INCREASE IN OSMIOPHILIA OF AXONAL MEMBRANES OF CRAYFISH AS A RESULT OF ELECTRICAL STIMULATION, ASPHYXIA, OR TREATMENT WITH REDUCING AGENTS

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ABSTRACT

Certain axonal membranes of crayfish abdominal nerve cord display ultrastructural changes if the axons are fixed, during electrical stimulation, by aldehydes followed by osmium. Such changes are characterized by an increase in electron opacity and thickness of the unit membranes' dense strata in the axon surface, endoplasmic reticulum, and outer mitochondrial membranes. The electron opacity completely disappears if the sections are treated with hydrogen peroxide solutions. This suggests that the changes represent an increase in the membranes' reactivity for osmium. An unmasking of SH groups could explain such increased osmiophilia, since SH groups are very reactive with osmium, while disulfide bonds are considerably less reactive. This hypothesis was tested by treating control, glutaraldehyde-fixed nerve cords with disulfide reducing agents. In these preparations an increase in electron opacity and thickness was observed to be localized in the same axonal membranes which reacted as a result of electrical stimulation. The phenomenon did not appear if the SH groups were blocked by maleimide or N-ethylmaleimide before treatment with osmium. These findings seem to suggest that certain axonal membranes of crayfish contain proteins rich in sulfur whose SH groups are unmasked as a result of electrical stimulation. In preliminary experiments an increase in osmiophilia localized in the same membranes with the same characteristics and distribution was observed also in axons from nerve cords asphyxiated either in vitro or in the living animal.

INTRODUCTION

Most of the information concerning changes in nerve fibers as a result of activity comes from electrophysiological studies. Nevertheless, some studies on morphological modifications are also available. Early in this century changes in the histological staining of neurofibrils were seen in nerve fibers subjected to polarizing current (1), and such observations were later confirmed and found to appear only in live, unanesthetized nerves (2).

Slow changes in light scattering were recorded from axons stimulated in vitro and were interpreted in relation to changes in volume of the examined axons (3–7). Recently, however, changes occurring at the speed of the action potential have also been recorded, suggesting that rapid modifications, possibly associated with excitation, take place (8, 9, 10).

Changes in birefringence were recorded in stimulated axons also synchronously with the action potential (9, 10), as well as an increase in fluores-
cience in axons treated with 8-anilinonaphthalene-1-sulfonic acid (ANS) (10). ANS seems to bind to the hydrophobic groups of macromolecules (11) and to change the fluorescence properties in relation to conformational modifications in various macromolecules (12). It was suggested, therefore, that these changes could be related to conformational modifications occurring in membrane macromolecules during excitation.

In previous reports we have briefly described ultrastructural modifications occurring in crayfish nerve fibers as a result of electrical stimulation, which are characterized by an increase in electron opacity and thickness of certain axonal membranes (13). These changes were interpreted as caused by an unmasking of SH groups in membrane proteins (14).

Modifications that could be of the same nature were described in stimulated frog sciatic nerves (15). In this case a deposition of electron-opaque granules was observed on axonal as well as Schwann cell membranes. The dense granules were absent in the anodic segment of nerves submitted to polarizing current.

METHODS

Crayfish (Procambarus clarkii), 10-15 cm long, were obtained from Carolina Biological Supply Co., Burlington, N. C., and kept in well oxygenated aquaria at about 20°C. The crayfish were anesthetized for about 10 min by cooling in crushed ice and water. The sternal artery was dissected, cannulated with a polyethylene tubing (outer diameter, 0.038 inches) and perfused, by syringe, with 50-100 U.S.P. units of heparin (Liquaemin Sodium "10") (Organon Inc., West Orange, N. J.) in 10 cc of Van Harreveld's salt solution (16). During the subsequent dissection and stimulation the crayfish were perfused with the salt solution using an intravenous system at a rate of 20-30 drops per minute at room temperature. In some experiments the perfusion with heparin was not performed. Oxygen was bubbled continuously through the salt solution.

