Movements of the Schwann Cell Nucleus Implicate Progression of the Inner (Axon-related) Schwann Cell Process During Myelination

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Abstract. Although it has been known for several decades that peripheral myelin is formed from an extended, spiraled, and compacted sheet of Schwann cell (SC) plasma membrane, the mechanism by which this unique spiraling is accomplished remains unknown. We have studied the movements of SC nuclei before, during, and subsequent to myelin formation (over periods of 24–72 h) to determine if this nuclear motion (noted in earlier reports) would provide useful insights into the mechanism of myelinogenesis. We used rodent sensory neuron and SC cultures in which initiation of myelinogenesis is relatively synchronized and bright field conditions that allowed resolution of the axon, compact myelin, and position of the SC nucleus. Observed areas were subsequently examined by electron microscopy (EM); eight myelinating SCs with known nuclear movement history were subjected to detailed EM analysis.

We observed that, prefatory to myelination, SCs extended along the length of larger axons, apparently competing with adjacent SCs for axonal surface contact. This lengthening preceded the deposition of compact myelin. SC nuclear circumnavigation of the axon was found to attend early myelin sheath formation.

This movement was rarely >0.25 turns per 3 h; on the average, more nuclear motion was seen in relation to internodes that formed during observation (0.8 ± 0.1 turns/24 h) than in relation to those that had begun to form before observation (0.3 ± 0.1 turns/24 h). Nuclear circumnavigation generally proceeded in one direction, could be in similar or opposite directions in neighboring myelinating SCs on the same axon, and was not proportional to the number of major dense lines within the myelin sheath. A critical finding was that, in all eight cases examined, the overall direction of nuclear movement was the same as that of the inner end of the spiraling SC process, and thus opposite the direction of the outer end of the spiral. We conclude that the correspondence of the direction of nuclear rotation and inner end of the spiraling cytoplasmic lip implicates active progression of the inner lip over the axonal surface to form the membranous spiral of myelin, the nuclear motion resulting from towing by the advancing adaxonal lip. This interpretation fits with finding basal lamina and macular adhering junctions associated with the external lip of SC cytoplasm; these attributes would imply anchorage rather than movement of this region of the SC.

T HIS study was undertaken to determine if observations on Schwann cell (SC)[1] nuclear movement during myelination might aid in understanding how the peripheral myelin sheath is formed. While several previous reports (see below) indicated that substantial nuclear movement occurs in myelin-related SCs, a relationship between this nuclear motion and the process of myelination was not estab-

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1. *Abbreviations used in this paper:* MAG, myelin-associated glycoprotein; MDL, major dense line; SC, Schwann cell.

lished. The absence of related work during the past several decades may reflect the difficulty in obtaining appropriate serial observations on the slowly maturing rodent dorsal root ganglion explant culture commonly used for the study of peripheral myelin in vitro (Bunge et al., 1967). Alternate culture methods now allow preparation of purified populations of dissociated rodent sensory neurons and SCs that are maintained in incomplete medium until the point of neuronal maturity and full SC population of the axonal outgrowth (Eldridge et al., 1987). Shift to enriched medium then allows myelination to begin in relative synchrony and to progress rapidly, thus enabling repeated observations of SCs during myelin deposition.

The earliest electron micrographs obtained in the 1950s established that a cylindrical sleeve of myelin is formed around an axon when a spiraled extension of the SC plasma membrane, the mesaxon, is compacted following the loss of

SC cytoplasm (Geren, 1954; Robertson, 1955). The spiral (rather than concentric) configuration of this membranous assembly prompted tissue culture experiments to determine if SC circumnavigation of the axon could be visualized during myelination. The ensuing observations documented that the position of SC nuclei associated with myelin internodes shifts in relation to the enclosed axon (Peterson et al., 1958; Murray, 1959, 1965; Peterson and Murray, 1965). Pomerat and colleagues (Pomerat et al., 1967) obtained a time-lapse sequence showing one complete revolution of a SC nucleus around a myelinated axon during a 48-h interval in a long-term (8-wk) culture of rat sensory ganglion. These experiments, as well as those reported earlier, did not claim to establish that SC nuclear circumnavigation is related to myelin sheath formation. Nonetheless, the concordance between the electron microscopic revelation of the spiral deposition of the SC plasmalemma and the observation of nuclear circumnavigation by myelin-related SCs led to the general assumption that the two processes are related.

The configuration of the myelin sheath–SC unit revealed by electron microscopy led to other assumptions as well. The SC nucleus is always observed to be located in the SC cytoplasm external to the compact myelin. If nuclear circumnavigation occurs during myelination, it seemed reasonable to assume that the outer lip of SC cytoplasm tows the nucleus as the lip moves forward during spiral elongation to prevent the SC nucleus from becoming trapped within compact myelin. According to this view, then, the outer end of the spiral is the best candidate for movement, providing the impetus for spiral elongation and explaining the SC nuclear movement accompanying myelination.[2]

In the present paper we shall show that SC nuclear circumnavigation was commonly observed during the early phases of myelin formation and that this movement slowed as the myelin sheath matured. This nuclear movement was seldom seen to exceed several turns, did not correlate with the number of myelin lamellae deposited, and was observed to occur in opposite directions in neighboring SCs related to the same axon. Electron microscopic observation of SCs with known nuclear circumnavigational history indicated that nuclear movement apparently reflects "towing" not by progression of the outer lip of the SC spiral but by the inner lip, that portion of the SC directly related to the axonal surface. This implies that the spiral membrane deposition results from progression of the inner lip around the axon surface. Implications of these observations for understanding the molecular events of myelination are presented in the Discussion.

2. The relative positions of the inner and outer lips and the SC nucleus as myelinogenesis begins are diagrammed in Fig. 8.

Materials and Methods

Observations were made on both explant cultures from neonatal mice (containing a mixture of cell types from the developing sensory ganglion) and cultures containing purified populations of dissociated sensory neurons and SCs prepared from embryonic rat tissue.

Preparation of Cultures

Explant cultures were prepared by placing whole dorsal root ganglia from neonatal mice on 22-mm-diam, no. 1 round glass coverslips that had been previously coated with collagen extracted from rat tail tendon and gelled with ammonia vapors (Okada et al., 1980). These cultures were carried for several weeks in medium consisting of human placental serum (15%), chick embryo extract (6%), MEM (77%), nerve growth factor (25 U/ml), and glucose (6 g/L). Observations were made on myelin segments forming in the outgrowth region; subsequently, these cultures were fixed for staining with a lipid stain (Sudan black) that allows exceptional definition of compact myelin.

Cultures of purified populations of neurons and SCs were prepared from embryonic rat tissue by methods previously described (Eldridge et al., 1987). Briefly, sensory ganglia from 15-d rat embryos were dissociated with trypsin and seeded onto the center portion (only) of 24-mm Aclar mini-dishes previously coated with collagen (as above); this confines neuronal somata to a central region from which axons grow radially to provide an extensive outgrowth well suited for high resolution light microscopic observations. Nonneuronal cells were eliminated by a 10-d pulsed treatment with fluorodeoxyuridine. Subsequently, the culture was reseeded with a pure population of SCs prepared by the Wood method (Wood, 1976). These SCs then proliferated in response to the underlying axonal network until (over several weeks) the entire axonal outgrowth was populated. This proliferation occurred in a defined medium deficient in vitamin C and serum that allows proliferation but does not support differentiation (Eldridge et al., 1987). Substitution of medium containing serum and ascorbate initiated SC differentiation, leading in 6–8 d to basal lamina formation and the initiation of myelination by SCs (Eldridge et al., 1987). In an attempt to further accelerate myelination (Eldridge et al., 1989), a basal lamina extract (Matrigel; Collaborative Research, Inc., Waltham, MA; Kleinman et al., 1986) was gelled on top of some cultures.

