

STRUCTURAL COMPLEXES IN THE SQUID GIANT AXON MEMBRANE SENSITIVE TO IONIC CONCENTRATIONS AND CARDIAC GLYCOSIDES

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ABSTRACT

Giant nerve fibers of squid *Sepioteuthis sepioidea* were incubated for 10 min in artificial sea water (ASW) under control conditions, in the absence of various ions, and in the presence of cardiac glycosides. The nerve fibers were fixed in OsO₄ and embedded in Epon, and structural complexes along the axolemma were studied. These complexes consist of a portion of axolemma exhibiting a three-layered substructure, an undercoating of a dense material (~0.1 μm in length and ~70–170 Å in thickness), and a narrowing to disappearance of the axon-Schwann cell interspace. In the controls, the incidence of complexes per 1,000 μm of axon perimeter was about 137. This number decreased to 10–25% when magnesium was not present in the incubating media, whatever the calcium concentration (88, 44, or 0 mM). In the presence of magnesium, the number and structural features of the complexes were preserved, though the number decreased to 65% when high calcium was simultaneously present. The complexes were also modified and decreased to 26–32% by incubating the nerves in solutions having low concentrations of sodium and potassium. The adding of 10⁻⁵ M ouabain or strophanthoside to normal ASW incubating solution decreased them to 20–40%. Due to their sensitivity to changes in external ionic concentrations and to the presence of cardiac glycosides, the complexes are proposed to represent the structural correlate of specialized sites for active ion transport, although other factors may be involved.

Spaced thickenings of the inner leaflet of the axon membrane (axolemma), in addition to the three-laminar substructure and to the regions of globular repeating arrangement, have been observed in the nerve fibers of several squid species (24, 27). Recently, electron-opaque deposits adjacent to the axolemma of squid nerve fibers, different from the thickenings and related to the presence of calcium and phosphorus, have also been described (9–11).

The axolemma thickenings have been compared to the postsynaptic density (7, 12), as well as to the

opaque axoplasmic material underlining the nodes of Ranvier of the peripheral and central nervous systems (1, 5, 17). These postsynaptic densities have been related to the sites where permeability changes occur (4). The opaque axoplasmic material of the nodes of Ranvier has been thought to be related to specialized zones of membrane permeability (5), to areas of activity (17), or to diffusion barriers (1). A dense layer of fine granular material undercoating the plasma membrane of the axon initial segment, resembling that found at the

node of Ranvier, has also been described in different neurons (13, 22). It has been suggested that this undercoating represents a structural modification of the cell surface involved in the origin and propagation of the action potential (13). The distribution of the thickenings along the axolemma has been compared to that found, with the aid of histochemical techniques, in connection with sites of $\text{Na}^+\text{-K}^+\text{-Mg}^{++}\text{-ATPase}$ (19) and of acetylcholinesterase activities (26). It has been suggested that the thickenings might be related to areas of the axolemma capable of accomplishing specific functions (27).

Electron-dense material attached to the axonal membrane has been reported by Peracchia and Robertson (16) in crayfish nerves after electrical stimulation or asphyxiation. This electron-dense material does not appear as plaques but as an increase in electron density and thickness of the complete axolemma, as well as of some other membranes (mitochondria and endoplasmic reticulum). The osmiophilia is not observed in the control fibers and it has been attributed to conformational changes of the membrane proteins that unmask the SH groups and consequently increase the formation of osmium polymers.

The present work deals with the ultrastructural details of such thickenings and their modifications by different external concentrations of monovalent and divalent cations, as well as by the action of cardiac glycosides. The results show that the thickenings are part of structural complexes. Each one consists of a portion of axolemma that exhibits the three-layered substructure, an undercoating of electron-dense material attached to or forming part of its inner leaflet, and a narrowing to disappearance of the interspace separating the axon from the Schwann cell. The preservation of the structural complexes seems to require external magnesium, sodium, and potassium, but not calcium. The complexes are also modified by cardiac glycosides. Evidence of the same complexes in giant nerve fibers of other marine invertebrates is also presented.

MATERIAL AND METHODS

General Procedure

Giant nerve fibers dissected out of the first stellar nerve of squid *Sepioteuthis sepioidea* were used. In a first group of experiments, three nerve fibers, each from a different animal, were fixed immediately after isolation from the mantle (within 8 min of the decapitation of the animal) in an ice-cold, veronal-buffered, 1% OsO_4 in

artificial sea water, at pH 7.9–8.0. Fixation was carried out for 1 h at 4°C, and afterwards the specimens were dehydrated in a graded ethanol series starting at 50%, and embedded in Epon 812. Ultrathin sections, obtained with a Porter-Blum MT-2B ultramicrotome equipped with a diamond knife, were mounted on Formvar-coated copper grids, double stained with uranyl acetate and lead citrate, and examined in a Siemens Elmiskop 101 electron microscope.

