

# Lectin Labeling of Sprouting Neurons

## II. Relative Movement and Appearance of Glycoconjugates during Plasmalemmal Expansion

KARL H. PFENNINGER and MARIE-FRANCE MAYLIÉ-PFENNINGER

*Department of Anatomy and Cell Biology, Columbia University College of Physicians and Surgeons, New York 10032*

**ABSTRACT** To study the dynamics of membrane components during neuritic growth, we carried out a series of pulse-chase experiments with ferritin-conjugated and unconjugated lectins on sympathetic neurons sprouting in vitro. Labeling of aldehyde-prefixed cultures with wheat-germ agglutinin or with the galactose-specific lectin of *Ricinus communis* is consistently dense near the distal end of the neurites. By contrast, if live cultures are labeled with these lectins and chased for 3–20 min, label-free plasmalemmal areas appear in the most peripheral regions of the growth cone, on filopodia and, furthermore, over vesicle clusters (SPVs). These marker-free areas, however, contain lectin receptors, as can be shown by relabeling the chased cultures with the same lectins after the aldehyde fixation. In a further set of experiments, cultures are labeled with a saturating concentration of native lectin, chased, aldehyde-fixed, and then relabeled with the ferritin conjugate of the same lectin. In this case, the surfaces of filopodia and of SPV clusters are selectively labeled with the ferritin conjugate, indicating the insertion of new lectin receptors into the plasma membrane in the growth cone periphery. These results indicate that plasmalemmal expansion in the neuron occurs by a mechanism of polarized growth, possibly involving SPVs as plasmalemmal precursor vesicles.

Recent studies on a variety of eukaryotic cell systems have produced a considerable body of data on mechanisms and location of synthesis of membrane components (e.g., references 41 and 42). However, only in a few cases are there data that suggest how newly synthesized membrane components, lipids and proteins, may reach the plasmalemma, or where and by what mechanism insertion of these components into the plasmalemma occurs. Results relevant to these questions have been obtained from studies on the assembly of viral membranes and have suggested that the transfer to the cell surface of certain membrane components occurs in particulate, rather than soluble, form (3, 40, 41). In another system, the retinal rod (e.g., reference 54), disk membrane components have been demonstrated to migrate distally into the outer segment, presumably after localized insertion near the connecting stalk (cf. references 4 and 5). Similarly, localized insertion of new membrane components may occur during egg cleavage. In that situation, fusion of vesicles with the plasma membrane of the cleavage furrow is thought to lead to its localized expansion (2, 7). The fusion hypothesis has also been proposed for hyphal growth in

root tips (19). This view of insertion of preassembled membrane into the plasmalemma is analogous to the well-established concept of exocytosis during secretion (cf. reference 29). Yet, it has not been possible so far to follow ultrastructurally the insertion of specific membrane components into the plasmalemma and, thus, to provide conclusive evidence for this hypothesis. Furthermore, incorporation of preassembled membrane into the plasmalemma appears to contradict the finding that membrane biogenesis is a stepwise phenomenon, i.e., one that involves the successive incorporation of individual membrane components (see, e.g., reference 44). Nevertheless, in the Discussion of this paper we will explain how these two views—insertion of preassembled membrane and stepwise membrane biogenesis—need not be mutually exclusive and could be combined in a single hypothesis.

One of the most dramatic examples of cellular growth is the sprouting of neurites in developing neurons. The formation of these processes may lead to a 1,000-fold expansion of the plasmalemmal surface of the neuron (43) so that, at this stage of differentiation, one of the neuron's main functions is mem-

brane synthesis. By measuring average neuritic elongation (0.5–1 mm/d for mammalian sympathetic neurons; 22, 25, 30) and the average diameter of newly formed neurites in such cells (~0.5  $\mu\text{m}$ ), the rate of plasmalemmal expansion can be estimated at 0.5–1.0  $\mu\text{m}^2/\text{min}$  per neurite. The mechanisms involved in neuritic elongation and plasmalemmal expansion have been dealt with in various studies that have suggested distal growth of the neurite. These investigations include the studies by Hughes (22), who found that the distal tip of a severed neurite continues to grow for a few hours, Bray's (8, 9) measurements of the distance of advancing growth cones relative to neuritic branch points or light microscopic markers on neurites, and the freeze-fracture analysis by Pfenninger and Bunge (35). However, none of these studies is conclusive.

