

Stages in Axon Formation : Observations of Growth of *Aplysia* Axons in Culture Using Video-enhanced Contrast-Differential Interference Contrast Microscopy

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Abstract. The regenerative growth in culture of the axons of two giant identified neurons from the central nervous system of *Aplysia californica* was observed using video-enhanced contrast-differential interference contrast microscopy. This technique allowed the visualization in living cells of the membranous organelles of the growth cone. Elongation of axonal branches always occurred through the same sequence of events: A flat organelle-free veil protruded from the front of the growth cone, gradually filled with vesicles that entered by fast axonal transport and Brownian motion from the main body of the growth cone, became more voluminous and engorged with organelles (vesicles, mitochondria, and one or two large, irregular, refractile bodies), and, finally, assumed the cylindrical shape of the axon branch with the organelles predominantly moving by bidirectional fast axonal transport. The veil

is thus the nascent axon. Because veils appear to be initially free of membranous organelles, addition of membrane to the plasmalemma by exocytosis is likely to occur in the main body of the growth cone rather than at the leading edge.

Veils almost always formed with filopodial borders, protruding between either fully extended or growing filopodia. Therefore, one function of the filopodia is to direct elongation by demarcating the pathway along which axolemma flows. Models of axon growth in which the body of the growth cone is pulled forward, or in which advance of the leading edge is achieved by filopodial shortening or contraction against an adhesion to the substrate, are inconsistent with our observations. We suggest that, during the elongation phase of growth, filopodia may act as structural supports.

THE tip of a growing neuronal process is a specialized motile structure, the growth cone. It has been recognized since the original identification of the growth cone by Ramón y Cajal (36) that this structure is likely to be important in causing and directing neurite elongation, but the mechanisms by which these roles are achieved are still unclear. Two motile protrusions of the growth cone are common: the digitate filopodium (or microspike) and the sheet-like veil (or lamellipodium) (12, 23). Filopodia of neuronal growth cones in culture can adhere to a suitable substrate and are thought to exert tension on the neurite (9). Most commonly, it has been suggested that this tension either pulls the growth cone forward (9, 11, 12, 15, 35, 43) or facilitates the addition of precursors of the plasma membrane and cytoskeleton (9, 12, 25), and does so directionally to determine the path of neurite growth. The role of the lamellipodium (veil) has been ill- or undefined (4, 29, 31).

Neurite elongation involves increase of the surface area of the plasma membrane. The net addition of membrane that this entails occurs primarily in or near the growth cone (6, 19, 33), probably by exocytosis of cytoplasmic vesicles containing membrane precursors (32). It is not known where in the growth cone this addition of membrane occurs, whether

at the base (28, 42) or, as more commonly suggested, at the leading edge (4, 9, 10, 12, 25).

We report here observations of the growth in culture of the axons of two giant neurons from the central nervous system of *Aplysia californica* that help to clarify the roles of the veil and the filopodium and that are also pertinent to the question of where in the growth cone new membrane is added. We have observed growth in real time using the technique of video-enhanced contrast-differential interference contrast (VEC-DIC)¹ microscopy. This technique, which is a modified form of differential interference contrast (Nomarski) microscopy, allows the visualization in living cells of structures smaller (≥ 30 –50 nm) than the conventional limit of resolution of the light microscope (200–250 nm), thus permitting the detection of all the types of membrane-bound organelles in the axon, vesicles as well as the larger mitochondria and prelysosomal organelles (1, 2). Also, we found that because of the high magnification and contrast and the shadowing provided by the technique, veils and filopodia were exceptionally well-defined and easy to detect in real time.

1. *Abbreviations used in this paper:* LIRB, large, irregular, refractile body; VEC-DIC, video-enhanced contrast-differential interference contrast.

