ULTRASTRUCTURE OF THE PRAWN NERVE SHEATHS

Role of Fixative and Osmotic Pressure in Vesiculation of Thin Cytoplasmic Laminae

C. F. DOGGENWEILER and JOHN E. HEUSER

From the Harvard Medical School, the Departments of Neurology and Psychiatry, the Biophysics Research Laboratory, McLean Hospital, Belmont, Massachusetts. Dr. Doggenweiler's present address is Facultad de Medicina, Universidad Catolica de Chile, Casilla 114-D, Santiago, Chile

ABSTRACT

The sheaths from freshly teased nerve fibers of the prawn exhibit a positive radial birefringence, consistent with their EM appearance as highly organized laminated structures composed of numerous thin cytoplasmic sheets or laminae bordered by unit membranes and arranged concentrically around the axon. The closely apposed membranes in these sheaths are fragile and often break down into rows of vesicles during fixation. Desmosome-like attachment zones occur in many regions of the sheath. The membranes within these zones resist vesiculation and thereby provide a "control" region for relating the type of vesicles formed in the fragile portions of the sheaths to the specific fixation conditions. It is proposed that during fixation the production of artifactual vesicles is governed by an interplay of three factors: (*a*) direct chemical action of the fixative on the polar strata of adjacent unit membranes, (*b*) osmotic forces applied to membranes during fixation, and (*c*) the preexisting natural relations between adjacent membranes. It is found that permanganate best preserves the continuity of the membranes but will still produce vesicles if the fixative exerts severe osmotic forces. These results support other reports (19) of the importance of comparing tissues fixed by complementary procedures so that systematic artifacts will not be described as characteristic of the natural state.

INTRODUCTION

The stability of unit membranes during preparation of tissues for observation with the electron microscope has been considered recently by Rosenbluth (19). He described a system in which osmium tetroxide and permanganate fixatives each produce consistent artifacts that are different and characteristic. Osmium tetroxide fixatives produce the breakdown of cell invaginations into rows of membrane-bounded vesicles that enclose extracellular material and are buried in the cytoplasm. In con-

trast, permanganate fixatives produce the breakdown of thin cytoplasmic processes or evaginations into rows of membrane-bounded vesicles that contain cytoplasm and are surrounded by extracellular space. In his study the results of one method of fixation were used as a control for the results of the other.

Franzini-Armstrong and Porter (5) reported that the intermediate element in the triad of skeletal muscle appears to be a continuous invagination from the extracellular space only in tissues prefixed with glutaraldehyde followed by osmium tetroxide, and not in tissues fixed with osmium tetroxide alone. Since there are good physiological reasons (12, 13) for the continuity of the intermediate element in the natural state, it appears again that osmium tetroxide fixatives can produce artifactual breakdown and discontinuity of cell invaginations.

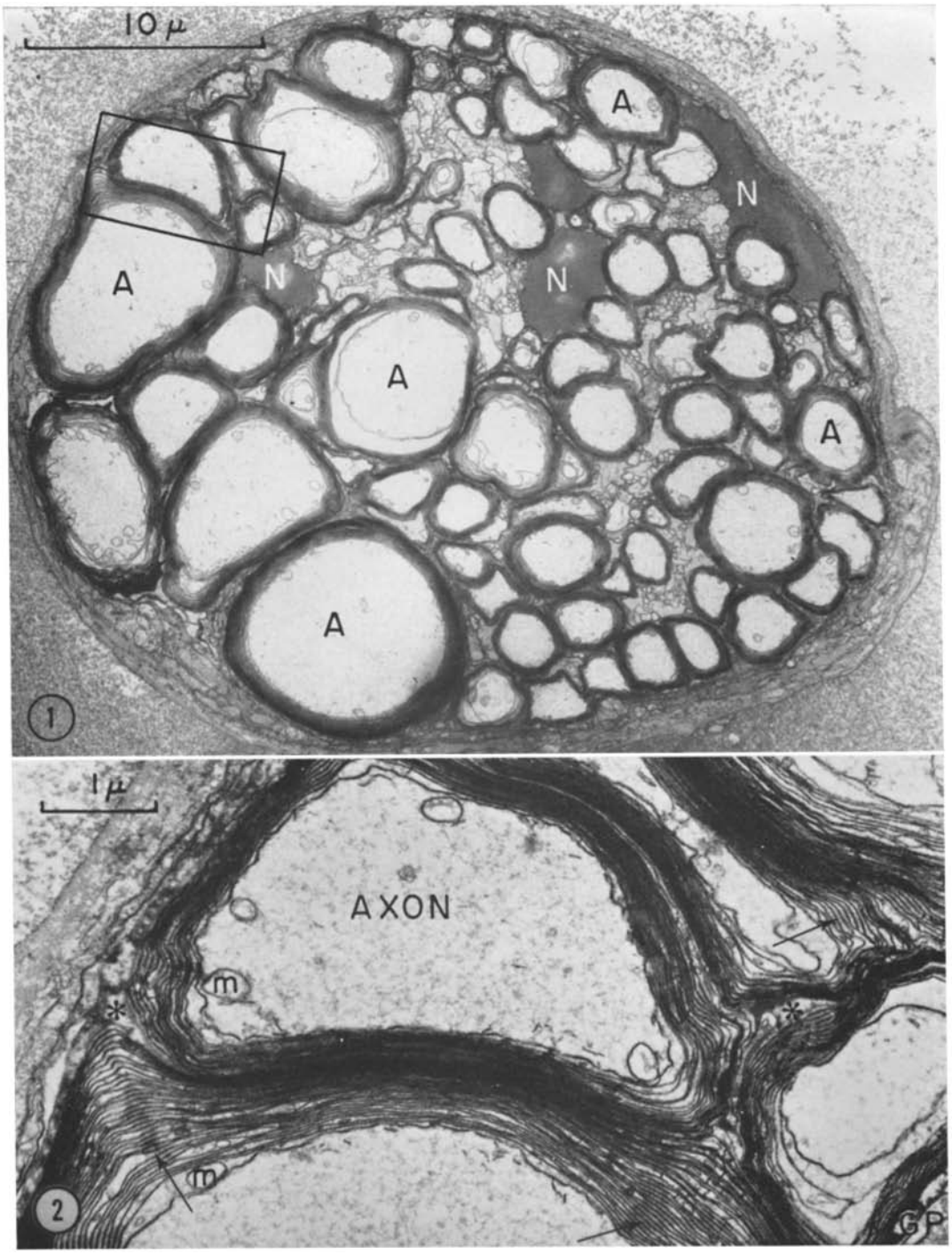
It seems improbable that membrane-bounded vesicles of cytoplasm could exist in the extracellular space of unfixed tissues, for this cytoplasm would have no access to the information carried in the nucleus. So it is easy to accept the idea that these vesicles are artifacts. On the other hand, the occurrence of isolated, membrane-bounded vesicles within cell cytoplasm is generally considered representative of the natural state. In fact, many of the proposed mechanisms for the uptake, transport, and release of material by cells are based on the observation of orderly rows of vesicles within the cytoplasm of fixed cells (2). Such vesicles are interpreted as a frozen representation of the natural activity of membranes: breaking and recombining to form dynamic cavities in which material is transported. The recognition that such vesicles may be artifacts in certain tissues under certain fixation procedures has been less easily accepted. For example, Vial and Orrego (28) proposed that the vesicles observed in the inactive vertebrate gastric parietal cell coalesce in the cells' secretory stages to form the long channels characteristic of the active cell. In contrast, Sedar (23) found that with permanganate fixation fewer vesicles appeared in gastric parietal cells. This suggests that the appearance of vesicles in this cell type depends not only on the functional state of the cell but also on the fixative used. These considerations cast some doubt on the belief that the commonly observed intracytoplasmic vesicles are permanently isolated from the extracellular spaces, and supports Robertson's suggestion (18) that there may be continuity between extracellular material and the lumens of intracytoplasmic vesicles which can be demonstrated only by a very careful control of fixation.

