FINE STRUCTURAL ALTERATIONS ASSOCIATED WITH VENOM ACTION ON SQUID GIANT NERVE FIBERS

RAINER MARTIN and PHILIP ROSENBERG

From the Department of Neurology and Psychiatry, Harvard Medical School, Boston, Massachusetts, the Research Laboratory, McLean Hospital, Belmont, Massachusetts, and the Department of Neurology, Columbia University, College of Physicians and Surgeons, New York, New York 10032. Dr. Martin's present address is Stazione Zoologica di Napoli, Villa Comunale Naples, Italy. Dr. Rosenberg* is at Columbia University College of Physicians and Surgeons

ABSTRACT

(1) Block of conduction and marked increase in permeability of the squid giant axon, when surrounded by adhering small nerve fibers, is caused by the venoms of cottonmouth, ringhals, and cobra snakes and by phospholipase A (PhA). This phenomenon is associated with a marked breakdown of the substructure of the Schwann sheath into masses of cytoplasmic globules. Low concentrations of these agents which render the axons sensitive to curare cause less marked changes in the structure of the sheath. (2) Rattlesnake venom, the direct lytic factor obtained from ringhals venom, and hyaluronidase caused few observable changes in structure, correlating with the inability of these agents to increase permeability. (3) Cottonmouth venom did not alter the structure of giant axons freed of all adhering small nerve fibers. This is in agreement with previous evidence that the venom effects are due to an action of lysophosphatides liberated as a result of PhA action. Cetyltrimethylammonium chloride, a cationic detergent, produces effects that resemble those of venom and PhA. (4) The results provide evidence that PhA is the component of the venoms that is responsible for their effects. It also appears that the Schwann cell and possibly the axonal membrane are the major permeability barriers in the squid giant axon.

INTRODUCTION

The effects of various venoms on the permeability and function of the squid giant axon have been extensively studied over the past years (1–9). Relatively low concentrations of cottonmouth moccasin venom and certain other venoms do not affect conduction but will increase the permeability of this giant axon preparation to many poorly penetrating compounds (5–8). Higher concentrations of these venoms will irreversibly block conduction (1–3). The component responsible for the action of the venom seems to be phospholipase A (2, 4, 14).[1] In venom-pretreated squid giant axons, it was possible to obtain evidence in support of an essential role of acetylcholine (ACh) in axonal conduction (10, 11). ACh, curare, and other lipid-insoluble compounds known to interact with ACh receptors at synapses are inactive on the intact squid axon. This inactivity could be due to

*Address request for reprints to Dr. Rosenberg.

[1] Condrea, E., and P. Rosenberg, Correlation of effects on axonal conduction and phospholipid splitting induced by venoms and venom fractions. II. Squid axon. *Biochim. Biophys. Acta.* In press.

an inability to reach the axon membrane (5, 12, 13). After venom pretreatment, however, ACh and curare penetrate the axon membrane, enter the axoplasm of the squid giant axon, and reversibly block conduction (5, 8).

Electron microscopic studies were undertaken in order to determine whether structural modifications may be observed in squid nerves treated with various venoms or with venom phospholipase A.

MATERIALS AND METHODS

Venoms: Fractionation and Measurement of Phospholipase A Activities

The venom of *Hemachates haemachatus* (ringhals) was purchased from Pierce Chemical Co., Rockford, Ill. The venoms of *Crotalus adamanteus* (Eastern diamondback rattlesnake), *Naja naja* (hooded cobra), and *Agkistrodon p. piscivorus* (cottonmouth moccasin) were purchased from Ross Allen Reptile Institute, Silver Springs, Fla.

Paper electrophoresis of the ringhals venom, and the identification and elution of the phospholipase A (PhA) and "direct lytic factor" (DLF) fractions have been described by Condrea et al. (15). The protein contents of these fractions were determined according to the method of Lowry et al. (16), and measurements of PhA activities were determined as previously described (15) by titrating the amounts of free fatty acids liberated from egg yolk according to the method of Dole (17). The PhA activity of electrophoretically separated PhA fractions ranged between 1 and 2 μEq. of free fatty acids liberated per μg of PhA protein per 5 min. This PhA activity is, respectively, about 3, 10, and 150 times as great as that of the venoms of ringhals, cottonmouth, and rattlesnake. The DLF fraction of ringhals venom had no PhA activity.

Dissection and Treatment of the Giant Nerve Fibers

The giant nerve fibers of the squid (*Loligo pealii*) were dissected as previously described (1, 2). In some preparations, the giant axons still contained many adhering small nerve fibers of the stellar nerve together with the interspersed connective tissue, whereas in others the giant axons were carefully cleansed and had no small adhering nerve fibers. After dissection, the specimens were placed in a chamber containing natural, filtered sea water and Ag, AgCl electrodes for external stimulation and recording of electrical activity of the giant axon (1, 2). Control axons were exposed to sea water for varying lengths of time prior to fixation, whereas the experimental axons were exposed for 30 min to solutions of the various agents. As a test of whether the axons were rendered sensitive to curare, the pretreating agent (venoms, etc.), in a concentration which by itself did not affect the action potential, was applied to the axon for 30 min. The axon was then placed in fresh sea water for at least 10 min and then exposed to curare for 30 min unless block of electrical activity occurred earlier. To check reversibility of the block, we added fresh sea water.

Fixation and Electron Microscopy

Control and treated axons were cut into three pieces about 10 mm in length, each of which was submerged into a different cooled fixative. Control nerves containing adhering small nerve fibers were fixed immediately after dissection (three nerves) or after standing at room temperature in Tris-buffered sea water (pH 7.5–8.0) for ½ hr (one nerve), 1 hr (two nerves), or 2 hr (two nerves). In addition, two axons free of adhering small nerve fibers were fixed immediately after dissection. The fixatives used were as follows: (1a) 1.5% $KMnO_4$ in veronal-buffered squid physiological solution (18) or (1b) 0.6% $KMnO_4$ in sea water; (2a) 2.5% OsO_4 in veronal-buffered squid physiological solution or (2b) 1% OsO_4 in sea water; (3) formaldehyde-glutaraldehyde in phosphate buffer (19). The osmolality of the fixatives 1a and 2a was adjusted to 900 milliosmols by reduction of the amount of NaCl in the physiological solution. No significant differences were found between fixatives 1a and 1b or between 2a and 2b.