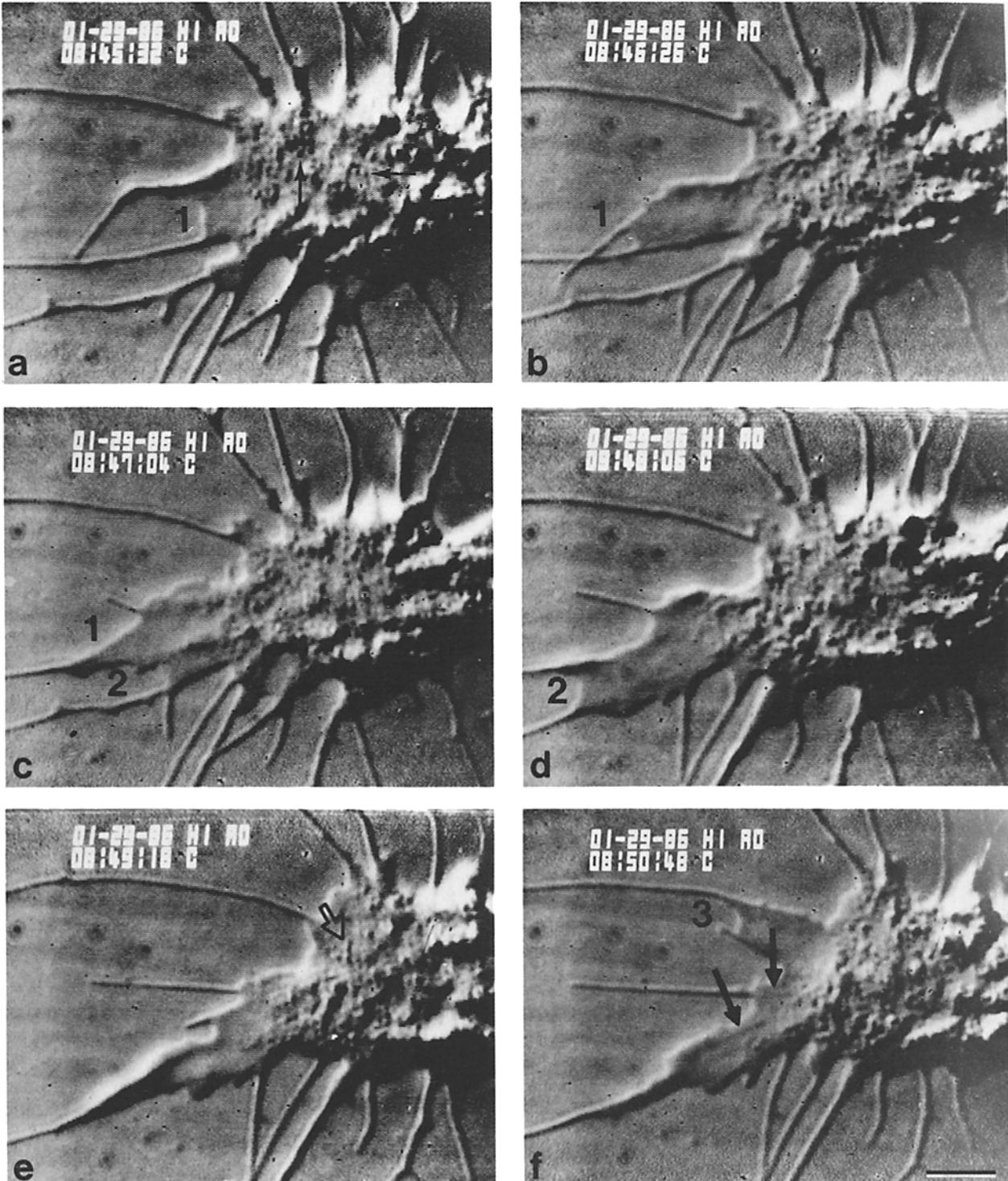


Figure 1. Veil advance and remodeling. Veil 1 advances between straight and curved filopodia (*a-c*), then veil 2 advances on other side of straight filopodium (*c* and *d*). That filopodium then lifts off the surface (*e*) and fuses with an adjacent filopodium, obliterating most of the veil (*f*). As this is happening, veil 3 advances between an established and a growing filopodium. Riblike extensions of two filopodia can be seen in the veil region (\uparrow in *f*). Numerous vesicles, a few of which are indicated (\uparrow in *a*), and a mitochondrion (\downarrow in *e*) are visible in the main body of the growth cone. Many more are visible when the videotape is viewed, as is true of subsequent figures. The veils are smooth and initially free of organelles. This and all subsequent figures except the last are 35-mm photographs of the television monitor displaying the videotape. Bar, 3 μ m.

Materials and Methods

Cell Culture

We used two large identified neurons, B1 and B2, in the buccal ganglia of juvenile (1–5 g) sea hares, *Aplysia californica*, obtained from the *Aplysia* mariculture facility at the Howard Hughes Medical Institute of the Woods Hole Oceanographic Institute in Woods Hole, MA. The cells were removed from the ganglia and placed in culture as previously described (38). The cell bodies were removed from the animal with 0.1–1.0-mm lengths of axon attached. The cells were placed on a glass coverslip embedded in a plastic culture dish. The dish with coverslip had been coated with poly-L-lysine. Growth of neurites, mostly from the ends of the axons, commenced within several hours of explant.

Video Microscopy

6–30 h after culturing, growth cones that were relatively isolated from other growth cones, cells, or axons were selected for observation. Observation periods generally ranged from 1 to 3 min followed by 5–10-min rest periods during which there was no illumination of the cell. Some growth cones were observed for up to 3 h without apparent injury or decline in growth rate. The cultures were maintained at room temperature (18–20°) and medium in the plate was changed manually or by constant perfusion. VEC-DIC observations were made using an inverted light microscope (IM 35; Carl Zeiss, Inc., Thornwood, NY) with a 50-W mercury arc lamp, 63× (1.4 NA) oil immersion planapochromat objective and 1.4 NA oil immersion condenser. The image was projected to a television camera (model 68 MK II; Dage-MTI, Wabash, MI) equipped with newvicon image tube and contrast enhancement circuit. The magnification of the specimen on the 9" television monitor (650–700 lines horizontal resolution, model PM 950; Ikegami, Tokyo, Japan) was 7370×. Video images were recorded by a Panasonic NV-9240XD U-matic video cassette recorder (>500 lines horizontal resolution), and photographs (except Fig. 4) were taken on 35-mm film directly from the monitor screen without further enhancement. Digital enhancement of Fig. 4 was kindly performed by Dr. S. Siegelbaum (Columbia University, New York) using a PC Vision Frame Grabber (Image Technology, Waltham, MA) and an IBM PC-XT computer.

Results

Structure of the Growth Cone

The B1 and B2 neurons of *A. californica* quickly attached to the poly-L-lysine substrate. A halo of neurites began to extend from the cut end of the main axon within several hours. Each of these neurites was 2–6 μm in diameter, supported directed vesicle movement (not shown), and was tipped by a 5–15-μm wide growth cone. The growth cone of the B1 and B2 neurons displayed an overall structure similar to that observed for other growth cones in culture (12, 23), but with VEC-DIC microscopy both vesicles and the diaphanous veils on the periphery of the growth cone were clearly and consistently visualized. Because we could observe these structures in the living growth cone, we were able to clearly define the events leading to the growth of the axon. Briefly, we saw thin, vesicle-free veils forming between growth cone filopodia, the veils subsequently filling with vesicles and other organelles from the body of the growth cone and eventually transforming into the mature axon.

Filopodia. All of the growth cones examined had filopodia (Figs. 1–10). Most of the filopodia lay parallel to the substrate and, because they did not display any lateral movements, appeared to be adherent to it along most or all of their length.