To shed some light on the mechanisms involved in plasmalemmal expansion in the neuron, we have carried out a series of surface-label pulse-chase studies<sup>1</sup> with carbohydrate-specific ligands, plant lectins (cf. references 1 and 10, and the studies with cationized ferritin by Wessells et al. [50]). The lectins have been applied either in unconjugated or in ferritin-conjugated form. Our experiments were tailored specifically to study (a) the relative movement of existing plasmalemmal lectin receptors and (b) the appearance of new plasmalemmal lectin receptors during neuritic growth. The studies have been carried out at the electron microscopic level for optimal resolution and identification of cytological detail, and because initial studies with fluorescent lectins produced too small a signal on growth cones compared with the culture substratum surrounding them. Brief reports on some of the experiments described here have been presented elsewhere (32, 36–38).

## MATERIALS AND METHODS

### The Neurons

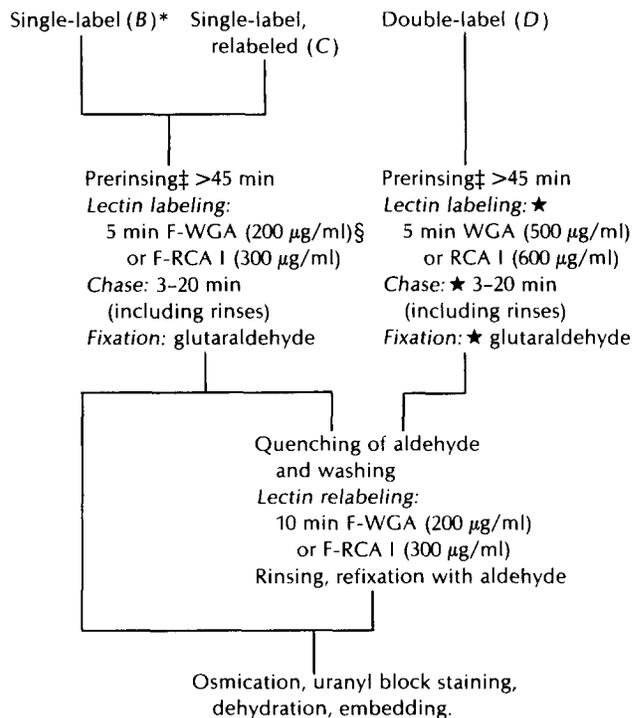
For easy access to cell surfaces and efficient sampling of different parts of the growing neuron, especially nerve growth cones, all experiments were carried out in culture. Superior cervical ganglia (SCG) from newborn or late gestation rats were cut into small pieces and grown for 3–4 d in collagen-coated Aclar wells (33C Aclar, gauge 5, Allied Chemical Co., Morristown, N. J.) according to the method of Bunge and Wood (13). The culture medium was modified from that of Bray (8). It contained 10% human placental serum, 600 mg of glucose, and nerve growth factor (calibrated to produce a maximal growth effect) in 100 ml of Leibovitz's (L15) medium. No antibiotics were used. The cultures were incubated at 36°C in air. The use of L15 medium made exposure to raised CO<sub>2</sub> levels unnecessary, which greatly facilitated handling of the cultures and reduced proliferation of nonneuronal cells. After 3–4 d in vitro, halos of neuritic sprouts were observed around the explants. Explants with maximal outgrowth and with neurites tipped by spread-out growth cones were selected for pulse-chase experiments.

### Lectin Labeling

Lectins from wheat germ and *Ricinus communis* were isolated and purified by affinity chromatography (27). These lectins and concanavalin A (Con A; obtained from Sigma Chemical Co., St. Louis, Mo.) were conjugated with ferritin according to the method described by Maylié-Pfenninger and Jamieson (27). Conjugates were then purified by affinity and gel chromatography to obtain a fraction that was free of denatured or unconjugated lectin and of unconjugated ferritin, and that contained active lectin and ferritin in a ratio close to 1:1. In various labeling studies on nerve tissue cultures, optimal binding conditions were determined (see below and the companion paper [39]). Under these conditions, nonspecific binding (in the presence of 0.2 M hapten sugar) was found to be negligibly low.

Just before the actual experiment (cf. flow diagram, Scheme 1), the cultures were rinsed (39) in a very gentle but continuous stream of fresh medium in which

<sup>1</sup> Although these lectin experiments are not strictly analogous to pulse-chase studies with radiolabeled precursors, the term "pulse-chase" has been adopted because of the timed exposure of live cells to the label, followed by various survival periods in the absence of the label.



SCHEME 1 Protocols of pulse-chase experiments.