Variation of fixation time from 3–5 hr had no significant effect on fine structure. The aldehyde-fixed specimens were rinsed in Tris-buffered sea water and postfixed for 2 hr in 2.5% OsO_4 as above. The specimens were dehydrated in acetone and embedded in Araldite or Durcupan. The permanganate fixatives gave by far the best preservation of the membranes and the layered substructure of the sheath, while the aldehyde fixatives gave relatively poor preservation of membrane structure; these results will not be considered further in this study. The nerves were transversely cut into thin sections, the section face being trimmed so that it contained the longest possible segment of the giant axon. The sections were stained with lead citrate (20) and examined in a Siemens Elmiskop 1b or an AEI EM 6b. For the estimation of the degree of effect of the treatment, a segment of the axon surface greater than 200 μ long was examined at 20,000 times magnification.

RESULTS

Ultrastructure of the Schwann Sheath of Giant Axons

As has been described previously (21–24), the Schwann sheath of the squid giant axon is com-

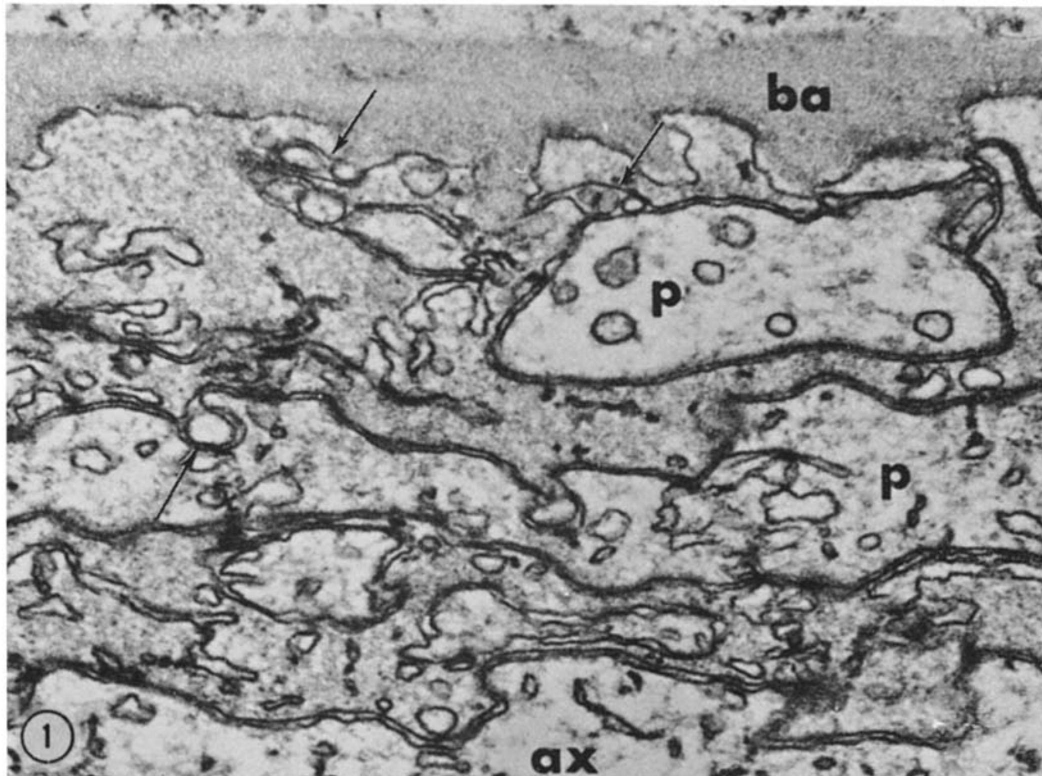


FIGURE 1 Schwann sheath of a control giant axon (*ax*) fixed in permanganate (1a) immediately after dissection. The sheath is formed by several processes (*p*) and surrounded by a prominent basement membrane (*ba*). At the arrows, there are either transversely cut finger-like extensions or globular postmortem artifacts. × 50,000.

posed of one to several layers (Figs. 1–3, 5). Up to six Schwann cell nuclei may be found in a transverse section of the axon. The formation of the layers can best be explained if we assume that each Schwann cell has a number of processes and branches that spread over the surface of the axon. Presumably by superposition and interdigitation of these processes, the layers of the sheath are formed. The layers are closely packed, the apposed membranes being separated by a regular gap 100–150 A wide. An equal gap separates the inner-most membrane of the sheath from the axon membrane. The external side of the sheath is surrounded by a prominent basement membrane and more externally by connective tissue. The extracellular spaces between two apposed layers are seen mainly to run parallel to the axon membrane although they can be traced from the external to the internal surface of the sheath. Occasionally, invaginations of the membranes of the layers can be found that appear to end blindly in the cytoplasm of the layer. These invaginations account for the "channels" of Villegas and Villegas (22, 23). Their undisturbed course in thin sections indicates that these channels are not tubelike structures, but long flat compartments of extracellular space, which could be explained by assuming that two branches of the same process are superposed.