In all the other experimental groups, the nerve fibers were incubated for 10 min, either in normal artificial sea water (controls) or in one of the various experimental solutions (described in detail below), before their processing for electron microscopy. The OsO_4 fixatives were prepared in their respective experimental solutions used for incubation, except in two groups of nerve fibers in which, after incubation in the corresponding experimental solutions (see below, solutions II *c* and III *b*), the fixation was carried out in the fixative prepared in normal artificial sea water similar to the one used for controls. These last two groups were studied to determine whether the structural changes were produced by the incubation or by the fixation.

Three types of control nerve fibers were used: (a) nerve fibers from a different animal, (b) the paired nerve fiber from the same animal, and (c) one half of the same nerve fiber used to test the effect of a given solution.

Experimental Solutions

(I) Artificial sea water (ASW) was used as control medium. The mM concentrations of its components were as follows: 442 NaCl; 10 KCl; 11 CaCl_2 ; 53 MgCl_2 ; 2.5 NaHCO_3 ; pH 7.9–8.0.

(II) Sea water solutions with different calcium and magnesium concentrations were prepared by replacing either the divalent cations by choline chloride (low Ca and low Mg solutions) or part of the sodium chloride by magnesium or calcium chloride (high Ca and high Mg solutions). The pH of these solutions was 7.9–8.0. The mM concentrations of Ca and Mg in the six different sea water solutions thus prepared (a–f) were: (a) 0 Ca, 0 Mg, 2 EDTA; (b) 0 Ca, 0 Mg, 0 EDTA; (c) 44 Ca, 0 Mg; (d) 88 Ca, 0 Mg; (e) 0 Ca, 75 Mg; and (f) 44 Ca, 53 Mg.

(III) Sea water solutions with low potassium and low sodium concentrations were prepared by replacing, mole per mole, potassium chloride and sodium chloride by choline chloride. The mM concentrations of potassium and sodium in the two different sea water solutions thus obtained were: (a) 0.1 K, 442 Na; and (b) 0.1 K, 4 Na. The pH of the solutions was 7.9–8.0.

(IV) Sea water solutions containing 10^{-5} M K-strophanthoside or 10^{-5} M ouabain were obtained by adding the necessary amount of drugs to control ASW.

Counting Nerve Membrane Structures

In order to examine most of the perimeter of the giant nerve fibers, complete cross sections were placed in grids having a few holes only. The whole perimeter of the

section was observed at a 50,000 diameter magnification, while the number of structural complexes was recorded. Afterwards, seriated pictures of the axon-Schwann cell boundary were taken at a 10,000 diameter magnification and the axon contour was measured directly on projections of the negative film in a Nikon Profile Projector with a K and E Map Measure.

RESULTS

Nonincubated Nerve Fibers

In the giant nerve fibers fixed immediately after isolation from the squid mantle, the axolemma showed the trilaminar and the globular substructures, as well as the spaced thickenings of the inner leaflet of the membrane, that have been reported in giant nerve fibers from other squid species (24, 27). The thickenings appeared as electron-dense zones, $\sim 0.1 \mu\text{m}$ in length and $\sim 70\text{--}170 \text{ \AA}$ in thickness, attached to the internal surface of the axolemma. At the level of the thickening, the axolemma always exhibited the three-layered substructure, and in a majority of the cases a reduction of the intercellular space between the axolemma and the Schwann cell plasma membrane was evident (Figs. 1 and 2). Neither a special modification nor a special substructural pattern was observed at this level in the Schwann cell plasma membrane. Often, the intercellular space appeared obliterated by the close apposition of both plasma membranes (Fig. 2).

From here on, we denote as "structural complex" the coexistence of these three features at the same level in the axon-Schwann cell boundary: (a) the three-layered substructure of the membrane, (b) the dense material attached to it, and (c) the reduction of the intercellular space. The structural complexes were not regularly spaced; however, when all the perimeter available for observation in a single cross section was surveyed, the number of complexes recorded, expressed per unit length, was very similar. An incidence of 182 complexes per $1,000 \mu\text{m}$ of axon perimeter was obtained in three different non-incubated nerve fibers, the individual values being $130/710 \mu\text{m}$, $142/775 \mu\text{m}$, and $135/753 \mu\text{m}$, which correspond, respectively, to 183, 183, and 179 complexes per $1,000 \mu\text{m}$ of perimeter.