\* Letters in parentheses refer to Fig. 1.

‡ All steps of these experiments up to and including the onset of the first glutaraldehyde fixation are performed at 36°C. Thereafter, processing takes place at room temperature. However, in the double-label control experiment, the steps marked by stars are carried out on ice.

§ Concentrations in micrograms per milliliter refer to the amount of lectin, not including ferritin. This quantitation is possible because lectins are radioiodinated before conjugation with ferritin, and their specific radioactivities are known. For F-WGA, experiments are also carried out with lectin concentrations of 50, 200, and 500  $\mu\text{g}/\text{ml}$ .

human placental serum had been replaced by 1% bovine serum albumin (BSA; fraction V), and in which the hapten sugar for a specific lectin, if included in the regular medium, was replaced by another sugar as follows: in experiments with agglutinin I from *Ricinus communis* (RCA I), which is specific for galactosyl residues, the galactose normally contained in L15 medium was replaced by glucose, and in experiments with Con A, specific for glucosyl and mannosyl residues, glucose was replaced by galactose. No such adjustments were necessary in experiments with wheat-germ agglutinin (WGA), specific for *N*-acetylglucosamine and, to some degree, for sialic acid residues. During the rinsing procedure, which was carried out at 36°C, growth cones remained spread out and neurites continued to grow without signs of being disturbed. The cultures were then labeled with lectin or lectin-ferritin conjugate for 3–5 min at 36°C. Subsequently, cultures were rinsed three times with the BSA medium for periods of 0.75–3.0 min each (depending upon the duration of the entire chase period). After chase periods of 3–20 min, the cultures were fixed at room temperature by slow infusion of 1.5% glutaraldehyde in phosphate buffer according to methods described elsewhere (33, 39). Aldehyde-fixed samples were either processed directly for electron microscopy or relabeled with ferritin-conjugated lectin after washout of the fixative. Removal of aldehyde was accomplished by washing the cultures first with phosphate-buffered saline (PBS: 0.15 M NaCl, 0.05 M phosphate buffer, pH 7.3), then with PBS containing 1 mM glycine to quench remaining aldehyde groups and, finally, with PBS to which 1 mM glycine and 1% BSA had been added (39). These carefully washed cultures were then subjected to a second labeling step with a ferritin-conjugated lectin (10 min), were rinsed three times (5 min each) with BSA-containing PBS, then refixed with glutaraldehyde, osmicated, uranyl block-stained, and processed for thin sectioning by embedding in Epon 812 (39). For the control experiments, carried out in the cold, prerinsed cultures were placed on a black aluminum block sitting in ice-ethanol slush so that the surface in contact with the culture dish was at 0° to –2°C. Labeling, rinsing, and fixation steps were carried out on this block in the cold room. Only

after 10 min in 1.5% glutaraldehyde were these cultures warmed up to room temperature for the subsequent steps of the fixation and embedding procedure.

After polymerization of the Epon, the Aclar dishes were peeled off the epoxy disks containing the cultures. With the aid of a phase-contrast light microscope, areas containing numerous spread-out growth cones were selected, marked with a diamond marker, cut out, and mounted on Epon blocks. The remounted specimens were then serially sectioned parallel to the culture substratum, and the sections were picked up on Formvar-coated grids and poststained with uranyl acetate and lead citrate. The sections were examined with a Philips 301 or a JEOL JEM 100C electron microscope.

In all the pulse-chase experiments, the maintenance of neuritic growth turned out to be particularly critical. In many of the initial experiments, in which rinsing and labeling techniques had not been perfected, nerve growth cones were often no longer palm-shaped but had started to retract by the time fixation was begun. Electron microscopy of such retracting neurites revealed a paucity of filopodia, an abundance of secondary lysosomes, a lack of clusters of subplasmalemmal vesicles (SPVs), and the absence of the changes in lectin receptor distribution described below. The observations described below were made on vigorously growing neurites with spread-out growth cones, in four experiment series involving WGA (20 individual experiments) and two series involving RCA I (15 individual experiments). The results were consistent.