The cephalic carapace was then dissected out, the green glands were carefully removed, and the circumoesophageal connectives with the supraoesophageal ganglion were exposed. One of the two circumoesophageal connectives was isolated and placed in contact with a pair of platinum electrodes for the stimulation. The abdominal chain was exposed between the fifth and the sixth ganglion and placed in contact with a similar pair of electrodes for external recording. Square pulses (1-5 v) (0.1-0.5 msec) repeated at frequencies of 2-60 cps were applied for various lengths of time (from 30 sec to 30 min).

Fixation was performed during the stimulation, perfusing by syringe, through the sternal artery, 40-60 cc of 3-6% glutaraldehyde solution buffered with 0.1 m (Na, K) phosphate to pH 7.4 at room temperature. The electrical activity, recorded on the oscilloscope screen, disappeared after 30-35 sec of perfusion with the fixative, corresponding to 15-20 cc of injected fixative.

The chain of abdominal ganglia was then dissected from the animal and kept for 2 hr in the same fixative at room temperature. It was washed for 15 min in 0.2 m phosphate (pH 7.4) and postfixed for 2 hr in 2% osmium tetroxide buffered by 0.1 m phosphate to pH 7.4 at room temperature. The dehydration was carried out in graded alcohol or acetone and the embedding was made in Epon or Araldite.

In control experiments, the animals were prepared in the same manner, in the sense that the sternal artery was dissected, cannulated, and perfused with heparin followed by Van Harreveld saline, and the electrodes were placed in contact with the nerve cord but the stimulator was left off. The fixative was perfused after lapses of time corresponding to those of different stimulations, and the experiment was carried on as described above.

Sections (~500 A thick) were cut on an LKB Ultratome microtome, collected on uncoated 400 mesh grids, and observed either unstained or stained by a 3 min immersion in a 3% solution of lead salts (17). On certain occasions, before lead staining, the sections were stained by a 10 min immersion in a saturated solution of uranyl acetate in 50% alcohol. In some experiments unstained sections from stimulated nerve cords were collected on uncoated grids and immersed in a 3% solution of hydrogen peroxide in order to remove the osmium (18). The specimens were observed with an AET EM 6B electron microscope. For examination at magnifications higher than 20,000, the sections were coated with a thin carbon film.

Experiments were performed using sulfidryl reagents as follows: chains of unstimulated ganglia were dissected from the animals and fixed for 2 hr by immersion in a 6% solution of glutaraldehyde buffered by 0.1 m (Na, K) phosphate to pH 7.4 at room temperature. After a 15 min wash in 0.2 m phosphate, the chains were cut into two portions. One was immersed for 1 hr in 0.4 m sodium thiosulfoclate (K & K Laboratories Inc., Plainview, N. Y.) (19, 20) or 5 mm DTE (Dithioerythritol) (Sigma Chemical Co., St. Louis, Mo.), both in 0.1 m phosphate buffer (pH 7) at room temperature. The other was placed in 0.2 m phosphate (pH 7) as a control. After a 30 min wash in 0.2 m phosphate buffer (pH 7), the specimens were postfixed, dehydrated, and embedded as described above. In certain experiments ganglia treated with the reducing agents...
were washed for 15 min in phosphate buffer (pH 7), and were treated for 30 min-1 hr with a 0.1 M solution of maleimide (K & K Laboratories Inc.) or N-ethyl-maleimide (NEM) (Mann Research Labs Inc., New York) (19, 20, 21) in 0.1 M phosphate buffer (pH 7.6) at room temperature and postfixed, dehydrated, and embedded as described above.

Preliminary experiments were performed on asphyxiated nerve cords. Chains of abdominal ganglia, dissected from the animals, were kept for 30 min in closed bottles filled with Van Harreveld's saline, through which nitrogen or carbon dioxide was bubbled. Control chains were kept for the same length of time in Van Harreveld's saline through which oxygen was bubbled. The saline solutions were each then rapidly replaced by glutaraldehyde, and fixation, dehydration, and embedding were carried out as described above.

In certain experiments, living animals were asphyxiated for 30 min in closed flasks filled with nitrogen gas. The abdominal chain was then rapidly dissected and fixed, dehydrated, and embedded as described above.

