Structural Alterations in Nerve Fibers Produced by Hypotonic and Hypertonic Solutions

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(Received for publication, December 9, 1957)

ABSTRACT

In sections of $KMnO_4$-fixed, developing mouse sciatic nerves, the central gap of mesaxons in myelinating fibers is normally closed with close apposition of the *outside* ~20 A dense strata of the two ~75 A Schwann cell membranes. The two combined outside strata make the intraperiod line bisecting each myelin lamella. The ~150 A mesaxon is elaborated spirally around the axon in either a right hand or left hand spiral, and its *inside* (cytoplasmic) ~20 A strata in apposition form the major dense lines of myelin. In hypotonic solutions the lamellae of adult frog sciatic myelinated fibers split apart along the *outside* membrane strata apposed at the intraperiod line throughout the spiral. Under similar conditions the *inside* (cytoplasmic) strata of the membranes, in apposition at the major dense lines, do not separate. The ~150 A membranous structure resulting from this is called an "internal compound membrane."

The double membranes of normal and control frog sciatic unmyelinated fibers have a central gap ~100 to 150 A wide. After soaking in 4 to 10 times normal strength Ringer solution or 10 N sucrose-Ringer solution, this gap closes and a membranous structure ~150 A wide resembling developing mouse mesaxons results. This is designated by the term "external compound membrane." The latter membranes resemble internal compound membranes, but their central dense zones, each consisting of two apposed *outside* membrane strata, are less dense.

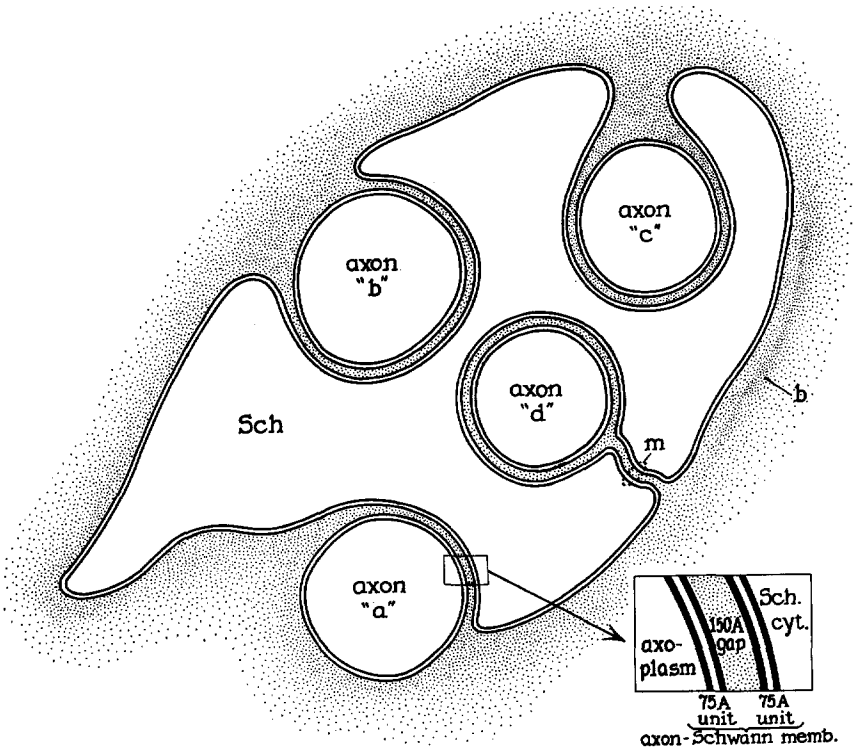
INTRODUCTION

This paper deals with alterations in the normal ultrastructure of frog sciatic nerve fibers brought about by experimental means. The findings have been reported in brief form (39) and are given here in detail. It seems useful to review briefly current general conceptions of the normal structure of vertebrate peripheral nerve fibers before proceeding. Certain new observations from this laboratory, either unpublished or published in brief form (36, 40), are important in interpreting the results and are included in this introduction.

Adult unmyelinated nerve fibers, as shown by Gasser (18, 19), consist of axons partially or completely embedded in Schwann cells. In Text-fig. 1 these relationships are shown with certain recently described new details (40).

Four commonly observed relationships of axons and Schwann cells in adult unmyelinated fibers are indicated. Axon "*a*" is merely in apposition with the Schwann cell, the two apposed cell membranes making a double membrane referred to as the axon–Schwann membrane. A segment of this double membrane is enlarged to show its components. It consists of two distinct cell membranes separated by a gap ~ 150 A across. As recently reported (36, 40) each of the cell membranes[1]

[1] The unqualified term membrane is applied to this structure with awareness that it is in a sense itself a double structure and may be proven later to have more layers (*e.g.* see Fig. 31). It is the structure elsewhere called a "unit" membrane (40). To call it a "double" membrane would be confusing, since this term is now in general use for two of these stratified membranes separated by a narrow gap. It will be reported separately that this stratified membrane structure is of very wide occurrence in cells, and it is shown here that it is a rather stable component resisting violent osmotic changes. The various strata seen in this membrane are connected with structural details within the membrane itself and are not the result of layering, folding, or



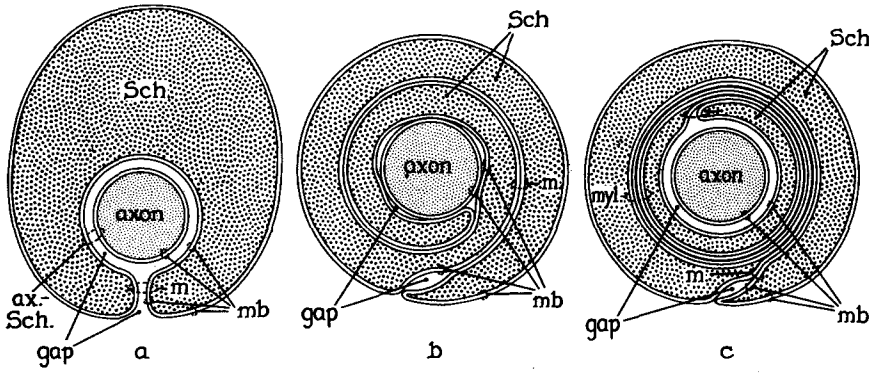
TEXT-FIG. 1. Diagram of typical normal adult unmyelinated fiber. One Schwann cell (*Sch*) is shown with four related axons. The ~75 A membranes of both axons and Schwann cells are indicated. The enlarged inset shows details of one of the axon-Schwann double membranes. The four axons show the different degrees of association of axons and Schwann cells commonly seen. Axons "*a*," "*b*," and "*c*" are only partially enveloped by the Schwann cell, but axon "*d*" is completely surrounded and the two enveloping lips of Schwann cytoplasm extending around it meet to produce a "mesaxon" (*m*). The extracellular matrix material filling the double membrane gaps and surrounding the entire Schwann cell is indicated by the stippling. In one place (*b*) this material is aggregated into a "basement membrane."

appears in slightly underfocused electron micrographs as two parallel dense strata ~ 25 A wide separated by a light interzone ~ 25 A wide, the whole measuring ~ 75 A across (see Figs. 21 to 23). In micrographs closer to exact focus the dense strata each measure about 20 A and the light interzones ~ 35 A. Axons "*b*" and "*c*" are almost completely embedded in the Schwann cell. Axon "*d*" is completely submerged, and the two enveloping lips of the Schwann cell extending around it are in apposition, producing a double membrane connecting the axon with the outside. This is referred to as a mesaxon (19).[2]

At an early stage of development of a myelinated fiber one axon is embedded in a Schwann cell as shown in Fig. 1 *a* and Text-fig. 2 *a*. At a later stage (Fig. 1 *b* and *c*, Fig. 3, and Text-fig. 2 *b*) the mesaxon, as shown by Geren (20), is elongated and extended around the axon in a simple spiral. Geren's observations of immature nerve fibers led her to postulate that adult myelin consists of a greatly elongated mesaxon spirally wrapped about

apposition of distinct separate membranes, as is the case with those structures referred to in this paper as "double membranes," compound membranes, or by the special term "mesaxon."

[2] Gasser originated this term in describing unmyelinated fibers (19). The same term is used here for the double membrane connecting compact myelin internally with the axon and externally with the outside. Previously (32) the term "surface-connecting-membrane" was used instead of mesaxon in describing myelinated fibers. The term "meso," meaning "in the middle of" was suggested to the author by Prof. René Couteaux, as a substitute for "mesaxon" since it can be applied generally to surface-connecting-membranes not involving axons. This is occasionally used instead of "mesaxon."



TEXT-FIG. 2. *a*, *b*, and *c* show three different stages in the formation of myelin in developing mouse sciatic nerve fibers. All these stages may be seen in Figs. 1 to 3. For convenience the relative cross-sectional areas of Schwann cytoplasm (*Sch*) and axoplasm are not drawn to scale. At an early stage of development single axons are seen embedded in Schwann cells, as in *a* and Fig. 1 *a*. A short mesaxon (*m*) is present. At a later stage, as in *b* and in Figs. 1 *b* and *c* and Fig. 3, the mesaxon (*m*) is elongated and wrapped spirally around the axon. Both the mesaxon and the axon–Schwann membrane (*ax.-Sch.*) gaps are largely obliterated at this stage (Fig. 3) by contact of the cell membranes (*mb*). At a later stage *c* and Fig. 2, the loops of the spirally wound mesaxon are in contact with one another to form "compact" myelin (*myl*) and the axon–Schwann membrane gap is restored. The major dense lines of the compact myelin result from the contact of the *cytoplasmic* surfaces of the mesaxon loops, and the intraperiod lines from that of the *outside* surfaces of the membranes (*mb*) forming the mesaxon.

the axon, but the manner in which the membrane loops might be combined in the compact myelin structure remained obscure. The author (32) first observed the inner and outer mesaxons (*i.e.*, the two ends of the primitive mesaxon from which myelin is formed) in adult myelinated fibers (confirming Geren's hypothesis) and showed that the major dense lines of compact myelin were formed by the apposition of those dense layers of the mesaxon bordering on cytoplasm. More recent findings (40, 41) have added new and significant details. The two layers originally observed in the mesaxons of myelinated fibers as single dense lines <100 A thick now each appear as stratified membranes ~75 A thick like those of unmyelinated fibers (above). These two membranes are separated in adult mesaxons by a variable gap, opening at its outer end to extracellular space. This gap is virtually absent in the ~150 A wide primitive mesaxon in immature fibers (Fig. 3 and Text-fig. 2 *b*).

The Schwann cytoplasm between the mesaxon loops is ultimately obliterated[3] and the ~150 A wide membranes come together along their cytoplasmic surfaces to make compact myelin. The previous assertion (32) that the major dense lines of myelin result from apposition of the cytoplasmic surfaces of the mesaxon is thus confirmed. The earlier conception (32) that the light zones between the major dense lines were identifiable with the light zone of the 200 to 300 A wide adult mesaxon (38) and other double membranes (49) is, however, incorrect, since this zone is obliterated at an early stage of development. The only trace of it remaining in adult myelin is in the intraperiod lines which result from very intimate apposition of the outside strata of the ~75 A component membranes of the mesaxon (Fig. 2 and Text-fig. 2 *c*). The ~120 A wide structure (48, 11) from center to center of each major dense line of compact myelin is referred to by the term "lamella," and it is thought (38) that this structure represents the shrunken and dehydrated repeating unit detected by x-ray diffraction (46, 47, 13).

Finean and Millington (17) have reported that this 171 A period detected in fresh frog nerve by x-ray diffraction can be reversibly increased to 270 A by soaking nerves in hypotonic Ringer solution. Furthermore, they found a slight increase in the period (to 190 A) in hypertonic solutions. The results reported here indicate that under conditions similar to those used by the above authors the increase in the period in hypotonic solutions is due to a separation of the myelin

[3] In several places it is stated that the gap of double membranes is "closed" or "obliterated." By this it is merely meant that the gap is no longer visible in the micrographs at moderate magnifications. As indicated further on, there is, in fact, a very thin gap ~ 15 A wide visible at high magnification in some instances.

lamellae along the intraperiod lines. No such definite explanation has been found for the increase in hypertonic solutions, though it appears probable that a similar structural alteration occurs. The central gap of the mesaxons and the axon–Schwann and inter–Schwann membranes of unmyelinated fibers is consistently closed in some regions after soaking in hypertonic solutions. These findings bear directly on the nature of the gap substance of double membranes and the ultrastructure of cell membranes in general.

Materials and Methods

Frog (*Rana temporaria*) sciatic nerve fibers were used exclusively. The animals were chilled in ice water and pithed. The sciatic nerves were removed with their connective tissue sheaths intact and placed in ice cold Ringer solutions of the following concentrations (based on an isotonic (I) 0.120 M Ringer solution containing NaCl (0.116 M), KCl (0.002 M), and $CaCl_2$ (0.002 M)): isotonic (I), I/4, I/6, I/10 isotonic, zero (distilled water), 1.25 I, 1.5 I, 2 I, 4 I, 6 I, and 10 I. In another experiment, sucrose was dissolved in normal frog Ringer solution to give hypertonic values of 10 I and 20 I. The 10 I solutions in each set of experiments were diluted with water to give values of 6 I, 4 I, 2 I, and 1.5 I. The nerve fibers were soaked in these solutions in vials bathed in ice water for the following times: ½, 1, 2, and 4 hours. Two hours gave the best results. The solutions were then replaced by ice cold permanganate fixative (26) isotonic with normal Ringer at pH 7.6 for 3 hours. In one series of experiments, diluted fixative solutions of concentrations corresponding to the solutions in which the fibers had been soaked, were used for the specimens treated with hypotonic solutions. Despite increases in the time of fixation proportional to the dilutions, extremely poor fixation resulted. It was soon found however, that definite effects were demonstrable with an isotonic fixative, and it was decided to use the same fixative solution for all the initial experiments reported here.

A total of 93 specimens were fixed with permanganate sufficiently well for interpretation. A few specimens were discarded because of grossly inadequate preservation of the membrane structures. The 93 specimens were distributed as follows among the various concentrations of Ringer solution: H_2O-10; I/10-4; I/6-3; I/4-5; controls-17; Ringer solution 1.25 I-1; 1.5 I-1; 2 I-8; 4 I-5; 6 I-3; 10 I-9; sucrose-Ringer solution 1.5 I-1; 2 I-6; 4 I-4; 6 I-6; 10 I-9; 20 I-1. About 1000 micrographs were made of these specimens. OsO_4 was used for fixation in a separate series of experiments, but only a few specimens were examined.