At the axon-Schwann cell boundary, two structures were commonly seen in the axoplasm alongside the axolemma and close to it. They were: mitochondria, some of which appeared almost touching the inner surface of the axolemma; and membranous vacuoles. The vacuoles seem to be

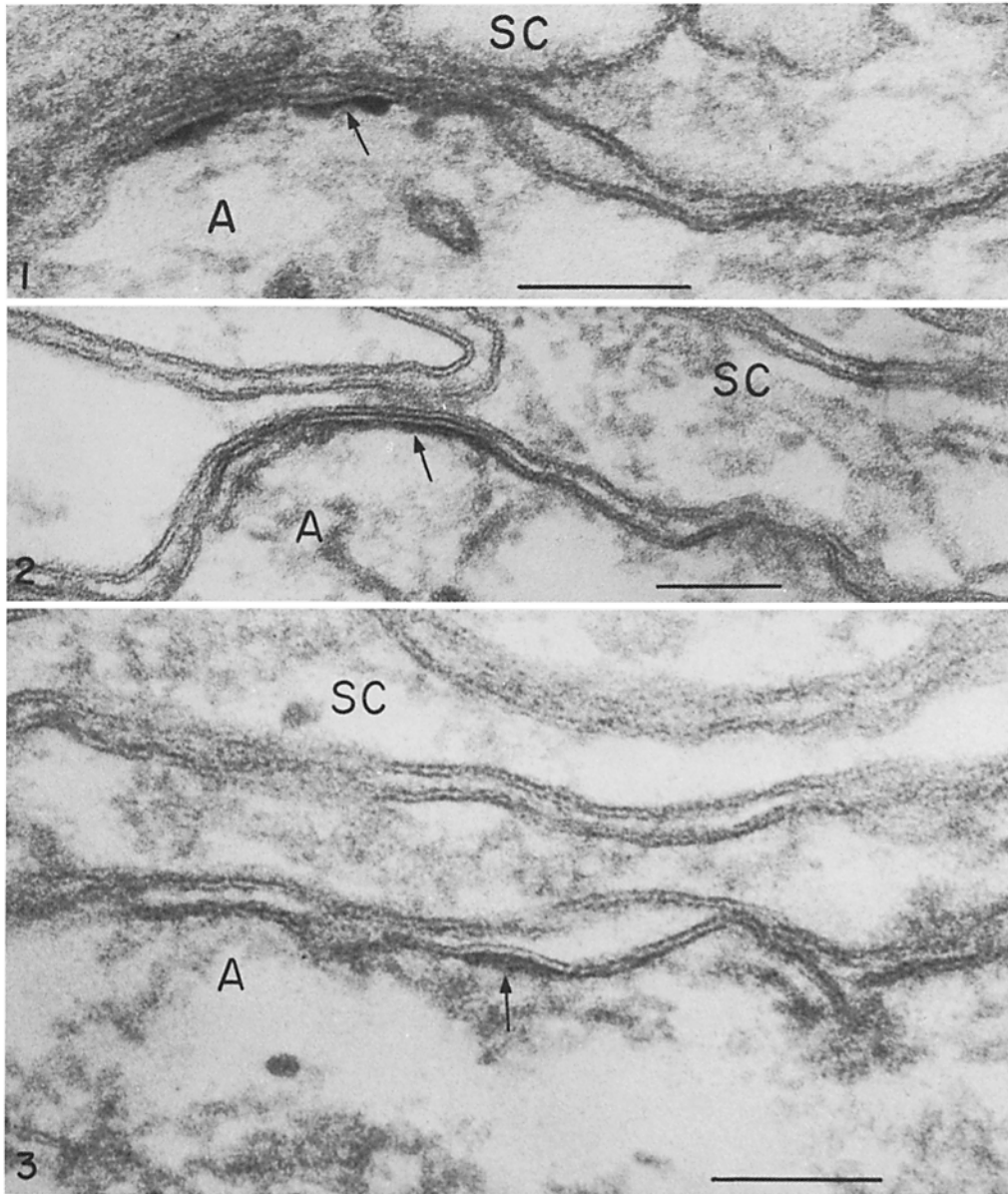
part of the axolemma, since their walls appeared to be continuous with this membrane in certain sections. Some undulations of the axolemma possibly penetrate more deeply into the axon, and, depending on the plane of the section, they appeared mostly as independent membranous profiles. No relationship was observed between the structural complexes and the zones in which the mitochondria or the membranous profiles lay close to the axon membrane.