## RESULTS

The rationale for these pulse-chase experiments was as follows: Membrane components that are essentially uniformly distributed throughout the plasmalemma of the sprouting neuron are likely to be inserted continually as plasmalemmal expansion occurs (Fig. 1A). A surface label for such a membrane component, e.g., a lectin, is suitable for pulse-chase studies relevant to the problem of plasmalemmal expansion (cf. reference 39). In a first experiment (Fig. 1B and Scheme 1), live cultures are labeled initially with a cell surface marker, then grown in the absence of the marker (chase), and subsequently fixed and processed for electron microscopy. In this experiment, insertion of patches of new membrane might initially lead to the lateral displacement of labeled, already existing, plasmalemmal components. If insertion has indeed occurred, relabeling of a similar preparation with the same cell surface probe, after aldehyde fixation (Fig. 1C and Scheme 1), is expected to reveal new receptors for the surface probe in the previously bare areas. Consequently, the result of a relabeling experiment would be a uniform rather than a patchy distribution of the marker. Such a relabeling experiment can also be used to demonstrate selectively the newly inserted receptors for the ligand: Existing membrane receptors are first saturated with the unconjugated, invisible cell surface probe. The neurons are subsequently allowed to grow in the absence of the label, fixed, and finally labeled with the ferritin conjugate of the same lectin. This experiment would demonstrate only those receptors for the ligand that appeared in the plasmalemma during the chase period (Fig. 1D and Scheme 1).

### Lectin Labeling of Immobilized Growth Cones

As reported in the companion paper (39), the labeling with certain lectins of aldehyde-prefixed or cooled cultures of growing nerve cells results in almost uniform labeling of the entire surface of the neuron including perikaryon, neuritic shaft, and growth cones (cf. Fig. 1A). This has been demonstrated for neurons from rat spinal cord and SCG, labeled with F-WGA, F-RCA I (F- designates the ferritin conjugate of the lectin). Despite the slight proximo-distal decrease in F-WGA labeling of the SCG neuron (13%), F-WGA labeling of the growth cone and distal shaft areas is rather dense; the labeling with F-RCA I is uniformly dense throughout the SCG neuron (39). Fig. 2A shows a nerve growth cone whose surface could be studied over a length of many microns. The enlarged details of this

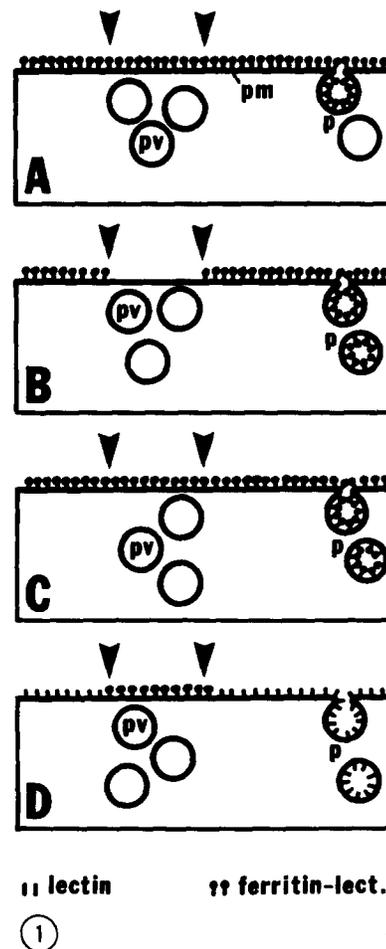


FIGURE 1 Rationale of pulse-chase experiments for the study of membrane expansion. A, labeling of cold- or fixative-immobilized neurite. B, result of single-label pulse-chase experiment. C, single-label pulse-chase experiment, relabeled. D, double-label pulse-chase experiment. pm, Plasma membrane; the arrowheads mark between them a putative area of insertion of new membrane components; p, pinocytotic vesicles, one of which is open to the extracellular space; pv, putative plasmalemmal precursor vesicle. For further explanation, see text.

growth cone (Fig. 2B-E) demonstrate that the surfaces of growth cone filopodia, SPVs (11, 33, 53), proximal and distal parts of the body of the growth cone, and neuritic shaft exhibit a consistently dense pattern of receptors for WGA. However, the fixation of SPVs is often less than ideal and may cause ballooning and ejection of membrane vesicles. The surfaces of such blisters are devoid of lectin receptors. This may lead to the erroneous conclusion that glycoconjugates are absent from part or all of the plasmalemma covering SPV clusters.