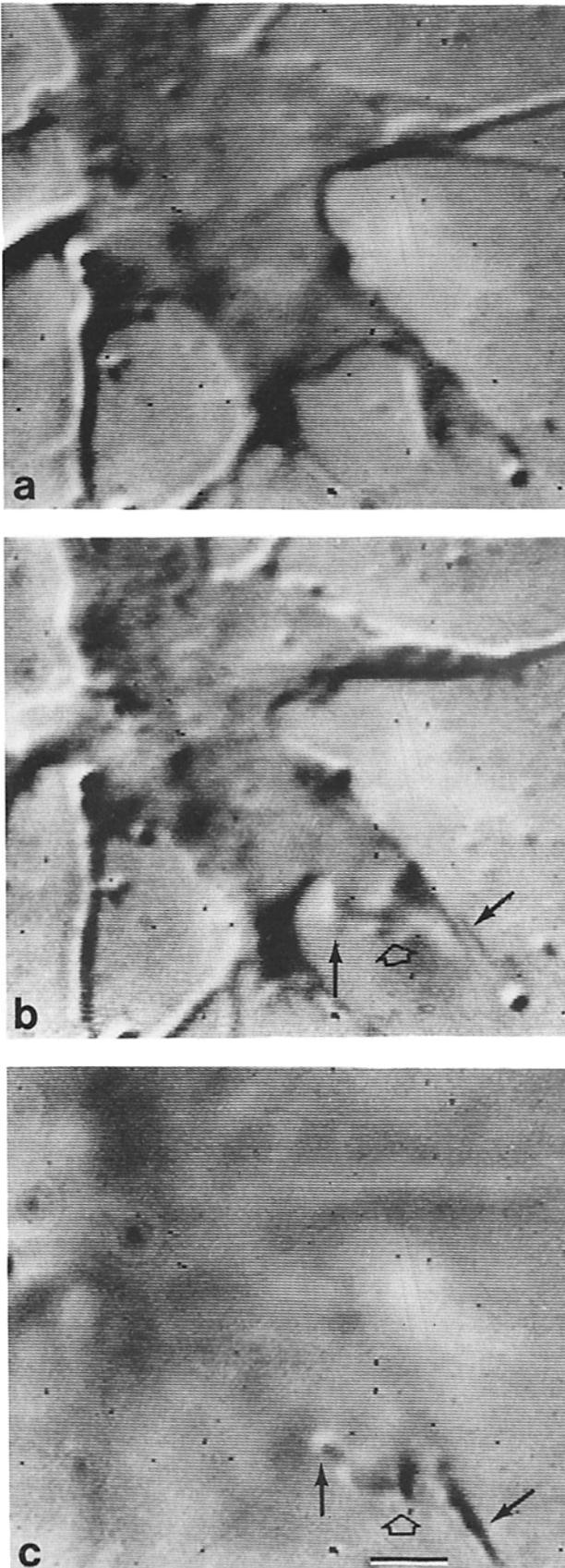


Figure 2. Veil advancing up into medium. Veil (\blacktriangleleft) advances between filopodia (\blacktriangledown) growing into the medium. The main body of the veil is not clearly visible in *c* because the plane of focus has been shifted upwards from *b* to show the leading edge of the veil.

The tips of both filopodia are not resolved in *c* because they project even higher. Bar, 2 μm.

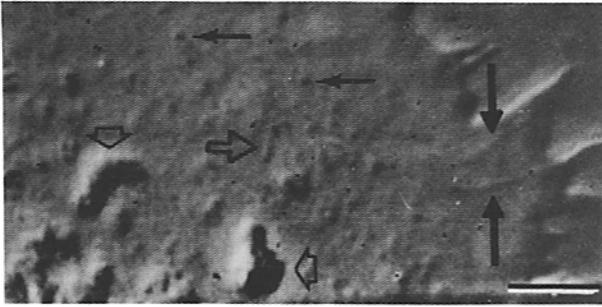


Figure 3. LIRBs (\diamond) in the main body of a growth cone. Small vesicles (\uparrow) and a mitochondrion (\uparrow) are also in the main body of the growth cone and riblike extensions of filopodia (\uparrow) can be seen in the veil region. Bar, 3 μ m.

Some filopodia projected from the growth cone directly into the medium at an angle to the substrate (Fig. 2). These filopodia could be either straight or bent and appeared to be rather stiff. They exhibited both a rapid, vibratory motion that appeared to be caused by movements of the medium, and larger swaying motions, but did not otherwise differ in appearance from attached filopodia. Long filopodia appeared adherent to the substrate in their proximal sections but often had distal sections that were not adherent. These sections displayed the same stiff, vibratory movements seen in completely nonadherent filopodia. We did not observe any instance where the tip of a filopodium was adherent and stable while the proximal regions were not. The stiff nature of the filopodium and the relatively flat substrate, however, made this type of observation problematic.

Veils. Many of the growth cones displayed distinct veils along their periphery. With VEC-DIC microscopy, the veils were clearly defined as regions with little or no vesicle activity, and were nearly always bound by two filopodia. In some growth cones veils extended across a wide area of the periphery, apparently spanning several filopodia (Fig. 1, *c-f*). In examining the development of the veils, however, (discussed in detail below), it was clear that the veils on either side of a filopodium could develop independently of their neighbors, so the wide expanse of membrane should be thought of as a group of veils rather than as a single structure. Occasionally, thin vesicle-free areas were not closely associated with filopodia; however, the formation of these areas was either a prelude to new filopodial growth or the result of previously formed filopodia being obscured.

The proximal border of the veils was demarcated by the organelles of the main body of the growth cone engaged in apparently random motion, presumably Brownian (Fig. 1), while the distal border was often a smooth concavity extending between two filopodia. Riblike extensions of the filopodia were often observed in the veils (Figs. 1 *f*, 3, 6, and 7).

The terms lamellipodium and veil have generally been used interchangeably to refer to flat extensions at the front of the growth cone (12, 23). We have often seen, however, that a region that appears uniformly flat and featureless with phase-contrast microscopy and that might formerly have been labeled a lamellipodium in its entirety, actually contains numerous vesicles in some or all of its area. We will therefore use the term veil exclusively to refer to regions that are initially devoid of visible organelles.

Main Body of the Growth Cone. The center of the growth

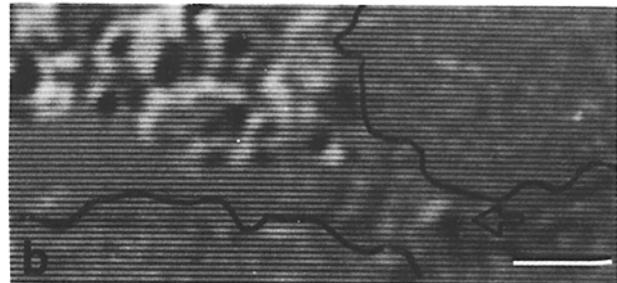
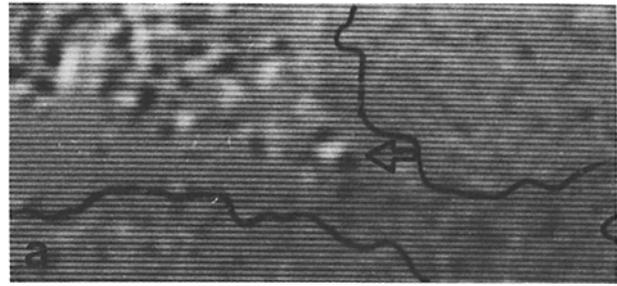


Figure 4. Movements of vesicles within the growth cone. Frame subtraction has been performed by use of a computer to eliminate all stationary objects in the sequence. The group of black-and-white spots in the upper left is the main group of organelles engaged in Brownian motion. A solitary vesicle (\uparrow) moves away from this group into the veil by fast axonal transport. The outline of the growth cone with veil is drawn in black. *b* is 0.4 s after *a*. Bar, 2 μ m.

cone was characterized by a tumultuous accumulation of membranous organelles. Some can be seen in Fig. 1 and subsequent figures, but many more are evident when the moving image is viewed. Small spherical vesicles predominated in the more distal areas of the growth cone body (exclusive of the veils and filopodia), but in more proximal regions mitochondria, recognizable by their smooth sausage shape, and a type of large irregular refractile body (LIRB) were also evident (Figs. 3 and 7). We are uncertain of the correlate of the LIRB in conventionally fixed tissue examined with the electron microscope, but we routinely observed one or a few of them in the living growth cone. They measured from 1 to 3 μ m across and exhibited small drifting movements. We never observed LIRBs in the cell body or any region of the axon except near the growth cones, nor did we observe them to move retrogradely from the growth cone. In these respects they differ from organelles of the lysosomal family.