Incubated Nerve Fibers

After a 10-min incubation in artificial sea water, the image presented by the axon-Schwann cell boundary of the giant nerve fiber is apparently no different from the one observed in the non-incubated fibers. The axolemma substructure and the structural complexes appeared unchanged. The mean \pm SEM incidence of structural complexes counted in $13,628 \mu\text{m}$ of axolemma measured in 16 different fibers was $137 \pm 17/1,000 \mu\text{m}$ of perimeter. These results are not given separately, as the range of complex density in the different types of controls was approximately the same.

Relationship between the Structural Complexes and the External Concentrations of Calcium and Magnesium

The incubation for 10 min in Mg-free, high Ca (44 or 88 mM) sea water solutions produced a decrease in the number of structural complexes, as well as a change in their morphological aspect. Few typical complexes were observed. The majority of them appeared as remnants of the dense material attached to zones in which the membrane conspicuously exhibits the trilaminar substructure and is in close apposition to the cell membrane facing it. Also occasionally seen were five-layered membrane compounds formed by the fusion of the axon and Schwann cell plasma membranes with no dense material attached to their inner surfaces. Such a close apposition of the membranes was found more frequently with the highest calcium concentration (88 mM). In the presence of 44 and 88 mM Ca, the lack of Mg caused a decrease in the incidence of structural complexes (even including the atypicals) from $137/1,000 \mu\text{m}$ of perimeter in the controls to $34/1,000 \mu\text{m}$ and to $14/1,000 \mu\text{m}$, respectively (Table I, Fig. 4). That the change in number of the structural complexes was induced by the incubation in the Mg-free solution and not by the fixation procedure was established because



FIGURES 1 and 2 Part of control nonincubated squid giant nerve fibers showing at the axon (A)-Schwann cell (SC) boundary, a structural complex (\rightarrow) formed by a dense material attached to the inner aspect of the axolemma, the conspicuous trilaminar substructure of this membrane, and the reduction to disappearance of the intercellular space at the same level. Bar indicates $0.1 \mu\text{m}$. $\times 224,000$; $\times 160,000$.

FIGURE 3 Giant nerve fiber of squid incubated for 10 min in ASW containing 10^{-6} M K-strophanthoside. Electron micrograph shows one of the few complexes (\rightarrow) observed at the axon (A)-Schwann cell (SC) boundary. Note the diminution of the dense material and the separation of both apposed plasma membranes. Bar indicates $0.1 \mu\text{m}$. $\times 224,000$.

of the almost identical results obtained in those fibers fixed in normal fixative after incubation in the same Mg-free, 44 mM Ca experimental solution.

Results like those obtained in the Mg-free, high-Ca solutions were seen in paired nerve fibers in Mg-free, Ca-free solutions. Thus, when the nerves were incubated for 10 min in the latter

TABLE I
Effect of External Cations on the Incidence of
Structural Complexes in the Giant Nerve Fiber

No. of nerve fibers	Na	K	Ca	Mg	EDTA	Complexes
	mM	mM	mM	mM	mM	no./1,000 μ m
16	442	10	11	53	0	137 \pm 17
2	414	10	88	0	0	14
2	442	10	44	0	0	34
2	389	10	44	53	0	84
2	442	10	0	0	0	13
2	442	10	0	0	2	11
4	425	10	0	75	0	117 \pm 19
2	442	0.1	11	53	0	36
3	4	0.1	11	53	0	58 \pm 15

Values are mean \pm SEM.

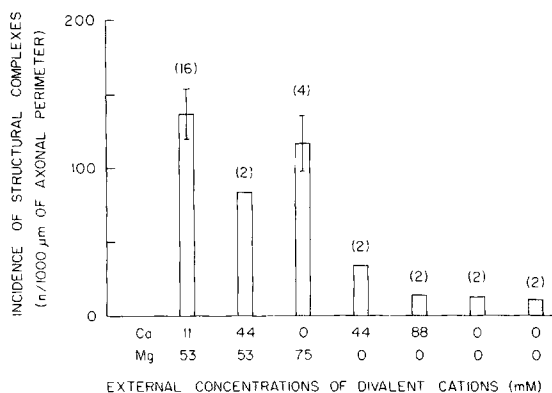


FIGURE 4 A histogram showing the number of structural complexes in the axolemma per 1,000 μ m of axon perimeter at different external concentrations of calcium and magnesium. The SEM was calculated when the complexes were measured in more than two axon perimeters. The shaded column indicates the addition of 2 mM EDTA. The numbers of fibers used are in parentheses.

solutions, with or without EDTA, the number of structural complexes observed was 11/1,000 μ m and 13/1,000 μ m of perimeter, respectively (Table I, Fig. 4), and their general aspect was that of a vestige.