### Single-label Pulse-chase Experiments

These experiments, also described in Fig. 1B and Scheme 1, are a first attempt to study the dynamics of membrane components in the growing neurite. Often as early as 3 min, but always after chase periods of 15-20 min, the original, nearly uniform lectin distribution has changed. As shown in Fig. 3 (F-WGA, 20-min chase) and in Fig. 4 (F-RCA I, 12-min chase), the most distal portions of nerve growth cones are frequently seen to be free of the surface marker, whereas neighboring, somewhat more proximal areas exhibit an un-

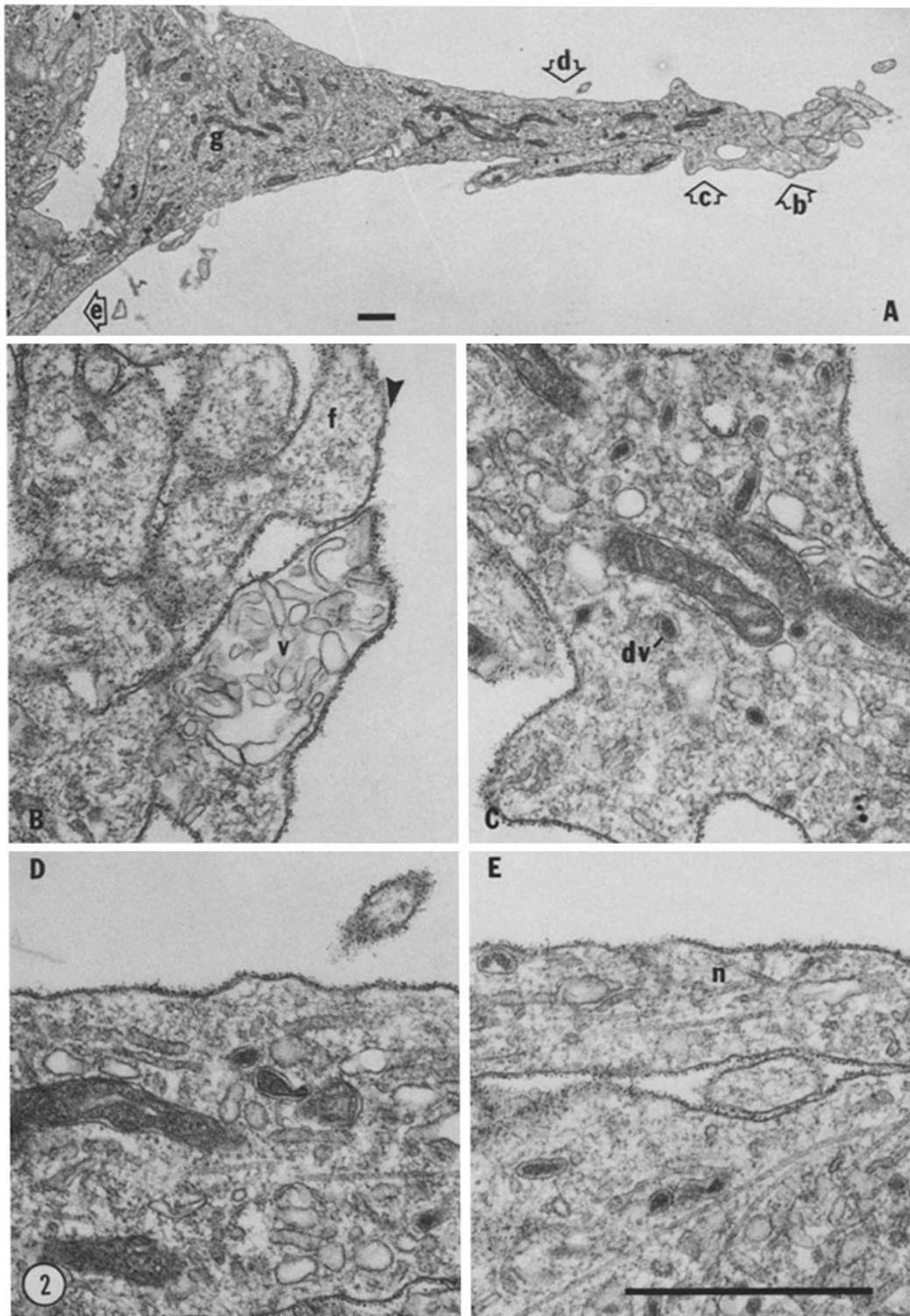


FIGURE 2 Nerve growth cone (*g*), aldehyde-fixed and labeled with F-WGA. Note the uniform distribution of lectin receptors in the various proximal and distal membrane regions shown at higher power in *B-E* (these micrographs show in greater detail *b-e*, respectively, in *A*). *f*, Filopodia; *v*, cluster of SPV; *dv*, dense core vesicles; *n*, neuritic shaft. Bar, 1  $\mu$ m. *A*,  $\times 5,800$ ; *B-E*,  $\times 37,700$ .

changed lectin labeling pattern. It is important to note that there is not a sharp boundary between bare surface areas of filopodia and the labeled, more proximal growth cone body region (Fig. 3); rather, there is a gradual increase in labeling density. Another area, in fact the one that is most consistently found free of ferritin in these pulse-chase experiments, is the surface of well-fixed SPV clusters and their immediate sur-

roundings (Fig. 11). These are found predominantly on growth cones but, occasionally, on neuritic shafts as well. Other neuritic shaft areas, by contrast, are consistently found to exhibit unchanged lectin receptor density and distribution, even after chase periods of 20 min (Fig. 5).