In order to clarify the role of vesicles as cytoplasmic organelles, it is necessary to distinguish those which exist in the natural state from those which arise artifactually during preparation of tissues for electron microscopy. For this purpose it would help to study a tissue that satisfies three criteria: (*a*) its native structure can be accurately determined by techniques that do not require its fixation, such as polarized light microscopy and x-ray diffraction; (*b*) it forms consistent artifacts during fixation that can be identified; and (*c*) these artifacts can be shown to result from specific conditions during fixation. In the course of studying the structure of invertebrate nervous systems we have fortuitously found a tissue that satisfies these criteria rather well: the nerve sheaths of the common sea prawn (*Palaemonetes vulgaris*).

Each prawn nerve sheath is a laminated structure constructed of many thin cytoplasmic sheets that extend along the axon and wrap concentrically around it in a highly organized manner. A detailed report of this sheath has been presented earlier (9). One might predict that the instability of unit membranes during fixation would be most evident in tissues with many thin cytoplasmic processes; here membranes could easily come close together and interact to form vesicles. In fact, the prawn nerve sheath is very fragile; its many closely apposed membranes break and recombine during most fixation procedures. This has been reported by us earlier (4). These sheaths are good tools for studying the formation of vesicles because their fragile membranes are differentiated in certain regions into desmosomal or attachment structures that are unusually stable during fixation. The stable membranes of these regions are continuous with the fragile membranes composing the rest of the sheath and thus offer an internal control for interpreting the effects of various fixatives. The purpose of this report is to describe the differing effects of certain currently used fixatives on this nerve sheath.

MATERIALS AND METHODS

Small sea prawns (*Palaemonetes vulgaris*) were obtained from the Woods Hole Marine Biological Laboratories. They were kept in a small sea water aquarium and fed fish food. Under these conditions the animals can survive indefinitely. Before fixation, each animal was anesthetized by cooling. It was then



All magnification markers indicate 0.1 μ unless otherwise stated.

FIGURE 1 Cross-section of a nerve fiber in the prawn, containing many axons (*A*) with electron-opaque sheaths, and a few interspersed glial cell nuclei (*N*). The area in the rectangle is magnified in Fig. 2. Permanganate at 800 mosmols. × 3600.

FIGURE 2 Portions of several axons and their laminated membranous sheaths which contain radial attachment zones among adjacent laminae (arrows) (see Fig. 3). Extracellular spaces (*) and glial processes (*GP*) are found between some nerve fibers. Poorly fixed mitochondria (*m*) are present in axons and a sheath lamina. Permanganate at 800 mosmols. × 16,000.

pinned upside down on a paraffin tray in preparation for dissection.

Various procedures for introducing the fixatives were attempted, including injection into the dorsal aorta and simple immersion either before or after injection. The best preservation was achieved when the abdominal portion of the ventral nerve cord was rapidly dissected out while still attached to the hard ventral exoskeleton and was immersed in cold fixative. This procedure prevented the cord from contracting during fixation and minimized direct damage. The fixatives used were potassium permanganate, osmium tetroxide, glutaraldehyde, and formaldehyde. These were dissolved in a variety of carriers, including unbuffered natural sea water, unbuffered artificial sea water made according to Nicol (14) with the concentration of NaCl partially reduced in order to adjust the osmolarity[1] to desired values several different buffers, and distilled water. The specific composition of different fixatives will be presented as their effects are described.

After fixation the specimens were dehydrated in acetone, embeddded in araldite according to Glauert et al. (6), and sectioned with an LKB Ultrotome I. Sections about 1 μ thick were examined with a Zeiss Ultraphot II phase contrast microscope. Thin sections (gray or silver interference hue) were mounted on carbon-coated slit grids and stained with lead citrate according to Venable and Coggeshall (27). They were observed in a Siemens Elmiskop I equipped with a pointed filament, a condenser aperture of 200 μ, and an objective aperture of 50 μ. Electron micrographs were taken on Kodak Contrast lantern slide plates (Eastman Kodak Co., Rochester, N. Y.) and enlarged. The microscope was calibrated according to the manufacturer's specifications and assumed to be good to $\pm 5\%$.

Over 350 shrimp were observed over a 3 yr period during this study. The results of various fixation procedures were consistent in nearly all cases; thus sampling errors were considered significant and no attempt was made to evaluate these observations statistically.

Birefringence studies of freshly teased axons of the prawn nerve cord were made with a Zeiss Ultraphot II microscope provided with a strain-free polarization optics. A Red I or a Kohler compensator was used to determine birefringence axes. The axons were observed submerged in artificial sea water at different osmolarities in a milled slot in a sheet of metal provided with glass windows. If the specimen stayed at the bottom of the well and in contact with the glass window, it became anchored so that its movement was negligible during the exchange of imbibing media. This allowed the observation of changes in birefringence occurring during fixation or during changes of osmolarity.

[1] By osmolarity we designate here the effective concentration of all solutes including the buffer, when used, as measured by the freezing depression method in an osmometer from Advanced Instruments, Inc., Newton Highlands, Mass.