As the above results show, when magnesium was not present during the incubation, the values decreased to 10–25% regardless of the calcium concentration. On the other hand, in solutions containing magnesium, the complexes were preserved even when the calcium was removed. In fact, the incubation of nerve fibers in Ca-free, high-Mg (75 mM) sea water solution had no

appreciable effect on the morphological aspect of the complexes or in their incidental number. The complexes were also well preserved in the nerve fiber halves left for 2 h in the same solution. The incidence calculated from the four individual values of the two nerve fibers was 117 ± 19 SEM/1,000 μ m of perimeter (Table I, Fig. 4). This value is similar to that of the controls (137 ± 17 SEM/1,000 μ m).

As has already been found for other squid species (32), the axon electrical potentials of *S. sepioidea* giant nerve fibers are not appreciably modified when all the divalent cations in the ASW medium are replaced either by 44 mM calcium or by 75 mM magnesium.

Other types of dense structures, such as the deposits shown by Oschman et al. (10) and Hillman and Llinás (9) in the presence of high calcium, were not observed in the groups of nerve fibers incubated with or without calcium. They were not observed in the nerves incubated in the presence of magnesium either, nor even in those two groups of nerves which, in a confirming experiment, were incubated in the presence of 44 mM Ca added to a normal magnesium concentration (53 mM). In this latter group, an incidence of 84 complexes/1,000 μ m of perimeter was obtained. This value is higher than that (34/1,000 μ m) found in the nerves incubated in sea water containing the same amount of calcium (44 mM) but no magnesium (Fig. 4). Note that the presence of a high calcium concentration rather tends to lower the number of complexes to 65%.

Relationship between the Structural Complexes and the External Concentrations of Potassium and Sodium

As shown in Table IV, the incidence of the structural complexes observed in two different nerve fibers incubated in low-K (0.1 mM) sea water, was 36/1,000 μ m of perimeter, while the number of complexes found in three different nerve fibers exposed to the low-K (0.1 mM), low-Na (4 mM) sea water solution was 58 ± 15 SEM. As compared to the values for control nerves incubated in ASW, these values represent a reduction to 26 and 32% of the number of complexes, respectively. In their morphological aspects, the persisting complexes were modified the same as when magnesium was absent: they appeared as vestiges (Fig. 5). A similar reduction in the number of complexes was observed in those fibers incubated in the same low-K, low-Na experimental