OBSERVATIONS

Control Axons

A portion of a control giant axon is shown in Fig. 1. The axoplasm contains cross sectioned mitochondria, endoplasmic reticulum, and microtubules scattered without a particular organization. The matrix is very clear and does not show any structure. Each giant axon is surrounded by several Schwann cells whose thin, sheetlike cytoplasmic processes overlap each other either in close contact or separated by branches of connective tissue. Desmosomes are frequently seen joining together adjacent sheets. Enlargements of portions of Schwann cells frequently contain membranous tubular lattices (22, 23). In these structures, tubules ~400 A in diameter are interconnected every ~0.1 µ, forming complex lattices. At their points of connection the tubules enlarge into spherical cavities ~500 A in diameter. The membranes of the tubules are continuous with the cell membrane, and the lumen of the channel system is open to the extracellular space on both sides of the cell (Fig. 1, inset). Similar lattices have also been observed in the prolamellar body of the chloroplast (24) and in the forming T-system of cultured skeletal muscle cells (25).

Fig. 2 shows a cross section through medium- and small-sized axons. In such axons the mitochondria are located primarily at the periphery. The axoplasm is rich in microtubules but contains little endoplasmic reticulum. The matrix is denser than in giant axons and is frequently occupied by fuzzy, unoriented filaments which sometimes seem to bridge adjacent microtubules. Medium-sized axons are surrounded by few Schwann cells, while small axons are generally enveloped by just one cell. Tubular lattices are also sometimes seen in these Schwann cells, but with a smaller extension.

In Fig. 3 a portion of a medium-sized axon at higher magnification is seen. In this axon, as well as in control axons of different size (Figs. 1, 2), all of the membranes display the same electron opacity. It is important to consider this element in comparison with treated specimens in which, on the contrary, the electron opacity of certain membranes was found to change.

Small portions of "fenestrated septa" (26) were seen in all different-sized axons, although their preservation was found to be very poor unless the specimens were fixed by means of H2O2-treated glutaraldehyde as previously reported (26, 27).

Electrically Stimulated Axons

Ultrastructural changes were seen in certain axonal membranes of stimulated fibers (Figs. 4-12). These changes consisted of an increase in electron opacity and thickness associated with the axon surface membranes, the membranes of the endoplasmic reticulum, and the outer membranes of the mitochondria. The inner membrane of the mitochondria (Figs. 8, 9) and the membranes of the Schwann cells (Figs. 4, 5, 6, 7) were not altered. The increase in electron opacity and thickness was found, in all experiments, in the membranes of the lateral giant fibers (Fig. 4, 5) as well as the membranes of many of the medium-sized fibers (Fig. 7), while the median giant fibers were rarely reactive (Fig. 6). The phenomenon frequently appeared more pronounced in outer mitochondrial and endoplasmic reticulum membranes than in axon surface membranes.

In giant axons the increased electron opacity was more intense in those membranes located in dorsal and peripheral areas of the axon. Such areas are primarily surrounded by blood vessels, while the ventral areas come in contact with nerve fibers and synaptic processes. It seems logical, therefore, to expect that the dorsal areas would be the first to receive the perfused fixative.

Not all of the axons were reactive. On the con-
FIGURE 1  Control giant axon. The axoplasm contains few mitochondria, vesicles, or flat cisterns of endoplasmic reticulum and microtubules. The matrix is clear and unstructured. A thick sheet surrounds the axon. The sheet is composed of long and narrow Schwann cell cytoplasmic expansions that overlap each other, either in close contact or separated by branches of connective tissue (C). Desmosomes (D) are frequently seen joining Schwann cell membranes. Inset (a) shows the desmosome at higher magnification. Enlargements of Schwann cell cytoplasm contain a system of interconnected tubules called "tubular lattice" (L) (22). The membranes of the tubules are continuous with the surface cell membrane, and occasionally tubules are seen running uninterrupted from side to side of the Schwann cell cytoplasm (inset [b], double pointed arrow). In the lower left corner giant-to-motor electrical synapses (S) are shown. X 18,000. Inset (a) X 36,500. Inset (b) X 60,000.
FIGURE 2  Control axons of medium and small size. The axonal mitochondria are located preferentially at the periphery and frequently are regularly spaced (arrowheads). The axoplasm is very rich in microtubules. The sheath is thin and Schwann cell cytoplasmic processes overlap each other in close contact. "Tubular lattices" are also seen sometimes, but not as extensively as in giant axons. X 12,750.