Degree of Preservation of Control Specimens

Previous studies have shown that squid axons maintain an approximately normal-sized action potential for at least 4 hr after dissection (16–18°C) (1, 2). The control axons in this work were conducting normally immediately prior to fixation. As may be expected, the fine structure of the control specimens that were fixed at least 30 min

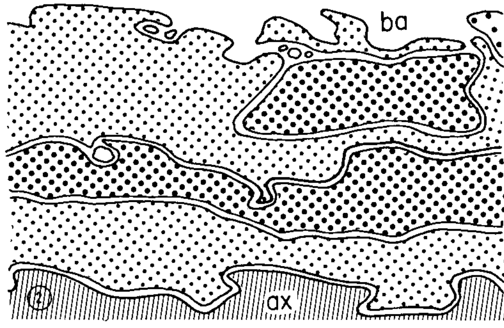


FIGURE 2 Diagram of the micrograph in Fig. 1 which separates the different layers of the sheath by different patterns. It is not intended to indicate that the elements marked by one pattern originate from the same cell.

after death of the animal shows varying degrees of "bad fixation," especially in the cytoplasm. However, the membranes and the layered substructure of the Schwann sheath of the giant axons appear to be well preserved (Fig. 1). In a few instances, it cannot be decided whether or not a postmortem fragmentation of the small processes has occurred. A similar state of preservation is found in specimens fixed 1 hr after dissection (Fig. 3).

Distinct signs of postmortem decomposition of the membranes and the layered structure of the sheath are found in specimens that were fixed 2 hr after dissection. In permanganate-fixed specimens, thin layers of the sheath are fragmented into cytoplasmic globules (Fig. 5). In osmium tetroxide-fixed specimens, there are areas in which two apposed membranes are split into chains of vesicles and in which extracellular space is included. Both phenomena have been interpreted as fixation artifacts (25, 26). However, these alterations are limited to small areas and occur rather rarely.

The fair preservation of the laminar structure as shown by the sheaths of giant axons up to 2 hr after dissection is not shown by the sheaths of the smaller nerve fibers that surround the giant axons. In permanganate-fixed specimens, the sheath of these fibers, which normally consists of one layer only, may show a large amount of cytoplasmic globules; in osmium tetroxide-fixed material, there are areas in which the apposed membranes of the sheath and the axon membrane form chains of vesicles and in which extracellular space is included (Fig. 4).

Venom Effects

After the giant axon preparations are treated with PhA or certain venoms, the layers of the Schwann sheath of the giant axons show a fragmentation into nests of cytoplasmic globules. The globules often contain cytoplasmic elements such as mitochondria or fragments of endoplasmic reticulum. They are entirely limited by membranes that show the trilaminar structure. There are large extracellular spaces in and around the nests of globules. Since the surface of a spherical cytoplasmic globule is larger than the surface of a finger-like cytoplasmic process of equal volume, it must be assumed either that a considerable amount of the cytoplasmic volume is lost during the fragmentation or that the amount of membrane is increased. At least some of the globules appear to be covered by membranes from the endoplasmic reticulum.

The effects of venoms are manifested in varying degrees of decomposition of the Schwann sheath as follows. 1. A relatively small effect (Fig. 6) is one in which the changes exceed distinctly the postmortem changes as observed in control sheaths. There are nests of a few cytoplasmic globules, and some of the layers seem to be broken up by transverse divisions. The extracellular space in the sheath is increased in some areas. 2. A moderate effect consists of a large number of nests, each with more than ten cytoplasmic globules, at the external as well as internal side of the sheath (Figs. 7–10). 3. A marked effect consists of the breakdown of the laminar substructure of the sheath into masses of globules that fill the space between the basement membrane and the axon membranes over some length (Fig. 11). These three degrees of structural change are indicated in Table I by the numerals 1, 2, and 3, respectively.

Varying degrees of fragmentation exceeding the postmortem and fixation changes of the control specimens were found after treatment with different concentrations of PhA and of venoms of cottonmouth, cobra, and ringhals snakes (Table I). As seen in Table I, rattlesnake venom, DLF, and hyaluronidase, even in relatively high concentration, had little or no effect on the structure of the Schwann sheath. It is of special interest that, even in high concentration, cottonmouth venom had no effect on axon ultrastructure when tested on giant axons freed of all adhering nerve fibers. This finding parallels the previous finding that cottonmouth venom has no effect on the electrical