RESULTS

Polarized Light Microscopy

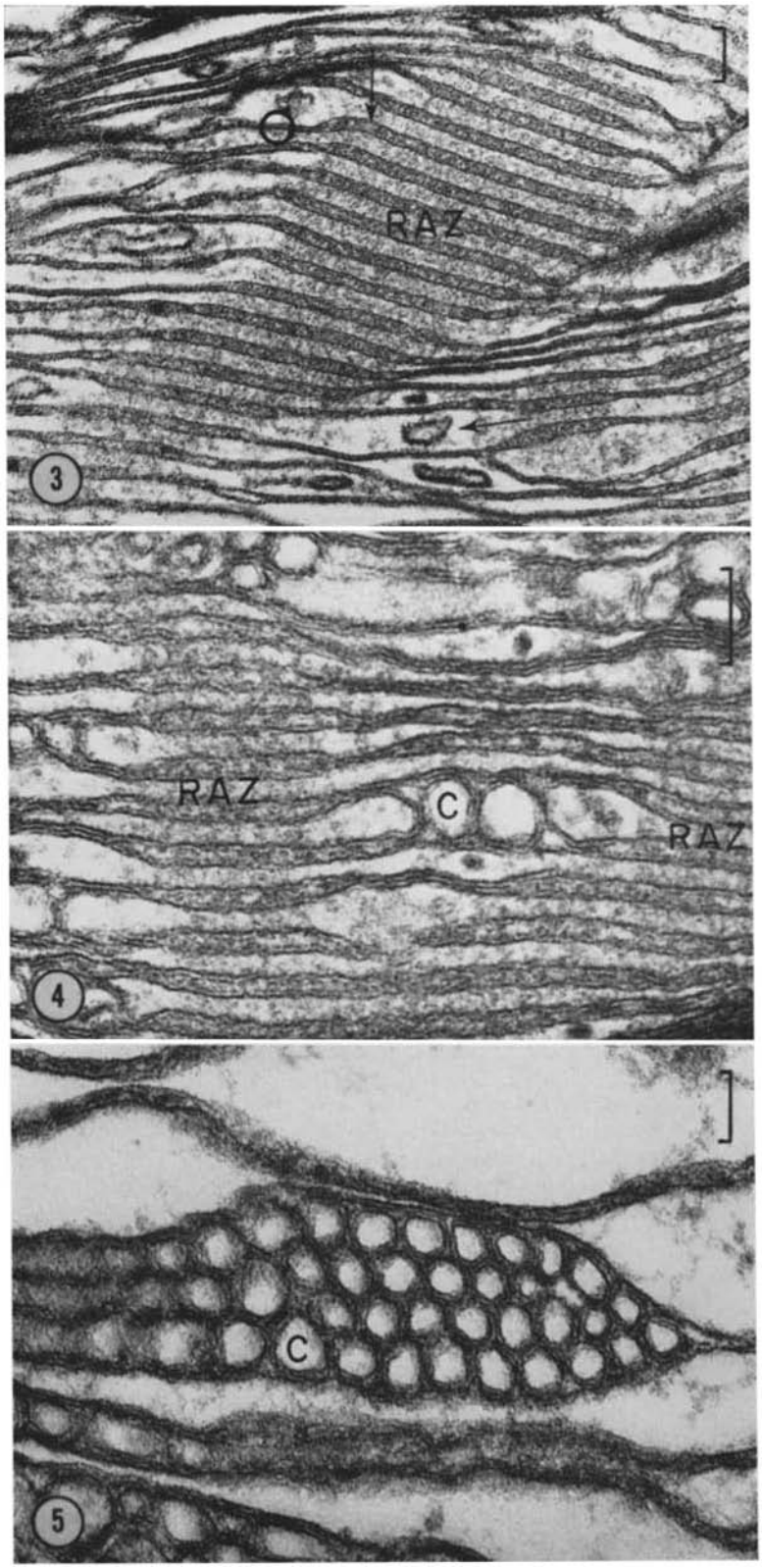
Sheaths of fresh nerve fibers isolated by teasing from the abdominal nerve cord show a distinct radially positive birefringence. This birefringence is not altered by prolonged bathing (1 hr or more) of prawn nerves in full strength sea water; nor is it altered by bathing in sea water diluted with distilled water to an osmolarity as low as 300 mosmols.

Substitution of the sea water bathing media by osmium tetroxide or permanganate fixatives dissolved in sea water results in a change in the sign of birefringence to negativity within 30 sec. The

FIGURE 3 A portion of a sheath around an axon. It contains a radial attachment zone (*RAZ*) among several sheath laminae. In this zone the laminar cytoplasm becomes more dense, and the membranes bounding each lamina proceed continuously through this zone. Membranes of adjacent laminae are separated in this zone by a characteristic 150 A gap (arrow) while outside this zone they often adhere to form external compound membranes (*ECM*) (circle). Permanganate at 800 mosmols. × 66,000.

FIGURE 4 A portion of a nerve sheath including two radial attachment zones (*RAZ*). One lamina has broken down to form two vesicles that contain cytoplasm (C vesicles, *C*) (See Fig. 12). The unit membranes of these vesicles form ECM with each other and with adjacent laminae. Permanganate at 1000 mosmols (hypertonic). × 120,000.

FIGURE 5 A portion of a sheath containing a cluster of vesicles identified as C type (*C*), whose unit membranes form a complicated array of ECM. Permanganate at 1000 mosmols (hypertonic). × 96,000.


RAZ
3
RAZ
C
RAZ
4
C
5

change occurs simultaneously along the fiber and does not seem to be related to the osmolarity of the fixing solution. A similar reversal of the sign of birefringence from radial positivity to radial negativity has been described by W. J. Schmidt in vertebrate myelin (20, 21). Chinn and Schmitt (3) have provided reasons to believe that this reversal is due to an enhancement of the form birefringence of the concentrically arranged lipoprotein strata within the myelin sheath.

Permanganate Fixation

Tissues were fixed for 3 hr in cold $KMnO_4$ dissolved in artificial sea water (14). Concentrations of fixative from 0.6 to 2.5% were used; best results were obtained at 1.5%. The membranes in these tissues were distinct and dense, and they satisfied the criteria of smoothness and continuity which Palay et al. (15) proposed as indications of good preservation. The membranes contrasted sharply with the cytoplasmic regions in this material which appeared light and amorphous and contained few well-preserved organelles (Fig. 1).