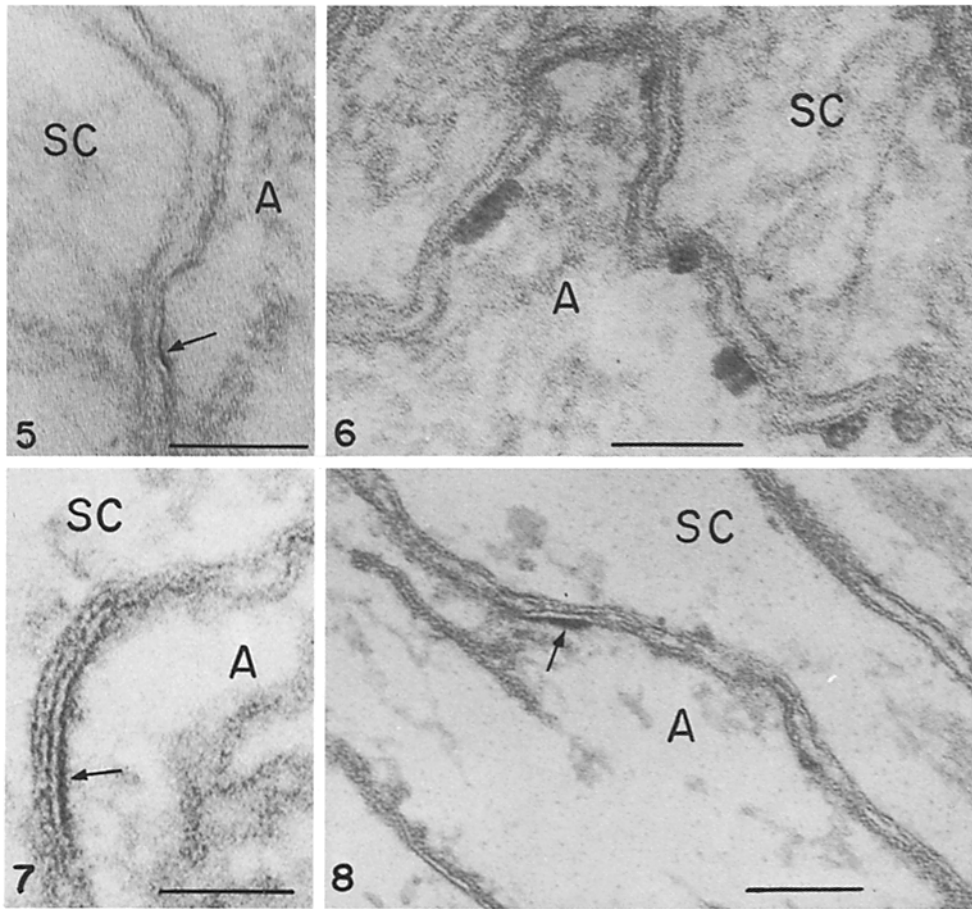


FIGURE 5 Electron micrograph showing part of the axon (A)-Schwann cell (SC) boundary of a squid giant nerve fiber incubated for 10 min in low-K (0.1 mM), low-Na (4 mM) sea water. A remnant of one of the structural complexes of the axolemma (→) is observed. Bar indicates 0.1 μm . $\times 173,400$.

FIGURE 6 Control giant nerve fiber showing, in part of its axon (A)-Schwann cell (SC) boundary, an accumulation of dense globules of different sizes. No close apposition between the axolemma and Schwann cell plasma membrane is observed at their levels. Bar indicates 0.1 μm . $\times 167,850$.

FIGURE 7 Electron micrograph of a giant nerve fiber of the lobster (*P. argus*) walking leg nerve. One structural complex (→) is observed at the axon (A)-Schwann cell (SC) boundary. Bar indicates 0.1 μm . $\times 182,000$.

FIGURE 8 Electron micrograph of a giant nerve fiber of polychaete showing a structural complex at its axon (A)-Schwann cell (SC) boundary. Bar indicates 0.1 μm . $\times 155,200$.

solutions and fixed in OsO_4 solution prepared in normal artificial sea water. This indicates that the changes were induced by the incubation and not by the fixation procedure.

In some of the nerve halves incubated in low sodium and low potassium sea water, as well as in some of their control halves, electron-dense deposits, similar to those induced by calcium as

reported by Oschman et al. (10), were occasionally observed (Fig. 6). In such cases, the deposits outnumbered the complexes found in normal non-incubated fibers by up to three times, and they were sometimes concentrated in about one-half of the perimeter. These deposits appeared as large granules, attached either to the axoplasmic side of the axolemma or to both surfaces of this mem-

brane, or also as large granules housed in dilata-tions of the axon-Schwann interspace. Since they were observed in both control and experimental nerve fiber halves, their appearance should be related to a cause distinct from the variables under control in the present experiments.

Relationship between the Structural Complexes and Cardiac Glycosides, Inhibitors of the Active Transport

The incubation of nerve halves for 10 min in ASW containing either 10^{-8} M ouabain or 10^{-8} M K-strophanthoside produced a marked diminution of the number of the axolemma structural complexes, with an incidence of 52/1,000 μm and 30/1,000 μm of perimeter, respectively. These values represent a diminution to 20–40%. Moreover, in the few complexes that persisted there was a decrease in the size and electron density of the attached material (Fig. 3). The three-layered sub-structure of the axolemma was preserved, but the narrowing of the axon-Schwann cell interspace and the close apposition of the plasma membranes facing each other were not observed. Table II illustrates the data obtained in the nerve fibers incubated with the cardiac glycosides, as compared to the data from the controls incubated during the same period in ASW.

DISCUSSION

The present results show the existence of structural complexes in the axon-Schwann cell boundary, each exhibiting an electron-dense zone attached to the axon plasma membrane by its axoplasmic side, the conspicuous three-layered pattern of this membrane at the same level, and a tapering-off of the axon-Schwann cell interspace. No precise limit

was detected between the inner leaflet of the membrane and the dense material. These structural complexes were observed to be spaced irregularly along the axolemma.

The experiments herein reported also show that both the incidence and the pattern of the structural complexes depend upon the external concentration of magnesium. When this divalent cation was not present, the number of complexes decreased significantly and their ultrastructural features were modified as to appear as mere vestiges. These alterations occurred either in the presence of high concentrations of calcium or in the absence of such a cation. On the other hand, when calcium was substituted by magnesium, or when a normal concentration of magnesium was present together with a high calcium concentration, the structural complexes were preserved without apparent modifications, and their incidence was close to that found in the controls.