Light Microscopic Observation

Observation periods of 33–78-h (in one case 288-h) duration were undertaken using an IM35 inverted microscope (Carl Zeiss, Inc., Thornwood, NY). The culture was transferred into a sealed chamber, closed below with a coverslip (no. 1) taking care to maintain pH at 7.4 or below (as judged by phenol red indicator) and temperature at 36°C with an air curtain device. Use of a coverslip (no. 1) as the culture dish bottom allowed observations with a Zeiss 40× planapochromat bright field lens. Efforts to use phase lenses were judged less advantageous because the nature of the phase image along the edge of the axon–SC unit does not allow judgment of when compact myelin is formed. In the bright field image, however, compact myelin provides a distinctive refractive image. Cultures were photographed at appropriate intervals at magnifications up to 400× and fixed at the end of the observation period for light and electron microscopy. Microscope focusing on SC nuclei by the investigator every 2–3 h with photographic documentation was considered to be a more effective analysis than time-lapse filming in these experiments.

Figure 1. Comparison of light microscopic myelin refractivity with the number of myelin lamellae discerned by EM. a illustrates a field observed in the living state by bright field microscopy; myelin sheaths appear as paired parallel refractive lines that resemble railroad tracks. Bright field microscopy of living cultures has clear advantages over phase microscopy; in phase micrographs, the distinctive refraction of compact myelin is not easily distinguished from the edge effect seen along all cell borders. Myelin internodes 1, 2, and 3 were studied in electron micrographs (b–f). The thickest sheath (sheath 3, b) was found to have 17 MDLs. Note in a that the line representing the inner half of the sheath underneath the nucleus (arrow) is as dense as the line that represents the SC surface to the left; this provides one way to detect compact myelin. Sheath 2 (c), thinner than sheath 3, contains only six MDLs. This sheath is separated from sheath 1 by a node (n), which is unclear in this photograph. Sheath 1 (in which the line between the nucleus and the axon is not as dense as that outlining the SC over the nucleus) did not develop characteristic myelin refractivity during the 34-h observation period (a). In fact, the clockwise nuclear movement ceased after 16 h of observation. Electron microscopic examination at the nuclear level (e) revealed a clockwise turn of the inner SC lip (e, arrow). At levels on either side of the nucleus ~50 μm apart (d and f), however, there was not a predominant direction of inner lip; SC processes appeared to extend in both clockwise and counter-clockwise directions, apparently precluding the development of a mesaxon long enough to initiate systematic spiraling. Bars: (a) 10 μm; (b–f) 0.5 μm.

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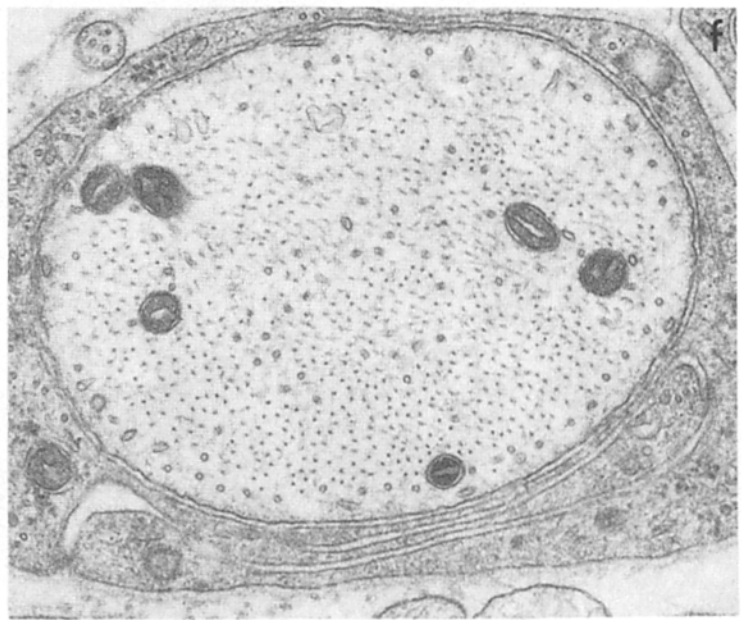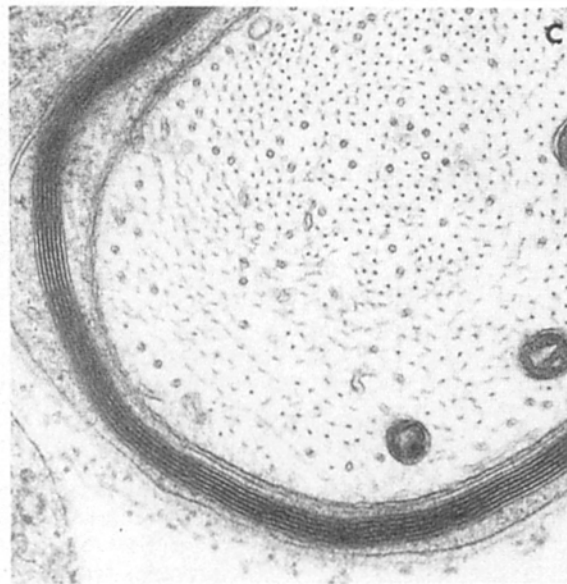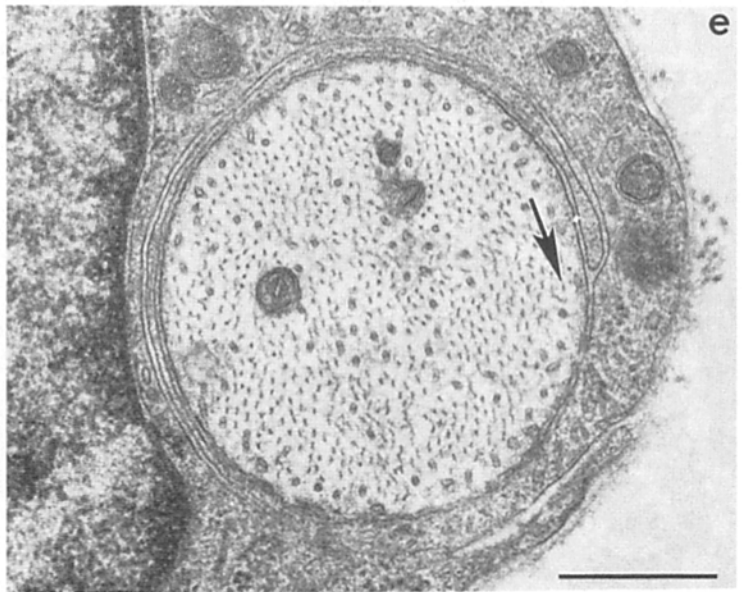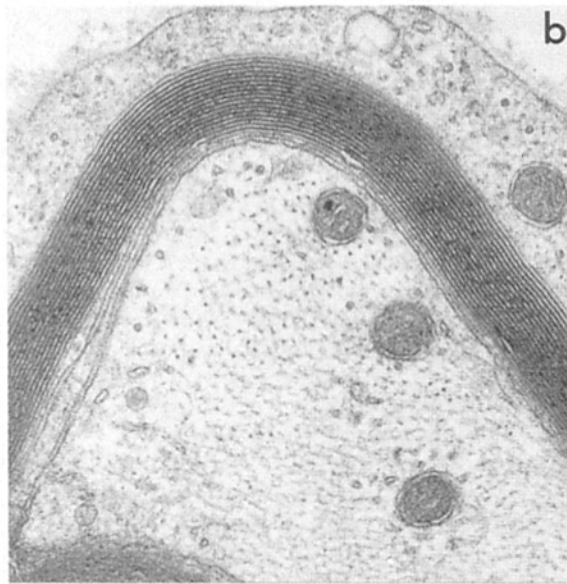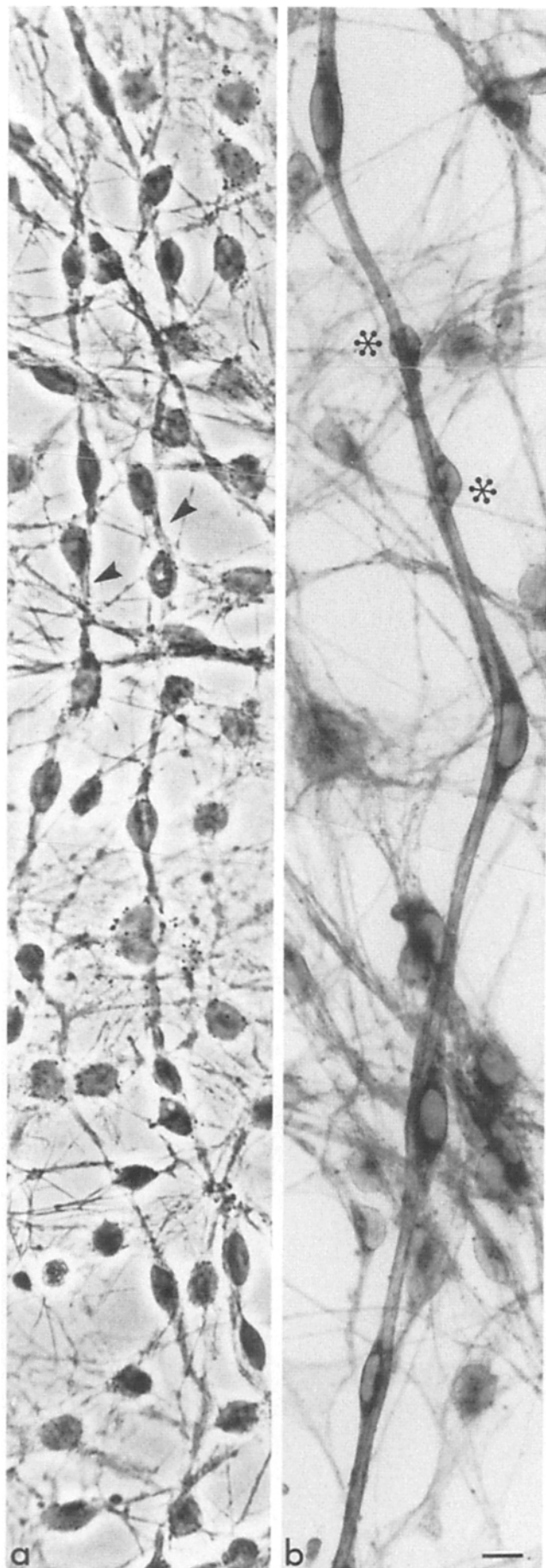
Electron Microscopic Observation