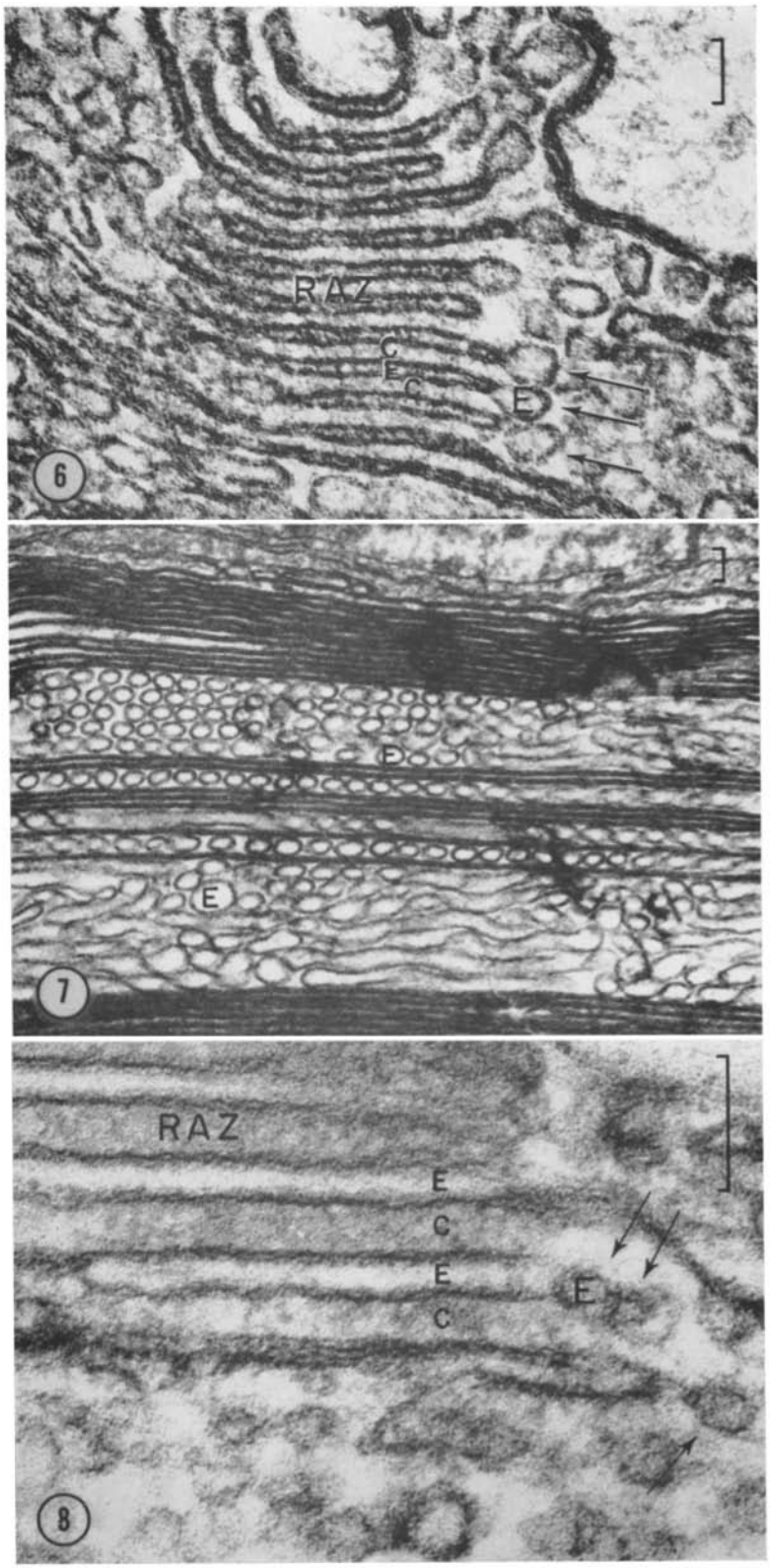
In tissues fixed with permanganate, prawn nerve fibers are seen to be composed of many axons that are surrounded by sheaths several microns thick (Figs. 1 and 2). As the osmolarity of this fixative is varied, the appearance of the nerve sheaths varies considerably. At a fixative osmolarity of 800 mosmols, each sheath appears, as in Fig. 2, to be a laminated structure composed of many thin cellular sheets which extend along the axon and wrap concentrically around it. Each of these sheets or laminae is composed of a thin layer of cytoplasm with two limiting unit membranes. The cytoplasm sometimes contains vesicles (Fig. 3) or mitochondria (Fig. 2). The sheath laminae vary in thickness between 2000 and 100 A; those in the inner regions of each sheath are generally thicker. Adjacent sheath laminae are attached to each other in a characteristic manner (Fig. 9). In the inner portions of the sheath, near the axon, the laminae are usually separated by a 100–200 A extracellular gap. In the outer regions of the sheath the laminae are so closely apposed that all extracellular space between them is displaced and the external dense strata of their adjacent unit membranes merge, forming a single intraperiod line characteristic of external compound membranes (ECM) and myelin sheaths (18). Also, in these outer regions of the sheath the cytoplasm within each lamina is largely displaced so that the external compound membranes become closely approximated and the overall appearance of the sheath approaches that of compact vertebrate myelin (Fig. 10). Nevertheless, in tissues fixed with permanganate the closely approximated external compound membranes never merge, so their adjacent dense strata remain split and do not form a major dense line like that in vertebrate myelin (18).

At osmolarities of fixative far from 800 mosmols (either below 600 mosmols or above 1000 mosmols), the nerve sheaths look very different. Some cytoplasmic laminae in every sheath are replaced by concentrically oriented rows of vesicles (Figs. 4–9). These vesicles vary in diameter from 500 A to more than 1000 A and are bounded by a typical unit membrane about 90 A thick.

FIGURE 6 A portion of a sheath containing several vesicles (arrows) which can be identified as containing extracellular material (E vesicles) by their direct continuity with the extracellular portions (*E*), not the cytoplasmic portions (*C*), of the adjacent radial attachment zone (*RAZ*). (See Fig. 12.) Permanganate at 260 mosmols (hypotonic). × 88,000.

FIGURE 7 A portion of a sheath around an axon. Many sheath laminae are intact and bounded by unit membranes which have adhered to adjacent laminae to form ECM. Several laminae have broken down to form vesicles (upper portion) and elongated irregular forms (lower portion) which are thought to contain extracellular material (E vesicles). Permanganate at 500 mosmols (hypotonic). × 48,000.

FIGURE 8 A portion of a sheath fixed with osmium tetroxide at 750 mosmols. The membranes are less distinct and dense than in permanganate-fixed tissue. Several vesicles (arrows) are formed in proximity to the adjacent radial attachment zone (*RAZ*) and can be identified as containing extracellular material (E vesicles) by their direct continuity with the extracellular portions (*E*), not the cytoplasmic portions (*C*), of the adjacent radial attachment zone (RAZ). × 174,000.


RAZ
C
E
C
E
6
D
E
7
RAZ
E
C
E
C
E
8

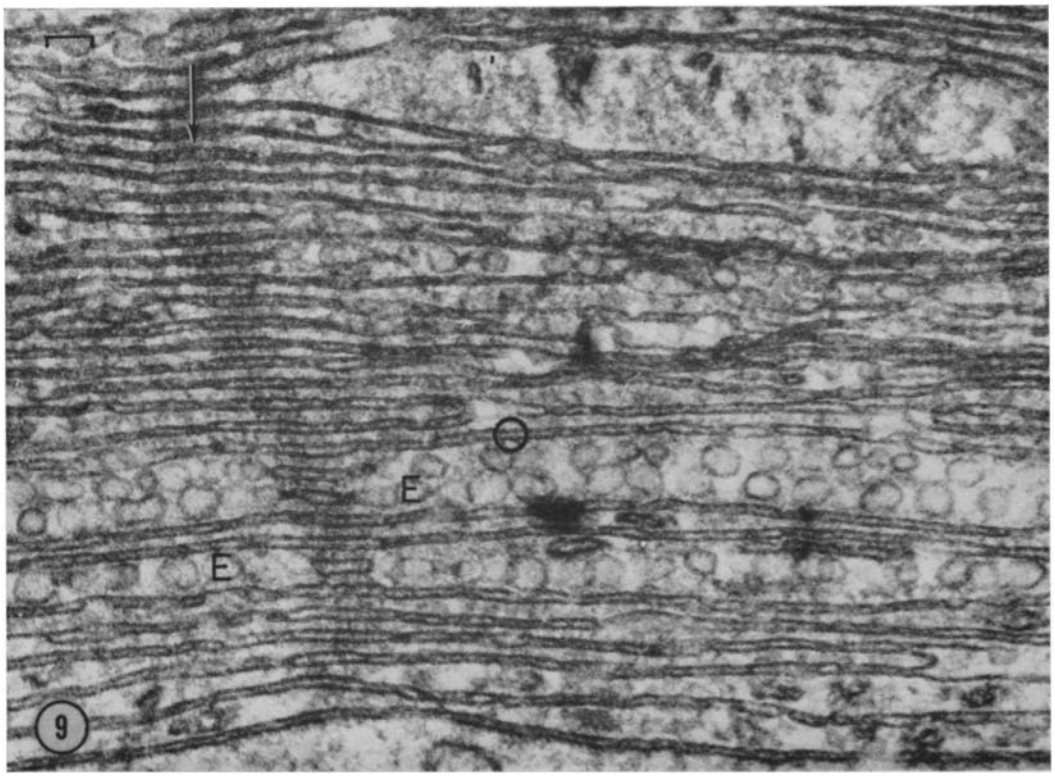


FIGURE 9 A portion of a sheath showing the formation of E vesicles (as in Fig. 6) in continuity with a radial attachment zone in the sheath (arrow). Note that these vesicles, formed in the inner region of a sheath where adjacent laminae do not adhere to each other (circle), also do not adhere to each other as do those vesicles in Figs. 4 and 5. Permanganate at 500 mosmols (hypotonic). × 56,000.