Internalization of ferritin-lectin conjugates is observed but seems not quite as prominent as the tracer intake suggested by

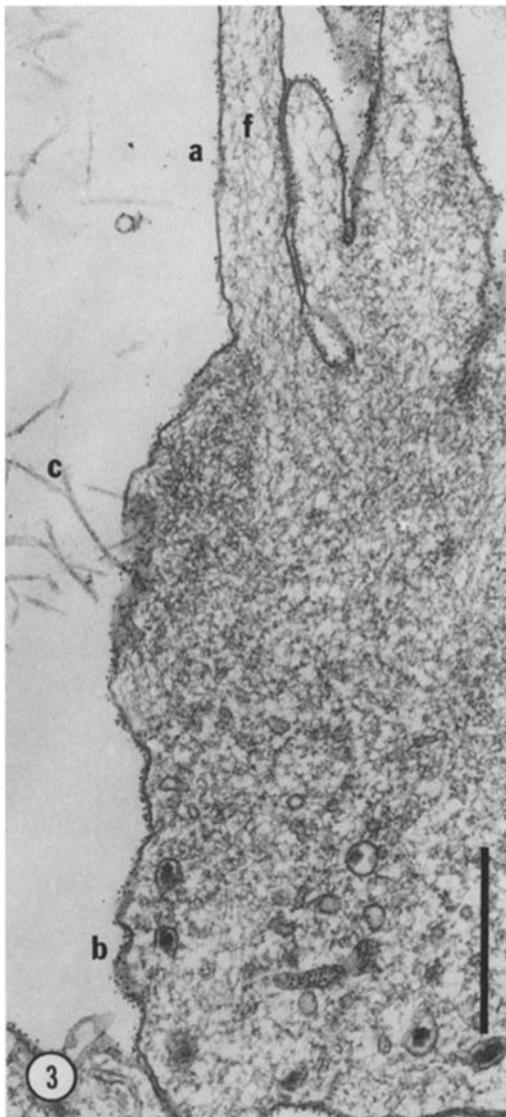


FIGURE 3 Single-label pulse-chase experiment with F-WGA, 20-min chase time. Note the reduction in label density (about threefold) on the most distal parts of the growth cone, the filopodia ( *f*, area *a*), and the gradual increase in ferritin density more proximally (area *b*). *c*, collagen fibers of the culture substratum. Bar, 0.5  $\mu\text{m}$ .  $\times 48,900$ .