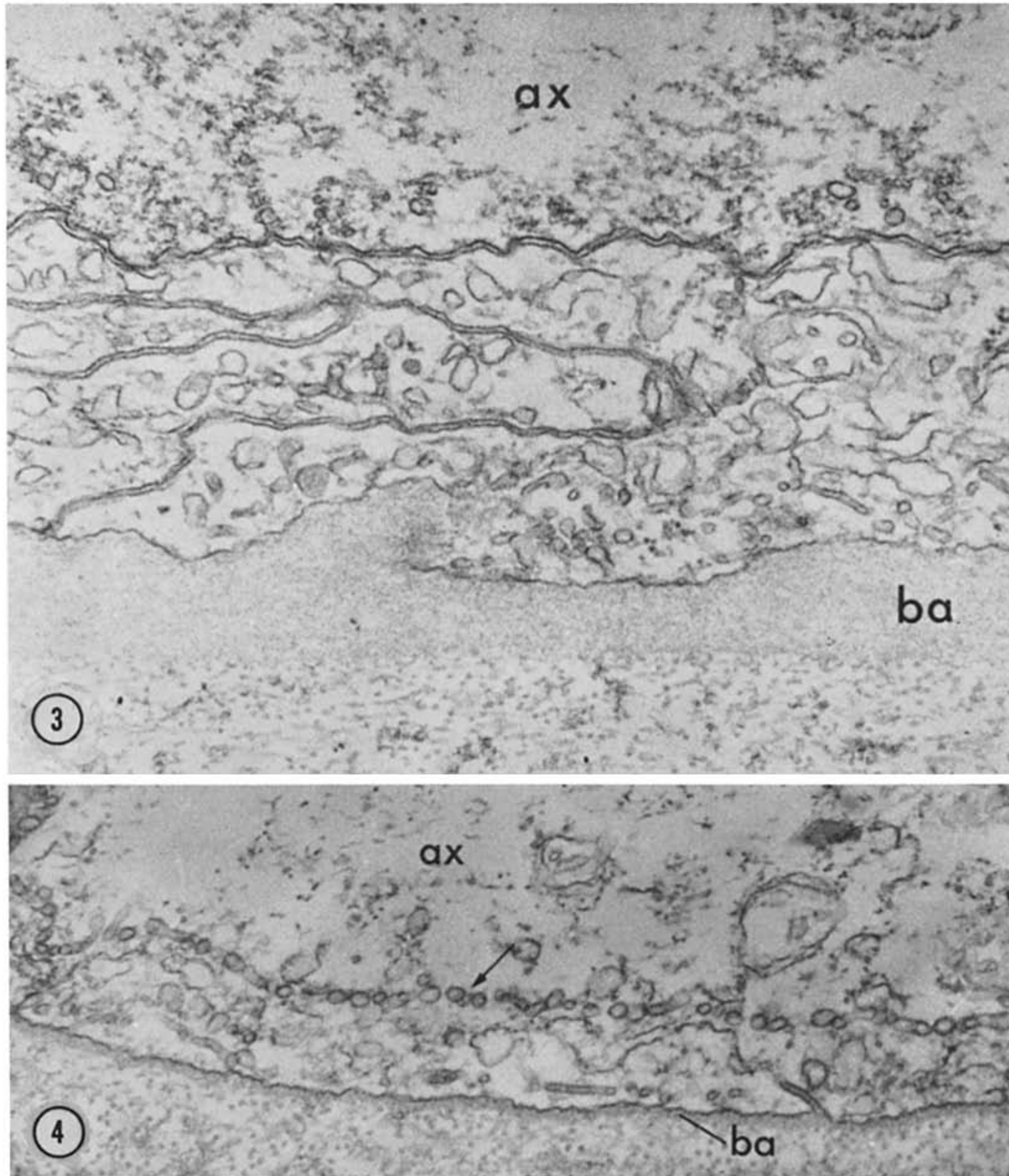


FIGURE 3 Schwann sheath of a control giant axon (*ax*) fixed in osmium tetroxide (2b) 1 hr after dissection. There is an advanced decomposition of the cytoplasm, but the membranes and the general arrangement of the layers are well preserved. × 50,000.

FIGURE 4 Schwann sheath of a small axon running parallel to the giant axon. Same specimen as in Fig. 3. The axon membrane and the apposed membrane of the sheath are fragmented as a fixation artifact into vesicles, and extracellular space (arrow) is included. × 50,000.

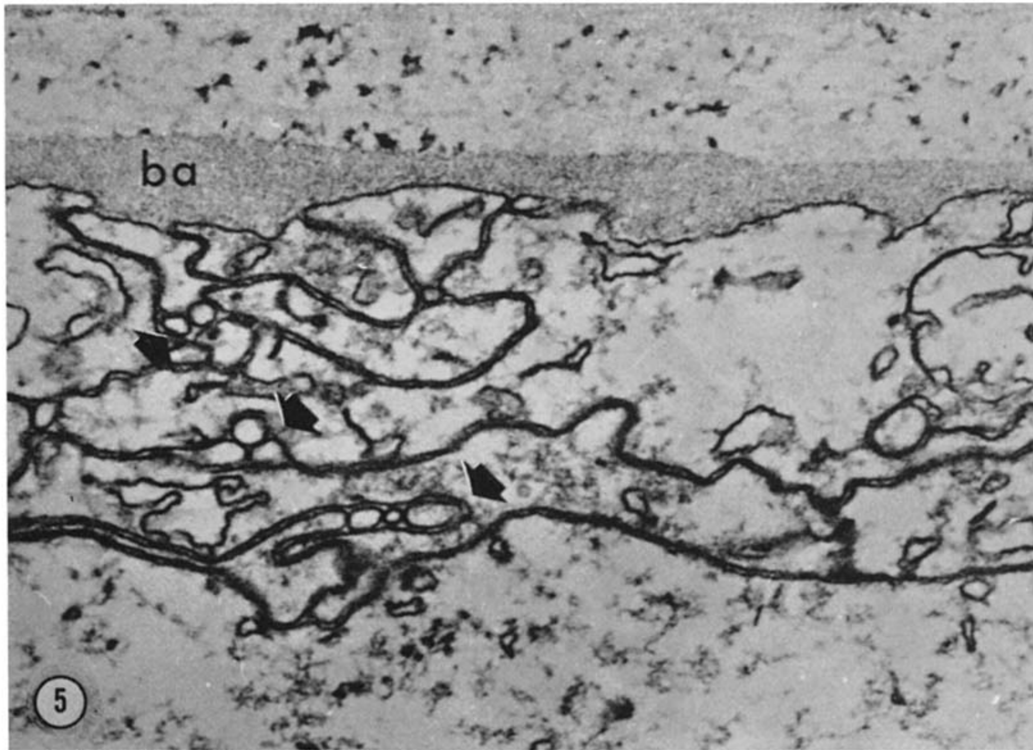


FIGURE 5 Schwann sheath of a control axon fixed 2 hr after dissection in permanganate (1b). At the arrows, laminae of the sheath are fragmented into cytoplasmic globules as fixation artifact. × 50,000.

or permeability properties of giant axons freed of surrounding nerve fibres (2, 8). Disruption of the Schwann sheath was produced by the cationic detergent cetyltrimethylammonium chloride (Fig. 11). The effects of the detergent were, however, not so consistent as those of the other treatments, and ranged from small to marked (Table I).

After all treatments, the ability of the nerves to conduct an action potential was checked. The results are indicated in Table I. More detailed studies on the effects of these agents on electrical activity have appeared (1–3, 9),[1] the findings of which are indicated in Table I. In a few cases, the effects of curare were checked on the axons actually used for electron microscopy.

DISCUSSION

Since in various tissues the fragmentation of thin cytoplasmic processes into chains of membrane-limited cytoplasmic globules has been observed to be an artifact, especially after permanganate fixation (25, 26), it is important to consider the extent to which postmortem or fixation artifacts may have contributed to our findings. Artifactual fragmentation has been found in cells that contain thin cytoplasmic laminae, e.g. the satellite cells of toad spinal ganglia and the Schwann layers of the prawn nerve. An equally drastic effect occurs in the sheath of the small fibers that run parallel to the giant fiber in our control specimens. However, the sheaths of the giant axons in our control specimens, even those kept at room temperature for 2 hr prior to fixation (See Methods), were found to be fragmented to only a very slight degree.