In order to vary the osmolarity of these fixatives, the permanganate was dissolved either in natural sea water diluted with distilled water or in artificial sea water (14) prepared with lowered NaCl content. Nevertheless, the same sensitivity of these sheaths to the osmolarity of $KMnO_4$ is observed in tissues fixed with permanganate dissolved in 0.01 M phosphate buffer with NaCl added to adjust the osmolarity to the desired values. Again, the fixative prepared at 800 mosmols shows the best preservation of the laminated structure in the sheath. This suggests that the osmolarity of the permanganate fixative, rather than a particular ionic species in it, is responsible for the observed structural alterations.

It is worthy of mention that another kind of alteration is observed when phosphate or cacodylate buffers are used as carriers. Permanganate dissolved in these buffers at pH 7.2–7.5 at 800 mosmols results in an increase in the granularity of the dense strata of the unit membranes and in the appearance of cross-hatching between dense strata in many membranes. This appearance is very similar to that reported by Sjöstrand (24) in mitochondria and interpreted to represent a micellar substructure in the biological membrane. It may equally well be due to better preservation of a mosaic substructure in the dense strata of the membranes, with cross-hatching resulting from a very slight degree of tilting of the plane of the membrane with respect to the optical axis of the microscope. This has been discussed by Robertson (17).

An important feature of these sheaths is the occurrence of "radial attachment zones" (RAZ) intermittently in all sheaths (Figs. 2 and 3). Similar zones have been described in the sheaths around the earthworm giant nerve fibers (7). We have described these zones in detail earlier (9). They resemble stacks of desmosomes or maculae adherentes In these zones adjacent sheath laminae are separated by a very constant 150 A gap containing a small amount of densely staining material, the

membranes are very distinct and regular, and the cytoplasm within them is more dense (diagrammed in Figs. 12 and 13).

The radial attachment zones are of particular interest since the membranes within them are not noticeably altered when different fixing agents are used or when the osmolarity of the fixative is varied. This resistance to damage suggests that these RAZ provide mechanical strength and rigidity to the living sheath, as discussed elsewhere (9). But what is more important to the present study is that this stability of the RAZ during fixation permits us to use intact RAZ to determine the original location of intracellular and extracellular spaces in sheaths that have otherwise been broken down into vesicles during fixation. By tracing the relations of intact membranes in the RAZ to the vesicles in the remainder of the sheath, we are able to learn something of the nature of the vesicles formed. In this manner we have found that two different types of vesicles may occur in the sheaths. These are diagrammed in Figs. 12 and 13. Some vesicles appear to contain cytoplasm and have been termed "C" vesicles (Fig. 12). Other vesiclees are embedded in cytoplasm and appear to contain nothing or extracellular material; these have been termed "E" vesicles (Fig. 13). By such relationships we are able to identify the vesicles in Fig. 4 as C vesicles and those in Figs. 6–9 as E vesicles.

In permanganate-fixed material we have observed that both types of vesicles may be produced but there is a definite relationship between the type formed and the fixative osmolarity. In Figs. 6, 7, and 9, tissues were fixed with hypotonic (600 mosmols) fixatives and display vesicles of the E type. In Fig. 4, the tissue was fixed with a hyper-

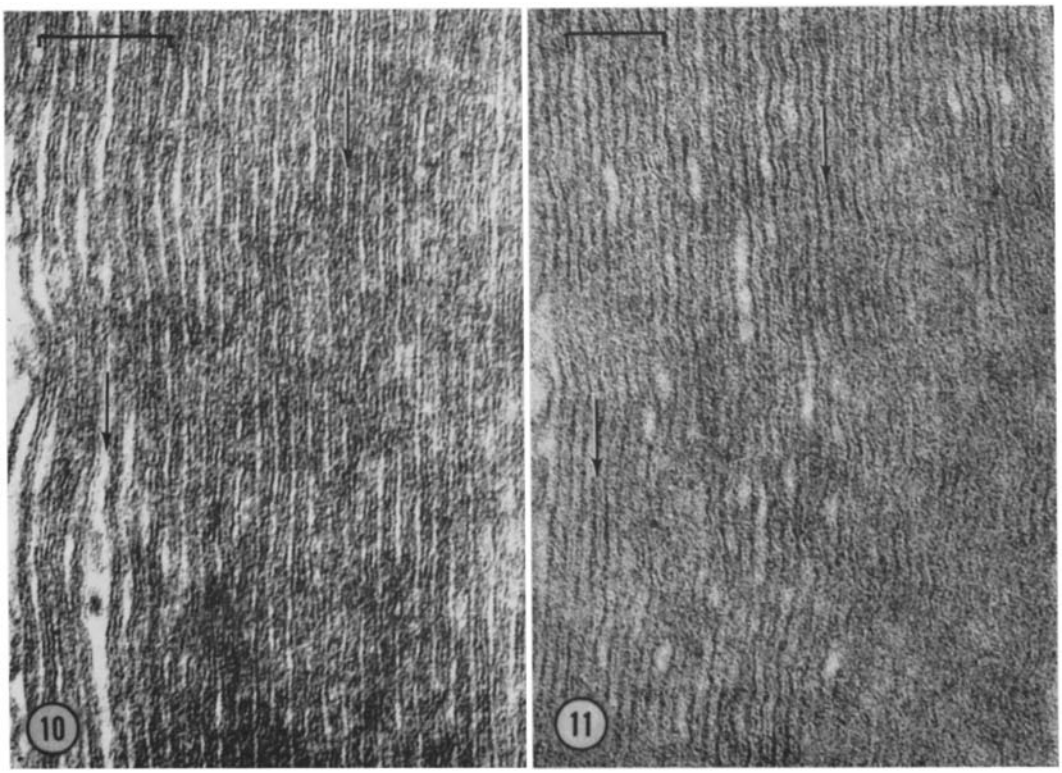


FIGURE 10 Outer portion of a sheath fixed with permanganate, showing the narrow cytoplasmic spaces between adjacent external compound membranes (arrows). 800 mosmols. × 170,000.

FIGURE 11 Outer portion of a sheath fixed with osmium tetroxide, showing that no space remains between adjacent ECM's (arrows) as the cytoplasmic dense strata of their unit membranes have merged to form major dense lines. In some regions the intraperiod lines can be seen. × 120,000.