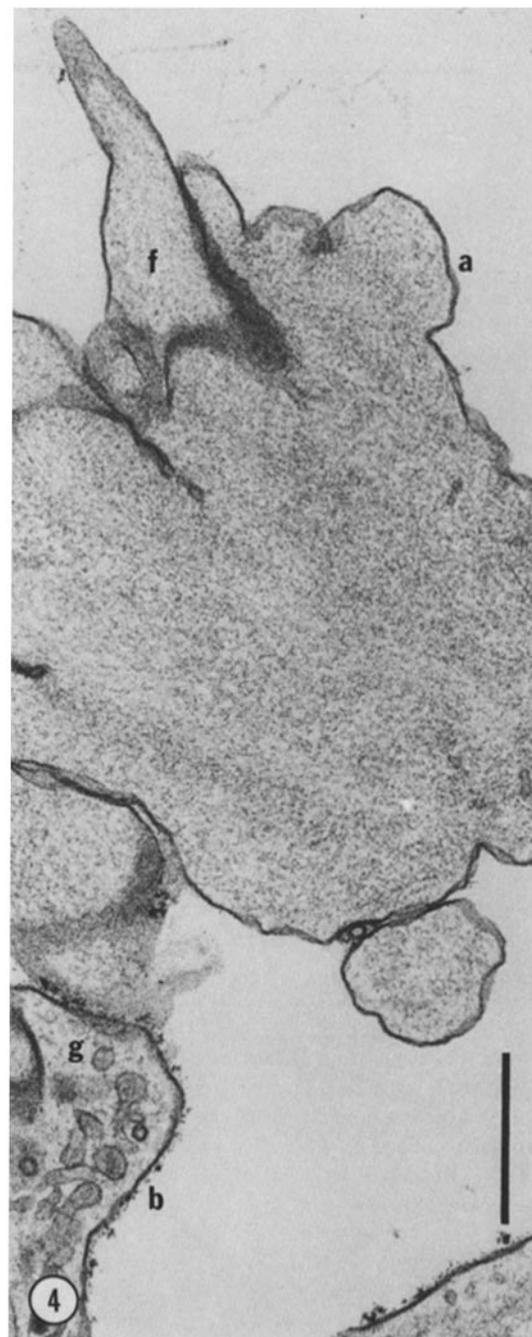


FIGURE 4 Single-label pulse-chase experiment with F-RCA I, 12-min chase time. Whereas the body of a growth cone ( *g*) is labeled (area *b*), microfilament-filled veils and filopodia ( *f*) of the same or of a neighboring growth cone are free of the marker (area *a*). Bar, 0.5  $\mu\text{m}$ .  $\times 44,500$ .

the studies of Birks et al. (6), del Cerro (15), and Bunge (12). This is consistent with the observation that, during chase for 15 min, the number of plasmalemmal lectin receptors at the growth cone is reduced only by a marginally significant 12% (39). Internalized label is frequently seen in coated vesicles (Fig. 5 *B*), which are common organelles in nerve growth cones and axonal shafts. Irregular smooth membrane cisternae are even more often seen to contain internalized tracer (Fig. 5). The internalized tracer is observed mainly within the growth cone body but rarely in filopodia (Figs. 3, 4, and 6) and not in

SPVs (Fig. 11). As a function of chase time, the changes in surface labeling and the internalization of the ligand become more extensive but do not differ in their patterns. Furthermore, they are identical for F-WGA and F-RCA I. Very similar observations are made for F-Con A, but this label tends to form clusters. Because of this irregular ligand distribution, which is suggestive of patching (cf. reference 24), F-Con A is not used as a label for the experiments described below.

No signs of toxic effects of the label can be detected during 30-min (or shorter) periods of exposure to the lectins. However,