Specimens were dehydrated with cold ethyl alcohol or acetone, embedded in araldite (22), and sectioned with a Porter-Blum microtome. The sections were placed on carbon films and examined with a Siemens Elmiskop Ib electron microscope equipped with molybdenum apertures.[4]

RESULTS

1. Controls:

(*a*) *Myelinated Fibers.*—Figs. 4 and 22 show cross-sections of the control myelinated nerve fibers soaked in normal Ringer solutions before fixation. These fibers are not noticeably different from freshly fixed ones as previously described (48, 11, 32, 40). There does, however, appear in the myelinated fibers to be a slight increase in the occurrence of cross-sectional shearing defects of the Schmidt-Lanterman type (Fig. 4, arrows *1*, 41), and there is frequently some degree of fragmentation of the myelin sheath (Fig. 4, arrows *2*), which is less common in freshly fixed material. At high magnification, after prolonged (8 hours or more) permanganate fixation, the myelin sheath shows heavy dense lines ~30 A thick, repeating radially at a spacing of ~60 A (12, 36). After shorter (~3 hours) fixation, the lines are not all alike. Every other one is less dense, though remaining as thick as its neighbors. The period is then ~120 A, and the less dense line is called the "intraperiod line." The denser layers are called "major dense lines." These layers correspond to the dense layers seen in myelin after OsO_4 fixation, but the intraperiod lines in OsO_4-fixed material are thinner and less regular (48, 11, 36).

Figs. 17 to 19 show a small segment of the Schwann surface membrane in an untreated freshly fixed myelinated fiber. It is seen as a pair of dense strata bordering a light zone as described above (40), making a unit ~75 A wide. The stratification of the cell membranes in the control material is not affected by soaking in isotonic Ringer solution, as indicated by the fact that the strata seen in this material (*e.g.* Fig. 5) are identical with those found in freshly fixed nerve fibers. The internal strata of the two membranes of a control myelinated fiber mesaxon may be seen near the arrow (*m*) in Fig. 22. The membranes are here separated by a small gap. The gap is of variable width in the controls, but is often present.

[4] Molybdenum apertures may be obtained from Baker Platinum Ltd., London, England, or Aeon Laboratories, Egham, Surrey, England. These apertures may be cleaned by heating in a molybdenum boat *in vacuo* at a temperature near the melting point of Mb for about 15 minutes.

TABLE I

(Each figure represents the average of the number of measurements in parenthesis; the range of the measurements is given in brackets.)

	Widths of gaps in unmyelinated fibers	Myelin period
Control	121 A [65–197] (79)	115 A [95–123] (57)
Hypotonic	177 A [77–316] (54)—I/4-H_2O	175 A [154–208] (28)—I/o 208 A [154–308] (45)—H_2O (relatively intact regions)
Hypertonic	90 A [15–140] (26)—6–10 I Ringer (excluding zero values)	117 A [114–118] (22)—4 I Ringer 110 A [99–126] (18)—6 I Ringer 122 A [108–142] (62)—10 I Ringer 145 A [108–216] (101)—4 I to 20 I sucrose-Ringer

A very significant point of general interest in relation to the ultrastructure of nerve myelin may be made by Fig. 4. Four myelinated fibers are shown in which the direction of entry of the mesaxons into compact myelin may be seen. From this direction the direction of the spiral is inferred. It appears that the direction of one of the spirals is opposite to that of the other three. One appears in the micrographs to be a left handed spiral, and the other three right handed. Similarly, in Fig. 30 three fibers appear to have right hand spirals, and two left hand spirals. No conclusions may be drawn from these samples about the relative frequency of the two types of spiral, but the important point can be made that the myelin spiral does not always turn in one direction. This matter will be amplified in a separate paper. It is mentioned here only because it is a fundamentally important point which is clearly made by the micrographs and which has not previously been established.

(*b*) *Unmyelinated Fibers.*—Two Schwann cells (*Sch.*) in apposition, each containing three *C* fiber axons (*ax.*), are seen in Fig. 4 (40). These control fibers were soaked 2 hours in normal Ringer solution before fixation. In Fig. 34 another unmyelinated fiber soaked 1 hour in Ringer solution is shown. Two axons (*ax.*) and three Schwann cells (*Sch.*) (or Schwann cell processes) are identifiable in it. In Fig. 5 a single *C* fiber axon (*ax.*) and Schwann cell (*Sch.*) are shown. The strata of the cell membranes and the gaps of the mesaxon and axon–Schwann membranes are visible (inset). The double membranes of all of the control unmyelinated fibers, where sectioned perpendicularly, showed definite gaps between the unit membranes. There was, however, a type of contact of the cell membranes which was seen in the controls indicated by the unlabelled arrows in Fig. 5. This seemed correlated with sectioning direction and was often associated with obliquity of the membranes. Hence, it is considered to be a sectioning artifact. Another type of gap closure not attributable to sectioning was seen in two control specimens (see below).

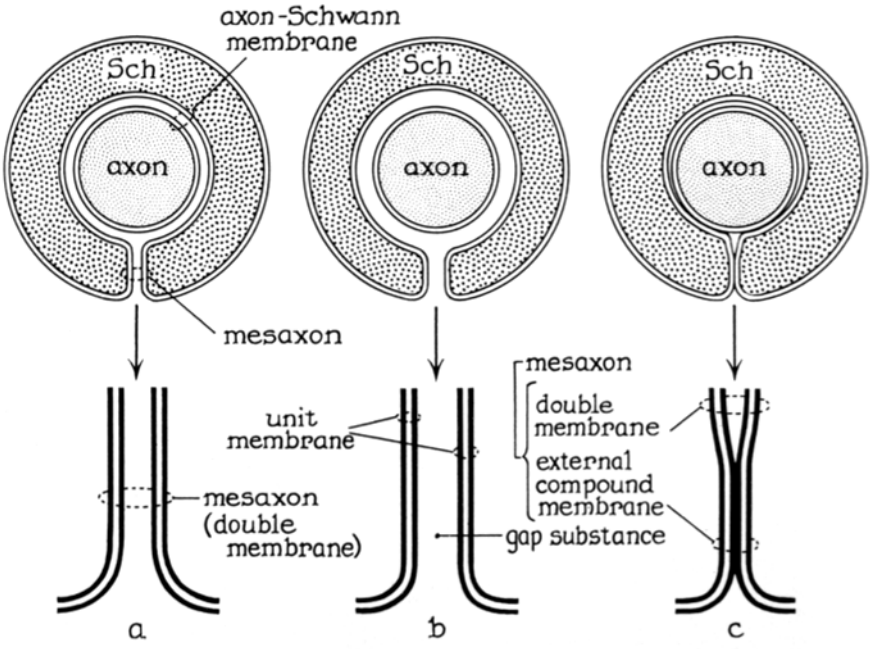
Table I presents a group of measurements of the average widths of the double membrane gaps of unmyelinated fibers in sections of the control material, compared with similar measurements in unmyelinated fibers soaked in hypotonic and hypertonic solutions (see below and Text-fig. 3).

2. Hypotonic Solutions:

(*a*) *Myelin.*—Sections of sciatic nerves soaked in distilled water for periods of ½, 1, 2, and 4 hours were examined. At the end of 2 hours, regular alterations in myelin structure seemed maximal. At shorter times more of the myelin seemed intact, and at longer times the fibers were more disordered and the membranes fragmented. The 2 hour preparations will be described first.

Figs. 6 to 16 and 20 and 21 show sections of myelinated fibers soaked in distilled water for 2 hours before fixation. A few of the fibers were grossly distorted as in Fig. 20. Here no definite axon is recognized, and it may be that such fibers represent myelin forms (*i.e.*, the minute birefringent cylindrical structures which grow out of myelin after damage). In the other examples, by far more common, the fibers were more nearly intact (Figs. 6 to 12). In some of these, the outer layer of Schwann cytoplasm seemed disproportionately swollen. In even the least distorted fibers, the myelin was not swollen entirely symmetrically. It was always buckled and distorted to some extent (Figs. 6 to 8). This implies unequal rates of intermembrane slippage in different parts of the myelin spiral.

The least distorted fibers are the most informative. It is immediately evident upon examining them (Figs. 6 to 10) that the myelin sheath is swollen and separated in a regular manner into membranous layers. The major dense lines are still clearly discernible and retain their radially repeating pattern. The period, however, is in-

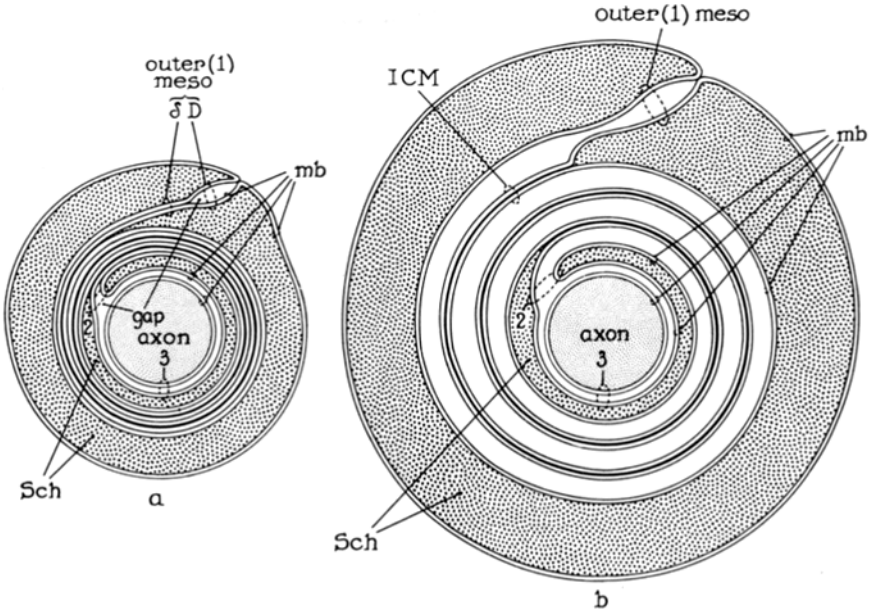


TEXT-FIG. 3. Three diagrams of an unmyelinated fiber, containing one axon with its mesaxon and showing the alterations produced by hypotonic (*b*) and hypertonic (*c*) solutions. The enlargement of the mesaxon below is drawn roughly to scale though the rest of the diagram is not. *a* shows the relationships of the membranes of the Schwann cell (*Sch*) and the axon in the control fibers soaked in isotonic Ringer solution. Note the dimensions of the gap relative to the width of the membranes. In *b* the width of the gap is increased in response to soaking in hypotonic solutions. In *c* the gaps are partially closed in both the mesaxon and the axon–Schwann membrane.

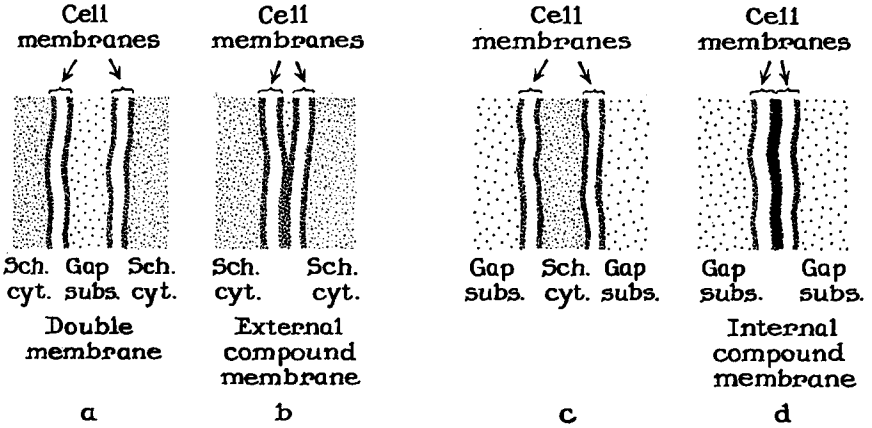
creased greatly. For example in the fiber in Fig. 6, enlarged in Fig. 7, the period measures ~200 A. In the inset enlargement of Fig. 7 certain details of the repeating layers may be seen. It is clear that each major dense line here measuring about 35 A across is bordered on each side by light zones about 35 A wide, which are in turn bordered by thinner dark lines. This whole membranous structure incorporating the major dense line measures about 150 A across (average of 29 measurements 147 A) (see also Figs. 8 to 11, 13 to 16, and 21). It appears that these ~150 A membranous structures originate by a splitting of the intraperiod lines of intact myelin.

This point is made clear by considerations of the relationships of the outermost layers of the swollen myelin, first, to the outer Schwann surface membrane, and second, to the component membranes of the mesaxon. First, if these layers originate by splitting of the intraperiod lines, one would expect to see a simple ~75 A membrane between the outermost one and the Schwann surface membrane. In Fig. 9 at the arrow *1* and in Fig. 21, it may be seen that this is the case. Second, it should be possible to see a region in which the cytoplasmic stratum of the outermost myelin membrane contacts the cytoplasmic stratum of one of the component membranes of the mesaxon to make one of the 150 A compound membranes. In Fig. 9 such a region may be observed at the arrow *2*. It seems clear, then, that the increase in the myelin period observed after treatment with distilled water is due to a splitting of the intraperiod lines. This effects a separation of the outside surfaces of the membranes originally brought into apposition in the primitive mesaxon to form the intraperiod line. The cytoplasmic surfaces of these membranes brought into apposition during development to form the major dense lines do not separate in adult fibers after treatment with distilled water. In essence, then, the alteration in distilled water is a reopening throughout the myelin spiral of the primitive mesaxon gap. These findings are summarized in Text-fig. 4.

It is interesting that Finean (15) deduced by x-ray analysis that myelin broke up into mem-



TEXT-FIG. 4. These two diagrams illustrate the effects on the structure of the myelin sheath of soaking fibers in hypotonic solutions. *a* is comparable to *c* in Text-fig. 2. The outer (*1*) and inner (*2*) mesaxons and the axon–Schwann membrane (*3*) are shown. A definite gap is present between the membranes of the latter two double (*D*) membranes (*2* and *3*), but the gap is only present in a part of the outer mesaxon; throughout most of its length the gap is closed and the two membranes (*mb*) are in contact making an external compound membrane (*δ*) of which myelin is formed. In *b* the myelin lamellae are separated throughout the spiral by a restoration of the gap between the membranes of the primitive mesaxon from which the myelin was formed during development (Text-fig. 2). The inner (cytoplasmic) surfaces of the membranes united during development to form the major dense lines of myelin remain united during this swelling process. The two membranes left united along their cytoplasmic surfaces form an internal compound membrane (*ICM*). Note that the outer ends of the mesaxons in both diagrams remain in contact. While this is not invariably found in the sections thus far examined, it occurs rather frequently.