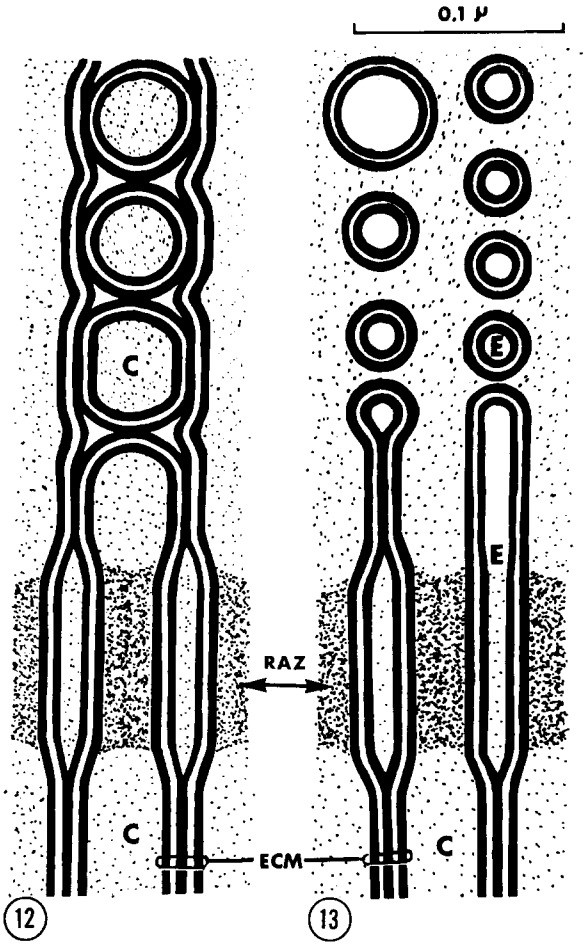


FIGURE 12 Diagram showing one sheath lamina (*C*) bounded by unit membranes that are adherent to the membranes of adjacent laminae to form ECM. In the region of the radial attachment zone (*RAZ*) these ECM split. Above, vesicles like those caused by hypertonic fixatives are formed; they are continuous with the cytoplasmic portion of the RAZ and so are identified as containing cytoplasm (C vesicles). Note that they form ECM with adjacent membranes, as in Figs. 4 and 5.

FIGURE 13 A diagram like Fig. 12; here vesicles like those caused by hypotonic fixatives are formed. They are continuous with the extracellular portion of the RAZ (*E*) and so are identified as containing extracellular material (E vesicles). Note that they do not form ECM with adjacent membranes, as in Figs. 7 and 9.

tonic (1000 mosmols) fixative and displays vesicles of the C type. This correlation between osmolarity and type of vesicle is not absolutely strict, since sometimes both types of vesicles occur in the same sheath. Nevertheless the E type predominates after hypotonic fixation and the C type predominates after hypertonic fixation.

In these tissues there also appears to be a relationship between the type of vesicle and particular regions in the nerve sheath. E type vesicles are commonly formed in the innermost portions of the sheath where the laminae are separated by significant extracellular spaces (Figs. 6 and 9) but they are only rarely found in outer portions of the sheath where adjacent laminae are adherent and form ECM (Fig. 3). Conversely, C type vesicles have never been seen in the inner portions of the sheath but are common in the outer portions (Fig. 4) where ECM are common. It is interesting to note that C type vesicles often appear in clusters, adhering by the external surfaces of their unit membranes to form short ECM among themselves as well as adjacent laminae Fig. 5. This may be related to a general tendency of membranes in these outer regions of the sheath to adhere along their external surfaces.

Osmium Tetroxide Fixation

Tissues were fixed for 3 hr in cold OsO_4 dissolved in artificial sea water (14). Concentrations of fixative from 1 to 4% were used with no appreciable difference. Prefixation with hypertonic solutions of glutaraldehyde or formaldehyde and rinsing with artificial sea water did not alter the results. The cytoplasm of both cells and axons appears better preserved than in permanganate-fixed tissues, but the membranes only vaguely show the unit-membrane pattern (Figs. 8 and 11) and are less distinct than in permanganate-fixed tissue. The nerve sheaths appear poorly preserved, with exten-

sive formation of disorderly rows of vesicles in all sheaths. Few distinct cytoplasmic laminae can be recognized. Nevertheless, the radial attachment zones (RAZ) and the membranes within them are still well preserved. It is found that, regardless of the osmolarity of the osmium tetroxide fixative, in all tissues the vesicles are in register with the extracellular portions of the RAZ, and thus are judged to be E type vesicles. This vesiculation is often so extensive that the axon membrane and the first sheath lamina are involved so that the normal 200 A axon–sheath gap is broken down into a row of vesicles. Only in tissues fixed with extremely hypertonic (1200 mosmols) osmium-tetroxide fixatives or organic prefixatives do C type vesicles appear. They are also confined to the outer regions of the sheath.

In contrast to the permanganate-fixed tissue described earlier, in the osmium tetroxide-fixed material the outermost layers of the nerve sheaths do form a kind of "compact myelin" with a periodicity of about 150 A (Fig. 11; compare to Fig. 10). This results from a complete displacement of cytoplasm from each sheath lamina and a merging of the cytoplasmic dense strata of its adjacent membranes to form a major dense line like that in vertebrate myelin (18). As in vertebrate myelin after osmium tetroxide fixation, the intraperiod line is very poorly defined and is discontinuous.

DISCUSSION

Determination of the Native Structure

In order to understand the causes and mechanisms leading to a change in a biological structure as seen with the electron microscope, it is necessary to determine the appearance which corresponds most closely to the condition in the living organism. In the case of the prawn nerve sheaths dealt with here, we had to decide whether in the living state the sheaths are composed of concentric membranes or of vesicles. The following points were considered in this decision.

(*a*) Observations of fresh, unfixed nerve fibers under polarized light showed that the axons are surrounded by a radially positive birefringent sheath. Since the unit membrane theory (18) proposes that the hydrocarbon chains of membrane phospholipids are oriented perpendicular to the plane of the membrane, this dominant radial positivity of the prawn nerve sheaths can best be interpreted as owing to an intrinsic birefringence of phospholipids present in *concentrically* arranged unit membranes. The destruction of this radial birefringence during fixation probably results from deposition of metal along the polar surfaces of the concentrically arranged unit membranes. Moreover, any distortion of the unit membrane pattern at the molecular level, or the presence of vesicles rather than intact concentric membranes, could be expected to produce reduction or cancellation of this intrinsic birefringence of the sheath. Since as birefringence is essentially a statistical effect, it cannot be excluded that a small proportion of long molecules oriented at any other angle remain undetected (1).

(*b*) The existence of permanent vesicles of isolated cytoplasm seems to be highly improbable, for this would provide no access to information carried in the nucleus. Such morphological considerations, like those used by Rosenbluth (19), support the view that the laminated structure of the sheaths is representative of the living state.

(*c*) The membranes of each lamina in a radial attachment zone are continuous with the membranes of the laminated sheath. But the attachment zones are isolated in sheaths where vesicular components predominate. If these zones are homologous to those described in earthworm nerve sheaths (7) and rat myelin sheaths (8), then they must be continuous with the membranes of the sheaths.