A further control consists in the comparison of the structural effects of the experimental treatment after use of OsO_4 fixatives. OsO_4 fixatives are known to produce occasionally, as an artifact, by fusion of two opposed membranes, chains of vesicles with encapsulated extracellular space. In the formation of vesicles by two opposed membranes, an outward bending of the cell membranes occurs, while in the formation of cytoplasmic globules an inward bending of the mem-

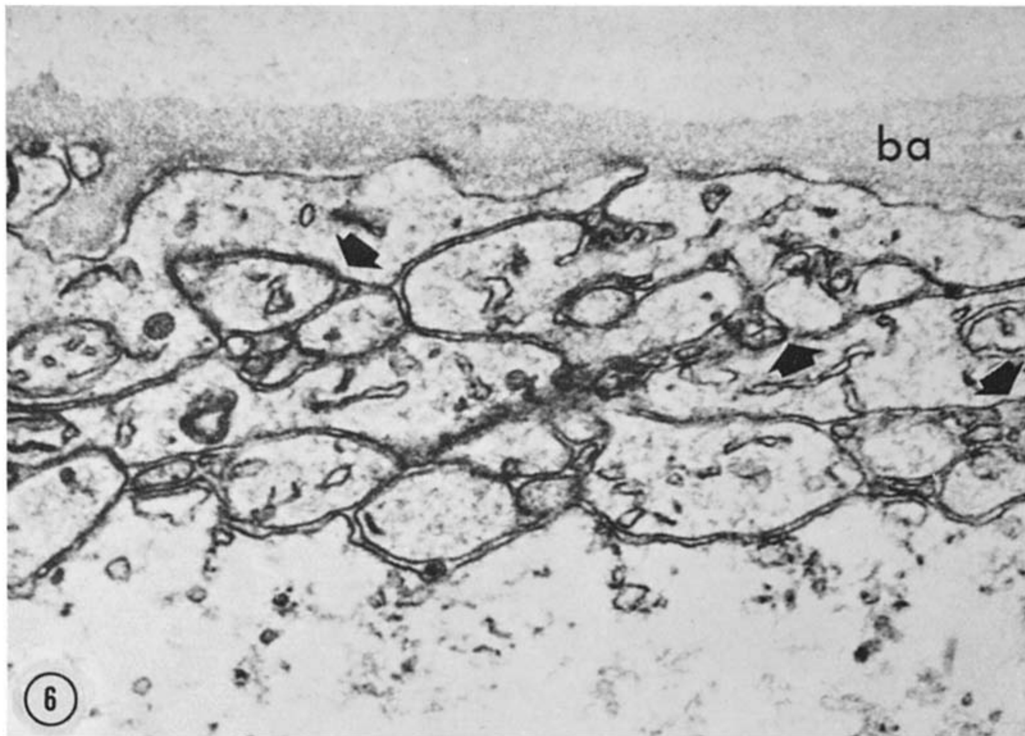


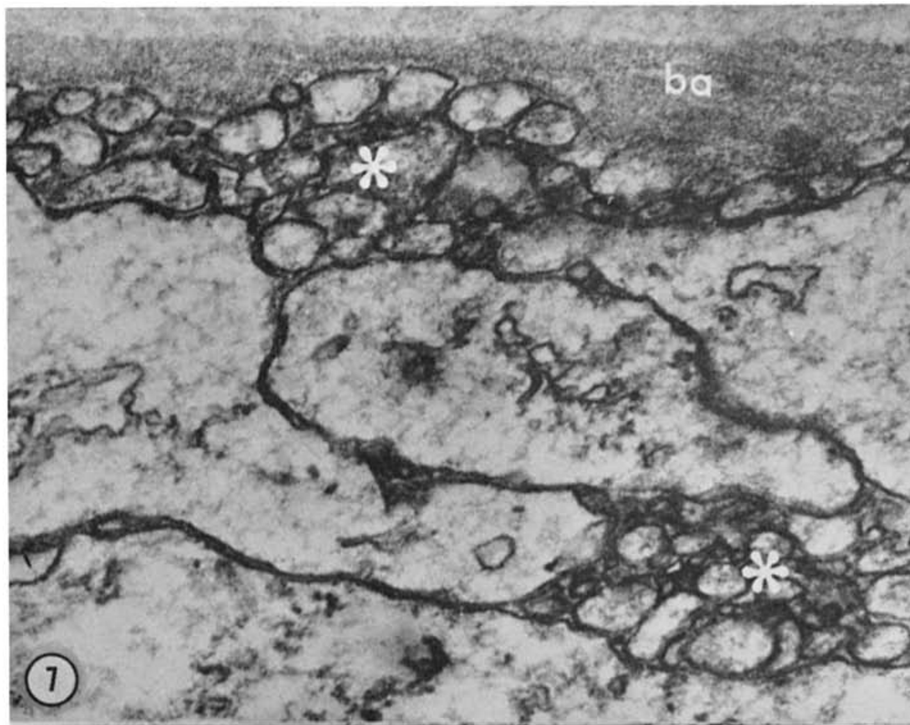
FIGURE 6 Effect of 15 $\mu g/ml$ of phospholipase A on the fine structure of the Schwann sheath of a giant axon fixed in permanganate (1a). At arrows, the layers are fragmented into cytoplasmic globules due to the experimental treatment. × 50,000.

brane takes place. The forces in these two processes thus are opposed. It may be speculated that permanganate fixatives enhance, to some extent, the effect of the experimental treatment, whereas OsO_4 fixatives reduce this effect, and that the degree of fragmentation of the sheath actually produced by the experimental treatment may lie in between that shown after OsO_4 fixation and that shown after permanganate fixation. As a matter of fact, the degree of globule formation in the experimental material often appears to be higher after permanganate than after OsO_4 fixation.