It was found also that the incidence and aspect of the complexes depend similarly upon the external potassium concentration. The number of complexes in the nerve fibers incubated in low potassium sea water is lower than the value obtained in the control fibers. No further reduction in the incidence of the complexes was produced by decreasing a hundred times the external concentration of sodium.

The present experiments also show the sensitivity of the complexes to cardiac glycosides. A three- to fivefold decrease in the number of complexes per 1,000 μm of perimetric length was found after a 10-min incubation in ASW containing either one of the cardiac glycosides ouabain and strophanthoside. In addition, the ultrastructural features of the complexes changed: the electron-dense zone was reduced and the apposed plasma membranes were separated.

The structural complexes of the axolemma seem to be a rather common feature in the unmyelinated nerve fibers of marine invertebrates. This has been pointed out in our previous work on other squid species, such as *Doryteuthis plei* (tropical squid from the Caribbean) (24), and *Dosidicus gigas* (giant squid from the Humboldt Stream) (27), and it can be seen in Plate 3 in the paper by Baker, Hodgkin and Shaw (2) that the complexes are also present in *Loligo forbesi*. The present authors have also observed the complexes in the giant nerve fibers of the lobster *Panulirus argus* (Fig. 7) and of polychaetes (Fig. 8).

TABLE II
Effect of Cardiac Glycosides on the Incidence of Structural Complexes in the Giant Nerve Fiber

No. of nerve fibers	Cardiac glycoside	Complexes
		no./1,000 μm
16	Control	137 \pm 17
3	K-strophanthoside, 10 μM	30 \pm 9
5	Ouabain, 10 μM	52 \pm 11

Values are mean \pm SEM.

Note that the structural complexes described in the present work are different from the electron-dense deposits found by Oschman et al. (10) in the giant axon of squid *Loligo pealii* and by Hillman and Llinás (9) in the giant synapse of the same squid. These differences are: (a) the electron-dense deposits were found when the nerves were fixed in glutaraldehyde containing 5 mM CaCl₂, and were not found when the tissue was prefixed in OsO₄ (9), whereas the structural complexes described in the present work are observed in nerve fibers fixed in OsO₄, and, furthermore, they remain relatively unmodified by variations in the external calcium concentration. Moreover, if any change occurs, it is a 35% diminution of the number of complexes observed in high calcium when all the other ions, including magnesium, were present in normal concentrations (Fig. 4). (b) The morphological features of both structures are somewhat different: the dense material in both is associated with the axonal membrane, but the dense deposits described by Oschman et al. (10) and Hillman and Llinás (9) are also localized in the intercellular space and in the Schwann cell membranes. Besides, these dense deposits appear as globules or plaques formed by subunits; and the three-laminar substructure of the axolemma and the narrowing of the intercellular space have not been pointed out in connection with them. Note, however, that electron-dense deposits similar to those of Oschman et al. (10) and Hillman and Llinás (9) appeared erratically in certain of our nerve halves (see Results and Fig. 6). This makes us think that there may be some special conditions, such as the so-called opaque axonal reaction pointed out by Hillman and Llinás (9), that induce the appearance of such deposits in our experiments.

In order to assess with certainty the significance of the axolemma complexes, further knowledge of their components, particularly of the membranes and the dense material, is required. Note, however, that the pattern of distribution of the complexes is rather similar to that observed for the deposits of reaction products indicating sites of acetylcholinesterase activity (26) and ATPase activity (19) in the squid giant nerve fiber. In both these cases, the structural complexes in the control tissue were modified by the histochemical procedure. It is likely that the reaction products are located in the same sites as the structural complexes, for two reasons: (a) the end product of the enzymatic reaction constantly lined sites of the axolemma

that conspicuously displayed the three-layered substructure, and (b) the complexes were drastically reduced in number when the nerve fiber slices were treated with specific inhibitors of these enzymes (26, 19).

Oschman and Wall (11) have reported that, since the opaque deposits induced by the presence of calcium in the fixative resemble the end product obtained when axons are reacted for ATPase localization, it is possible that the deposits may be formed by calcium trapped by intracellular phosphates hydrolyzed by membrane phosphatases. In the case of our experiments, calcium seems to be unrelated to the preservation of the structural complexes, while magnesium appears to be essential for it. Since divalent cations form insoluble phosphates, it can be surmised that, as in the case of the dense deposits of Oschman and Wall (11), magnesium and phosphorus are present in the dense material of the structural complexes, as an end product of the normal activity of the membrane ATPases. However, since no microprobe analysis has been carried out in the present experiments, it is not possible to identify the exact nature of such a dense material.