(*d*) Recently Thomas and Sheldon (26) demonstrated that vertebrate compact myelin can undergo a breakdown phenomenon that may be related to our findings. They showed that vertebrate myelin fixed after the onset of Wallerian degeneration becomes rearranged into tubular arrays. Since vertebrate myelin is known from many kinds of evidence to be a laminated structure in the native state, this finding indicates that such a breakdown of a laminated membranous structure can and does occur.

(*e*) Finally, the existence of isolated vesicles rather than continuous laminae in these sheath would seem to be inconsistent with the probable role of these sheaths in axon conduction. Holmes et al. (11) have reported that the prawn axons have a relatively high conduction velocity; these authors have proposed that this velocity depends in large measure on the maintenance of a good insulating sheath that can restrain radial ionic fluxes. A vesiculated sheath would satisfy this require-

ment poorly, as it would have numerous, low resistance, cytoplasmic, or extracellular pathways for ionic flow away from the axon.

For these several reasons, we consider the occurrence of the continuous cytoplasmic laminae in fixed tissues to be representative of the living structure and consider the occurrence of vesicles in the fixed tissues to be artifactual. The similarity found between prawn and earthworm nerve sheaths, and their similarity to vertebrate myelin, presents an interesting case of biological convergence with physiological implications (11). Nevertheless, our detailed study of the prawn sheaths shows that these sheaths are morphologically less similar to vertebrate sheaths and earthworm sheaths than was formerly believed (9).

In the course of this study, we were concerned with the problem of defining the tonicity of the tissue fluids of the prawn. The evidence presented here could be used empirically to define the isotonic fixative as that which preserves the structure of the nerve sheaths in the laminated form. It is known that the prawn maintains a fairly constant osmolarity in its tissue fluids, in spite of drastic changes of osmolarity in its environment (coastal sea water). This is accomplished by an active mechanism that maintains in its body a salt concentration at about 85% that of pure sea water (16). This value is in approximate agreement with our estimation that the sheaths are most stable at an osmolarity of 800 mosmols, which is about 80% that of pure sea water (1000 mosmols) (14). The significance of this coincidence is limited, however, since some of the particles which contribute to the measured osmolarity of the fixative may penetrate membranes and thereby fail to exert osmotic forces across membranes.

Role of Fixative versus Role of Osmotic Pressure

We have shown that the osmolarity of the fixative controls the presence or absence of artifactual vesicles as well as the kind of vesicles produced. Water movements elicited by osmotic pressure seem to be the most likely cause of the approaching and breaking of unit membranes. Rosenbluth (19) ascribed the same results to water movements produced during the dehydration of the specimen. This would require that there be no complete fixation of membranes until after the start of the dehydration. Because the vesiculation depends on the osmolarity of the fixative solution, we think that it happens during fixation itself.

The persistence of strong, positive, radial birefringence in nerve fibers immersed in sea water of different osmolarities suggests that little or no vesiculation occurs under these conditions. Since the solutions used in the birefringence experiments differed from the fixing solutions only in the lack of the fixative itself, we believe that the chemical action of the fixative is a necessary factor in producing artifactual vesicles.

The Effects of Permanganate Fixation

Rosenbluth (19) observed that in toad ganglia permanganate fixatives, but not osmium tetroxide fixatives, cause the breakdown of attenuated sheets of satellite cell cytoplasm into large membrane-bounded globules of cytoplasm. These are analogous to the C vesicles we have observed in prawn sheaths. Rosenbluth proposed that permanganate fixatives cause a selective delay or lack of fixation of the cytoplasmic dense strata of the unit membranes, and that this may leave these membrane surfaces reactive enough to interact by adhering to each other intermittently to form artifactual vesicles. This proposed effect of permanganate could account for the consistent 20 A gap found between adjacent ECM in the outer portions of sheaths fixed with permanganate, if we assume that this gap is an artifact resulting from the splitting of a major dense line.

In our material, however, we found three conditions which did not support Rosenbluth's proposed mechanism of artifact formation: (*a*) there is no single type of vesicle formed in permanganate-fixed tissues, as both C and E vesicles can be found in the same sheath; (*b*) there is an observed correlation between the osmolarity of the permanganate fixative and the type of vesicles formed, with an "optimum" osmolarity of 800 mosmols at which few or no vesicles occur; and (*c*) the type of vesicle formed depends partially on its location within the sheath. Thus our observations suggest that, even if permanaganate does produce vesicles by causing a lack or delay of fixation of unit membranes, there is no reason to regard permanganate as selective of either surface of the membrane. Instead, the type of vesicle formed with permanagnate fixatives seems rather to depend on the osmolarity of the fixative and the local environment of cytoplasm and extracellular space to which the membrane is exposed during fixation.

The Effects of Osmium Tetroxide Fixation

We have observed that osmium tetroxide fixatives extensively destroy the sheath laminae and produce great quantities of vesicles. Because of this extensive vesiculation it is difficult to ascertain whether the vesicles contain cytoplasm or extracellular material, but they most often appear to contain extracellular material and are considered E vesicles. Hama (7) has demonstrated the same kind of E vesicle formation in the loose myelin around earthworm giant nerve fibers fixed with osmium tetroxide but not in fibers fixed with permanganate. Rosenbluth (19) has observed that in toad ganglia osmium-tetroxide fixatives, but not permanganate fixatives, cause the breakdown of cell invaginations which become rows of artifactual vesicles enclosing extracellular material.

These results can suggest that osmium-tetroxide fixatives cause a selective delay or lack of fixation of the external, dense strata of the unit membranes, and that these surfaces are left sufficiently reactive to adhere, when closely apposed (as in slender invaginations), and to form artifactual vesicles. This is supported by the poor preservation, after osmium tetroxide fixation, of the intraperiod line (formed by two apposed external strata of membranes) in prawn sheaths and vertebrate myelin. Nevertheless this hypothesis, like that proposed for permanganate fixatives, is not fully supported by our observations. We also observed C type vesicles in the outer portions of sheaths fixed with hypertonic (1000 mosmols) osmium-tetroxide fixatives.

Glutaraldehyde Prefixation

We have consistently observed that glutaraldehyde prefixation does not prevent the vesiculation produced by osmium tetroxide fixation. These observations are inconsistent with Franzini-Armstrong's and Porter's (5) observation that glutaraldehyde fixation preserves the continuity of the T system in fish skeletal muscle. Clearly, the uniquely organized membranes in prawn nerve sheaths are unusually fragile. These inconsistencies stress that membranes of structures as different as crustacean glial cells and vertebrate muscle fibers can react quite differently to fixation and osmotic stresses.