FIGURE 3  Control axon. A small area of a cross-sectioned, medium-sized axon is seen. In control, well-oxygenated axons, all the membranes display similar electron opacity. The inset enlargement shows a mitochondrion (M) at higher magnification. X 25,500. Inset X 130,000.
FIGURE 4  Stimulated ganglion. An area of a lateral giant fiber is shown in the upper right corner. Here the axon surface membrane, the outer membrane of the mitochondria, and the membrane of the endoplasmic reticulum appear very electron opaque. Two small axons in the lower left corner display membranes of normal electron opacity. Between the lateral giant and the two small axons there is a thick sheath composed of Schwann cells and branches of connective tissue. \( \times 8500 \).

FIGURE 5  Stimulated ganglion. An area of lateral giant fiber particularly rich in endoplasmic reticulum. Axon surface, mitochondrial and endoplasmic reticulum membranes display very pronounced electron opacity, while inner mitochondrial and Schwann cell membranes are as in control axons. G, Golgi apparatus of Schwann cell. \( \times 36,000 \).
FIGURE 6 Stimulated ganglion. An area of a median giant fiber is shown. As seen in the two previous
figures, axon surface, outer mitochondrial and endoplasmic reticulum membranes appear more electron
opaque than inner mitochondrial and Schwann cell (Sc) membranes. In the inset, axonal and Schwann
cell surface membranes are shown at higher magnification. X 36,000. Inset X 72,000.

FIGURE 7 Stimulated ganglion. Only one (the superior) of these two medium-sized fibers displays mem-
branes of increased electron opacity and thickness. This finding (shown also in Fig. 4) suggests that the
reaction is not without specificity, but occurs only in certain axons while others remain as in control
preparations. X 25,000.
FIGURE 8 Stimulated ganglion. Axonal mitochondrion stained with uranyl and lead is seen in cross section. In the outer unit membrane the two dense strata show an increase in electron opacity and thickness. The latter is more pronounced on the axoplasmic side. The light central zone of both the inner and the outer mitochondrial membranes is approximately of the same thickness. Two small vesicles of the endoplasmic reticulum display changes similar to the outer mitochondrial membrane. In the inset an enlargement of inner and outer mitochondrial membranes is shown. The overall thickness of the outer membranes is 120–150 A. The axoplasmic stratum is one and one half to two times thicker than the inner stratum. × 160,000. Inset × 600,000.

FIGURE 9 Stimulated ganglion. Axonal mitochondrion cross sectioned and unstained. The increased electron opacity of the outer membrane is apparent also in unstained preparations. Here, also, the axoplasmic stratum is thicker than the inner one. The inset shows a higher magnification of inner and outer mitochondrial membranes. The profile of cross-sectioned unit membranes is visible, in unstained preparations, only in membranes of increased electron opacity. Here, therefore, the trilaminar appearance of the mitochondrial inner membrane is not seen. × 160,000. Inset × 600,000.

At high magnification the affected membranes displayed an increase in thickness and density of each of the two dense strata (Figs. 8, 9). The over-all thickness of the membranes increased to 120–150 A. In all three types of membrane showing changes, the dense stratum of the axoplasmic side was one and one half to two times thicker than the other (Figs. 8, 9, insets). The electron opacity of the affected membranes was higher than that of any other cellular material even in sections unstained (Fig. 9).

While in most of the cross sections the dense strata appeared very compact and did not display granularity, in tangential sections the membranes were granular because of the presence of dense particles (Fig. 10). In the best preparation these particles (~150 A in size) were clearly seen separated by a distance of ~200 A (Figs. 11, 12) and...
seemed to be located on the axoplasmic side of the unit membranes (Fig. 11). In septal electrical synapses (lateral giant fibers) particles of the same size were seen more clearly (Fig. 13). Here, in membranes seen in face view, they were organized in a hexagonal array in which the center-to-center distance between adjacent particles was ~200 Å (Fig. 14).