There were two types of vesicle movement in the growth cone. Most vesicles traveled short distances in an apparently random manner. Poisoning the cells with 20 mM NaCN to deprive them of ATP did not interfere with this movement, though it blocked fast axonal transport, indicating that the major visible movement in the growth cone is probably Brownian in nature. This movement was obvious during viewing of the growth cone live or on videotape, but is impossible to document in still photographs of the videotape. We thus subjected a segment of videotape to computer processing in which all stationary objects were eliminated from the pictures by frame subtraction (Fig. 4). A group of vesicles remains visible, indicating that these were in motion. Intermixed with the vesicles in Brownian movement were vesicles that displayed a smoother, directed movement similar to the fast transport of vesicles in the axon. Continuous move-

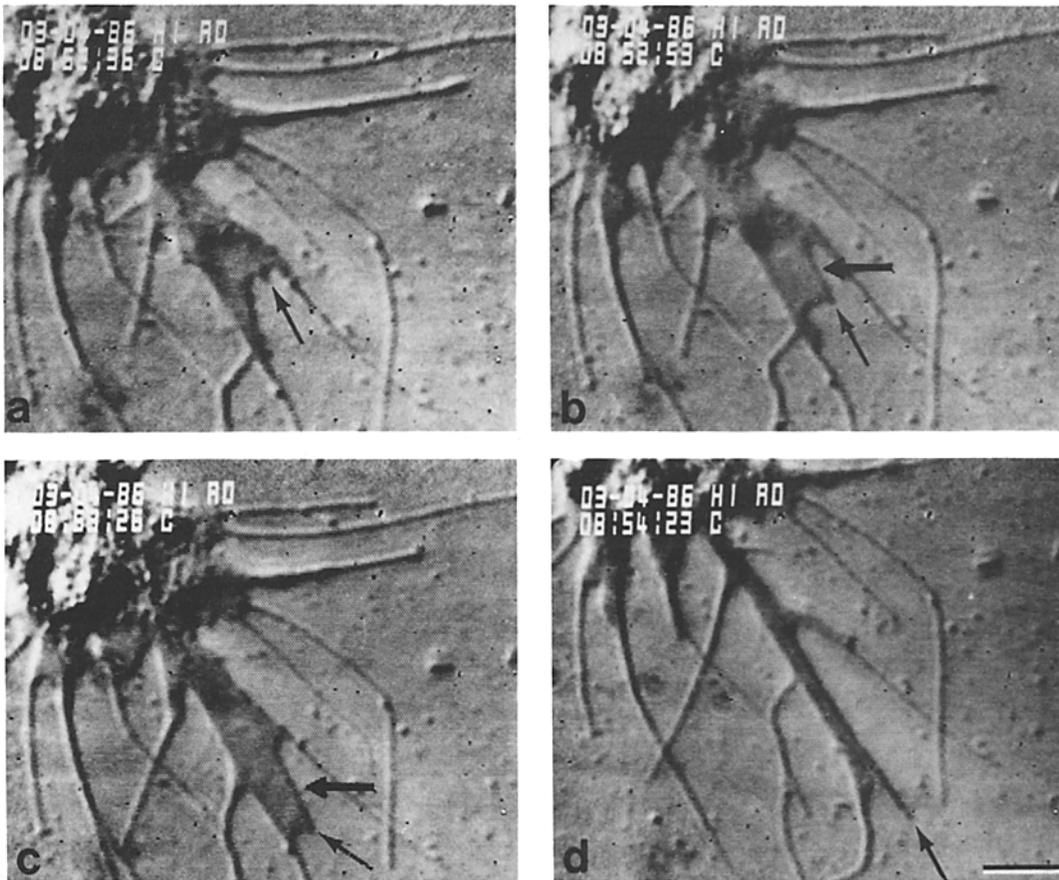


Figure 5. Coincident growth of a filopodium and a veil. Though what appears to be the core of a filopodium (\uparrow) is visible while the veil is growing (b and c), the nubbin at the leading edge of the veil (\uparrow) becomes a frank filopodium only when the veil stops advancing (d). Field of view has been moved a little to the right in d. Bar, 3 μ m.

ments of these vesicles across the main body of the growth cone were observed.

Growth of the Axon

Because we were able to clearly define the edges of the growth cone and to observe in real time, we have been able to closely observe the mechanism by which the axon grows. In reviewing our videotapes we carefully outlined the position of the growth cone at the start of each observation period (excluding the distal tips of the filopodia) and then noted every instance where there was growth in any direction, even if it was only transitory. In viewing 27 growth cones from 16 cells we observed 165 distinct instances of advance of the leading edge of the growth cone. In every instance the advance took place through the formation of a thin, vesicle-free veil.

Because of the central nature of the veils in the growth of the neurite, we will first describe veil formation and then the maturation of the vesicle-free veil into the mature neurite. All of our observations were on growth cones that were actively growing at the start of our observations, so that both formation and maturation were generally occurring simultaneously.

Veil Formation. The formation of veils was closely linked to the presence of filopodia (Table I). We observed 165 instances of veil formation; in 150 instances the veil advanced between two distinct filopodia, in 10 instances the veil

formed between a single filopodium and the main body of the growth cone, and in only 5 cases was there no direct evidence of filopodial involvement. In all five of these last cases the veil advanced in much the same manner as seen with filopodial guidance, but no filopodia were resolved during our period of observation. The filopodia clearly acted to limit the lateral spread of the veils, as illustrated when a veil advanced along a curved filopodial border (Fig. 1, a and b).

In more than half the instances of veil formation, the advance of the veil came about in close association with the lengthening of one or both of the bordering filopodia (Table I; Fig. 5). Often a new filopodium would emerge 0.5–1.5 μ m from an established filopodium. As the new filopodium advanced, the band of membrane extending between the filopodia would also advance, remaining a short distance behind

Table I. Filopodia Bordering Growing Veils

	No.	Percentage
No filopodia	5	3%
One filopodium (growing)	10	6%
Two filopodia (both growing)	14	8%
Two filopodia (one growing)	85	52%
Two filopodia (neither growing)	51	31%
Total	165	100%
Total with growing filopodia	109	66%