TEXT-FIG. 5. Illustration of similarities and differences between double membranes, external compound membranes, and internal compound membranes. Double membranes (*a*) consist of two cell membranes ~75 A wide separated by a gap substance (*gap subs.*) of variable width connected with the outside. The cell membranes are bordered by cytoplasm (*Sch. cyt.*). External compound membranes consist of the same structures with the gap between the membranes closed (*b*). Internal compound membranes (*d*) consist of two cell membranes in contact along their cytoplasmic surfaces and bounded on both sides by gap (outside) substance (*gap subs.*). The central density of internal compound membranes is accentuated. *c* shows a developmental stage in the formation of internal compound membranes. Double membranes measure from 200 to 300 A in over-all thickness; both internal and external compound membranes measure ~150 A in over-all thickness.

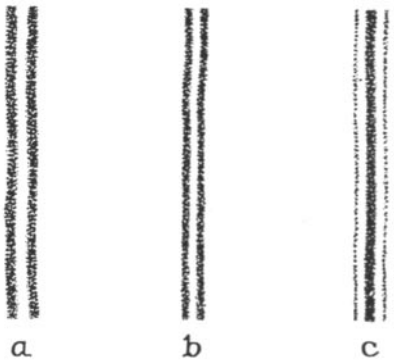
branous units ~150 A thick after freezing and thawing, though he could not determine their nature.

It seems useful to introduce a special term for the ~150 A membranous components appearing in swollen myelin for convenience of description and because it seems possible that in other situations this kind of structure will be observed. It is proposed that the term "internal compound membrane" be used.

A consideration of through focus images of these membrane structures (Figs. 13 to 16) brings up several important points relating particularly to instrumentation. Figs. 13 *a*, *b*, 14, 17, and 18, and all the micrographs mentioned above are slightly underfocused. In such micrographs the light zones of the simple membranes and the compound membranes contrast distinctly with the dense zones. In micrographs nearer to exact focus (Figs. 15 and 13 *c*) the contrast between the light zones and dense layers is markedly reduced. Indeed, in Fig. 15 the outer dense lines of the internal compound membranes are no longer seen, though the central dense lines are visible (see area between the unlabelled arrows). The structure in such micrographs as this resembles one reported by Hodge *et al.* in chloroplasts (24). In overfocused images (Figs. 13 *d* and 16) the central dense line of the compound membranes is doubled and the remainder of the structure blurred (unlabelled arrows). Figs. 17 to 19 show a through focus series of one of the simple membranes at the surface of a Schwann cell. Fig. 19 is overfocused. In the first two (underfocused) micrographs a pair of dense lines ~ 25 A wide are seen. The width of the dense lines is decreased in Fig. 18, which is less underfocused. In Fig. 19 three unequal dense lines and two light zones are seen (Text-fig. 6). Without a careful consideration of through focus images such as those in Figs. 17 to 23, this false image could be misinterpreted as due to a compound membrane, even though its dimensions are too small.

Density distributions like those in these images (Figs. 17 to 19) may be derived graphically by considering the interaction and summation of the overlapping Fresnel fringes from a hypothetical structure consisting of two dense lines separated by a light zone, making a unit ~ 75 A wide. If the anomalous distribution of Fresnel fringe intensities peculiar to electron optics is taken into account, it may readily be seen that the underfocused images are reasonably close representatives of the structure and the overfocused images are completely false. Even the former must not be interpreted too literally. Any measurements of the line widths are very sensitive to focus.

Most microscopists prefer slightly underfocused images because of their higher contrast. In this instance,



TEXT-FIG. 6. Diagrams illustrating the changes in the appearance of an ~ 75 A cell membrane in images at different levels of focus (Figs. 21 to 23). The image in *b* is very nearly in focus, and two thin dense lines are seen separated by a light central zone. To the left (*a*) an underfocused image is shown. Here the over-all picture is essentially the same as in *b*, but the dense lines are thicker and the over-all width of the paired structure increased. In *c* the overfocused image is shown. Here the pattern of two parallel lines is replaced by one of three parallel lines, with the central line much more dense and thicker than the other two. These images may be explained for a hypothetical structure like that in *b* by plotting graphically the anomalous Fresnel fringe intensities produced by each dense line and considering their interactions.

however, a rather important point is involved. The light zone in these membranes in slightly underfocused images might be interpreted as representing zones containing material of much less electron-scattering power than is actually the case. Examination of the outer Schwann membrane in Fig. 15 (arrow *u*) showed that even in exact focus where the ordinarily stratified structure is almost homogeneously dense, there is a very slight but definite rise in density at the edges. Further, when two of these membranes are in contact and their densities add in the images at nearly exact focus, a definite dense line (the major dense line) results, as in the membranes in Fig. 15 (between the unlabelled arrows) and Fig. 13 *c*. So there doubtless is material of greater scattering power at the borders. The point to be emphasized is that the scattering power of the material in the light zones is not very much less. It is certainly not nearly so low as the usual underfocused micrograph might suggest. These considerations are important in interpreting the dark and light layers seen in nerve myelin and at surfaces generally in terms of underlying molecular structure. Apart from the implication that the actual electron-scattering power of the light zones of myelin as usually observed may be higher than previously supposed, certain peculiar ribbon-like 70 to 80 A dense bands seen in local regions in OsO_4-fixed

myelin (36) may be explainable as focusing effects, despite their localized nature. These matters will be dealt with separately in detail.

In myelinated fibers soaked in distilled water for ½ hour (Figs. 11, 12), early changes are seen. By far the most common and general one is that shown in Fig. 11. Here the myelin is for the most part unaltered. However, the outermost few lamellae show a distinct splitting along the intraperiod lines. The detailed structure of the separated lamellae is here somewhat obscured, but an internal compound membrane may be made out at the arrow (inset). At arrow *2* (inset), two such compound membranes are in apposition, while the intraperiod lines on either side of this intact region are split. It might be inferred from the information given above that the splitting of the intraperiod line throughout the myelin spiral occurs by direct extension from the gap between the component membranes of the outer mesaxon on through the spiral. This may, in fact, be correct, but it does not seem to be the case in the plane of this section, since an intraperiod line is skipped. Furthermore, as shown in Fig. 12, splitting of the intraperiod lines sometimes seems to begin in the deeper, rather than in the more superficial layers of the spiral. These findings suggest that even though the earliest changes occur most frequently in the outer layers, the opening of the spiral does not of necessity simply propagate inward from the outer meso gap. The water which is presumably necessary for the expansion may, in part at least, be transported directly across the membranes. Alternatively, it might enter at nodes of Ranvier (35, 37) and dissect longitudinally along the internode myelin. This point, which may be a very significant one, is under further study.

Definite alterations in myelin of the type described above were seen after soaking in I/4 Ringer, but apparently unaffected fibers were seen fairly frequently. With I/6 Ringer after 2 hours, the effect was more consistent and qualitatively the same as that seen after distilled water treatment, though the average increase in the period was smaller (Table I).

A few nerve fibers were soaked in water and fixed with a veronal-acetate buffered OsO_4 fixative (Palade) isotonic with normal Ringer solution. The myelin was swollen and the period was increased due to its separation into layers like those observed in $KMnO_4$-fixed material. However, the layers appeared as very poorly fixed irregular thin dense lines or ribbons which were difficult to interpret in terms of normal myelin structure. This technique was, therefore, put aside for later investigation.

(*b*) *Unmyelinated Fibers.*—Unmyelinated fibers soaked in distilled water were sometimes so distorted as to be hardly identifiable. More commonly, however, they were relatively intact, as in Figs. 6 and 8. Here there is no gross distortion of the fibers. In Fig. 26 a portion of a large Schwann cell containing at least nine axons is seen. In this fiber, which was soaked for 2 hours in I/4 Ringer solution, the mesaxon gaps are enlarged to more than 300 A (see inset). Measurements of the average widths of the double membrane gaps in all of these unmyelinated fibers after soaking in hypotonic solutions are given in Table I. While no statistical analysis has been made, the figures suggest a significant increase in the gap width (Text-fig. 3).

3. Hypertonic Solutions:

(*a*) *Myelin.*—No dramatic changes such as the above were seen in myelin after treatment with hypertonic solutions. The axons and Schwann cells were well preserved, but shrunken after treatment for 2 hours (Fig. 28). In the lower range of concentrations the effect was principally one of a general sharpening of the images. After soaking for 1 to 2 hours in 6 to 10 I Ringer, the myelin most commonly appeared intact and no change in the period was detected. The mesaxon gaps were, however, consistently closed (Fig. 23).

After prolonged treatment (4 hours) with hypertonic solutions, even in the lower ranges of concentrations (4 I Ringer) there was a distinct change in the myelinated fibers. This involved anomalous swelling and fragmentation of myelin associated with shrinkage of the axon (Fig. 24 *b*). It seemed to affect the larger fibers more than the smaller ones. In some regions, interpreted as showing early fragmentation changes, vacuoles were seen in the major dense lines (Fig. 24 *a*). The fragmentation was typically more pronounced on the axonal side. This change involving both myelin and the axons was only widespread, in the lower concentration ranges, after soaking for 4 hours (Fig. 24 *b*). It was the most prominent feature of fibers soaked for only 2 hours in 20 N sucrose-Ringer. In this case the axons were very greatly shrunken.

Another type of change, seen in the fibers

treated with sucrose-Ringer, occurred in myelin that remained relatively intact. The average period was increased (Table I). In some instances, the spacing was increased to 140 to 160 A without any obvious alterations in the dense layers. In other regions it seemed that the width of the intraperiod line was increased to over 40 A, and in some areas it was definitely split into two layers (Figs. 25 *a* and *b*). This alteration occurred in some specimens in all ranges of concentration of sucrose-Ringer. Perhaps in the lower range (<10 I) it was due to dilution of salt ions since the solutions were made by dilution of 10 I sucrose-Ringer. This could not, however, explain its occurrence in the 10 I and 20 I solutions. Possibly two mechanisms are involved.

(*b*) *Unmyelinated Fibers.*—After 1 to 2 hours soaking in 4 to 10 I Ringer solution or 10 I sucrose-Ringer solution, a distinct change was seen in the unmyelinated fiber double membranes as compared with the control fibers. This alteration consisted of a complete closure of the gaps of mesaxons or axon–Schwann membranes for distances in the plane of section of up to 0.3 to 0.7 micron (Fig. 27). Near these gap closures, the three strata of the individual ~75 A cell membranes could often be seen clearly. A structure about 150 A wide containing dense and light strata was produced by the close apposition of the two cell membranes (Figs. 27, 29 to 33, and Fig. 21 (upper inset)); (see Text-fig. 3). The possibility that these regions of membrane contact might result from compression during sectioning was eliminated by the fact that they were frequently seen running in directions both parallel and perpendicular to the direction of sectioning. Similar gap closures were seen, though infrequently, in mitochondrial double membranes (Fig. 32), but not in those of endoplasmic reticulum or nuclear membranes.

At moderate electronic magnifications (Figs. 27, 29, and 32), the ~150 A compound membranes resulting from the gap closures appeared as five layered structures consisting of a central dense stratum bordered by two light zones and two somewhat thinner dense strata (Text-fig. 3). At the two apposed arrows in Fig. 27, the central dense stratum measured ~40 A across. The two light strata each measured ~35 A, and the two dense strata at the borders of the structure measured ~20 A. Further photographic enlargement of areas such as that between the arrows in Fig. 27 enlarged in the lower inset often failed to yield more information and served only to emphasize the degree of arbitrariness involved in making the individual measurements quoted above.

In micrographs taken at high initial electronic magnifications (Figs. 30, 31, and 33), it was sometimes possible to distinguish the four individual dense strata of the two cell membranes making up the compound structure, though in Fig. 33 this is only true because the membranes are in one place (arrow in inset) slightly parted. In Fig. 30 (inset), on the other hand, in one region all four dense strata may be seen without such parting of the membranes. Here the dense strata each measure about 20 A across, and the light zones over 30 A. The two cell membranes are separated by a light zone in the middle measuring less than 15 A across. In Fig. 31 one of the compound membranes is seen in an image at almost exact focus. Here each of the dense strata of the membranes measures about 20 A across, the light zones about 35 A. In the region between the two arrows in the inset enlargement, each of the two dense strata in apposition in the middle of the compound membrane structure is resolved into further subdivisions. Here there is an intermembrane space ~15 A wide, and each of the apposed membrane strata measuring ~20 A across appears itself to consist of two parallel dense lines separated by a light zone. Each of these dense lines and the intervening light zones measure less than 10 A across. Unfortunately, a through focus series was not obtained in this instance, and thus the exact significance of the finer strata cannot be assessed accurately, even from the electron optical viewpoint.[5]

[5] It has been stated previously (40) that the cell membrane appears in sections as two parallel dense strata measuring about 25 A across, separated by a light zone of about the same width. This statement was intended only to indicate orders of magnitude. It was based principally on measurements made in micrographs taken at fairly low electronic magnifications (~ 40,000) and slightly underfocused. Micrographs such as that shown in Fig. 31 taken at higher electronic magnifications and closer to exact focus suggest that, in fact, the central zone is wider and the dense strata more narrow. However, until a larger number of micrographs showing this degree of resolution have been obtained, it seems best to state the measurements merely as orders of magnitude. Furthermore, in interpreting these measurements it must not be forgotten that the preparatory procedures (15, 12) have undoubtedly induced certain alterations of undetermined nature in the structure which affect the measurements appreciably.

The double membrane gap closures were quite obvious in sections viewed at 400,000 magnification (~40,000 electron optical) directly on the fluorescent screen of the electron microscope. It was usually necessary to examine no more than one or two unmyelinated fibers to see the effect in any one of the specimens treated with hypertonic Ringer solution (4 to 10 I). All these test specimens were easily segregated from the controls. The following criteria were used in deciding whether or not the gap closure effect was present. First, the membranes must be well fixed. Second, in the regions showing the gap closures the membranes must be sectioned perpendicularly so that their internal strata could be seen clearly. Third, the gap closures must extend for at least 500 A in the plane of the section. Fourth, the closures must be present in over half of the well fixed perpendicularly sectioned fibers examined. Within these criteria none of the seventeen control specimens showed the effect. Nor have such closures been observed in normal adult freshly fixed vertebrate unmyelinated nerve fibers. It should be mentioned, however, that two of the control specimens did each after considerable search, show one fairly convincing gap closure extending for a few hundred angstrom units. All the other controls were negative.