The described changes appeared after prolonged stimulation as well as after stimulation for as short as 30 sec at the frequency of 2 cps. In material perfused with fixative immediately after the suspension of stimulation, the membranes were always normal. The heparin treatment did not influence the appearance of the phenomenon.

The electron-opaque material was not visible in specimens of ganglia fixed with glutaraldehyde alone and unstained, which suggests that it does not have intrinsic electron opacity but needs to be stained with osmium in order to be seen by electron microscopy. This finding was also confirmed by the observation that the electron opacity of the material disappears completely in sections in which the osmium was removed with hydrogen peroxide solutions.

Axons Treated with SH Reagents

In specimens treated with S-S reducing agents, the axonal membranes showed an increase in thickness and electron opacity with the same characteristics and distribution as in the electrically stimulated axons (Figs. 15, 16, 17). In this case all of the axons of the observed ganglia were reactive but the increased electron opacity was more pronounced in axonal membranes of large axons. In giant axons the intensity of the electron opacity was the same in dorsal and ventral areas. In specimens treated with S-S reducing agents followed by treatment with SH blocking agents, the increase in electron opacity and thickness was not observed and the axonal membranes appeared exactly as in control fibers (Figs. 18, 19).

Asphyxiated Axons

In ganglia asphyxiated by immersion in Van Harreveld's solution through which CO2 or N2 were bubbled as well as in ganglia dissected from animals asphyxiated in N2 atmosphere, the axonal, endoplasmic reticulum, and outer mitochondrial membranes displayed increased electron opacity and thickness with the same characteristics as in preparations stimulated electrically or treated with reducing agents. However, in asphyxiated ganglia, as in those treated with reducing agents, all the axons presented the change in electron opacity. Here also, the intensity of the reaction was more pronounced in the larger axons. As in specimens treated with reducing agents, in asphyxiated giant axons the increased electron opacity was the same in dorsal and ventral areas.

DISCUSSION

It was at first believed that the electron-opaque material observed on the two dense strata of axonal membranes could be a calcium precipitate because of the very high electron opacity which it displays even in unstained sections (13). However, the fact that the material is not visible in specimens of ganglia fixed with glutaraldehyde alone, suggests that it does not have intrinsic electron opacity as would be expected in a calcium salt (28). It needs to be reacted with OsO4 in order to be seen by electron microscopy. This finding was also confirmed by the observation that the electron-opaque material completely disappears in sections in which the osmium was removed with hydrogen peroxide solutions. The phenomenon, therefore, seems to be the consequence of some changes in the membrane that result in an increase in osmiophilia.

It is well known that membrane osmiophilia is due primarily to the reaction of both lipids and proteins with osmium tetroxide. In the case of the lipids, osmium tetroxide reacts first with the double bonds of the hydrocarbon chains forming cyclic osmic esters (monoesters and diesters) (29, 30, 31, 32, 33). The monoesters may then be cleaved and osmate ions migrate to the lipid polar ends (30, 33). There a reduction to osmium dioxide may occur as a result of reactions with the proteins or the reagents used for dehydration (33).

Very little is known about the reactions occurring between proteins and osmium tetroxide. Among the amino acids, diamino acids, sulfur-containing amino acids, and amino acids containing cyclic groups proved to be reactive with osmium (34). If incorporated in proteins, however, only a few of these amino acids seem to be able to react with osmium, and it has been suggested that the osmiophilia of proteins can be roughly forecasted knowing their content of three amino acids: tryptophane, cysteine, and histidine (34). The reactivity of tryptophane and histidine is probably related to their content in unsaturated bonds,
FIGURE 10 Stimulated ganglion. Axonal mitochondria obliquely cut. Outer membranes, tangentially or obliquely cut, display granularity, suggesting that the electron opaque material is not a homogeneous, continuous layer. × 76,000.