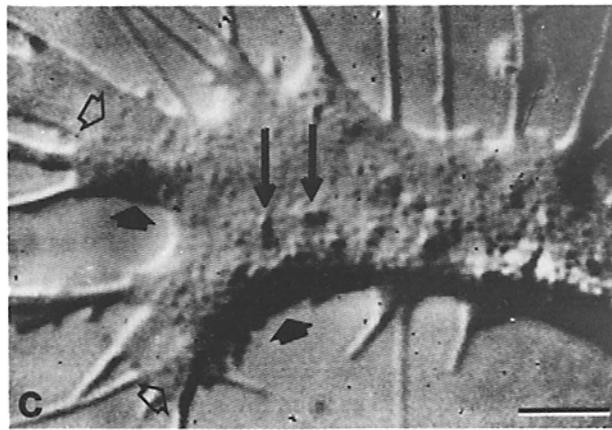
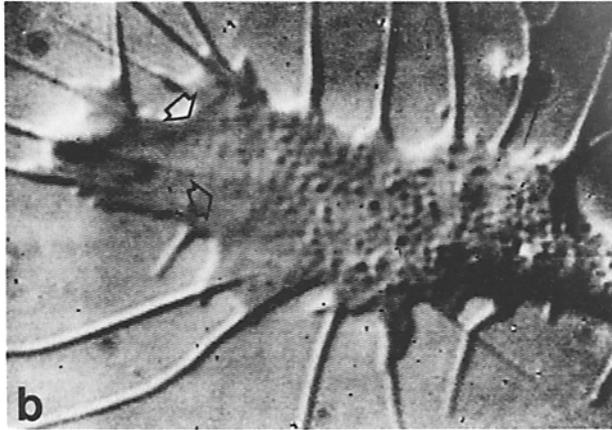
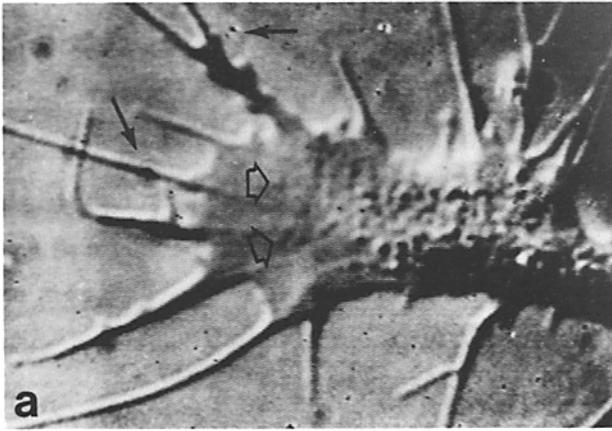


Figure 6. Maturation of veils by incremental advance of main group of growth cone vesicles. (a) Broad veil with riblike extensions of filopodia. The approximate front of the main group of vesicles is marked (◊) here and in subsequent panels. Forward movement of this group in subsequent panels can be judged by comparison with fixed dirt spots in the picture (↑). (b) 2 min later, veils have projected farther forward and vesicles (◊) have invaded previously vesicle-free veil areas. Riblike extensions of filopodia are visible. (c) Several minutes later there has been further veil growth as the axon is beginning to branch. Vesicles (◊) now fill all but the most distal veil regions, larger organelles (↑) are appearing and former veil regions have become more voluminous, as indicated by the thick shadows on the edges (●). Bar, 3 μ m.

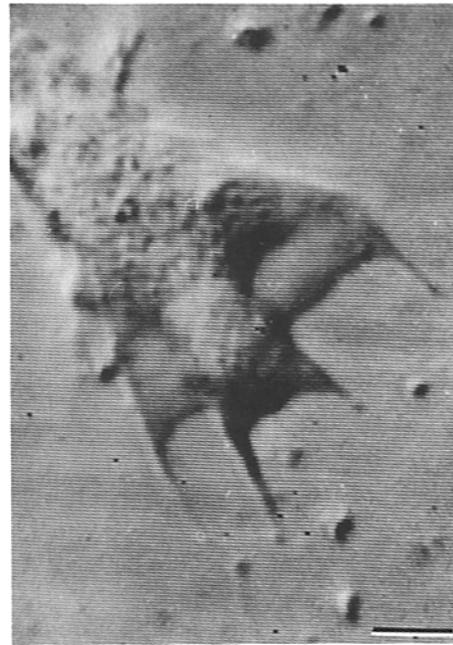


Figure 7. Inhomogeneous incremental filling of veils with organelles. Central veil has matured by gaining vesicles and volume, while the two neighboring veils remain flat and free of vesicles. Bar, 2 μ m.

the growing filopodial tip. Sometimes the distance was so short that there was just a nubbin, rather than a definite filopodium, on one side of the veil. In all of those instances, though, a frank filopodium formed from the nubbin when the veil advance either slowed or stopped. Although most advances occurred with coincident filopodia lengthening, in 31% of the instances counted, the veil advance took place between two established filopodia that were not evidently extending (Fig. 1, *a-d*). (In some of these cases the ends of both filopodia were not in the field of view so we report only that there was no close association between veil advance and filopodial growth.)

The formation of veils was episodic, with periods of quiescence and of advance. Of our sample of 165 veil advances, 48 were seen in their entirety, and 75% of these lasted no longer than 30 s (overall range 9–135 s). The growth of veils was also localized. Although many filopodia extended from the growth cones, veil advance occurred only between some. Often a veil would form on only one side of a filopodium (Fig. 1, *a* and *b*), and when growth was evident on both sides it was often asynchronous and unequal (Fig. 1, *c* and *d*). The adhesion of the filopodia was not always necessary for veil formation, since veils were occasionally seen to advance along filopodia projecting into the medium (Fig. 2).

Veils were often transient structures. In many instances, advance of the veil was followed by retraction, revealing the underlying filopodial framework (4). In other instances, one of the bordering filopodia would detach, resulting in the eventual fusion of the veil and the detached filopodium with the adherent filopodium (Fig. 1, *d-f*).

Maturation of the Veil. Growth of the axon occurred only by maturation of the veil. The flat veil would fill gradually with organelles, become more voluminous while still retaining a growth cone morphology and displaying Brownian or-