Moreover, the increased osmiophilia of the axonal membranes of crayfish, which is induced by electrical stimulation, asphyxia or reducing agents (16), has been explained as being due to the unmasking of the SH groups of certain membrane proteins. Specifically, SH groups belonging to ATPase molecules have been signaled by Peracchia and Robertson (16), since Mg-dependent ATPase, which is sensitive to SH reagents, has been found in crustacean nerve microsomal fractions.

On the other hand, as described above, the structural complexes are sensitive to the addition of cardiac glycosides to the external medium. Cardiac glycosides are known to block the active transport of ions across the plasma membrane of different cells (3, 6, 20). Furthermore, the Na⁺, K⁺, Mg⁺⁺-dependent ATPase is inhibited by cardiac glycosides, and such inhibition has been histochemically demonstrated in the squid giant nerve fiber (19). In the case of the structural complexes being discussed, a 10-min incubation in the physiological solution containing 10⁻⁵ M ouabain or 10⁻⁵ M K-strophanthoside was accompanied by: (a) a three- to fivefold decrease of the incidence of the complexes; (b) a reduction of the electron density of the complexes, and (c) a

separation of the apposed plasma membranes. These experimental findings, taken together with the fact that the structural complexes depend upon the potassium and magnesium external concentrations, and with the fact that the presence of a high external calcium concentration tends to lower the number of complexes, strongly suggest that such structures are possibly related to the active transport of ions and molecules across the axon plasma membrane. However, the fact that the number of complexes decreased, though not drastically, when the nerves were incubated in a high concentration of external calcium (44 mM), together with normal concentrations of all the other cations, suggests that some other factors, in addition to the ATPase system, are responsible for the existence of the complexes.

It is known that the Na^+ , K^+ -stimulated ATPase is inhibited by calcium (21). On the other hand, a Ca^{++} -stimulated ATPase has been found in the excitable membrane preparation from the same squid *S. sepioidea* giant nerve fiber (F. Proverbio, M. Condrescu-Guidi, F. V. Barnola and R. Villegas from our laboratories, personal communication). If it is assumed that the structural complexes are related to transport ATPases, it is possible that the inhibitory effect of calcium on the Na^+ , K^+ -stimulated ATPase may be masked by the stimulatory effect of calcium on the Ca^{++} -dependent ATPase.

The close apposition of the axon and Schwann cell plasma membranes at the site of the structural complex poses the question of whether these specialized zones are also related to the functional interaction between the axon and the Schwann cell that has been already described in squid giant nerve fibers (29) and that seems to be mediated by a cholinergic system (30, 31). In this way, the structural complexes may be related to the so-called gap junctions (18) which represent the morphological correlates for the low resistance junctions that serve as sites of electrotonic coupling between cells (14). The existence, at the surfaces of the apposed membranes, of a lattice formed by globules or particles closely packed in a hexagonal array is, at present, the well-known ultrastructural feature of the gap junction (17, 18). These junctions have also been pointed out in nerve tissue of vertebrates and invertebrates (15, 23). In the cerebellum of the gymnotid-fish (23), the gap junctions exhibit an asymmetrical arrangement due to the existence of an electron-

dense material underlining the membranes on their cytoplasmic surfaces. This material is more abundant in the dendritic side than in the axonal side of the junction. On the other hand, in frog cerebellum, the distribution of the dense material is symmetrical at both sides of the junction (23), and in the crayfish lateral giant fibers the junction is also symmetrical due to the presence, on both of its sides, of rows of synaptic vesicles (15). To ascertain whether the structural complexes described in the present work are also truly gap junctions, it will be necessary to carry out tracer experiments with markers such as lanthanum or peroxidase because, with the simple OsO_4 -fixation, it has not been possible to observe any modification of the closely apposed membranes which might suggest the existence of a lattice arrangement between them.

Whatever the correct interpretation is, the facts reveal the existence of structural complexes at the axon surface which are sensitive to a decrease of the external ionic concentrations, particularly of magnesium, potassium, and sodium, and to the presence of cardiac glycosides.

The authors are indebted to Doctors Raimundo Villegas and Guillermo Whittembury for valuable discussions of the experiments and the critical reading of the manuscript, and to Dr. F. Proverbio for useful suggestions. They also thank Messrs. J. Aristimuño, Freddy Paredes, and Mrs. Belkiss Pizani for their technical assistance, Mr. Pedro Medina for the photographic work, Mr. J. Mora for the drawings, and Ms. Olga F. Agosto for the secretarial help.

Some of these results have been presented as communications at the Fourth and Fifth International Biophysics Congresses (25, 28).

Received for publication 24 July 1975, and in revised form 3 December 1975.

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