We can conclude, therefore, on the basis of comparison of micrographs of control and treated giant axon preparations, that the venoms and PhA have altered the membrane structure in such a way that, upon fixation, masses of cytoplasmic globules are formed in the Schwann sheath. We cannot, however, conclude as to whether the vesiculation is the actual alteration in membrane fine structure produced by these treatments or whether some other change in membrane structure predisposes the Schwann cell membrane to globule formation during dehydration and fixation.

However, we should like to emphasize the difficulty in estimating quantitative differences, since in each specimen only a small percentage of the length of the axon could be examined because of the ultrathin sectioning used for electron microscopy. In order to get comparable results we checked, in each sample, a sheath segment of equal length. Our results are consistent in that, whenever the action potential of the giant axons is impaired or absent after venom treatment, there is a distinct structural decomposition of the Schwann sheath. It is of special interest that the membrane of the giant axon is less affected by the treatments than the membrane of the Schwann cell, indicating perhaps a difference in chemical composition.

Our studies suggest that the increase in permeability and the block in conduction produced by certain venoms or phospholipase A are caused by an alteration of the membranes of the Schwann



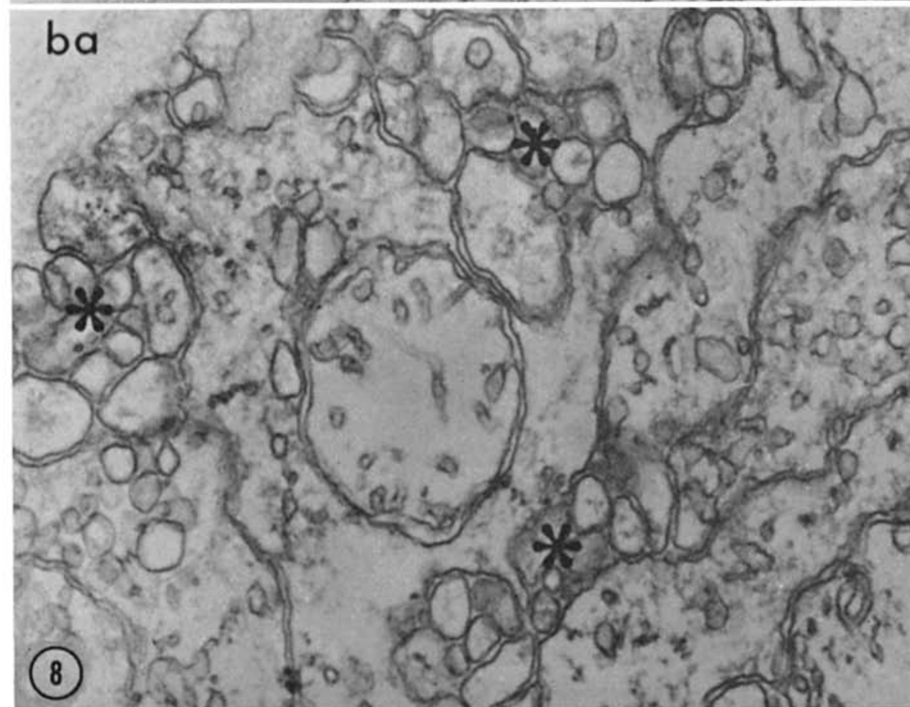


FIGURE 7 Effect of 100 μg/ml of cottonmouth venom on the structure of the sheath of a giant axon fixed in permanganate (1a). At (*) there are nests of cytoplasmic globules produced by the treatment. $\times$ 50,000.

FIGURE 8 Same specimen as in Fig. 7, but fixed in osmium tetroxide (2a). $\times$ 50,000.

TABLE I

Effects of Various Agents on the Fine Structure of the Schwann Sheath of the Squid Giant Axon

Every concentration was tested on one or more (indicated by number in parenthesis) separate giant axons with surrounding small nerve fibers (* giant axon free of adhering small nerve fibers). Structural change indicated by: 0, none; 1, small; 2, moderate; 3, marked (see text for further details). — is little or no effect on action potential (AP); + is block or marked decrease in AP. Abbreviations are as follows: CM, cottonmouth venom; CV, cobra venom; RI, ringhals venom; RS, rattlesnake venom; PhA, phospholipase A; DLF, direct lytic factor; HY, hyaluronidase; and CTA, cetyltrimethylammonium chloride.

		Effect on AP of			
Treatment	Conc.	Treatment	Curare after treatment	Fixation	Structural change
	$\mu g/ml$				
PhA	15	—	+	OsO_4	0–1
				$KMnO_4$	1
	50	+		OsO_4	2
				$KMnO_4$	2–3
CM	15–25	—	+	OsO_4 (2)	1–2
				$KMnO_4$ (3)	2
	100	+		OsO_4	2
				$KMnO_4$	2
	250*	—	—	$KMnO_4$ (3)	0
CV	30	—	+	$KMnO_4$	1–2
	100	+		$KMnO_4$	2–3
RI	100	+		$KMnO_4$	2–3
RS	500	—	—	OsO_4	0–1
				$KMnO_4$	1
DLF	100	—	—	$KMnO_4$	0
	500	+		$KMnO_4$	0–1
HY	1000	—	—	$KMnO_4$	0
CTA	200	+		$KMnO_4$	1–3

