. Section doubly stained with uranyl acetate and lead citrate. *A*, axon; *SC*, Schwann cell.

Control Experiments on ATPase Specificity

No deposits were observed (*a*) in the nerve fibers fixed in osmium tetroxide prior to incubation in the Wachstein-Meisel medium containing ATP as substrate, (*b*) in the nerve fibers incubated without substrate, or (*c*) in those nerve fibers incubated with adenosine diphosphate, adenosine monophosphate, Na β-glycerophosphate or guano-

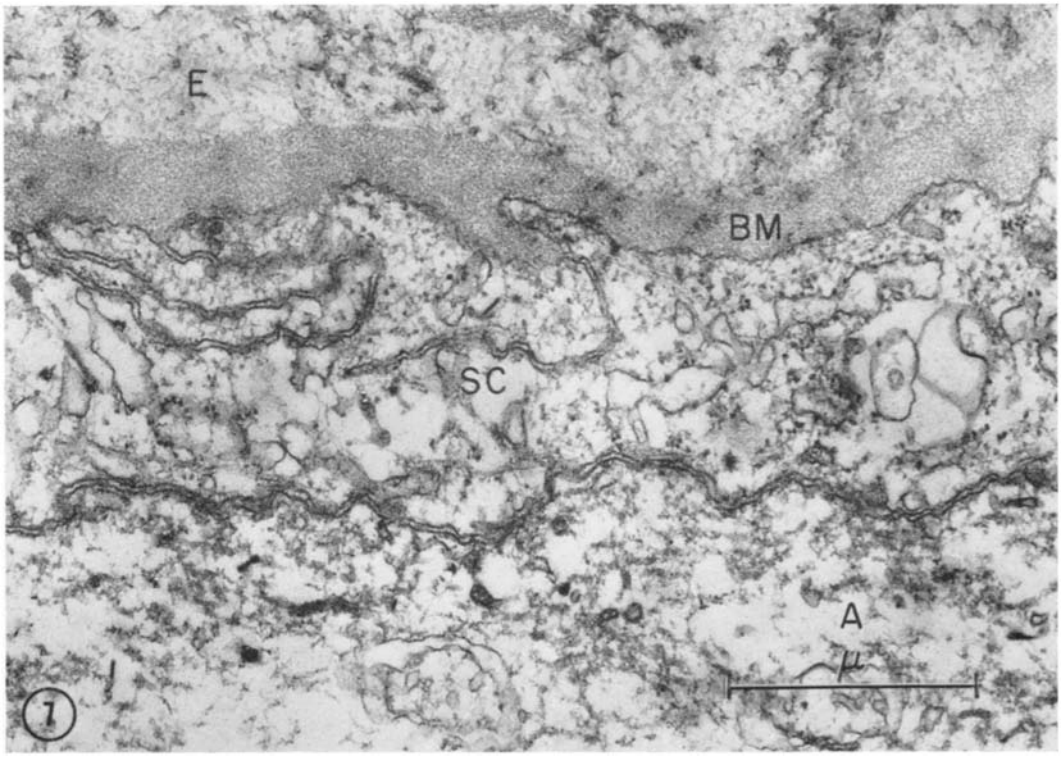


FIGURE 7 Section of a nerve fiber treated with K-strophanthoside prior to glutaraldehyde fixation. No ATPase activity is observed. The procedures of slicing, postfixation, and embedding were the same as those used for the nerve fibers shown in preceding figures. Section doubly stained with uranyl acetate and lead citrate. *A*, axon; *SC*, Schwann cell; *BM*, basement membrane; *E*, endoneurium.

sine triphosphate as substrate. These results indicate that in all probability the reaction product we observed was due to the effect of the nerve fiber ATPase on the ATP added as substrate. Thus, it seems unlikely that the deposits were due to either a nonspecific acid phosphatase or a nonenzymatic hydrolysis of ATP of the type observed by Moses and Beaver (34) and Rosenthal (35) in renal tubular tissue.

Effect of Cardiac Glycosides on ATPase Activity

No deposits were found, as shown in Fig. 7, in the axolemma of the majority of the sections of six nerve fibers treated with G-strophanthin and of six treated with K-strophanthoside before incubation in the Wachstein-Meisel medium, or in nerve fibers incubated in Wachstein-Meisel medium containing G-strophanthin. In a few of the remaining sections of these cardiac glycoside-treated nerve fibers, the number of deposits was markedly reduced. The disappearance and diminution of the deposits are considered to be caused by the inhibitory effect of the cardiac glycosides on the transport ATPase. The largest inhibitory effect was observed in the nerve fibers treated with the cardiac glycosides prior to the glutaraldehyde fixation.

In the Schwann cell layer the cardiac glycosides also reduced the number of deposits, especially those situated in contact with the cell membrane. In only one case, a nerve fiber treated with G-strophanthin, were many deposits found in the lumen of the channels.

Novikoff et al. (36), Moses and Beaver (34), and Rosenthal (35) have found that the usual lead concentrations cause marked ATPase inactivation. Though the level of enzyme inactivation was not measured in the present experiments, it should be similar in all the nerve fibers used,

since the same Pb $(NO_3)_2$ concentration was employed in all the experiments. Thus, it appears that the relative diminution of the sites of ATPase activity observed in the nerve fibers treated with G-strophanthin or K-strophanthoside as compared with the normal nerve fibers should be due to the glycosides. It should be pointed out also that the inhibitory effect of K-strophanthoside appeared always to be greater than that of G-strophanthin. The demonstration of a large cardiac glycoside inhibition may be taken to indicate a large ratio of transport ATPase to Mg^{2+}-activated ATPase in the axolemma and the Schwann cell membrane. This conclusion is in agreement with the results of Canessa (25), who found that this ratio in the microsomal fraction of the "axon sheath" is 5.6.

Thus, the present results allow a correlation of the inhibitory effects of the cardiac glycosides on the membrane-bound transport ATPase and on the active ionic movements in the axon (22) and Schwann cell (23).

The largest deposits of reaction product in the normal nerve fibers were found at the axolemma, and the greatest inhibitory effect of the cardiac glycosides also was observed in this membrane. Thus, it may be suggested that the highest content of ATPase, especially of transport ATPase, is in the axonal surface. The finding of the deposits at the innermost portion of the axolemma suggests that in order to maintain the active sodium-potassium transport mechanism in perfused axons it is necessary to preserve the integrity of the axolemma and possibly also of the neighboring axoplasm. This latter suggestion may explain the success of Brinley and Mullins (37) in preserving the active ion transport mechanism in their dialyzed axons.

As Farquhar and Palade indicated (27), the results obtained with the present method should be considered subject to a number of important limitations. These limitations are due to the essentially qualitative character of the method, the loss of a large part of the ATPase activity during fixation, and the inhibitory effect of the lead ions used as phosphate-trapping agents. However, the findings herein reported agree with the results of previous works on the mechanism of active transport in the squid nerve fiber (1–6), the ATPase content of the axon sheath and its fractions (24, 25), and the effect of the cardiac glycosides on the axon and Schwann cell (22, 23).

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