FIGURE 11 Stimulated ganglion. Axonal mitochondrion cross sectioned. In several places particles ~150 Å in size, ~200 Å distant center-to-center (arrows), are seen on the axoplasmic side of the membranes of increased electron opacity. × 125,000.

FIGURE 12 Stimulated ganglion. Axonal mitochondrion cross sectioned. Where the outer mitochondrial membrane is obliquely cut (area between the two arrows), a beaded image is seen. The beads are spaced at intervals of ~200 Å. The beaded appearance is seen more clearly if the picture is held at an arm’s length and tilted sharply. × 125,000.

FIGURE 13 Control ganglion. Septal electrical synapse (lateral giant fiber). Cross-sectioned membranes of electrical synapses frequently display a beaded appearance due to the presence of particles with the same characteristics as those seen in Figs. 11, 12. This specimen was fixed with H₂O₂-treated glutaraldehyde (37). The inset shows a higher magnification of the synaptic membranes. × 80,000. Inset × 180,000.

FIGURE 14 Stimulated ganglion. Septal electrical synapse (lateral giant fiber) tangentially cut. In septal synapses the membranes, seen in face view, display a hexagonal array of particles ~150 Å in size, with center-to-center spacing of ~200 Å. × 80,000.
FIGURE 15  Ganglion treated with thioglycolate. Medium sized axons. Increase in electron opacity is seen in axonal, outer mitochondrial, and endoplasmic reticulum membranes as in stimulated axons. \( \times 8500 \).

FIGURE 16  Ganglion treated with thioglycolate. Median giant fiber. At higher magnification the increase in electron opacity and thickness in the axonal membrane (\( Am \)), outer membrane of mitochondria (\( M \)) and endoplasmic reticulum membranes (the latter are not in the micrograph) appear as in stimulated fibers even to an exaggerated degree. Inner mitochondrial membranes and membranes of the Schwann cell (\( Sc \)) do not show a change. The inset shows inner and outer mitochondrial membranes at higher magnification. \( \times 39,000 \). Inset \( \times 120,000 \).

FIGURE 17  Ganglion treated with thioglycolate. Median giant fiber. Unstained section. Also in unstained preparations the increased electron opacity is apparent as in stimulated preparations (Fig. 9). \( Am \), axonal membrane, \( M \), mitochondrion, \( Sc \), Schwann cell. \( \times 52,000 \).
FIGURE 18 Ganglion treated with thioglycolate followed by maleimide. Medium-sized axons. SH groups, unmasked by thioglycolate, were blocked by maleimide. All the membranes display the same electron opacity as in control preparations. The granules seen in the axoplasm are glycogen. X 13,500.

FIGURE 19 Ganglion treated with thioglycolate followed by maleimide. Median giant fiber. At higher magnification, no difference in electron opacity is noticeable among different axonal and Schwann cell membranes. In the Schwann cell cytoplasm (Sc) the tubular lattice (L) is seen. M, mitochondria. X 27,000.
while the reactivity of cysteine is due to the presence of the SH group. Free SH groups of different compounds such as cysteine, glutathione, and thioglycolic acid proved, in fact, to react readily with osmium and precipitate with it, while disulfide bonds were found less reactive (34). In this reaction the sulphydryl groups \( (E_0 = -0.389 \text{ V}) \) in the case of cysteine \( (35) \) reduce the osmium tetroxide \( (E_0 = +0.640 \text{ V}) \) \( (36) \) to insoluble compounds \( (34) \).

Usually cell components are stained quite weakly by osmium, and different cell structures fixed in osmium and observed in unstained sections appear only faintly electron opaque. On a few occasions, however, a very electron-opaque osmium precipitate called "osmium black" was obtained in specific cell regions by means of particular techniques.

Precipitates of osmium black were seen in Golgi apparatus of different cells \( (37, 38) \), in endoplasmic reticulum and mitochondria of "zona fasciculata" cells of adrenal cortex \( (39) \), and in retinal rods \( (40) \) as a result of prolonged osmication in unbuffered solutions at 40°C \( (37, 41) \). Osmium black was again produced as a result of reactions between osmium tetroxide and tissue-bound cytochemical reagents containing sulfur groups such as SH, thioarabamyl, and diazothioether \( (42) \) as well as azo groups \( (azoindoxyl) \) \( (43) \). Osmium black could also be the precipitate observed in synaptic vesicles as a result of zinc iodide–osmium impregnation \( (44) \).