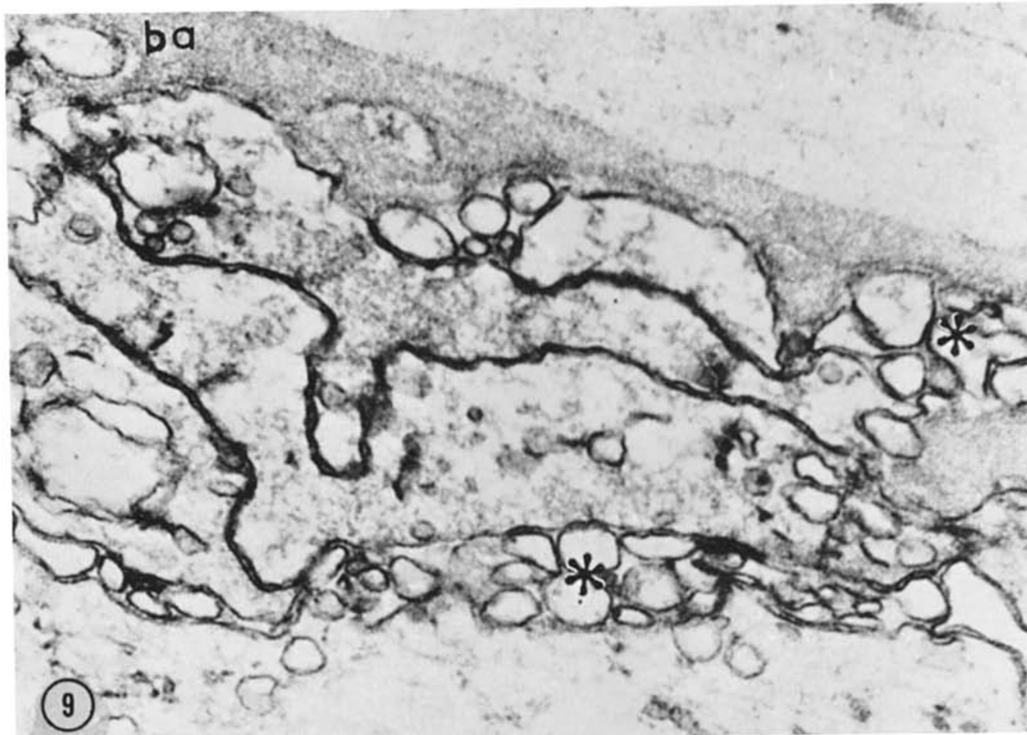
sheath. The effects of the isolated PhA fraction mimicked those of whole venoms of cottonmouth, cobra, and ringhals snakes, which is confirmatory evidence that PhA is the factor in venoms which is responsible for their effects on the squid giant axon (2, 4).[1] Other preparations may differ from squid giant axons in their response to venoms or PhA, since Tobias has reported that block of conduction in the lobster giant axon is not associated with observable structural changes (27). Concentrations of PhA and venom which did not block conduction, but which rendered the squid axons sensitive to curare (Table I) caused either no or small or, at most, moderate effects on Schwann sheath structure, depending upon the agent and the fixative employed. It was, of course, to be expected that these lower concentrations of venom and PhA, which increase permeability of the squid giant axon much less than higher concentrations which block conduction (5, 6, 8),[1] would also cause much less structural change. Even these relatively small changes in structure of the Schwann sheath appear sufficient, however, to allow small molecules such as curare and ACh to penetrate to some extent, i.e. concentrations sufficient to block conduction. Marked structural changes permit ACh and curare to penetrate to a much greater extent.

Since high amounts of venom and PhA which markedly increase permeability had no noticeable effect on the basement membrane or connective tissue, it would appear that these structures are not the sites of the permeability barrier. Earlier studies, which showed that little ACh penetrated giant axons whether or not they were surrounded by smaller nerve fibers, had also indicated that adhering small nerve fibers and associated connective tissue do not constitute a strong permeability barrier for the giant axon (8).

The direct lytic factor (DLF) is a component of certain venoms which has been shown to render membrane phospholipids, in some preparations, susceptible to the action of PhA even though DLF itself has no PhA activity (15, 28). In the squid axon, however, DLF has no effect on permeability nor does it enhance the effects of PhA on permeability or phospholipid splitting.[1] Although DLF in relatively high concentrations blocks conduction, this effect is not associated with observable structural changes in the Schwann sheath (Table I).

Rattlesnake venom, in contrast to venoms of cottonmouth, cobra, and ringhals, has little or no effect on the structure of the Schwann sheath, even though it was tested in higher concentrations. This finding agrees with the finding that this higher concentration of rattlesnake venom is unable to affect electrical or permeability properties of the squid giant axon (2, 5).[1] As noted in Results, and as has been reported elsewhere (14),[1] rattlesnake venom has very low PhA activity, which correlates with its weak effects on the squid giant axon.