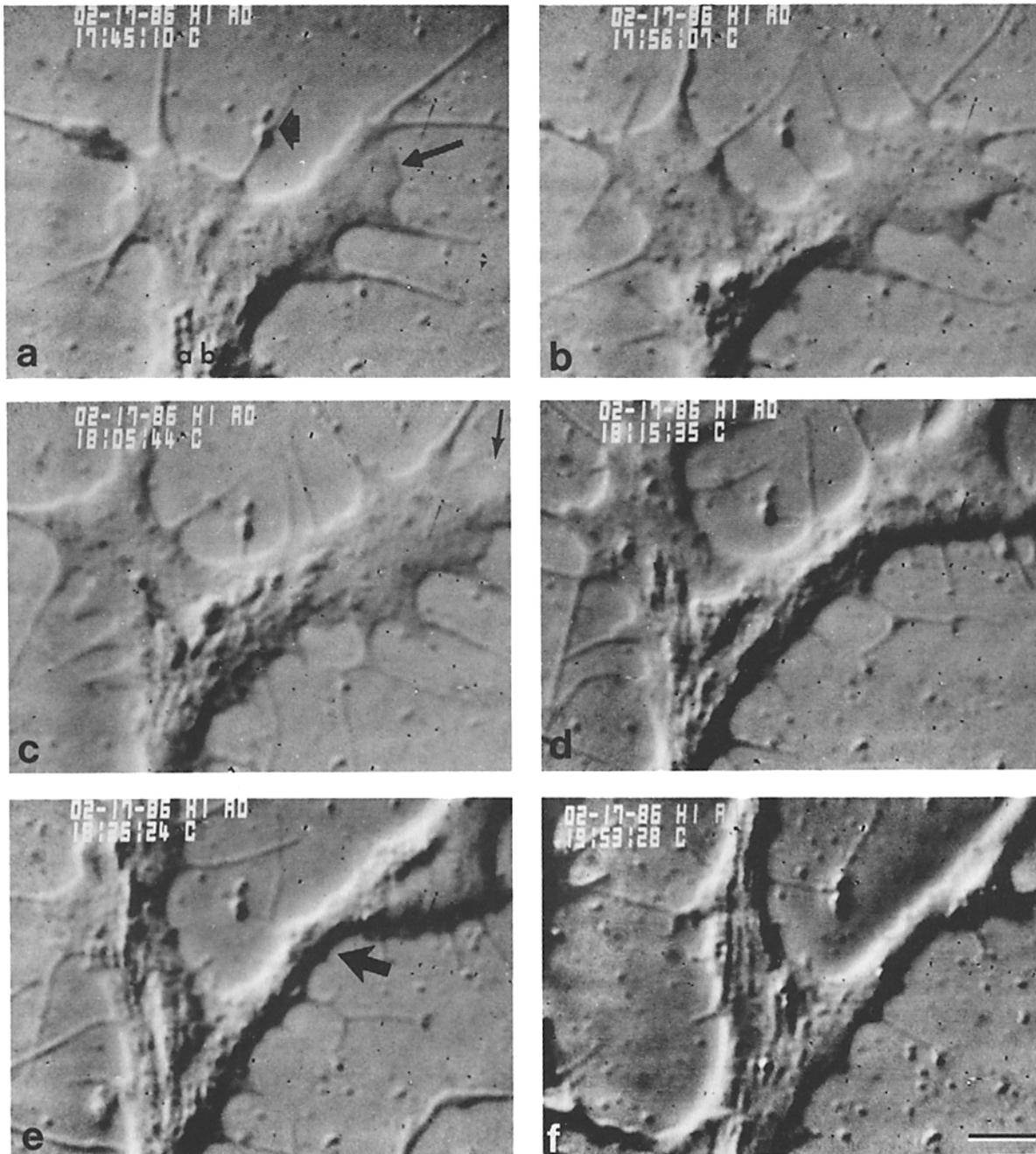
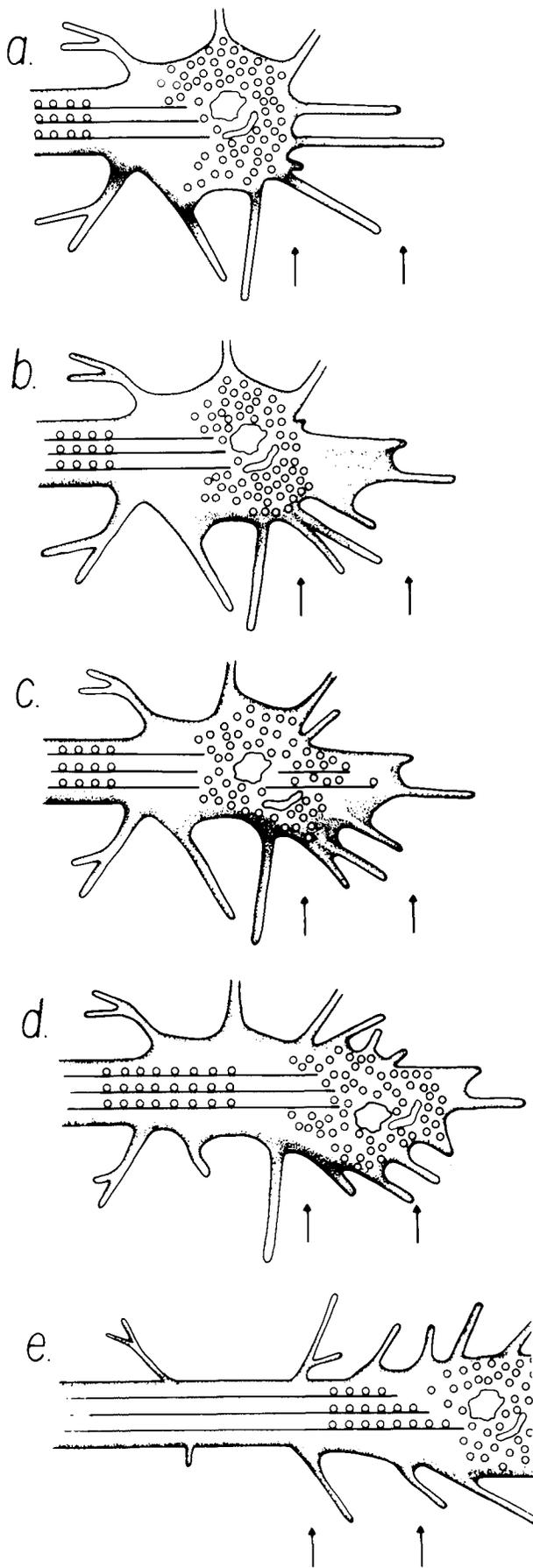


Figure 8. Maturation of veil into axon. Axon branch (*ab*) is bifurcating in this sequence. Veil (\uparrow) advances between filopodia (*a*), fills with vesicles (*b*), begins to become more voluminous as new flat veil (\uparrow) advances at leading edge (*c*) and then becomes even more voluminous and starts to round into cylinder in proximal regions as larger organelles appear (*d*). Proximal region (\blacktriangle) then completes maturation into neurite while distal region is still somewhat spread on surface (*e*), and then, finally, distal region completes the transformation (*f*). Pair of stationary particles (\blacklozenge) on the substrate can be used as landmarks. Bar, 3 μm .

ganelle movement, and then convert into a tubular neurite displaying predominantly directed movement of organelles.

Filling of the veil with organelles occurred in two ways. In some veils, vesicles could be seen traveling by directed transport through otherwise vesicle-free terrain to points deep within the veil (Fig. 4) where the vesicles tended to remain. The vesicles moved rapidly (mean, 1.5 ± 0.3 [SD] $\mu\text{m/s}$, $n = 12$) and seemed to follow defined paths, because subsequent vesicles could often be seen describing the same

trajectory. Even if this deep penetration took place, however, most filling occurred through small, incremental movements of vesicles from the border of the main body of the growth cone (Fig. 6). The advance of the front of vesicles into the veil occurred at a mean rate of 1.0 ± 0.5 (SD) $\mu\text{m/min}$ ($n = 10$). At the leading edge of the mass of organelles, vesicles could often be seen moving both in apparent Brownian motion and in slightly longer movements, as if being directed along short tracks.