Upon removal of the culture from the microscope stage, room temperature or slightly warmed fixative (containing 2% glutaraldehyde and 100 mM sucrose in 0.046 M phosphate buffer, pH 7.4; Ratner et al., 1986) was added slowly to replace the culture medium. After several rinses in fixative, the culture was stored in the cold overnight. The culture was then rinsed in 0.15 M phosphate buffer, further fixed in phosphate-buffered 2% OsO₄, dehydrated in ethanol, and embedded in Polybed (Polysciences, Inc., Warrington, PA). Observed areas in the embedded sample were relocated in the light microscope for sectioning. Semithin sections were compared with photographs taken in the living state to identify the position of each observed sheath. These sheaths were photographed (in a Philips Electronic Instruments, Inc. [Mahwah, NJ] model 300 electron microscope) in thin sections (stained with uranyl acetate and lead citrate), with notation of their position in relation to the substratum and neighboring cells in order to decide the orientation of the inner and outer lips of SC cytoplasm. Sheaths in three experiments were prepared for EM examination and most were photographed at more than one level, often at levels beyond both ends of the nucleus as well as in the nucleus.

Results

Appearance of Myelin

Myelin sheaths were defined as those SC-related sleeves that attained a distinctive refractivity when viewed by bright field microscopy. Our EM observations established that this refractiveness in bright field images was seen only when compact myelin was present. Sheaths 2 and 3 in Fig. 1 a illustrate the characteristic refractivity of compact myelin; they contained six (Fig. 1 c) and 17 (Fig. 1 b) major dense lines (MDLs), respectively. This refractive image characterizing compact myelin can be compared with the image of the axon–SC unit in the premyelination phase that lacks this refractivity (sheath 1, Fig. 1 a). Myelin sheaths appeared refractive when thinner than sheath 2; in a later experiment, compact myelin was observed to be distinctively refractive under the light microscopic conditions used here when only three MDLs were present (see Fig. 7, e, f, h, and i).

A second clue that aided in detecting the presence of myelin was as follows. Before compact myelin appeared, the outermost edges of the axon–SC unit were more dense than the edge of the axon adjacent to the SC nucleus. For example, the line between nucleus and axon in sheath 1 in Fig. 1 a is less dense than the refracting lines on the cell exterior. EM of this area revealed that compact myelin had not yet formed; only one turn of spiraled mesaxon without compaction was present (Fig. 1, d–f). On the other hand, when the line between nucleus and axon (arrow, sheath 3, Fig. 1 a) was as dense as the outer axon edge, the presence of compact myelin was indicated (Fig. 1 b; see also Fig. 7 b vs. f). This was clearer in the microscope during careful focusing than in photographs.