Seven of the eight specimens treated with 2 I Ringer satisfied the criteria for gap closure, but one did not. The specimen treated with 1.5 I Ringer showed the effect, but the one treated with 1.25 I did not.

In the case of the hypertonic solutions containing sucrose, the changes only became consistent at ten times the isotonic concentration. Even at this concentration, one specimen failed to show the effect. Only three of the six specimens treated with 6 I sucrose-Ringer fully satisfied the criteria for gap closure. Two were equivocal in that less than half the fibers examined showed the closures and one was entirely negative. Of the four specimens treated with 4 I sucrose-Ringer, only two showed any gap closures and these were too infrequently seen to satisfy the criteria. One of the six specimens soaked in 2 I sucrose-Ringer showed the effect. The one specimen treated with 1.5 I sucrose-Ringer could not be distinguished from the controls. The effect was, however, as distinct and almost as consistent after 10 I sucrose-Ringer (Fig. 31) as after 4 to 10 I Ringer (Figs. 27, 29, 30, 32, and 33). It appears then that, for given total molarities, increases in the total salt concentrations were distinctly more effective in bringing about the gap closures than increases in tonicity effected by sucrose alone.

It should be noted, in this connection, that the hypertonic sucrose-Ringer solutions except 10 I were all deficient in salt ions as compared with isotonic Ringer solutions, despite their higher total molar concentrations. This was the case because the 6 I, 4 I, 2 I, and 1.5 I solutions were made by dilution from a stock solution made by dissolving sucrose in isotonic Ringer solution to bring the total molarity to ten times the isotonic value. These experiments are now being repeated, using solutions of a constant salt concentration at all hypertonic values and solutions of sucrose containing no salt. In other experiments the mole fractions of specific ions have been varied at a constant total molarity equal to that of isotonic Ringer solution.

Interestingly, gap closures satisfying the criteria given above are present in developing mouse unmyelinated fibers, as well as immature mouse myelinating fibers (Fig. 3). They have frequently been seen in the mesaxons of adult frog and mouse myelinated fibers. They seem characteristic of the axon–Schwann membranes in the juxtaterminal internode regions near nodes of Ranvier (35, 37). They have also been seen in this laboratory in certain locust nerve fibers by Gray (23), though the frequency of their occurrence in this material is not yet known.

The appearance of the ~150 A layered structures resulting from apposition of the cell membranes bordering the double membrane gaps (Figs. 3, 27, and 29 to 33) should be compared with that of the internal compound membranes observed in myelin after treatment with distilled water (Figs. 7, 13 to 16, and 21) (see Text-fig. 5). In the former, the density of the central layer is not very different from that of the outer bordering layers, though its thickness is greater. In the internal compound membranes, on the other hand, the central lines are strikingly more dense as well as thicker than those at the borders. The density differences are like those between the major dense lines and the intraperiod lines of myelin with which these structures are directly related. While both these ~150 A membrane structures are composed of two cell membranes in contact, in the one case two inside (cytoplasmic) membrane surfaces are involved (internal compound membranes), and in the other two outside membrane surfaces are concerned. In the case of internal compound membranes, Finean's "difference factor" (13) (see Discussion) is split into two layers which border the whole structure, and in the other

case the "difference factor" is all located in the central dense zone. Thus, these two kinds of membrane structure are fundamentally different. The term "internal compound membrane" distinguishes the type seen in myelin involving contact of internal membrane surfaces. The term "external compound membrane" is used for the other type, involving contact of outer membrane surfaces described in this section.

4. The Surface-Membrane-Complex:

The relationship of the layers seen in the present study at the free surfaces of cells to those seen in previous studies with other techniques is interesting. The term surface-membrane-complex was introduced (33) for the three layers seen in OsO_4-fixed, phosphotungstic acid (PTA)-stained preparations at the surfaces of nerve and muscle fibers. This included a dense line next to connective tissue (basement membrane) and a light zone ~200 to 300 A thick between the two dense lines. Similar layers are now seen after $KMnO_4$ fixation without PTA staining, but the outer layers are less discrete. It is now clear that the single dense line previously observed next to cytoplasm was a part of the ~75 A cell membrane, and the rest of the surface-membrane-complex is extracellular. After $KMnO_4$ fixation, a hazy delicately granular or reticular material is now often seen extending continuously out from the cell membrane and into the gaps of double membranes (*b* in Fig. 29 and Figs. 4 and 5, 27, 32, and 34). In developing hair cells, a much denser extracellular material has been seen filling double membrane gaps by Birbeck and Mercer (6–8). In studies of neuromuscular spindles after OsO_4 fixation with or without PTA staining, similar material has often been seen extending out from the surfaces of muscle fibers for a distance of microns (34).

Such material as this is PAS-positive and probably contains polysaccharides, and perhaps also proteins. It seems likely that the gap substance also contains, at least in nerve fibers, similar material. It seems clear now that this material should be regarded as outside the cell, although a monolayer of similar material may be an integral part of the outer dense layer of the cell membrane. This latter component, which enters into the compact myelin structure in the intraperiod line, may represent Finean's "difference factor" (13) and occupy the central region of external compound membranes. The nature of this material in chemical terms remains unsolved.

DISCUSSION

The findings reported bring up general problems of mainly technical interest and more specific problems related to the molecular structure of myelin, unmyelinated nerve fiber double membranes, and cell membranes in the general sense. These will be dealt with in this order.

General:

Perhaps the most important aspect of the findings is not the details reported, but rather the general fact that techniques in electron microscopy are now adequate to permit experiments of the type presented here to be carried out at all. The recent work of Fernández-Morán and Finean (12) correlating x-ray diffraction techniques with electron microscopy of nerves fixed with OsO_4 and embedded in methacrylate or gelatin (21), provides another illustration of this fact. It is demonstrated again in the present work that it is possible, by the use of a chemical fixative, not only to preserve and visualize structures in tissues representing only a few organized molecular layers, but to bring about and visualize specific alterations in the relationships of such structures in an instance in which the findings may be checked with an independent technique on unfixed tissues (17). Furthermore, it now seems more feasible than hitherto to think of interpreting experimentally induced structural alterations in tissues at the macromolecular level in physical chemical terms. This has been attempted many times in the past, but with little success in the field of nerve ultrastructure because of technical barriers (31). At least as far as cell membranes are concerned, these barriers now seem less formidable. Apart from instrumental factors, the principal technical advances taken advantage of in the present work are the development of permanganate fixation by Luft (26) and of epoxy embedding techniques by Maaløe and Birch-Anderson (27) and Glauert, Rogers, and Glauert (22).

A more specific feature concerned with general technical matters is that the alterations in myelin induced by a non-isotonic medium were successfully preserved by an isotonic fixative. Finean and Millington (17) obtained an increase of the myelin period to ~270 A in hypotonic solutions. In the present findings in the regular areas of

myelin, which might be expected to contribute to an x-ray diffraction pattern, the spacing is ~200 A after similar treatment. At first glance, it might appear that some of the effect of the hypotonic medium was reversed by the isotonic fixative. However, when normal frog myelin is fixed, the period is reduced from 171 A to ~120 A (a reduction by ~30 per cent). If it be assumed that the period was increased by soaking in water to ~270 A, then the reduction by ~23 per cent to ~200 A after fixation is roughly comparable to that to be expected from the preparative procedures alone. It thus appears that the tonicity of the permanganate fixative solutions used here is of less importance than might be supposed, though no effort has yet been made to investigate this in detail.

It must be emphasized despite the relatively good agreement between the observed membrane structures and those deduced by indirect means, that many changes must have been introduced by the procedures required for observation of the membranes with the electron microscope. W. J. Schmidt demonstrated in 1935 (43, 44) that permanganate fixation reverses the birefringence of nerve myelin. Finean (15) and Fernández-Morán and Finean (12) demonstrated that definite alterations in myelin structure occur during OsO_4 fixation and during dehydration and embedding. Other alterations occur during bombardment with the electron beam (38). Furthermore, in dealing with closely spaced dense strata of dimensions approaching the practical resolution limit of the electron microscope, the apparent thickness of layers becomes extremely sensitive to the level of focus of the image. When it is stated that cell membranes are ~75 A thick and consist of two dense lines ~20 A wide bordering a central light zone, a very great deal of emphasis should be placed on the "~" sign in front of the figures and the adjectives "dense" and "light" must not be taken too literally. Until very exact through focus studies have been made, it is difficult to debate about the dimensions and densities, and any correlation or lack of correlation between them and the features to be expected of a bimolecular leaflet of lipide covered by monolayers of non-lipide material.

It has not yet been determined to what extent, if any, the structures observed in cell membranes may be directly related to the molecular structure present before fixation. They may be related only in the way that the charred remains of a burned piece of paper are related to the original paper. On the other hand, there may be much less alteration than this analogy implies. The important point is that the observed densities probably represent fairly accurately the general macromolecular pattern of structure existing before fixation. Precisely what alterations are introduced in the actual molecular layers responsible for the pattern remains to be determined.

Myelin:

Hypotonic Solutions.—The behavior of myelin in distilled water gives some qualitative information about the binding forces between the inside and outside surfaces of the Schwann cell membrane when in apposition in compact myelin. A consideration of the molecular structure of myelin is important in interpreting this information. W. J. Schmidt suggested in 1937 (45) that myelin consists of alternating fairly thick layers of lipide and protein with the lipide molecules oriented radially, and the protein molecules tangentially. Schmitt *et al.* (46, 47) presented evidence which indicated that each of the repeating lamellae in myelin contains only two smectic bimolecular leaflets of mixed lipides, with the non-polar lipide carbon chains radially oriented and some of the polar surfaces covered by monolayers of protein. Finean (13) has presented more recently collected evidence in agreement with this conception. His results indicate that each polar surface of the bimolecular leaflets is covered by monolayers of protein or material of similar x-ray scattering power. Since it has been shown directly that the Schwann surface membrane contains half of this repeating structure (40), it is reasonable to suppose, if the conceptions of myelin structure are correct, that the pattern observed in the Schwann cell membrane represents one bimolecular leaflet of lipide with its polar surfaces covered by monolayers of non-lipide material. It seems reasonable to guess that at least one of these non-lipide layers is protein, and the other something else. This latter idea has been based in the past chiefly on the pronounced differences in the way these layers react with fixatives (38, 14). Thus the major dense lines are very much more dense and regular after OsO_4 fixation than the intraperiod lines, and this difference may also be seen after $KMnO_4$ fixation. It seems, then, that the components at the intraperiod line are chemically dif-

ferent from those at the major dense lines. Since the former represent the two enfolded outside strata of the Schwann cell membrane and the latter the inside strata, it appears that the membranes, while looking symmetrical in electron micrographs, are actually chemically unsymmetrical.

Support for this concept is given by the contrasting behavior in distilled water of the inside and outside surfaces of the membranes united in myelin. It is tempting to draw an analogy between the behavior of the layers at the major dense lines, which do not separate in water, and that of some globular proteins in water. The polypeptide chains folded together in a globular protein molecule are held together by a variety of heteropolar forces, prominent among which are hydrogen bonds (30). At temperatures of 0–4°C., globular proteins are not easily denatured simply by immersion in water. The behavior of the major dense line constituents in water is then somewhat like that of closely approximated polypeptide chains in their resistance to hydration. The x-ray evidence suggests that at the intraperiod lines some other constituent is added to the lipide layers (13). The constituents at these layers are evidently not bonded in the same way as those at the major dense lines. Perhaps a different class of compound, such as a polysaccharide, is involved.

The observation that after prolonged soaking in very hypertonic solutions, myelin swells and axons apparently shrink is consistent with the distribution of water in these two structures. Axons contain ~90 per cent water (3), whereas myelin contains only 40 to 50 per cent water (16), despite the presence of large hydrophilic surfaces. It is not very surprising, then, that water would be removed from the highly hydrated axons by hypertonic solutions, while the lyophilic molecules and ions in the hypertonic solutions penetrate myelin and actually increase its volume by virtue of their own bound water carried in with them. Thus, while it may seem anomalous for a tissue constituent to swell in both hypo- and hypertonic solutions, viewed in this way it seems reasonable. This problem is discussed briefly by Finean and Millington (17), but is by no means well understood.

Unmyelinated Fibers:

The induced variations in the spacing between the apposed membranes in mesaxons and axon–Schwann membranes can be viewed in more than one way. One might consider the interaction of the closely apposed membranes as analogous to that of two macromolecules in a colloidal solution (1, 25, 47, 50), in which one deals with spheres, rodlets, or platelets, which interact with one another at distances comparable to that between the membranes. It is quite possible that the same general rules apply to extended surfaces such as those of the membranes, which in effect may act like macromolecules with respect to one another. One is forced to consider such factors as being involved in the stabilization of the smectic phospholipide bimolecular leaflets of lipide-water model systems (2, 29) and in tobacco mosaic virus sols (4, 5). According to such a conception, the membranes might maintain certain positions with respect to one another, determined by a potential energy minimum (47). Contact of the membranes in hypertonic solutions could then be considered analogous to precipitation of a colloid, with the membranes then occupying another position of minimal potential energy. All this ignores the serious problem of what happens during fixation.

Another way to look at the problem is to consider the gap to contain a very highly hydrated colloid gel. This might serve to hold the cells together, perhaps in conjunction with the above forces. It is well known that many kinds of gels containing up to 80 to 90 per cent water may behave in the manner in which the gap substance has been observed to behave here in response to variations in the concentrations of solvate salt solutions. Thus, gelatin gels behave in every way as if they were surrounded by a semipermeable membrane, with the gelatin molecules acting as the non-diffusible macromolecular constituent setting up a Donnan equilibrium (1). Michaelis describes a highly hydrated carbohydrate gel the volume of which is remarkably sensitive to the ionic constitution of its bathing medium in salt concentrations not very different from those used here (28). Such gels are also sensitive to uncharged highly polar lyophilic substances such as sucrose. The phenomenon of syneresis in which gels collapse and extrude their solvates occurs in response to the kind of treatment that brought about the closure of the double membrane gaps observed here.

This second possibility is more attractive than the first alone for several reasons. For example if a continuous gap structure is postulated, one might

expect the membranes to be stabilized more readily by a fixing agent. Furthermore, the material observed in the gaps is like that which one might expect to see at high resolution in a highly hydrated gel. However, the evidence now available does not permit definite conclusions to be drawn.