Filling sometimes occurred only in certain areas of a veil, often along riblike extensions of the filopodia into the body of the veil. Filling was selective among neighboring veils, proceeding in one but not another (Fig. 7), and filling was not inevitable. We observed one instance of a veil that was free of vesicles for more than 40 min.

The initial invasion of a veil was generally by small vesicles, followed later by the other membranous organelles (Figs. 6, 8, and 9, *c* and *d*). The region gradually became more voluminous, while still spread on the surface and displaying many filopodia (Figs. 6, 8, and 9 *d*). Movements of organelles were predominantly Brownian. At this stage, then, the veil had been converted to the main body of the growth cone, and other veils had often projected forward (Figs. 8 *c* and 9 *d*).

The main body of the growth cone converted to a neurite by losing some of its attachments to the substrate and assuming a more tubular shape, although many partially retracted filopodia remained. At this stage, the predominant visible organelle movement became bidirectional fast axonal transport, with little evident Brownian motion (Figs. 8, *d-f* and 9 *e*).

The rate of advance of the central growth cone ranged from 5 to 25 $\mu\text{m}/\text{h}$ in growth cones observed for 20 min or more. Both slow and rapid advance occurred through the same mechanisms, the formation of veils and their subsequent maturation, although during slow growth veils were often small and were rapidly filled, making them more difficult to detect (Fig. 10).

The entire sequence of axon formation is depicted diagrammatically in Fig. 9.

Discussion

Roles of Veil and Filopodium in Axon Growth

Our observations with VEC-DIC microscopy of the growth of the axons of *Aplysia* neurons B1 and B2 in culture allow us to reach certain straightforward conclusions about the roles of the veil and the filopodium. Growth always occurred by the same process: formation of a veil followed by gradual maturation of the veil into a neurite (Fig. 9). The veil is thus

Figure 9. Stages in axon formation. (*a*) Axon branch with organelles moving by bidirectional fast axonal transport widens into main body of growth cone, which has vesicles, mitochondria and LIRBs displaying Brownian motion. Filopodia project from the growth cone; one has just begun to grow. (*b*) Two large, flat, organelle-free veils, and a third tiny one, have advanced. (*c*) The largest of the veils has begun to fill with vesicles and gain volume. The most distal vesicle in this veil entered by a long translocation along a microtubule that has recently formed in the veil, while the others are entering mainly as a group, by Brownian motion and by short translocations on microtubules that are elongating slowly into the veil. Two smaller veils have advanced. (*d*) All of the veils have filled with vesicles and become voluminous. Mitochondria and LIRBs have entered the region. The organelles are moving predominantly by Brownian motion. (Microtubules are still present in this area, but we have removed their symbols.) A new veil has advanced. (*e*) The region has become cylindrical to form the axon. Most movement is now bidirectional fast axonal transport. We did not directly observe microtubules; their growth into the veils depicted in this figure is an inference from our observations of directed vesicle movements.

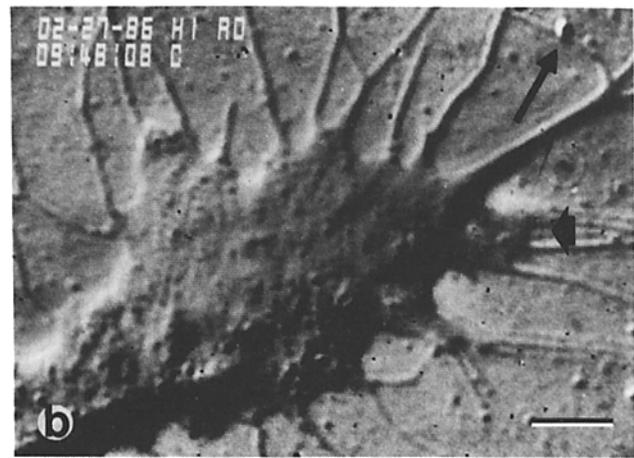
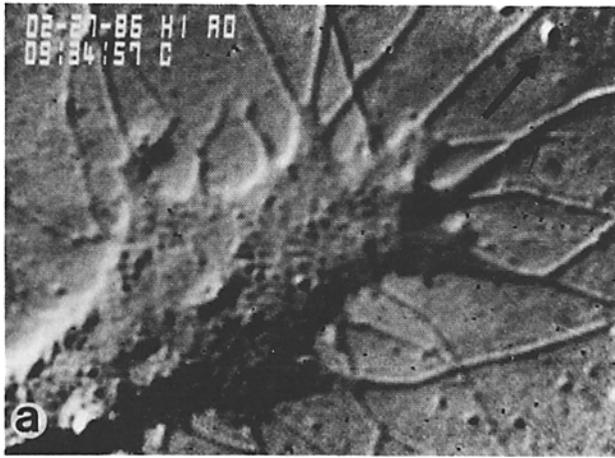


Figure 10. Slowly growing axon branch. Only small veils (●) can be observed. This axon branch grew approximately 1 μm in the 14-min interval between *a* and *b*, as can be seen by judging its position relative to a stationary particle (↑) on the substrate. Bar, 3 μm .

the nascent axon. Veils almost always protruded between or along filopodia, often obviously following the contours of the filopodia. Therefore, one important function of the filopodia in the growth of the B1/B2 axon on this substrate is to direct elongation by demarcating the pathway along which axolemma flows. When a growth cone has few filopodia (14, 27), the axon will then grow in their direction. When many filopodia are present (41), directionality might be determined through selective formation or maturation of veils, for we observed that not all of the filopodia of a growth cone supported veil protrusion and not all veils matured.

Identification of the veil as the immature axon has not been previously made, to our knowledge. It has sometimes been suggested that protrusion (formation of filopodia and veils) at the front of the growth cone is the manifestation of the membrane expansion underlying elongation, though the filopodium rather than the veil has usually been viewed as the neurite precursor (7, 13, 25, 26). More commonly, extension of filopodia and veils and elongation have been viewed as separate but linked events (23, 42). The function of the veil has largely been ignored.

Veils have been reported without associated filopodia (4). We suspect that in these cases the veil was masking an underlying filopodial skeleton. We found that during rapid axon growth a growing filopodium would often appear as merely a nubbin at the edge of the growing veil. The same phenomenon has been reported in spreading epithelial cells (16) and coelomocytes (17), with the filament bundle core of the filopodium present within the veil. In fact, in the case where veils were present in growth cones without obvious filopodia, the filopodial skeleton appeared when the veils retracted (4). At the other extreme, growth, albeit slow, has been reported when only filopodia were present (4). We wonder whether higher resolution optics and real time observation might have revealed the presence of small veils, which characterized our most slowly growing neurites. The phenomenon of lateral spreading of filopodia has been noted (44), and we think that this involves the movement of a thin (as opposed to a broad) veil, not recognized as such, along the filopodium.