Figure 2. SC elongation prefatory to myelination. In defined medium (a), SCs are irregularly and closely spaced along larger diameter (myelin-competent) neurites, as exemplified by the two SC-studded neurites designated by the arrowheads. When the medium is changed to one that supports SC myelination (b), SCs elongate to cover more axonal length. SCs apparently compete for the axonal surface because some SCs are pushed off; possible examples of SCs losing their hold on this myelin-competent axon are marked by asterisks. The end result is that there are fewer SCs along a given axon length. This SC spacing occurs before compact myelin forms. Sudan black–stained, whole-mount cultures. Bar, 10 μm.
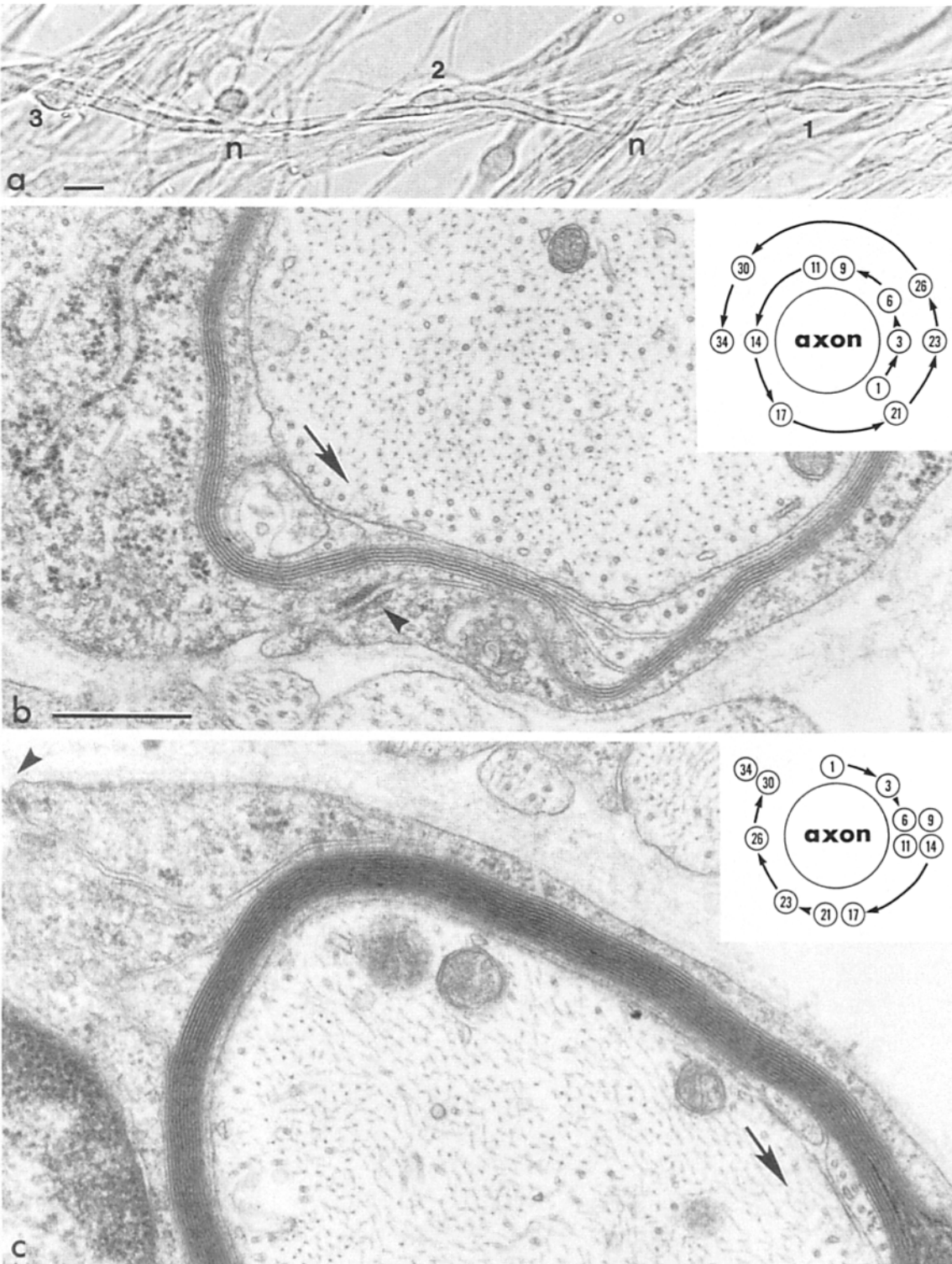
Figure 3. Occurrence of nuclear circumnavigation in opposite directions during formation of myelin internodes along the same axon. Myelin internodes 1–3 (*a*) were followed for 34 h; sheaths *1* and *3* formed during the observation period. Nuclear motion charted during the formation of myelin sheaths *1* and *3* was in opposite directions (*insets, b* and *c*); in addition to the direction, the nuclear position (given in hours elapsed) is indicated in each circle. Electron micrographs of sheaths *1* (*b*) and *3* (*c*) show that the orientation of the inner SC lip (*arrows*) is the same as the direction of nuclear movement. If the outer SC lip were to extend, it would be in a direction opposite that of the nuclear circumnavigation. Junctions are visible at the arrowheads. (*a*) Living culture; *n*, nodes of Ranvier. Bars: (*a*) 10 μm; (*b* and *c*) 0.5 μm.

SC Elongation Precedes Myelin Deposition

When cultures of pure populations of sensory neurons (~8,000 confined to the central 1/3 of a 22-mm dish) are seeded with a small number of SCs (~25,000) in defined medium, a mitogen (Wood and Bunge, 1975) on the surface of the neuronal somata and their neuritic outgrowth causes an extensive expansion of SC number (over a 21-d period) to 300,000–500,000 (Eldridge et al., 1987). In defined medium, these SCs are comparatively loosely attached to and closely spaced along axons (as in Fig. 2 *a*). Individual nerve fibers are difficult to resolve in neuritic networks in defined medium.

When these cultures are shifted to medium that allows myelination, a series of changes precedes the first appearance of myelin. As the axon–SC relationship shifts from one of casual contact observed in defined medium to engulfment by the attendant SC, the axon increases in diameter and appears to straighten and stiffen (cf. Fig. 2, *a* and *b*). We have documented elsewhere that the largest diameter of cultured rat sensory axons without SC ensheathment is 1.25 μm; with SC ensheathment, diameters of myelinated fibers average 1.67 μm (range, 0.7–3.4 μm) (Windebank et al., 1985).

In the present study, we observed elongation of SCs along the larger axons as the 1:1 relationship between axon and SC is established before the onset of myelination. During this spacing process, we observed SCs competing for the surface of larger axons; some SCs were pushed off the axon by adjacent lengthening SCs, a phase of which is illustrated in Fig. 2 *b*. In 10 observation sessions in which 89 SCs involved in myelin formation were under serial observation, we noted nine instances in which SCs were displaced from the axon by neighboring extending SCs. This number of examples is low because, in order to choose prospective myelinating SCs for this study, we sought out SCs that had already elongated (as in Fig. 2 *b*) and become hypertrophic (see below).

Although the full extent of the SC was difficult to discern during the spreading phase in the living state, it was our impression that SCs spread along the axon to establish the linear domain of the future internode before compact myelin deposition began. This impression was strengthened by observing that SC nuclei (which can be clearly seen) became regularly spaced along the axon before myelination began; the SC nuclear spacing reflects the extent of SC cytoplasmic spreading along the length of the axon. Figs. 2 *b* and 3 *a* illustrate this observation. In an area such as Fig. 2 *b*, where regular SC nuclear spacing had not yet been achieved, myelin deposition was not yet visible, whereas new compact myelin was present in an area where SC nuclei had attained regularity in spacing (as in Fig. 3 *a*; see nuclei *1*, *2*, and *3*). In all the illustrated examples of myelin internodes that formed during observation (Figs. 3; 4, *a* and *c*; and 5–7), the SC length (assumed from SC nuclear spacing) did not increase notably as myelin deposition began; Figs. 5, *a–e*, and
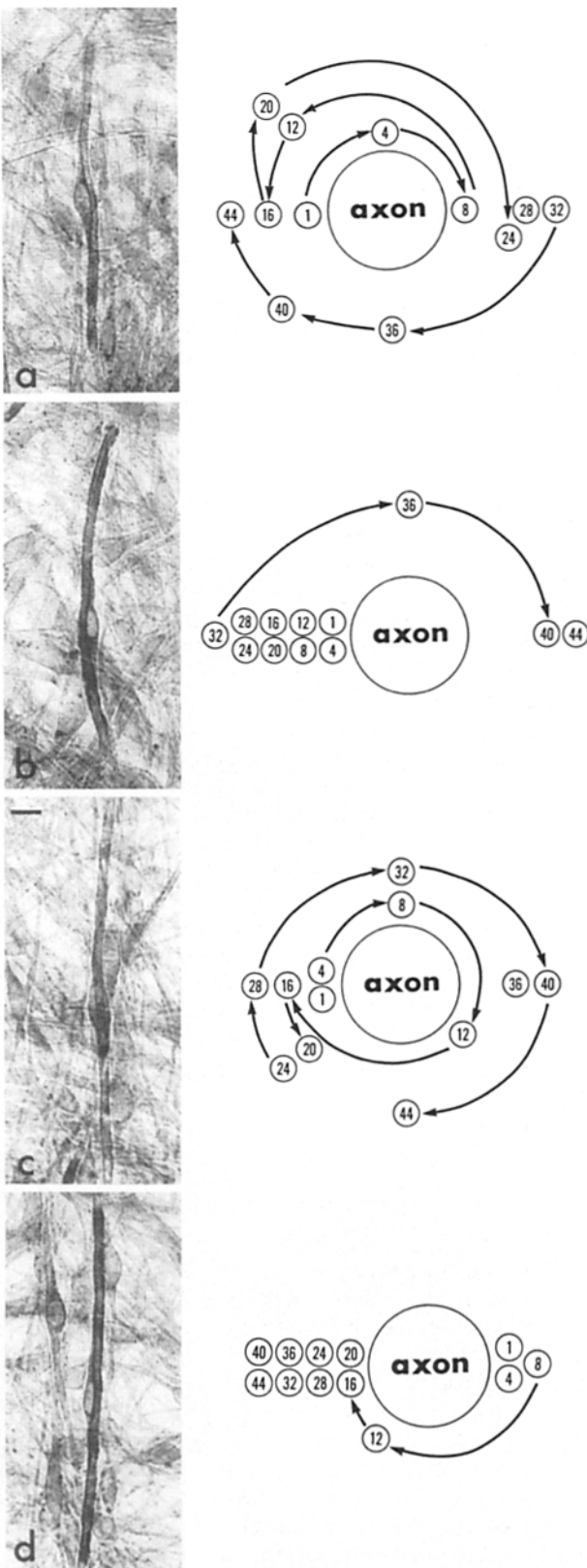
Figure 4. Greater nuclear movement during early stages of myelin formation than later. Sheaths in *a* and *c* formed (i.e., developed the distinctive refractivity illustrated in Fig. 2) during the observation period of 44 h. These two sheaths exhibited more nuclear movement (one to two turns) than did the sheaths in *b* and *d* (½ turn each) that had attained the characteristic refractive quality denoting my-