Cell Membrane Structure:

Quite apart from the problem of the nature of the gap substance, the findings presented are of general significance with regard to the cell membrane concept. As resolution has improved in micrographs of sectioned material, there has been a tendency on the part of microscopists to refer to any discrete dense line next to cytoplasm as the cell membrane. Thus the whole surface-membrane-complex as defined above (33) was first seen as a single dense line and thought of as the cell membrane. Later, the inner dense line of this complex was resolved separately and called the cell membrane (see 33). This trend reached a climax recently when Birbeck and Mercer (8) described an ~30 A dense line next to the cytoplasm of certain hair follicle cells and referred to this as the "plasma" membrane. Carrying this terminology on to its logical conclusion, one might expect to see the cell membrane defined as a single molecular monolayer. If the term "cell membrane" is to have physiological meaning, however, it seems more reasonable to look for some entity which is constantly present and thicker than a monomolecular film in view of the physical properties of cell membranes (10, 42). An entity such as this should be definable as a constant pattern of organization of discrete molecular species which is common to a variety of cell types. It has been shown here and elsewhere (36, 40, 41) that such a structural entity is present at the surface of Schwann cells. This entity behaves as a unit in the formation of nerve myelin and maintains its structural integrity in extremes of environmental conditions. It is of the correct dimensions to contain within it several layers of discrete molecular species, which might be expected to account for the observed physical characteristics of cell membranes (10, 42). It will be shown in a subsequent paper that a membrane with the general pattern of organization of the Schwann cell membranes is present at the surfaces of a wide variety of cell types in different animals of different phyla. This same kind of membrane is also the basic component of mitochondria, nuclear membranes, and the endoplasmic reticulum. It seems, then, that after fixation the cell membrane is represented by the pair of parallel dense lines making the ~75 A units described here. A problem for the future is the visualization and precise definition of the molecular components present within this structure.

The author wishes to express his appreciation to Professor J. Z. Young for his constant support throughout all phases of this work. He also wishes to acknowledge the technical assistance of Miss Rose Smith, and the photographic assistance of Mr. Alan Aldrich. He is indebted to Miss E. R. Turlington for the diagrams.

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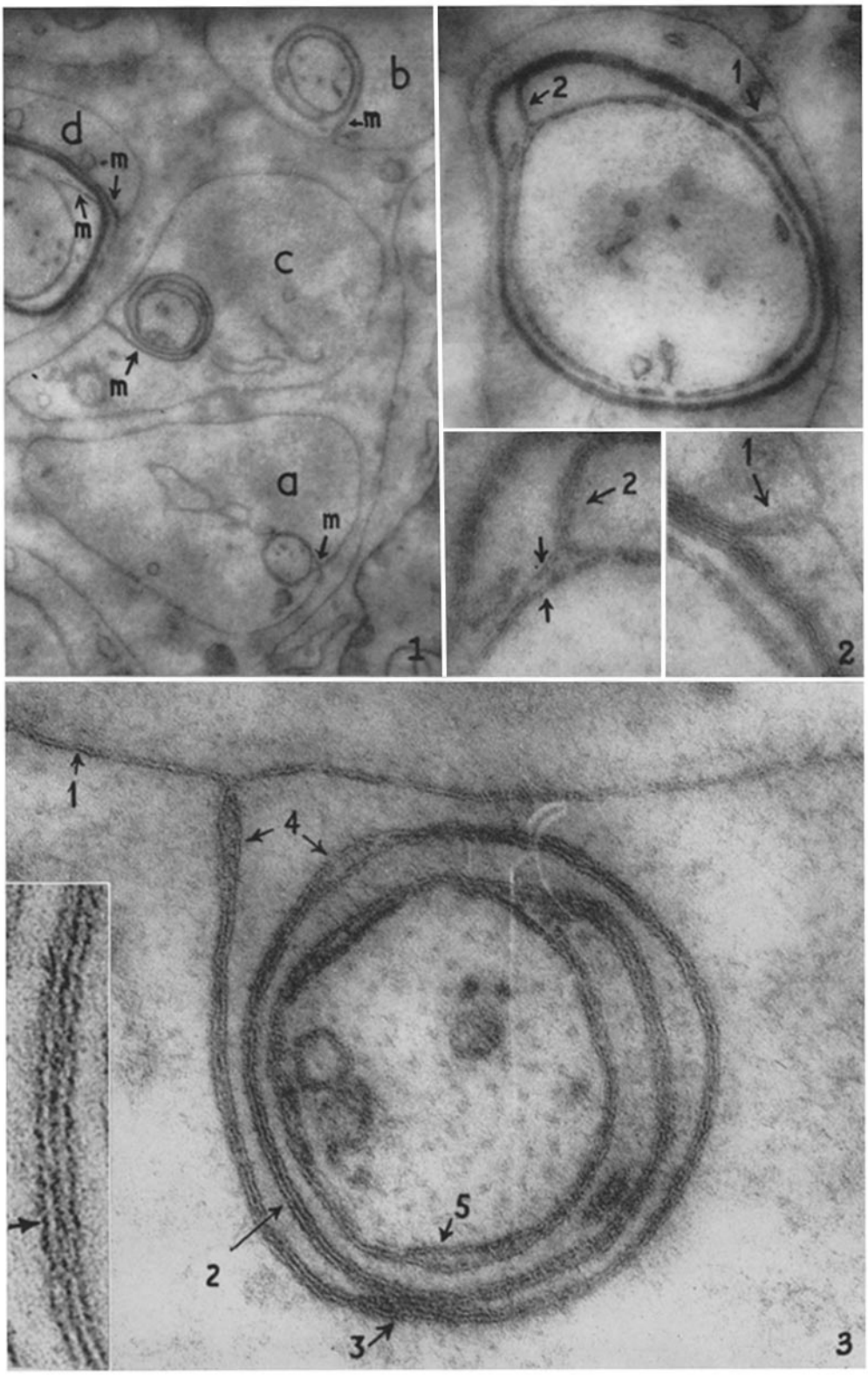
Explanation of Plates

Plate 194

Fig. 1. Section of a sciatic nerve fiber of a 2 day old mouse. Four developing nerve fibers are shown in different stages of the formation of myelin. At *a* a small axon is shown embedded in a relatively large Schwann cell (see Text-fig. 2 *a*). In *b* the mesaxon is elongated and wrapped around the axon in a spiral. In *c* a still later stage is shown in which the mesaxon is more elongated and wrapped spirally around the axon in almost two complete loops. This is enlarged in Fig. 3. At *d* a later stage is shown in which the loops of the mesaxon have come together and formed a few compact myelin lamellae. Note the two ends of the mesaxon (*m*). The three spirals in *b* to *d* here run in the same direction. Compare with Figs. 4 and 28, in which spirals running in opposite directions in the same field are shown. × 27,000.

Fig. 2. Higher magnification of a fiber like that shown in Fig. 1 *d*. Arrow *1* points to the outer mesaxon enlarged in the inset below. The inner mesaxon at arrow *2* is enlarged below to the left, and the component membranes of the axon–Schwann membrane are visible in one area (arrows). × 51,000. Insets, × 130,000.

Fig. 3. The axon of Fig. 1 *c* enlarged. At arrow *1* the Schwann membrane appears as two parallel dense lines making a unit ~75 A across. At arrow *2* two of these units are shown in contact in the mesaxon, making an external compound membrane ~150 A across (inset enlargement). Note the slight separation of the membranes at the arrow in the inset. Note that the densities of the component dense lines of the external compound membrane are almost the same (compare with the external compound membranes in Fig. 21 (upper inset) and Figs. 27 and 29 to 33, and with the internal compound membranes in Figs. 13 to 16 and 21). The cytoplasmic surfaces of two of the loops are in contact at arrow *3*, making a very short segment of compact myelin, but the membranes are oblique in the section at this site and their structure is not clearly defined. At arrows *4* the two membranes of the mesaxon are slightly separated. While the component membranes of the axon–Schwann membrane are for the most part in contact, they are in some areas separated (arrow *5*). × 145,000. Inset, × 340,000.

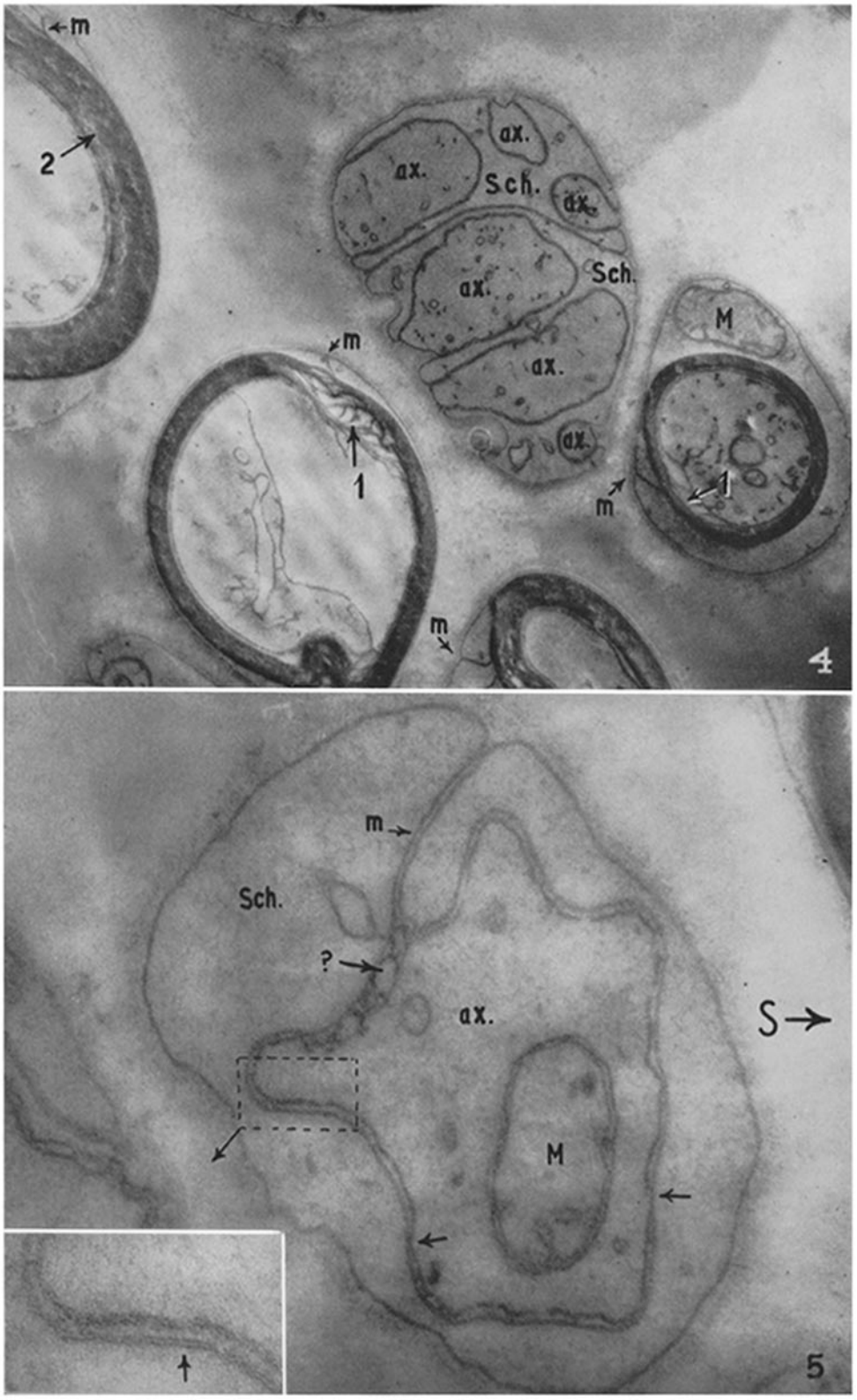


(Robertson: Structural alterations in nerve fibers)

PLATE 195

FIG. 4. Section of control nerve soaked in isotonic Ringer solution before fixation, showing one unmyelinated and portions of four myelinated fibers. Note (arrows *1*) the cross-sectional shearing defects of the Schmidt-Lanterman type (41) and the fragmentation of the myelin sheath (arrow *2*). The unmyelinated fiber consists of two Schwann cells (*Sch.*), each containing three axons (*ax.*). Outer mesaxons (*m*) are visible in each of the myelinated fibers. From the directions of entry of the mesaxons into the myelin, it is apparent that the myelin spiral of the fiber to the right turns in a direction opposite to that of the other fibers. A mitochondrion (*M*) is present in the outer Schwann cytoplasm of one fiber. × 15,000.