A reaction product of osmium tetroxide and carbon–carbon double bonds and polar ends, presumed to be hydrated osmium dioxide or osmium metal, was considered the primary compound of the osmium black observed in specimens treated with unbuffered osmium at 40°C \( (39, 40) \). However, in cases in which it was possible to study the chemical composition of osmium black, it was found that polymers of osmium are produced representing coordination compounds of osmium with organic sulfur ligands \( (45) \). In these cases, experimental data ruled out the presence of hydrated osmium dioxide or osmium metal. Reagents containing SH groups proved to be the most reactive in the synthesis of such polymers. The osmium black was found, in this case, to be insoluble in tissue components, in solvents used for dehydration, and in embedding compounds such as acrylic and epoxy monomers. These properties are consistent with the high electron opacity of the osmium black in sections, suggesting a preservation of the compound in high concentration throughout the various stages of dehydration, infiltration, and embedding.

Although these data do not necessarily imply that any observed precipitate of osmium black represents osmium polymers, the fact that osmium black could be synthesized by means of compounds containing SH groups suggested to us that, among different alternatives, an unmasking of SH groups in membrane proteins could explain the observed increase in osmiophilia in the axonal membranes of our preparations and could be tested experimentally. Moreover, an unmasking of SH groups occurring in axons as a result of stimulation would not be surprising, since it has been previously suggested in stimulated nerve tissue studied by means of different experimental techniques \( (46–49) \).

It seemed reasonable, therefore, to test at first such a hypothesis by treating control, glutaraldehyde-fixed nerve cords with disulfide reducing agents and looking for the effect. Such an experiment is reasonable because it has been shown that cleavage of disulfide bonds in aldehyde-fixed material can be performed \( (19, 20) \), and it is known that reaction between osmium and the SH groups of proteins can occur. The latter is illustrated in wool keratin, where treatment with thioglycolic acid causes the matrix of the fibrils, which contains a cystein-rich component \( (50) \), to become very osmiophilic \( (51) \).

Our experiments with sulphydryl reagents seem to support the hypothesis of unmasking of SH groups, since after treatment with thioglycolate or DTE the axonal membranes displayed an increase in osmiophilia with the same characteristics and distribution as in the electrically stimulated axons \( (14) \). Moreover, the same membranes appeared as in control axons, if the newly unmasked SH groups were blocked by maleimide or NEM before treatment with osmium.

It could be assumed, therefore, that in or very near the two dense strata of the axon surface membranes, the endoplasmic reticulum membranes, and the outer mitochondrial membranes of crayfish axons, there are proteins rich in sulfur whose SH groups are unmasked as a result of electrical stimulation.

The fact that the axoplasmic dense stratum of each of the reactive membranes appears thicker than the other, could mean that it contains a
higher number of SH groups available for the reaction with osmium. In this respect it is interesting to remember that, also in sarcoplasmic reticulum membranes, SH groups were found more numerous in the homologous layer (52). In this case, isolated membranes of sarcoplasmic reticulum were treated with a specific SH-reagent (Hg-phenyl azoferritin) before fixation. The ferritin particles were easily recognized in the sections and appeared considerably more numerous on the sarcoplasmic surface of the membranes. The binding of the mercury-ferritin compound could be prevented, as expected, by treatment with SH blocking agents (NEM). It has been suggested that these SH groups may belong to ATPase molecules, since treatment with ATP proved to prevent the SH blocking effect of NEM. In our case also, the sulfur-rich proteins could represent ATPase since Mg-dependent ATPase, sensitive to SH reagents, was found in crustacean nerve microsomes (53).