elin compaction before observation had begun. All the sheaths pictured were associated with the same axon and were, in fact, sequentially disposed in the order shown here. In the cases of the less mature sheaths (*a* and *c*), nuclear movement during myelination was in the same direction. These sheaths were fixed and stained with Sudan black at the end of the observation period. Bar, 10 μm.

Table I. Comparison of Nuclear Movement (No. Turns/24 h) in SCs Related to Compact Myelin

Compact myelin that appeared during observation period*	Compact myelin present at onset of observation period‡
1.4	0.7
1.3	0.6
1.1	0.6
1.1	0.6
0.8	0.4
0.6	0.4
0.6	0.4
0.6	0.3
0.5	0.3
0.4	0.3
0.4	0.3
0.8 ± 0.1§	0.2
	0.2
	0.2
	0
	0
	0
	0
	0
	0.3 ± 0.1§

* $n = 11$.
‡ $n = 19$.
§ Mean ± SEM; means are significantly different ($P < 0.001$, t test).

7, a–f, portray this although the entire internode is not in focus in every photograph. More recent immunostaining of premyelinating SC cytoplasm has confirmed that SC elongation precedes myelination (Owens and Bunge, 1989; see Discussion). As SCs attained their linear dimension, they became manifestly hypertrophic with the accumulation of substantial granular cytoplasm in the perinuclear region (Fig. 2 b).

Nuclear Rotation During Myelination

As the axon enlarged in diameter, it became possible to clearly discern both nuclear and individual axonal profiles in the living cultures and to chart SC nuclear position in relation to the ensheathed axon. Human observation was necessary to determine by careful focusing whether SC nuclei were passing above or below axons; this was critical for finding if SC nuclei shifted back and forth in a rocking motion or unidirectionally circumnavigated the axon. SCs selected for observation were often related to axons on which adjacent SCs had begun myelinogenesis, thus establishing that axon as capable of inducing myelin formation.

We charted SC nuclear movement related to 100 prospective, forming, or recently formed myelin internodes in 11 observation sessions. 30 of the internodes that were observed during or shortly after the onset of myelination were selected for more detailed analysis of the rate of nuclear movement. 40 of the internodes were subjected to analysis by EM (see below).

The light microscopic analysis showed that SC nuclear circumnavigation of the axon was common during myelination. No myelinogenesis was initiated without some (≥1/2 turn) nuclear movement, but movement was relatively slow (rarely >1/4 turn in 3 h) and in no cases exceeded 2 1/2 turns in

44 h. Of the 30 selected internodes, 11 formed during the period of observation and 19 appeared refractive (indicating the presence of compact myelin) when observation began. Internodes that formed during the observation period showed more nuclear movement (mean, 0.8 turns/24 h) than those that had started to form before observation began (mean, 0.3 turns/24 h) (Table I; Fig. 4). Compact myelin materialized uniformly along the internode, not from the nuclear level nodeward.

Although some reversal of direction of nuclear movement was observed (not exceeding 1/2 turn), in general nuclear circumnavigation exhibited a dominant direction (Figs. 3–5 and 7). In five instances we obtained records of two internodes forming along the same axon where adequate nuclear movement occurred to assess direction; in two of these cases, the direction of nuclear circumnavigation was the same (e.g., Fig. 4, sheaths 1 and 3) and, in three instances, it was opposite (e.g., as in Fig. 3). When nuclear rotation was in opposite directions, the directions of the myelin spirals were observed by EM to be opposite as well (Fig. 3, b and c), as would be expected.

In several cases substantial myelin deposition was noted with relatively little circumnavigation. The sequence of photographs in Fig. 5 shows the formation of a relatively thick refractive sheath within a period of 34 h when only one turn of the SC nucleus around the axon was noted. Subsequent EM revealed 13 MDLs within the compact myelin. Thus, it was clear that one turn of the SC nucleus did not occur during the formation of each myelin lamella.

The question may be raised whether substantial nuclear circumnavigation may occur before the appearance of refractive myelin, leaving behind uncompacted mesaxonal membrane that would later compact into visible myelin during a period of relatively little nuclear movement. Fig. 6 illustrates an instance in which this was found not to be the case. The SC nucleus related to this sheath had moved little during the 66-h period before myelin began to appear, as judged by its refractivity in the living state. Also, noncompacted spiral mesaxons of more than three turns have not been seen in this project or numerous other EM studies of comparable cultures in our laboratory.

Correlation of Nuclear Circumnavigation and Direction of Myelin Spiral

We undertook EM analysis of eight myelin sheaths for which the direction of nuclear movement before fixation was known; in each instance this movement was one turn or more. This required identification of specific internodes subsequent to embedding in plastic; this was accomplished by drawing detailed maps of the observed regions and surrounding areas in the living state. Because the cultures were embedded in a thin layer of plastic, the mapped regions could be relocated under phase microscopy and specific myelin internodes marked before mounting in the proper alignment for sectioning for EM.