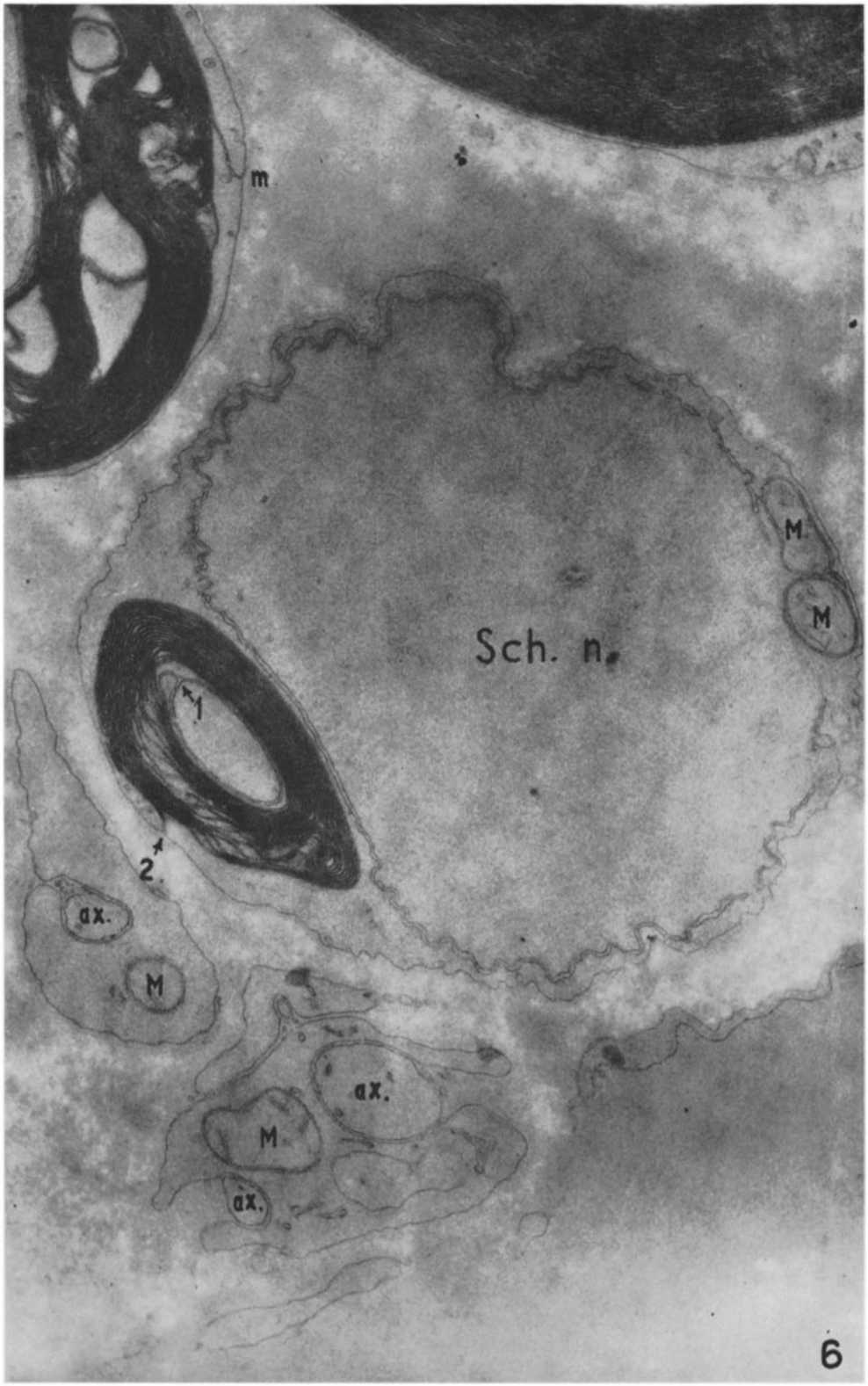
FIG. 5. Unmyelinated fiber from control specimen soaked in isotonic Ringer solution before fixation. One axon (*ax.*) is seen surrounded by one Schwann cell (*Sch.*). A mesaxon (*m*) is present, and its ~ 75 A membranes and central gap may be seen. The component membranes of the axon–Schwann membrane may be seen in the dotted rectangle (inset). A mitochondrion (*M*) is present in the axon. The direction of sectioning is indicated by arrow *S*. Note that the gap of the double membranes is present in most areas. In those designated by the unlabelled arrows the gap is closed, but the membrane structure here is obscured; this kind of gap closure is probably a sectioning artifact, and it is quite unlike that seen after hypertonic solutions. The structures bounded by ~ 75 A membranes present in the axon–Schwann membrane gap at arrow *?* are thought to represent finger-like evaginations of either the Schwann surface membrane or that of the axon. × 66,000. Inset, × 130,000.



(Robertson: Structural alterations in nerve fibers)

PLATE 196

FIG. 6. Cross-section of myelinated and unmyelinated fibers soaked in water. A small myelinated fiber containing a Schwann nucleus (*Sch.n.*) occupies the center of the field. The myelin of this fiber is separated into internal compound membranes, and the inner (*1*) and outer (*2*) mesaxons are visible. A cross-sectional shearing defect of the Schmidt-Lanterman type runs across the myelin between the two ends of the mesaxon. Two mitochondria (*M*) are present in Schwann cytoplasm beside the Schwann nucleus. The myelin of this fiber is enlarged in Fig. 7 to show the membrane structures. Two unmyelinated fibers containing axons (*ax.*) and mitochondria (*M*) are shown below, and segments of the myelin of two larger myelinated fibers are shown above. The outer mesaxon (*m*) of one of these is visible. From its direction of entry into the myelin, it is apparent that the spiral of this fiber turns in a direction opposite to that of the fiber below. × 24,000.

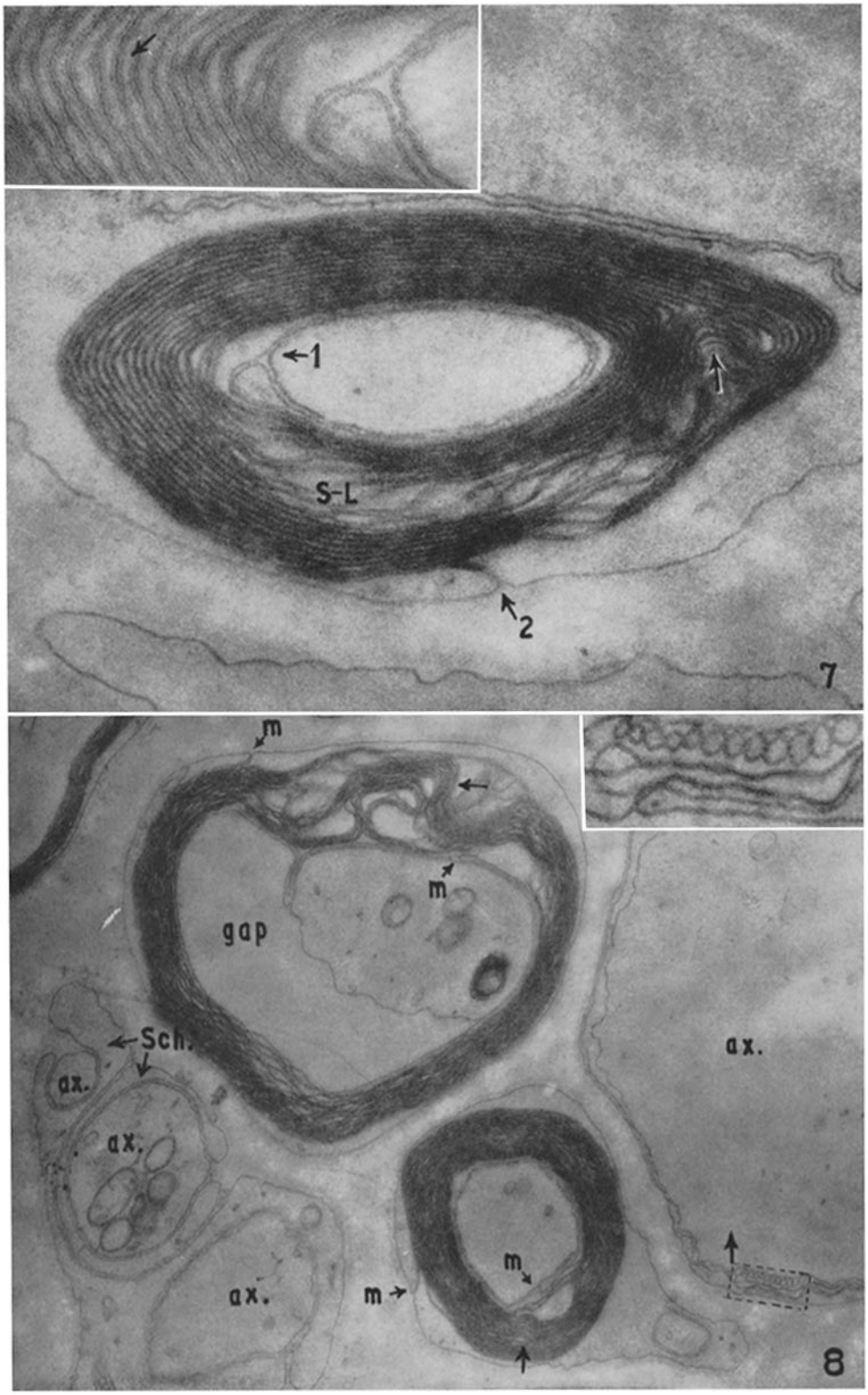


(Robertson: Structural alterations in nerve fibers)

PLATE 197

FIG. 7. Enlargement of the myelinated fiber in Fig. 6. Inner and outer mesos are shown at arrows *1* and *2* respectively. A shearing defect of the Schmidt-Lanterman type (41) runs through the myelin at *S-L*. Note the buckling of the myelin at the unlabelled arrow. The segment of myelin containing the inner mesaxon is enlarged in the inset. Here it may be seen that the increase of the period to $\sim$ 200 A is due to a splitting of the lamellae along the intraperiod lines. The unlabelled arrow points to one of the internal compound membranes which results from this splitting. Note the greater density and width of the central dense line as compared with the dense lines at the borders. $\times$ 51,000. Inset, $\times$ 110,000.

FIG. 8. Section of fibers soaked for 2 hours in distilled water. An unmyelinated fiber consisting of two Schwann cells (*Sch.*) and three axons (*ax.*) is seen to the left. Two myelinated fibers are seen. The myelin lamellae are split up into internal compound membranes, and the period is increased to $\sim$ 200 A. Note the buckling of the myelin at the unlabelled arrows. Inner and outer mesaxons (*m*) may be identified in both fibers. From their directions it may be seen that the two spirals turn in opposite directions. There is a greatly swollen axon–Schwann membrane gap (*gap*) in the upper fiber. To the right a large axon (*ax.*) is seen. A few distorted internal compound membranes were identified around this axon at high magnification near the row of vesicles or tubules with $\sim$ 75 A membranes in contact (inset enlargement). This fiber is probably sectioned near a node of Ranvier (35, 37). $\times$ 19,000. Inset, $\times$ 70,000.



(Robertson: Structural alterations in nerve fibers)

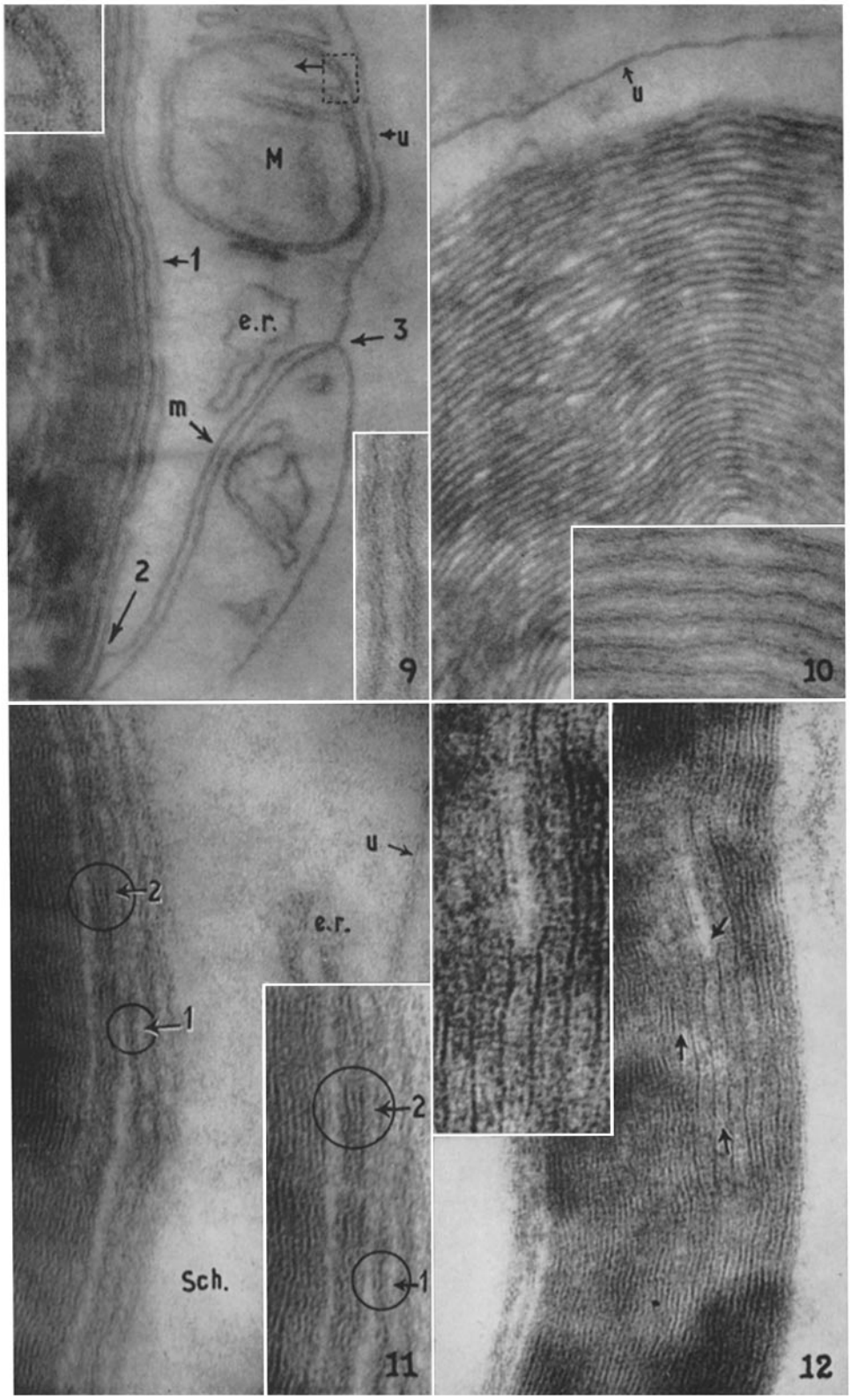
PLATE 198

FIG. 9. Myelinated fiber soaked in water for 2 hours. The intraperiod lines are split, and internal compound membranes are present throughout except for the outermost myelin membrane (arrow *1*), which is a simple ~ 75 A membrane. At the arrow *2* this outer membrane contacts the cytoplasmic surface of the upper membrane of the mesaxon (*m*) to make the outermost internal compound membrane. At the outer end of the mesaxon its two membranes are in contact before they separate as the Schwann surface membrane (*u*). A mitochondrion (*M*) and a few components of the endoplasmic reticulum (*e.r.*) are present in Schwann cytoplasm. A portion of the mitochondrion (*M*) is enlarged above to show its ~ 75 A membranes, and below a segment of the meso (*m*) is enlarged to show its component membranes. This micrograph is closer to focus than many others, and here the dense lines bordering the ~ 75 A membranes measure ~ 15 A and the light interzones ~ 50 A. × 82,000. Inset, × 254,000.

FIG. 10. Another example of a segment of myelin swollen in water. The outer Schwann surface membrane (*u*) is visible, and the internal compound membranes are shown in the inset enlargement. These measure ~ 150 A across. The central dense lines measure 30 to 35 A across, and the bordering dense layers ~ 15 A across. × 70,000. Inset, × 180,000.

FIG. 11. Small segment of myelinated fiber soaked in distilled water for ½ hour. The outer Schwann (*Sch.*) surface membrane (*u*) is shown to the right. The Schwann cytoplasm contains a component of the endoplasmic reticulum (*e.r*). The outer few layers of myelin show alterations. The intraperiod lines are split, and an internal compound membrane may be seen at arrow *1*. At arrow *2* two internal compound membranes are in contact at the intraperiod time, whereas on both sides of this lamella the intraperiod lines are split. The inset enlargement shows these features in more detail. × 150,000. Inset, × 220,000.