At least in the axoplasmic stratum of the unit membrane, these proteins could be organized in particles ~150 A in diameter, spaced in rows at intervals of ~200 A. Particles of the same size were seen in hexagonal arrangement, with a center-to-center spacing of ~200 A, in axonal surface membranes of crayfish lateral giant septal electrical synapses. It could be that also in the other axonal membranes the particles are arranged in a hexagonal pattern. In our present study, however, this has not been proven.

The presumed presence of proteins rich in sulfur in axonal membranes could be intriguing, since sulfhydryl groups seem to be important in nerve conduction. It is known, in fact, that the blockage of SH groups by different agents such as heavy metals, parachloromercuribenzoate (PCMB) and N-ethylmaleimide (NEM) rapidly stops nerve conduction (54–57). Such blockage was observed after external treatment (54, 55, 57) as well as after internal injection of the blocking agents (squinax giant axons) (56), and in all cases, except after NEM treatment, it was reversed by treatment with reducing agents.

Unmasking of SH groups may be expected to result in conformational changes in proteins. It is, therefore, interesting to notice that changes in the molecular arrangement of proteins in excitable membranes, during electrical activity, have been frequently proposed (58–63 et al.). The general hypothesis is that the proteins undergo reversible conformational modifications which may be important in the ion transport and electrical properties of membranes during excitation.

Increased membrane permeability could be correlated with the unmasking of SH groups in membrane proteins. It has been shown, in fact, that treatment of microsomal membranes with SH reagents results in lipid peroxidation associated with an increase in membrane permeability and a decrease in the ionic discrimination (64, 65). Moreover, this notion is particularly interesting if applied to axonal membranes, since such membranes, isolated in a quite pure fraction, were recently found to contain a high percentage of double bonds, suggesting a relatively high fluidity in the membrane structure.

In our study, however, no attempts to correlate the results with excitation can be made, since fixation cannot be timed in such a way that axonal membranes were reacted only in a certain stage of activity. It would be, moreover, puzzling to interpret the positive reaction occurring also in membranes which are not considered excitable, such as endoplasmic reticulum and outer mitochondrial membranes. Nevertheless the phenomenon seems to occur quite rapidly, as suggested by the observation that, in giant axons, the increased electron opacity is very pronounced only in membranes of dorsal areas. These areas arc, in fact, closer to the blood supply and therefore are expected to be fixed more rapidly than other regions in perfused preparations. Moreover, our findings are not due to hyperstimulation, since they occur already as a result of short stimulations at low frequency.

In preliminary experiments an increase in osmophilia was observed in the same membranes also as a result of asphyxia. It seems reasonable to suppose that asphyxia can be associated with reduction of disulfide bonds as a result of changes in the oxidation-reduction potential; hence this experiment is in agreement with the other results. Whether or not changes in the oxidation-reduction potential, sufficient to produce the phenomenon, could also occur in electrically stimulated axons, cannot be stated at present; this possibility, however, must be kept in mind.

In membranes of septal electrical synapses tangentially cut we observed particles organized in a

1 Canessa-Fischer, M., and S. Fischer. 1970. Personal communication.
hexagonal array with a center-to-center spacing of \( \sim 200 \text{ A} \). Hexagonal patterns in electrical synapses were first described in goldfish brain at the junctions between club endings and Mauthner cells (66). In these junctions, by means of potassium permanganate fixation, a hexagonal array of closely packed polygonal facets was seen. These facets repeated at a period of \( \sim 95 \text{ A} \). In crayfish septal electrical synapses also a hexagonal pattern with \( \sim 100 \text{ A} \) periodicity was recently found in the gap between the two synaptic membranes, by means of lanthanum hydroxide as a staining agent (67). The possible relationship between this 100 A pattern and our 200 A pattern is at present unknown.

In conclusion, the present study suggests that certain crayfish axonal membranes have the property of considerably increasing their reactivity towards osmium under various circumstances such as electrical stimulation, treatment with reducing agents, and asphyxia. Such increased osmiophilia seems to be best explained by assuming that SH groups become free in membrane proteins and subsequently react with osmium, producing precipitates of osmium black. Although the SH unmasking appears well supported by our present experimental data, we feel that alternative explanations of the phenomenon are still possible.

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