Fig. 7 illustrates a myelin sheath studied in this manner. In this example the nucleus circumnavigated the axon 2 1/2 turns during a 44-h observation period. The myelin internode that was formed was examined by EM at the level of the nucleus and beyond both ends of the nucleus. The micrographs (Fig. 7, a–f) illustrate the SC hypertrophy commonly seen during initial myelination; by EM the enlarged perinuclear region was seen to contain a predominance of granular
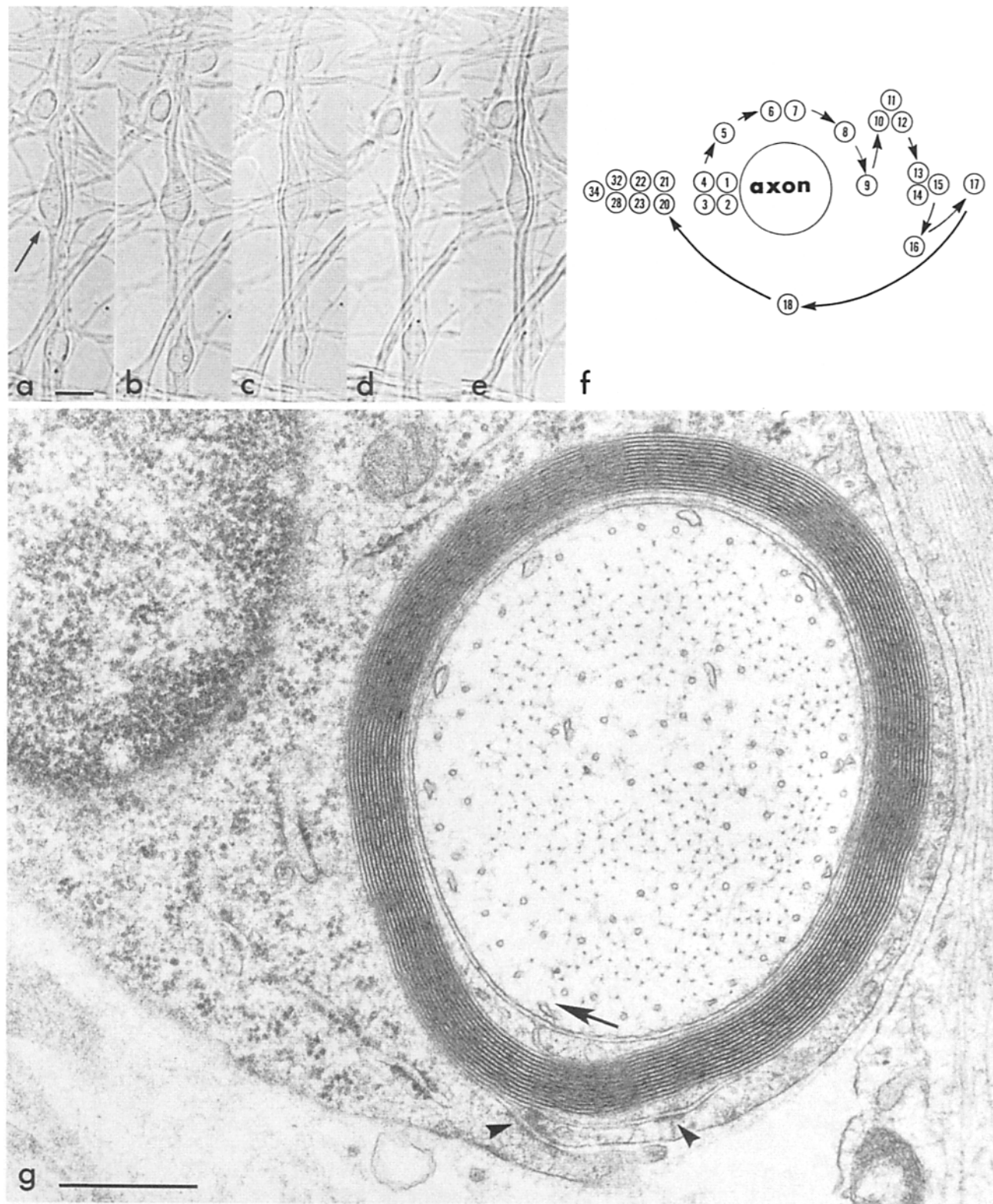
Figure 5. Lack of correspondence between the extent of nuclear circumnavigation and the number of myelin lamellae. *a–e* show different positions of one SC nucleus (*a, small arrow*) at 1 (*a*, west), 6 (*b*, above the axon), 12 (*c*, east), 18 (*d*, below the axon), and 34 (*e*, west again) h of observation. The nucleus made one turn around the axon in 34 h (*f*). At the beginning of the observation period, definitive myelin was not yet visible. After one turn of the nucleus around the axon in 34 h, the refractivity characteristic of myelin was clearly developed and 13 MDLs were visible in the electron micrograph of the newly formed internode (*g*). It can also be seen that the inner lip of the SC points clockwise (*g, arrow*), corresponding with the direction of nuclear circumnavigation (*f*). Junctions (*g, arrowheads*) are present in the outer mesaxon and the adjacent turn of the mesaxon. (*a–e*) Living culture. Bars: (*a–e*) 10 μm; (*g*) 0.5 μm.
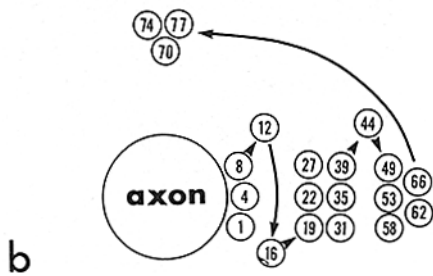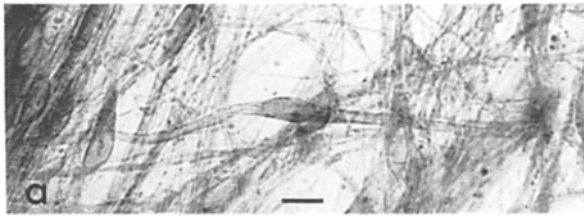
Figure 6. Lack of nuclear movement preceding observation of definitive myelin. *a* depicts a thin sheath that has just begun to show in the living state the definitive refractive properties of compact myelin at the time the experiment was terminated. The record of nuclear position reveals that there was little nuclear movement for the 66 h (*b*) before myelin first appeared. (*a*) Sudan black–stained, whole-mount culture. Bar, 10 μm.

endoplasmic reticulum. At every level examined, the direction of the myelin spiral and the number of MDLs were the same (as in Fig. 7, *h* and *i*). When we aligned the electron micrographs to correctly reflect the known history of nuclear movement, we found that the direction of movement (counter-clockwise) (Fig. 7 *g*) corresponded to the direction in which the inner (axon-related) lip of the SC process pointed (Fig. 7, *h* [*arrow*] and *i*). This direction was opposite that of the outer SC lip. The same analysis was undertaken for seven additional internodes that had shown at least one nuclear rotation during observation (Figs. 3 *b* and 5). In every case the known direction of nuclear movement corresponded to the direction of the inner lip of the spiral. Also, in all cases examined at several levels (e.g., Figs. 1, 3, 5, and 7), the direction of the spiral did not change along the internode. The implications that these observations have for the cellular mechanics involved in myelinogenesis are addressed in the Discussion.

One of the SCs chosen for survey by EM (Fig. 1, *a* and *d–f*) exhibited nuclear rotation in the early part of the observation period (the first 16 h) followed by a period (the next 18 1/2 h) in which the SC nucleus had seemingly "parked". (In the 1/2 h intervening between observation and fixation, the nucleus started to resume movement.) Compact myelin did not appear during observation though this area had been chosen because of its potential for myelination. Electron micrographs were obtained at four different levels, over a total distance of ~70 μm. A turn of mesaxon was observed only at the nuclear level (Fig. 1 *e*) and the direction of this corresponded to the direction of early nuclear movement. At other levels, however, one full turn of mesaxon was not present; only a partial turn or dovetailing lips of SC cytoplasm (Fig. 1, *d* and *f*) could be seen. It thus appeared that mesaxon spiraling had started but that one inner lip had not been able to predominate along the length of the cell, leading to a delay

in the initiation of myelin formation during the observation period.

Outer Mesaxonal Adhering Junctions

In many of the thin sections of myelinating SCs with known nuclear circumnavigational history, adhering junctions linked the membranes of the outer mesaxon (Figs. 3, *b* and *c*; 5 *g*; and 7 *h*) and sometimes the adjacent turn of mesaxon as well (Figs. 5 *g* and 7 *h*). Adhering junctions were seen in all sections at all levels of the sheath shown in Fig. 5 *g*. These adhering junctions consisted of apposed plasmalemmas with increased density along with contiguous cytoplasmic dense material; sometimes the extracellular gap between the membranes was widened and contained some material (Fig. 3 *b*). The junctions appeared macular rather than linear because their positions varied in neighboring sections. Similar junctions joining the membranes of the SC inner mesaxon on the innermost SC plasmalemma and adjacent axolemma were far less common and, when present, were in areas where cytoplasm had been retained, leading to less compaction than usual. A cistern of granular endoplasmic reticulum was usually seen near the outer mesaxon (e.g., Fig. 7 *h*). Basal lamina spanned the opening of the SC outer mesaxon onto the SC exterior (Figs. 1, *d–f*; 3, *b* and *c*; 5 *g*; and 7, *h* and *i*).