FIG. 12. Segment of myelin soaked in H_2O for ½ hour. Note the regions in which the myelin period is increased. The increase is due to a splitting of the intraperiod lines (arrows and inset). × 110,000. Inset, × 280,000.



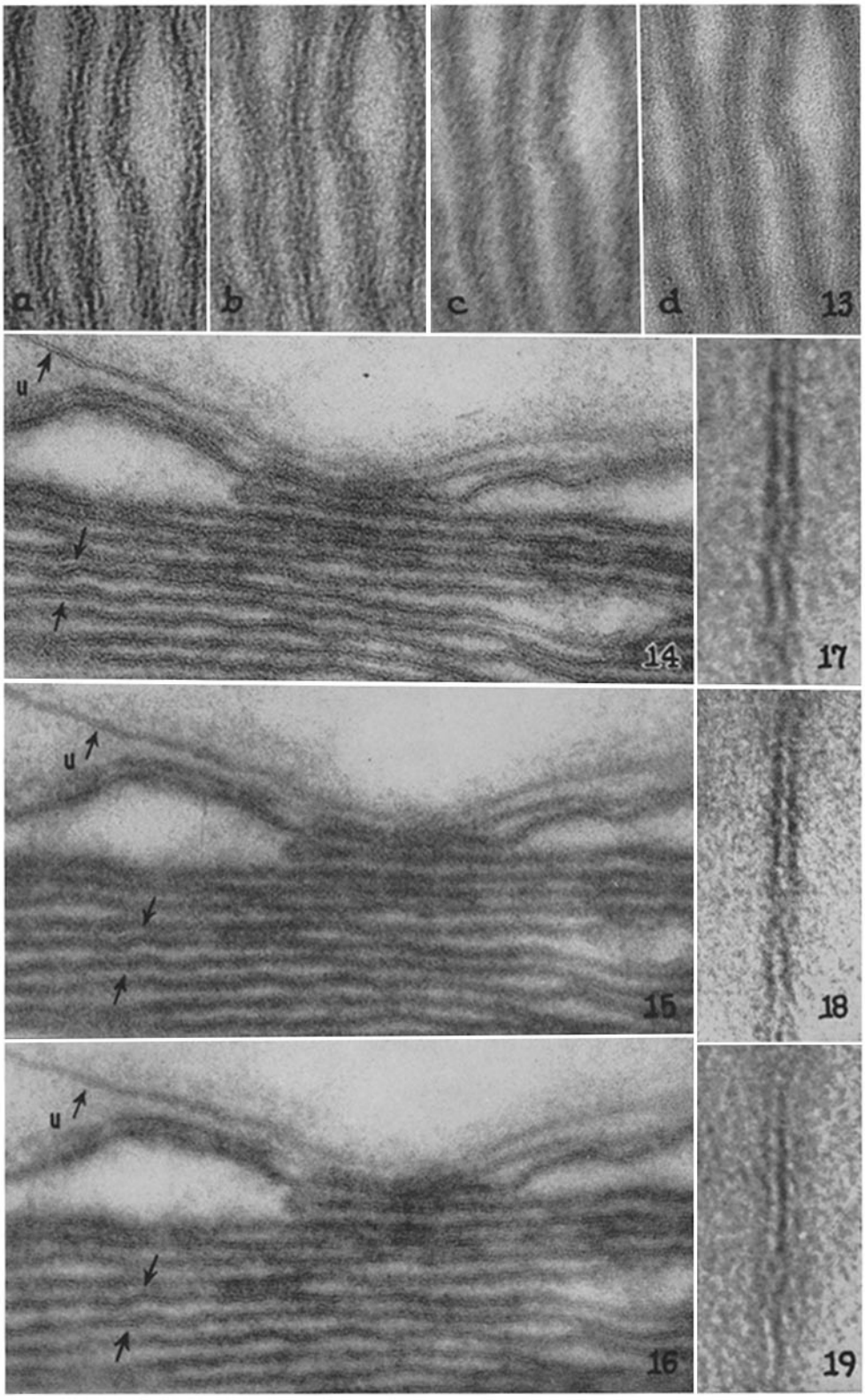
(Robertson: Structural alterations in nerve fibers)

PLATE 199

FIG. 13, *a* to *d*. Myelin of fiber soaked in water 2 hours before fixation, showing through focus series of internal compound membranes; *a*, underfocused; *b*, slightly underfocused; *c*, near exact focus; *d*, overfocused. In *a* and *b* the contrast is quite high. The five layers of the membranes are evident. In *c* the contrast is lower and the density of the central line less prominent. The light zones have almost disappeared here. In *d* the central dense line appears doubled and the remaining structures blurred. × 280,000.

FIGS. 14 to 16. Through focus series of a group of internal compound membranes in swollen myelin (H_2O, 2 hours) at lower magnification. Fig. 14, underfocused; Fig. 15, very close to exact focus; Fig. 16, overfocused. Note the changes in the density distributions in the outer Schwann surface membrane (*u*) and the internal compound membranes (unlabelled aligned arrows) in the three images. The light zones of the membranes are less light when in focus, but the central dense lines of the internal compound membranes still are visible. The dense lines bounding the ~ 75 A Schwann surface membrane are just visible in the in focus image. In the overfocused image the central dense lines of the internal compound membranes are doubled (area in Fig. 16 designated by the unlabelled arrows). The ~ 75 A surface membrane (*u*) in Fig. 16 in the overfocused image consists of three dense lines (compare with Fig. 19). × 130,000.

FIGS. 17 to 19. Through focus images of an ~ 75 A Schwann cell membrane. The level of focus was checked by the Fresnel fringes of a nearby hole in the support film. Fig. 17, underfocused; Fig. 18, less underfocused; Fig. 19, overfocused. In the underfocused image the over-all membrane width is ~ 90 A, and each dense line approaches 30 A. As focus is approached, the over-all membrane width decreases to ~ 75 A. The width of the dense lines decreases to about 20 A, and the light zone increases to over ~ 30 A. In the overfocused image three dense lines are seen. The central one appears much more dense. (See Text-fig. 6). × 472,000.



(Robertson: Structural alterations in nerve fibers)

PLATE 200

FIG. 20. Completely disorganized myelin seen in a nerve soaked for 2 hours in distilled water. No axon is identifiable here, and this structure may represent a myelin form. × 5,600.

FIG. 21. Small segment of swollen myelin in fiber soaked in distilled water 2 hours. The outer Schwann surface membrane (*u*) is visible. The next membrane beneath it is of the same kind, but all the visible deeper membranes are internal compound membranes. The difference in the density of the central dense line of the latter membranes as compared with the bordering dense lines is well shown here in the outermost internal compound membrane. This is enlarged in the lower inset (π), and an inset micrograph of an external compound membrane (δ) is inserted above for comparison. × 130,000. Insets, × 330,000.

FIG. 22. Outer meso in a control myelinated fiber. The outer myelin lamella consisting of two ~ 75 A membranes (see Fig. 23 and (40)) separates from the compact myelin by a splitting of the outer major dense line (*d*) (inset). The intraperiod lines (*i*) are here almost as dense as the major dense lines because fixation was prolonged slightly. The unit membranes do not show clearly either in the mesaxon (*m*) or the Schwann surface membrane. (See Fig. 27). × 200,000. Inset, × 350,000.

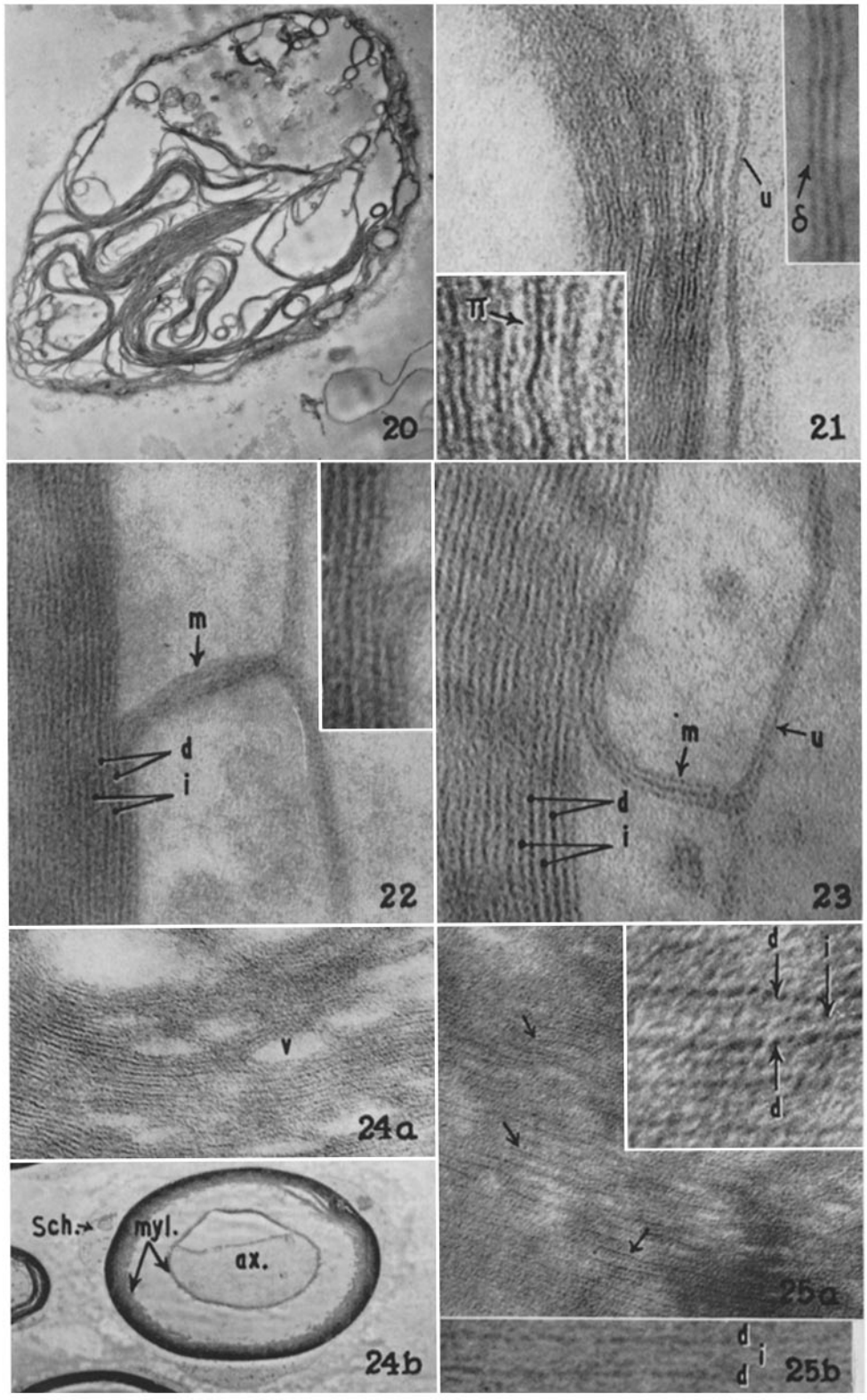
FIG. 23. Fiber soaked for 2 hours in 4 I Ringer solution. The two component membranes of the mesaxon (*m*) are in contact. The mesaxon separates from the compact myelin with a splitting of the outermost major dense line. The major dense lines (*d*) and intraperiod lines (*i*) are labelled because the density differences in this case are not marked due to prolonged fixation. *u*, the outer Schwann surface membrane. × 250,000.

FIG. 24 *a*. Myelin soaked in 4 I Ringer solution for 2 hours interpreted as showing early fragmentation changes. The vacuoles (*v*) seem to have developed by splitting of the major dense lines. × 140,000.

FIG. 24 *b*. Myelinated fiber soaked in 4 I Ringer for 4 hours before fixation. Note the relatively small diameter of the axon (*ax.*) and the relatively large diameter of the Schwann cell and myelin (*Sch.* and *myl.*). The myelin is fragmented on the axonal side. × 5,000.

FIG. 25 *a*. Segment of myelin soaked in 10 I sucrose-Ringer for 2 hours. The myelin period is increased in some regions, and the arrows indicate areas in which this appears to be due to splitting of the intraperiod lines. In the inset, two major dense lines (*d*) are separated and the intraperiod line (*i*) is split. × 110,000. Inset, × 465,000.

FIG. 25 *b*. One myelin lamella with two major dense lines (*d*) and a split intraperiod line (*i*) is shown. This specimen was soaked in 20 I Ringer solution for 2 hours, and the myelin was very extensively fragmented (like Fig. 24 *b*). × 375,000.