Although cottonmouth venom produced struc-



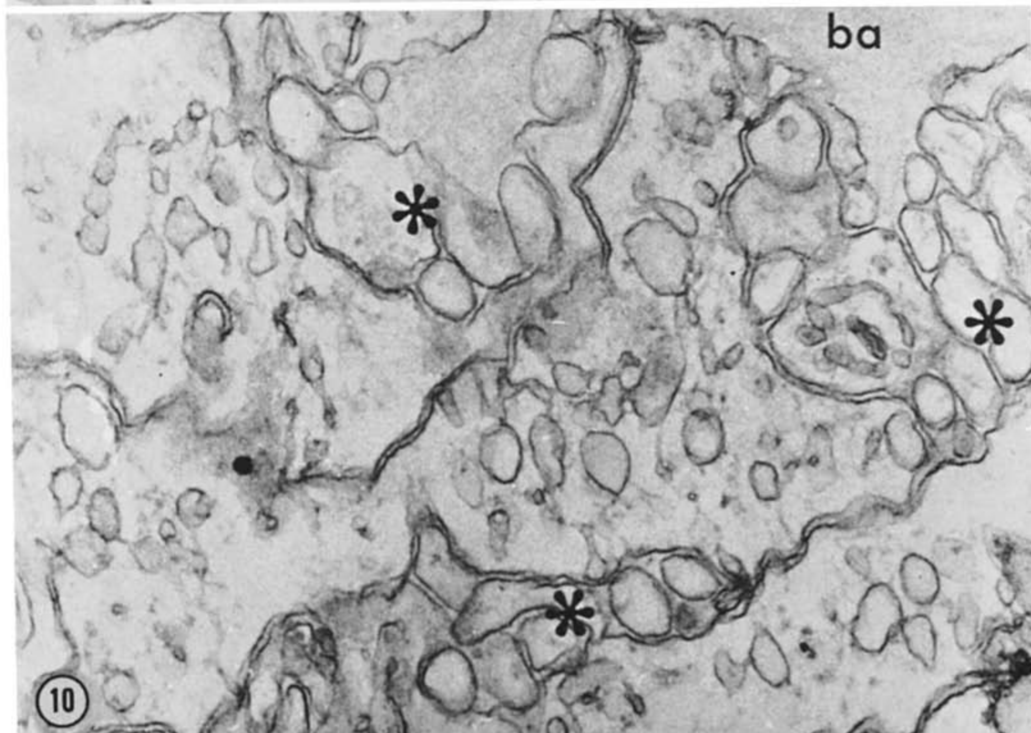


FIGURE 9 Effect of 50 μg/ml of phospholipase A on the structure of the sheath of a giant axon fixed in permanganate (1a). × 50,000.

FIGURE 10 Same specimen as in Fig. 9, but fixed in osmium tetroxide (2a). × 50,000.

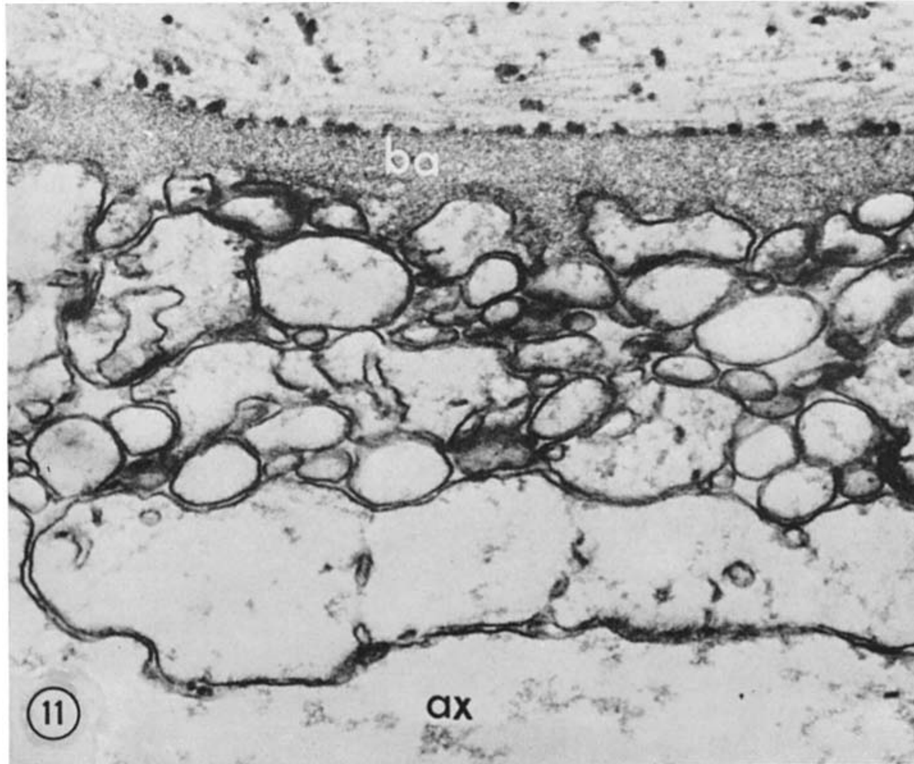


FIGURE 11 Effect of 200 μg/ml of cetyltrimethylammonium chloride on the structure of the sheath of a giant axon fixed in permanganate (1a). The sheath is fragmented into cytoplasmic globules. $\times$ 50,000.

tural alterations in giant axons surrounded by small nerve fibers, it had no effect on the structure of giant axons free of adhering nerve fibers, even when it was tested in relatively high concentrations. Similarly, cottonmouth venom has no effect on the permeability or electrical properties of giant axons free of small nerve fibers (2, 8). As has been previously discussed, the most likely explanation for these observations appears to be that in the axon preparation lacking small nerve fibers there is much less substrate on which phospholipase A can act and that, therefore, less lysophosphatides and free fatty acids are formed (2, 4, 8).[1,2] Lysophosphatides and free fatty acids, produced by the action of PhA on phospholipids, are known to have strong detergent properties. It is of interest that cetyltrimethylammonium chloride, a cationic detergent, has effects on structure of the Schwann cell that resemble the effects of venoms and PhA. Very recent studies involving phospholipid analyses of squid axons following venom application have also indicated that the effects of venom and of PhA are caused by toxic products liberated as a result of PhA action, since the action of venom or of PhA can be mimicked by the addition of lysophosphatides to the squid axon. In addition, phospholipase C, the action of which does not give rise to products with detergent properties, has no effect on axonal conduction or permeability, even though the extent of phospholipid splitting is even greater than with phospholipase A.[1,2]

[2] Rosenberg, P., and E. Condrea. Maintenance of axonal conduction and membrane permeability in the presence of extensive phospholipid splitting. In preparation.

We would like to thank Drs. D. Nachmansohn and J. D. Robertson for their interest in this study. Special thanks are due to Dr. E. Condrea for helping to provide preparations of phospholipase A and DLF.

For able assistance we thank Mr. George Frances

and Misses Jane Frick and Maxine Parsons. We thank the Marine Biological Laboratory, where some of these studies were carried out, for the fine facilities made available.

These studies were supported by Division of Research Grants and Fellowships, United States Public Health Service (NB-03304 and NB-02665), by the National Science Foundation (GB-6734 and GB-3128), and by a research grant from the National Multiple Sclerosis Society (499A). Dr. Rosenberg is recipient of a research career development award 5-K3-NB-21, 862-04.

Received for publication 11 August 1967, and in revised form 17 October 1967.

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