General Remarks

In conclusion, we believe that the artifactual production of vesicles can only be explained as the result of an interplay of several factors, including (*a*) the direct chemical action of the fixative on components of the unit membrane, (*b*) the osmotic forces applied to surrounding cytoplasm and extracellular spaces, and (*c*) the preexisting natural relations between adjacent membranes that may become subject to interaction during fixation. First, the chemical actions of the different fixatives appear to affect vesicle formation in prawn sheaths by leaving different areas of the membranes unfixed and reactive or by leaving "weak spots" along the surface of the membranes. Second, osmotic forces during fixation appear to affect vesicle formation by expanding or contracting the sheath cytoplasm in some manner that leads to disruption and recombination of membranes. For example, the enhanced formation of C vesicles by hypertonic fixatives in this study may have resulted from a shrinkage of the cytoplasmic sheath laminae that would draw the membranes together along their cytoplasmic surfaces (Fig. 12). Similarly, the enhanced formation of E vesicles by hypotonic fixatives may have resulted from a swelling of the sheath laminae that would push membranes together along their extracellular surfaces (Fig. 13). Third, the preexisting natural relations of membranes in the prawn sheath determine the type of vesicle formed, as membranes narrowly separated along their extracellular surfaces (in the inner portions of the sheath) tend to form E vesicles while membranes narrowly separated along their cytoplasmic surfaces and adherent along their outer surfaces (in the outer portions of a sheath) tend to form C vesicles which also adhere to each other along their outer surfaces.

Our results are consistent with the belief that potassium permanganate is the best fixative for unit membranes, although it is decidedly poorer for other cellular structures. This reinforces the current belief that in electron microscopy, as in light microscopy, there is no fixative for general use. The use of several kinds of fixatives and careful control of their carrier solutions will provide complementary observations that may make it possible to describe a structure in a state closer to the fresh, living condition.

The authors wish to thank Dr. J. David Robertson for his encouragement and criticism of this work. They also wish to thank all the personnel in his laboratory, especially Mr. Alfred Ley for the photographic work.

This work was supported by National Institutes of

Health Grant No. NB 02665 and National Science Foundation Grant No. 574.

Mr. Heuser is a member of the Class of 1968 at Harvard Medical School. During these studies Dr. Doggenweiler was on leave from Universidad Catolica de Chile.

Received for publication 30 November 1966.

REFERENCES

1. BEAR, R. S., and F. O. SCHMITT. 1937. Optical properties of the axon sheaths of crustacean nerves. *J. Cellular Comp. Physiol.* **9**:275.
2. BRANDT, P. W. 1958. A study of the mechanism of pinocytosis. *Exptl. Cell. Res.* **15**:300.
3. CHINN, P., AND F. O. SCHMITT. 1937. On the birefringence of nerve sheaths as studied in cross sections. *J. Cellular Comp. Physiol.* **9**:289.
4. DOGGENWEILER, C. F., and J. E. HEUSER. 1964. The myelin in Palaemonetes. *Anat. Record.* **148**:277.
5. FRANZINI-ARMSTRONG, C., AND K. R. PORTER. 1964. Sarcolemmal invaginations constituting the T system in fish muscle fibers. *J. Cell Biol.* **22**:675–696.
6. GLAUERT, A. M., G. E. ROGERS, and R. H. GLAUERT. 1958. Araldite as an embedding medium for electron microscopy. *J. Biophys. Biochem. Cytol.* **4**:191.
7. HAMA, K. 1959. Some observations on the fine structure of the giant nerve fibers of the earthworm *Eisenia foetida*. *J. Biophys. Biochem. Cytol.* **6**:61.
8. HARKIN, J. C. 1964. A series of desmosomal attachments in the Schwann sheath of myelinated mammalian nerves. *Z. Zellforsch. Mikroskop. Anat. Abt. Histochem.* **64**:189–195.
9. HEUSER, J. E., and C. F. DOGGENWEILER. 1966. The fine structural organization of nerve fibers, sheaths, and glial cells in the prawn. *J. Cell Biol.* **30**:381.
10. HOLMES, W. 1942. The giant myelinated nerve fibers of the prawn. *Phil. Trans. Roy. Soc. (London) Ser. B.* **231**:293–312.
11. HOLMES, W., R. J. PUMPHREY, and J. Z. YOUNG. 1941. The structure and conduction velocity of the medullated nerve fibers of the prawn. *J. Exptl. Biol.* **18**:50.
12. HUXLEY, A. F. and R. W. STRAUB. 1958. Local activation and interfibrillar structures in striated muscle. *J. Physiol. (London).* **140**:143.
13. HUXLEY, A. F. and R. E. TAYLOR. 1958. Local activation of striated muscle fibers. *J. Physiol. (London).* **144**:526.
14. NICOL, J. A. 1960. The Biology of Marine Animals. Sir Isaac Pitman & Sons Ltd., London.
15. PALAY, S. L., S. M. MCGEE-RUSSELL, S. GORDON, and M. A. GRILLO. 1962. Fixation of neutral tissues for electron microscopy by perfusion with solutions of osmium tetroxide. *J. Cell Biol.* **12**:385.
16. PARRY, G. 1954. Ionic regulation in the palaemonid prawn *Palaemon serratus*. *J. Exptl. Biol.* **31**:601.
17. ROBERTSON, J. DAVID. 1967. Design principles of the unit membrane. Ciba Symposium "Principles of Bimolecular Organization". In press.
18. ROBERTSON, J. DAVID. 1960. The molecular structure and contact relationships of cell membranes. *Progr. Biophys. Biophys. Chem.* **10**:343.
19. ROSENBLUTH, J. 1963. Contrast between osmium-fixed and permanganate-fixed toad spinal ganglia. *J. Cell Biol.* **16**:143.
20. SCHMIDT, W. J. 1936. Doppelbrechung und feinbau der markscheide der nervenfasern. *Z. Zellforsch. Mikroskop. Anat. Abt. Histochem.* **23**:657.
21. SCHMIDT, W. J. 1935. Der einfluss von kaliumpermanganat auf die doppelbrechung der markscheide der nervenfasern und der aussenglieder der sehzellen. *Z. Zellforsch. Mikroskop. Anat. Abt. Histochem.* **23**:261.
22. SCHMITT, F. O. 1959. Ultrastructure of nerve myelin and its bearing on fundamental concepts of the structure and function of nerve fibers. *In* The Biology of Myelin. S. R. Korey, editor. Harper & Row, Publishers, New York.
23. SEDAR, A. W. 1961. Electron microscopy of the oxyntic cell in the gastric glands of the bullfrog, *Rana Catesbiana*. III. Permanganate fixation of the endoplasmic reticulum. *J. Cell Biol.* **14**:152.
24. SJÖSTRAND, F. S. 1963. A new repeat structural element of mitochondrial and certain cytoplasmic membranes. *Nature.* **199**:1262.
25. SJÖSTRAND, F. S., and LARS-G. ELFVIN. 1964. The granular structure of mitochondrial membranes and of cytomembranes as demonstrated in frozen-dried tissue. *J. Ultrastruct. Res.* **10**:263–292.
26. THOMAS, P. K., and H. SHELDON. 1964. Tubular arrays derived from myelin breakdown during Wallerian degeneration of peripheral nerve. *J. Cell Biol.* **22**:715.
27. VENABLE, J. H., and R. A. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25**:407.
28. VIAL, J. D., and H. ORREGO. 1960. Electron microscope observations on the fine structure of parietal cells. *J. Biophys. Biochem. Cytol.* **7**:367.