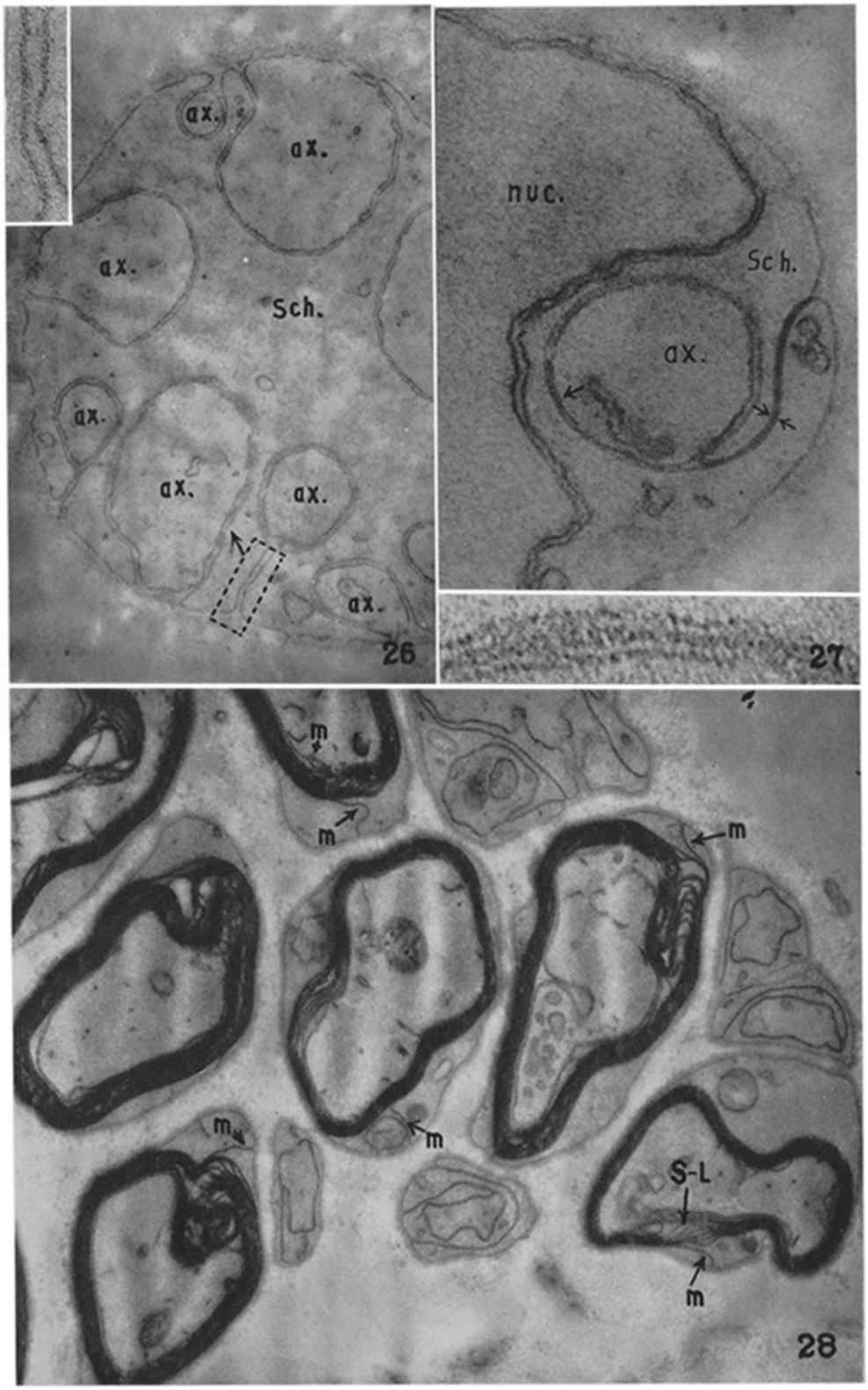
(Robertson: Structural alterations in nerve fibers)

PLATE 201

FIG. 26. Unmyelinated fiber consisting of one Schwann cell (*Sch.*), containing at least seven axons (*ax.*) soaked in I/4 Ringer solution for 2 hours. The mesaxon gaps measure 200 to 350 A. One mesaxon (dotted rectangle) is enlarged in the inset to show the prominent gap (<300 A). × 22,000. Inset, × 74,000.

FIG. 27. Unmyelinated fiber soaked in 4 I Ringer solution before fixation. A portion of the nucleus (*nuc.*) of the Schwann cell (*Sch.*) appears to the left. The nuclear double membrane does not show gap closures. The axon (*ax.*) is connected to the outside to the right by a mesaxon (arrows). The axon–Schwann membrane gap is closed at the unlabelled arrow. The mesaxon gap is closed throughout its entire length of about 0.4 to 0.5 micron. Between the aligned arrows the following measurements were obtained; over-all width ~150 A; width of central dense line ~40 A; width of each bordering light zone ~35 A; width of each outer dense line ~20 A. This area is enlarged in the inset below. Here it may be seen clearly that the central dense line is approximately twice the width of each of the outer dense lines. The arbitrariness of the measurements given above is made clear by the inset enlargement. × 74,000. Inset, × 470,000.

FIG. 28. Lower power view of myelinated and unmyelinated fibers soaked for 2 hours in 4 I Ringer. All of the fibers seem shrunken. The myelin is more folded and distorted than it is in the controls (Fig. 4). Outer mesaxons (*m*) may be seen in most of the myelinated fibers, and one inner mesaxon is seen above. From the directions of entry of the mesaxons into the myelin of five of the fibers, it is apparent that three spirals turn one way and two the other. Most of the myelinated fibers show cross-sectional shearing defects of the Schmidt-Lanterman type (*S-L*). × 163,000.



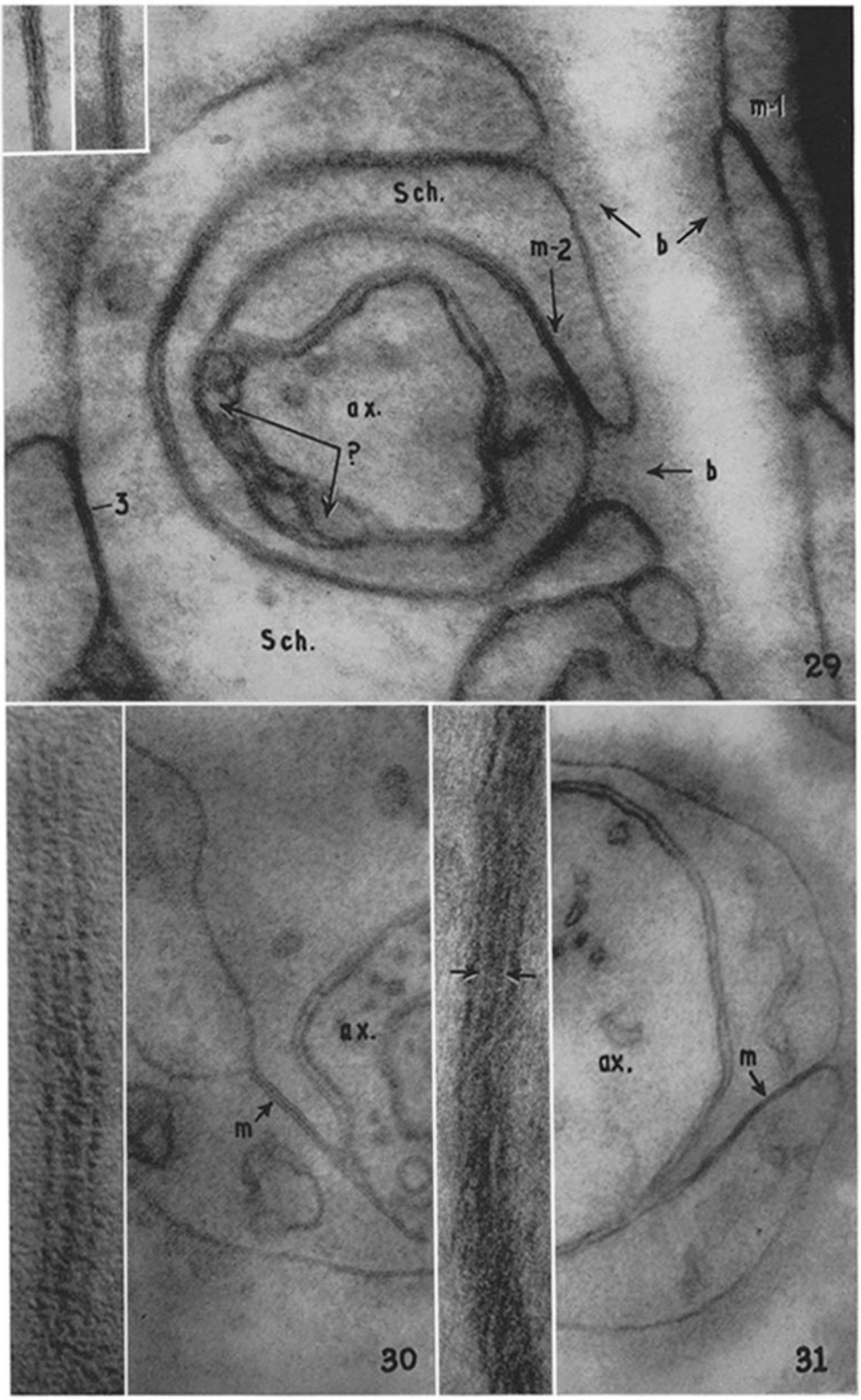
(Robertson: Structural alterations in nerve fibers)

PLATE 202

FIG. 29. Specimen treated with 10 I Ringer before fixation. Note the hazy, dense layer (*b*) which extends out for a few hundred angstrom units continuously from the membranes of the Schwann cells of both the unmyelinated and the myelinated fiber. This layer also extends down into the space between the two overlapping lips of Schwann cytoplasm (*Sch.*) near the origin of a mesaxon (*m-2*). In this region one Schwann cell containing an axon is almost completely enveloped by another Schwann cell. The gaps of the outer mesaxon (*m-1*) of a myelinated fiber and that of an inter-Schwann membrane (*3*) of the unmyelinated fiber are closed. (*m-2*) and (*3*) are enlarged in the insets. The structures designated by the arrows (*?*) lie in the axon–Schwann membrane gap and are tentatively considered to be small pseudopodial extensions of the Schwann cell. × 105,000. Insets, × 170,000.

FIG. 30. Unmyelinated fiber soaked in 4 I Ringer for 1 hour before fixation. Note the closed mesaxon gap (*m*) connecting with the axon (*ax.*). A segment of this is enlarged to the left. Note that the central dense line is about twice as thick as the other two and consists of two closely apposed dense lines. × 104,000. Inset, × 600,000.

FIG. 31. Unmyelinated fiber similar to that in Fig. 33, but soaked in 10 I sucrose-Ringer for 1 hour before fixation. Note the closed mexaxon gap (*m*) leading to the axon (*ax.*). This is enlarged in the inset. The two dense layers of the membranes in contact to form the central dense layer in the inset (arrows) seem to show some fine structure. They are separated by ~15 A. Each appears to be ~20 A across and composed of two dense layers <10 A wide separated by light zones. These images must not be interpreted too literally, however, since no through focus series was obtained in this instance. × 68,000. Inset, × 500,000.



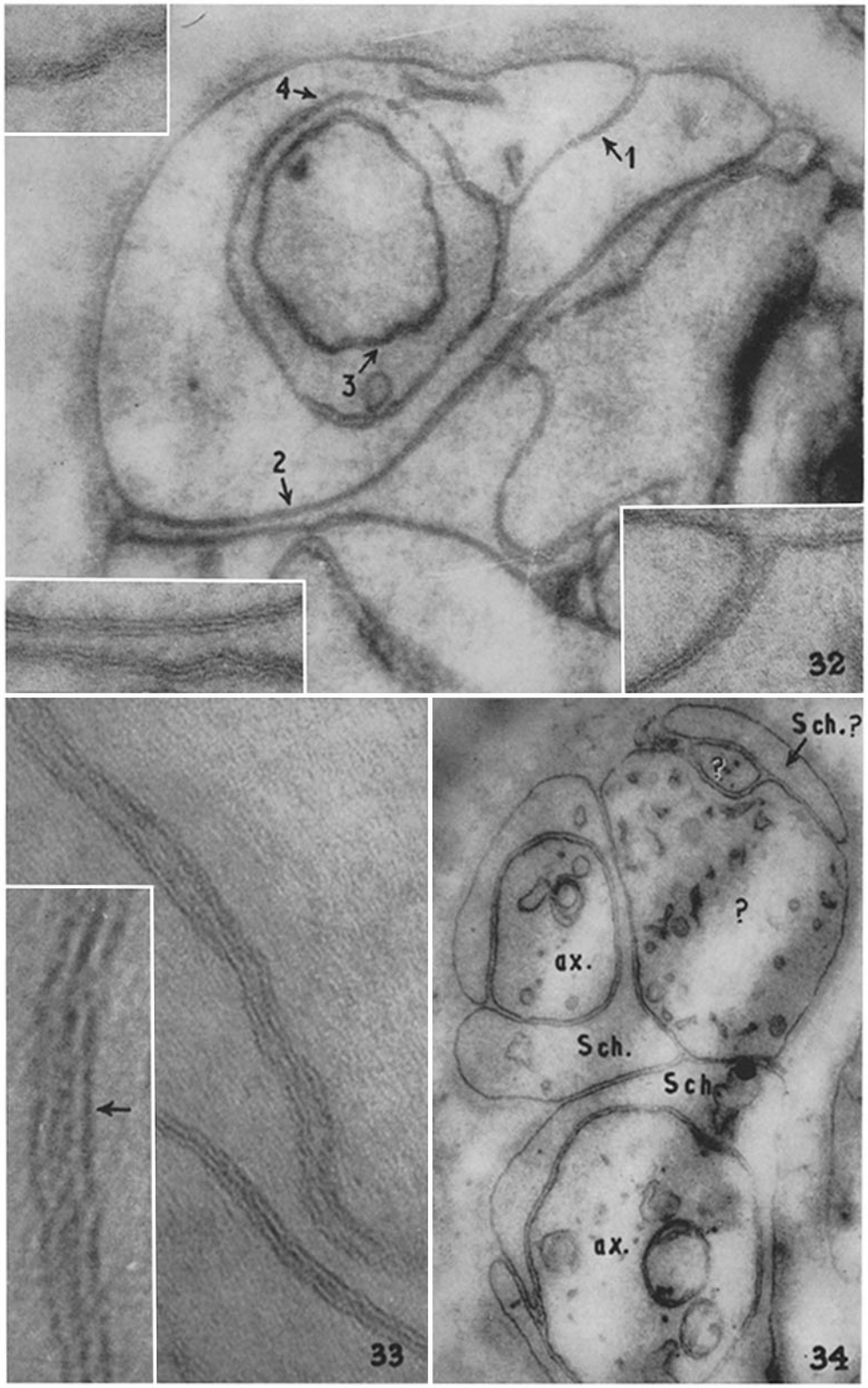
(Robertson: Structural alterations in nerve fibers)

PLATE 203

FIG. 32. Unmyelinated fiber treated with 6 I Ringer solution before fixation. Note the closures of the double membrane gaps of a mesaxon at arrow *1* (inset to lower right), an inter–Schwann membrane at arrow *2* (inset to lower left), and a mitochondrial double membrane at arrow *3* (inset to upper left). Arrow *4* indicates similar closures of an axon–Schwann membrane, but this does not show so clearly. × 84,000. Inset, × 186,000.

FIG. 33. Two double membrane structures in an unmyelinated fiber treated with 4 I Ringer solution before fixation. The ~75 A membranes of the double membrane above may be seen separated by a gap. Below two membranes are seen in contact forming an external compound membrane. A segment of this membrane is enlarged in the inset. Note that in one region (arrow) the central dense line is composed of two layers. In this region the membranes are somewhat separated. × 242,000. Inset, × 600,000.

FIG. 34. Micrograph of control unmyelinated fiber. Two Schwann cells (*Sch.*) containing two axons (*ax.*) may be identified. A questionable lip of Schwann cytoplasm (*Sch.?*) is seen associated with two structures (*?*) either or both of which could be axons, but which are not definitely identified. Note the absence of closures of the double membrane gaps. × 37,000.



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