Our results focus attention on veil protrusion for insights into the mechanism of elongation of the axon. Axon elongation has been commonly thought to occur through the exer-

tion of force by the filopodia (8, 9, 11, 12, 15, 23, 25, 35) acting either to pull forward the body of the growth cone (9, 12, 15, 35) or to pull or channel precursors of the plasma membrane and cytoskeleton (8, 9, 12, 25) towards the leading edge of the cone. Our observations are not in accord with either of these ideas. We never observed forward movement of the main body of the growth cone. Rather, advance occurred always by protrusion of veils. Nor did we ever observe advance of vesicles to the leading edge of the forming veils. The force exerted by the filopodia has been thought to be generated either by the shortening of filopodia (11, 12) or by their isometric contraction against a firm adhesion to the substrate (12, 25). We observed that veil protrusion was not accompanied by shortening of filopodial cores, but simply by their masking (Figs. 1 and 3; also see electron micrographs in references 24 and 42). Also, it seems that firm adhesion of filopodia to the substrate was not essential for veil protrusion (Fig. 2; 21, 28) though, since one mode of veil disappearance was caused by detachment from the substrate of a bordering filopodium (Fig. 1, *d-f*), adhesion of filopodia might be required for stabilization of a veil (24, 25).

An explanation that is in accord with, though unproven by, our observations is that filopodia serve only as a structural framework for veil protrusion, and thus for the elongation phase of axon growth. A veil could protrude because of rearrangements of the actin-containing meshwork underlying the membrane (42) or simply because of addition of membrane to the surface of the cone, with it being easier for the expanding membrane to project forward between rigid extensions of the surface (the filopodia) than from an unextended portion of the surface. Were this mechanism correct, one would expect that axon elongation could continue in the absence of filopodia, though new membrane would not project forward in the form of a veil; this has, in fact, been found to occur (29).

Movements of Organelles Within the Growth Cone

We never saw organelles in a newly formed veil. It is possible that organelles were present that were unresolved. We think this quite unlikely because we were able to see many organelles in the axon and the main body of the growth cone. Electron microscopic examination of another *Aplysia* neuron that we have studied indicates that we can detect organelles

as small as 40–50 nm with VEC-DIC microscopy (18), and the veil, because of its flatness and thinness, is an optically favorable structure, more so than the axon or the main body of the growth cone. Veils extended as far as 10–15 μm beyond the main body of the growth cone. Therefore, if expansion of the surface area occurs by exocytosis of cytoplasmic organelles (32, 33), this must be occurring, not at the leading edge, but in the main body of the growth cone. Tosney and Wessells (42) reached the same conclusion from electron microscopic examination of actively protruding veils, finding them devoid of organelles. They could not rule out the possibility, however, that vesicles are transported to the leading edge and fuse immediately. We can do so, and thus our results and theirs are complementary.

Neurite elongation always involved the progressive filling of a veil with organelles. This could have occurred either by Brownian motion or by fast axonal transport. Many instances of fast transport of vesicles from the main group of organelles well into the veil were observed and, since fast transport of organelles occurs only along microtubules (20, 39), we take those translocations as evidence of the formation of microtubules in the veil. More often, the main group of organelles engaged in Brownian motion moved forward slowly into the veil. Because we frequently saw short organelle movements, apparently by fast transport, at the front of this group, we think that microtubule extension may have been occurring concomitantly with the advance of the front of organelles. Extension of microtubules into the veil, either well in advance or in step with the front of organelles, could explain why filled veils were less likely to retract than veils that were devoid of organelles. We could never positively resolve microtubules, however, so that while it was evident that both long and short organelle translocations into the veil occurred along defined pathways, we can only assume those pathways were microtubules.

Several observations suggest that, although organelles moved into a veil by Brownian motion and fast transport, their ingress was limited by some other factor, or factors. First, the filling was not as fast as would be expected from diffusion in water of vesicle-sized particles. Second, veils often remained completely devoid of organelles for many minutes after formation. Third, organelles often filled a veil inhomogeneously, such that one region would fill while a laterally neighboring region remained unfilled. A possible limiting factor, though certainly not the only possibility, is the flatness of the veil. Perhaps it has to open somewhat to allow organelles to enter.

One way in which inhomogeneous filling took place was by the incremental advance of organelles along what appeared to be the core of a filopodium projecting into the veil. Long translocations into a veil, well beyond the main group of organelles, also sometimes followed this apparent filopodial core, though stopping well short of the filopodium proper. These observations substantiate results from electron microscopic examination of fixed mammalian growth cones that showed microtubules and membrane-bound organelles aligned along filament bundles projecting inward from filopodia (24, 42). It has been suggested that the bundles act to align and pull forward microtubules and serve as a pathway for the transport of membrane to the leading edge to be used for elongation (9, 24, 25). While the former may be true, it is clear that the movement of vesicles along the bun-

dles is associated not with the formation of a protrusion, but rather with its maturation.

The presence of a discrete organelle in the growth cone of the size and mobility of the LIRB was unexpected. Perhaps it is the same as the motile phase-dense body (intervening phase-dense inclusion) seen in neurites of goldfish retinal ganglion neurons in culture (22). This body has been suggested to contain smooth endoplasmic reticulum-like membranes used for plasmalemmal expansion in the growth cone. Electron microscopic examination of growth cones in developing chick optic tectum that had not been subjected to glutaraldehyde fixation revealed not endoplasmic reticulum but discrete stacks of lumenless membrane (multilamellated stacks) (14). Perhaps these are related to the LIRB. The LIRB was a regular constituent of the living growth cones that we observed, so electron microscopic examination of LIRBs previously identified by VEC-DIC microscopy should be worthwhile.

We do not feel that our observations on the mechanism of axon growth are peculiar to either the regenerative growth of *Aplysia* neurons B1 and B2 or to our culture conditions. Although poly-L-lysine coverslips are highly adhesive substrates for axon growth, we observed the identical sequence of events when the cells were grown on less adhesive uncoated glass (unpublished observations). Similarly, other neuronal types from *Aplysia* displayed the same outgrowth behavior. Veils are commonly found on the growth cones of neurons of other species in culture (11, 30, 31, 34, 43) and probably in vivo (5, 37, 41), and an inspection of published photographs reveals that they often have been observed between two filopodia and with a concave leading edge (9, 11, 25, 30), as we have observed. Also, veil formation has been found to directly precede the resumption of growth by neurites that had filopodia but were not elongating (3, 10, 40). Thus, although the B1 and B2 neurons may be particularly favorable cells for VEC-DIC observations, we feel that the sequence of veil formation and maturation that we have described is probably common to all axon growth.

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