Discussion

We began this study with considerable skepticism regarding the significance of SC nuclear movement during myelination. As indicated in the Introduction, nuclear circumnavigation would seem most likely to reflect progression of the outer lip of the forming membrane spiral that causes towing of the nucleus contained in the collar of cytoplasm external to compacting lamellae. But observations made concurrently in our laboratory (Bunge et al., 1986; Eldridge et al., 1989) indicated that the SC surface must acquire basal lamina in order for myelination to occur, suggesting anchoring of the surface membrane rather than active movement. Basal lamina was consistently present on the external surface (including the outer mesaxon region) of the myelinating SCs in the experiments reported here. Also, adhering junctions between outer mesaxon membranes were often observed, again suggestive of anchorage rather than movement. It has been reported from in vivo studies that zonula occludens junctions become established in external mesaxon areas before myelin compaction occurs (Schnapp and Mugnaini, 1978).

We report here that circumnavigation of the enclosed axon by the SC nucleus occurred commonly during early phases of myelination. We were puzzled by this observation until we discovered, after ultrastructural study of myelin segments with known precedent nuclear circumnavigational history, that in all eight cases examined the direction of nuclear movement was the same as that of the inner (adaxonal) lip of the spiraling SC process (Fig. 8).

We believe that this finding is best explained by proposing that when the inner lip of the SC advances around the axon during the earliest phases of myelin deposition (before acquisition of a well-developed basal lamina and collagen fibril framework over the external surfaces of the SC), the entire myelin–SC assemblage (including the nucleus) is towed around the axon. According to this view, the force exerted
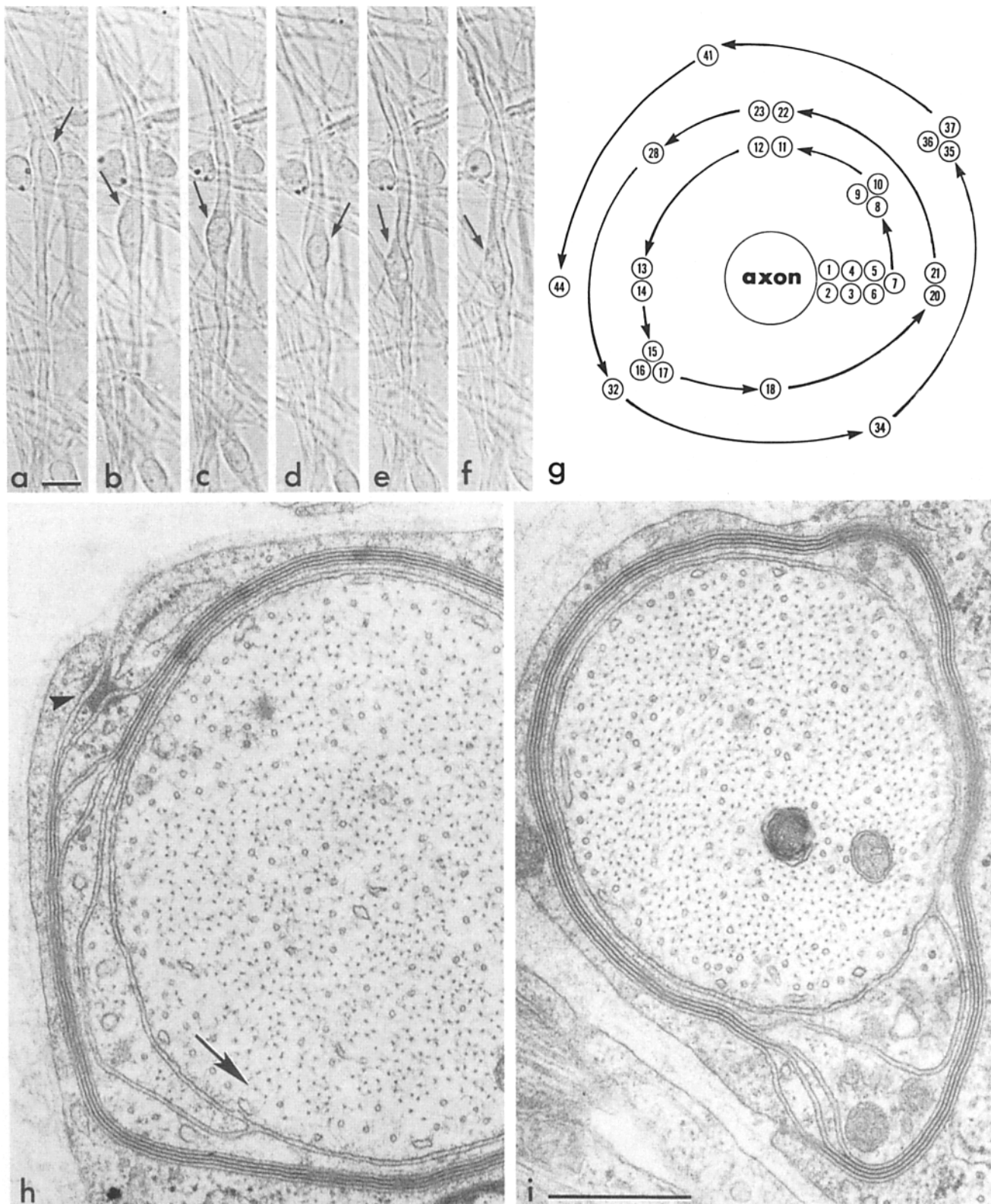
Figure 7. Correspondence of the direction of nuclear circumnavigation with that of the SC inner lip. *a–f* document the nuclear (*small arrows*) movement that occurred during the 44-h observation period; the positions at 6 (*a*, east), 15 (*b*, west), 18 (*c*, below the axon), 20 (*d*, east), 28 (*e*, west), and 44 (*f*, west) h of observation are shown. *a–e* illustrate 1½ rotations (east-west-east-west) and *f* shows the result of a further rotation (west-east-west). Myelin formed during the observation period; the distinctive refractivity of myelin is apparent in *e* and *f*. In all, the nucleus circumnavigated the axon 2½ times, illustrating that the nucleus can move substantially in early myelination and systematically in one direction (*g*). Despite this many nuclear turns, only three MDLs can be seen in the sheath at 44 h (*h*). The direction in which the inner SC lip is pointed (counter-clockwise; *h, arrow*) corresponds to the direction of nuclear circumnavigation. Moreover, the number of MDLs and this orientation of the inner lip extends throughout the forming internode as would be expected; *i* shows a level of the internode ∼100 μm away from that illustrated in *h*. Junctional material joins the outer mesaxon and the adjacent turn of mesaxon (*h, arrowhead*). (*a–f*) Living culture. Bars: (*a–f*) 10 μm; (*h* and *i*) 0.5 μm.
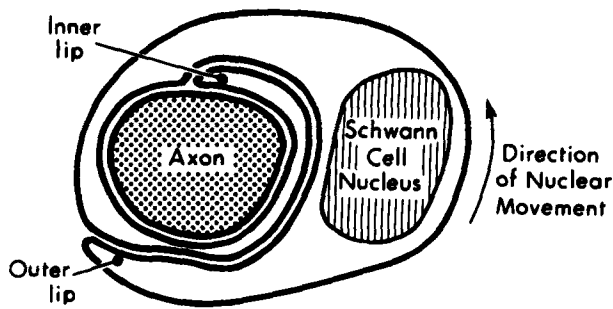
Figure 8. Diagram of a transversely sectioned, myelinating SC in the early phases of mesaxon formation preceding myelination. Circumnavigation of the axon by the SC nucleus is observed during early myelinogenesis to be in the direction of the inner lip of the spiraling SC process and opposite the direction of the outer lip.

to effect SC nuclear movement derives from the active progression of the inner lip across the axonal surface. The "trail" of membrane left behind by this migration is the extending mesaxon that will compact to form the myelin sheath. For this mesaxon to be drawn out, the external aspect of the SC must be fixed, otherwise the movement of the inner lip would continue to carry the entire SC assemblage with it and no extension of the mesaxon would occur. If the external aspect of the SC plasmalemma is anchored to the immediately surrounding basal lamina, this could explain (at least in part) the requirement that SCs organize a basal lamina before myelination begins.

SC nuclear movement may be particularly conspicuous in the culture system used because the cultures initially develop in a defined culture medium lacking vitamin C; these conditions prevent basal lamina assembly. After shifting to complete medium to initiate myelinogenesis, basal lamina formation begins but is not yet complete at the time spiral formation accompanying myelination begins. Under these conditions, the assertive advance of the inner SC lip may tow the entire SC assemblage during early stages of myelination. If the whole assemblage did not move, and the inner lip specifically towed the nucleus directly behind it (with the external SC surface held in place by an established basal lamina), the nucleus could end up underneath the compact myelin; this configuration has not been reported in either in vitro or in vivo studies.

New observations on the early appearance of the myelin-specific component, myelin-associated glycoprotein (MAG), during peripheral myelination (Owens and Bunge, 1989) support our proposed mechanism of myelinogenesis. These observations suggest that MAG may be the ligand responsible for (*a*) SC segregation of enlarging axons to be myelinated from smaller axons that will not be myelinated; and (*b*) SC longitudinal spreading along these axons in the premyelination stage before spiral formation begins. Immunostaining with anti-MAG and anti-Po antibodies shows that the SC achieves substantial elongation along the axon before myelin compaction is initiated (Owens and Bunge, 1989). This SC occupation of a substantial linear axolemmal domain defines the future length of the internode and regions of the intervening nodes of Ranvier. We have speculated in our present in vitro observations that this elongation appears to be an assertive maneuver, with SCs being stripped away from the axon by adjacent SCs competing more successfully for axonal space. Similar observations have been reported from studies

of developing peripheral nerve in the kitten (Berthold et al., 1984). The assertive nature of SC spreading along the axon surface preceding myelination is suggested by the observations of Kidd and Heath (1988). They found that under certain circumstances SCs in the premyelination phase were able to strip away previously formed myelin internodes by inserting themselves between the axolemma and the paranodal loops before initiating the spiral deposition of myelin membrane.

It seems reasonable to suggest that the aggressive advance of the inner SC lip over the axonal surface may be an extension of the earlier spreading behavior that defines internode length. If, after longitudinal extension, the SC directs its efforts to extending the inner lip over the axonal surface, this advance would generate the membranous spiral that with compaction forms the internode. It is of interest to consider the different immediate environments of the inner and outer lips of the myelin-forming SC (refer to Fig. 8). The outer lip is apposed on one side to the underlying turn of the SC membrane spiral; its external aspect does not have contact with another cellular surface but with the overlying basal lamina instead. In contrast, the inner lip lies between two cell surfaces: the axolemma on its inner aspect, and the next turn of SC plasma membrane on its outer surface. If both of these adjacent surfaces provide traction for forward advance of the inner lip, this unusual double surface contact could explain the exceptional propensity for continuous forward movement of the inner lip that would draw out the myelin spiral until the full thickness of the myelin sheath is achieved. The interpretation of our data presented here—that advance of the inner lip generates enough traction to tow the SC (and its nucleus) around the axon during early stages of myelin formation—supports this mechanism of myelinogenesis.

References

Berthold, C.-H., T. Carlstedt, and O. Corneliuson. 1984. Anatomy of the nerve root at the central-peripheral transitional region. *In* Peripheral Neuropathy. Second edition. P. J. Dyck, P. K. Thomas, E. H. Lambert, and R. P. Bunge, editors. W. B. Saunders Co., Philadelphia, PA. 156-170.

Bunge, M. B., R. P. Bunge, E. R. Peterson, and M. R. Murray. 1967. A light and electron microscope study of long-term organized cultures of rat dorsal root ganglia. *J. Cell Biol.* 32:439-446.

Bunge, R. P., M. B. Bunge, and C. F. Eldridge. 1986. Linkage between axonal ensheathment and basal lamina production by Schwann cells. *Annu. Rev. Neurosci.* 9:305-328.

Eldridge, C. F., M. B. Bunge, R. P. Bunge, and P. M. Wood. 1987. Differentiation of axon-related Schwann cells in vitro. I. Ascorbic acid regulates basal lamina assembly and myelin formation. *J. Cell Biol.* 105:1023-1034.

Eldridge, C. F., M. B. Bunge, and R. P. Bunge. 1989. Differentiation of axon-related Schwann cells in vitro. II. Control of myelin formation by basal lamina. *J. Neurosci.* 9:625-638.

Geren, B. B. 1954. The formation from the Schwann cell surface of the myelin in the peripheral nerves of chick embryos. *Exp. Cell Res.* 7:558-562.

Kidd, G. J., and J. W. Heath. 1988. Double myelination of axons in the sympathetic nervous system of mouse. II. Mechanisms of formation. *J. Neurocytol.* 17:263-276.

Kleinman, H. K., M. L. McGarvey, J. R. Hassell, V. L. Star, F. B. Cannon, G. W. Laurie, and G. R. Martin. 1986. Basement membrane complexes with biological activity. *Biochemistry.* 25:312-318.

Murray, M. R. 1959. Factors bearing on myelin formation in vitro. The biology of myelin. *Prog. Neurobiol. (Oxford).* 4:201–221.

Murray, M. R. 1965. Nervous tissue in vitro. *In* Cells and Tissues in Culture. E. D. Wilmer, editor. Academic Press, New York. 373–455.

Okada, E., R. P. Bunge, and M. B. Bunge. 1980. Abnormalities expressed in long term cultures of dorsal root ganglia from the dystrophic mouse. *Brain Res.* 194:455–470.

Owens, G. C., and R. P. Bunge. 1989. Evidence for an early role for myelin-associated glycoprotein in the process of myelination. *Glia.* 2:119–128.

Peterson, E. R., and M. R. Murray. 1965. Patterns of peripheral demyelination in vitro. Research in demyelinating disease. *Ann. NY Acad. Sci.* 122:39–49.

Peterson, E. R., S. M. Crain, and M. R. Murray. 1958. Activities of Schwann cells during myelin formation in vitro. *Anat. Rec.* 130:357.

Pomerat, C. M., W. J. Hendelman, C. W. Raiborn, and J. F. Massey. 1967. Dynamic activities of nervous tissue in vitro. *In* The Neuron. H. Hyden, editor. Elsevier Science Publishing Co., Inc., New York. 120–178.

Ratner, N., A. Elbein, M. B. Bunge, S. Porter, R. P. Bunge, and L. Glaser. 1986. Specific asparagine-linked oligosaccharides are not required for certain neuron–neuron and neuron–Schwann cell interactions. *J. Cell Biol.* 103:159–170.

Robertson, J. D. 1955. The ultrastructure of adult vertebrate peripheral myelinated fibers in relation to myelinogenesis. *J. Biophys. Biochem. Cytol.* 1:271–278.

Schnapp, B., and E. Mugnaini. 1978. Membrane architecture of myelinated fibers as seen by freeze-fracture. *In* Physiology and Pathobiology of Axons. S. G. Waxman, editor. Raven Press, New York. 83–123.

Windebank, A. J., P. M. Wood, R. P. Bunge, and P. J. Dyck. 1985. Myelination determines the caliber of dorsal root ganglion neurons in culture. *J. Neurosci.* 5:1563–1569.

Wood, P. M. 1976. Separation of functional Schwann cells and neurons from normal peripheral nerve tissue. *Brain Res.* 115:361–375.

Wood, P. M., and R. P. Bunge. 1975. Evidence that sensory axons are mitogenic for Schwann cells. *Nature (Lond.).* 256:662–664.