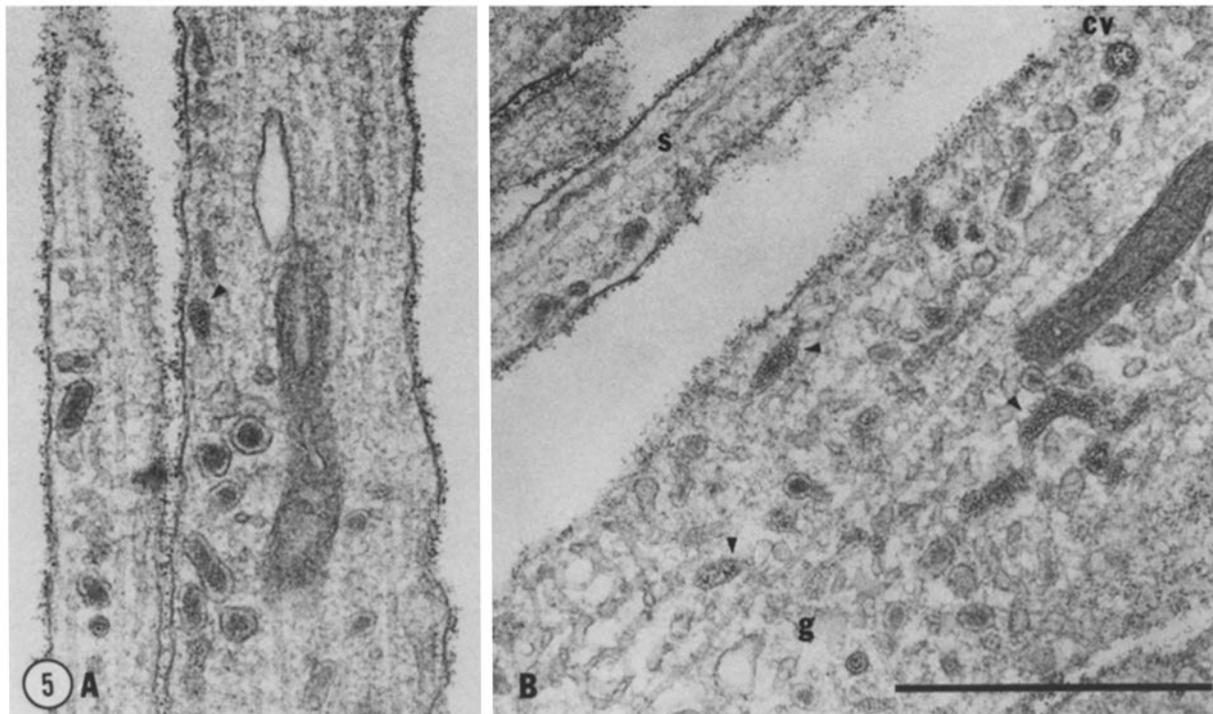


FIGURE 5 Single-label pulse-chase experiment with F-WGA, 15-min chase time. Note the completely uniform label distribution on the neuritic shafts (*A*; *s* in *B*). Some conjugate has been internalized into a coated vesicle (*cv*) and smooth membrane sacs (arrowheads). *g*, Growth cone body. Bar, 1  $\mu\text{m}$ .  $\times 42,500$ .

after longer chase times, neurites are seen frequently to retract and sometimes are phagocytosed by supporting cells in the cultures.

#### Single-label Pulse-chase Experiments, Relabeled

As explained above (Fig. 1 *C* and Scheme 1), preparations identical to the ones just described are relabeled after aldehyde fixation with the same ferritin-conjugated lectin. Fig. 6 illustrates the results obtained in such experiments. Proximal growth cone region, neuritic shaft, and the surfaces of filopodia and SPV clusters, which appear bare in the previous experiments, are completely and uniformly labeled. Densely labeled filopodia and SPV clusters may be seen on growth cones containing substantial amounts of internalized tracer that stem from the initial labeling step before the chase period (Figs. 6 *A* and *C*).

#### Double-label Pulse-chase Experiments

This last experiment (Fig. 1 *D* and Scheme 1) consists of an initial labeling step with a high concentration of unconjugated, invisible lectin, followed by a chase period (3–20 min), aldehyde fixation, and a second labeling step with a considerably lower concentration of the ferritin conjugate of the same lectin.

**CONTROLS CARRIED OUT AT 0°C:** In the absence of growth there is minimal ferritin labeling of neuritic shafts and growth cones, including the surfaces of filopodia and SPV clusters (Fig. 7; compare with Figs. 2 and 6). The sparse labeling in this control experiment shows that (*a*) under the conditions used, saturation with native lectin of existing plasmalemmal receptors is nearly complete, (*b*) the dissociation of bound native label from the membrane is slow, (*c*) exchange of ferritin-conjugated and unconjugated label during the second labeling step is not extensive, and (*d*) aldehyde fixation does

not induce the appearance of new, previously inaccessible lectin receptors.

**EXPERIMENTS CARRIED OUT AT 36°C:** Fig. 8 shows a small group of nerve growth cones at low power, chased for 3 min (compare with Figs. 2, 6, and 7; the estimated amount of growth during 3 min is 1–2  $\mu\text{m}$  of neurite elongation, corresponding to 1.5–3.0  $\mu\text{m}^2$  of surface expansion). Note that the more proximal plasmalemmal regions are quite poor in lectin label (triangles), whereas the most distal, filopodial regions are covered with ferritin particles at much higher density (asterisks). A small SPV cluster (*v*) can also be seen, and most of its surface is densely labeled with ferritin particles. Figs. 9 and 10 illustrate these results at higher magnification. High label density on a filopodium is evident in Fig. 9 *A* and is in contrast to the much lower label density on growth cone body (Fig. 9 *B*; cf. Fig. 2) and neuritic shaft (Fig. 9 *C*). The labeling of the surface of SPV clusters in this experiment (Fig. 10) is strikingly complementary to the result of the single-label experiment (Fig. 11): while the surfaces of SPV mounds are very densely labeled in the double-label pulse-chase experiments, these areas are found almost always label-free in the single-label pulse-chase experiment. Selective labeling of growth cone filopodia and of the surfaces of SPV clusters was found after chase periods of 3–20 min but was more frequently seen after the longer survival times of the cultures.

#### DISCUSSION

The almost uniform distribution of some lectin receptors on the growing SCG neuron enables us to use these carbohydrate-specific probes for studies of membrane dynamics, provided that experimental procedures are fast enough to overcome the problem of mobility of membrane components. Rapid lateral diffusion in the membrane and mixing of at least some of the

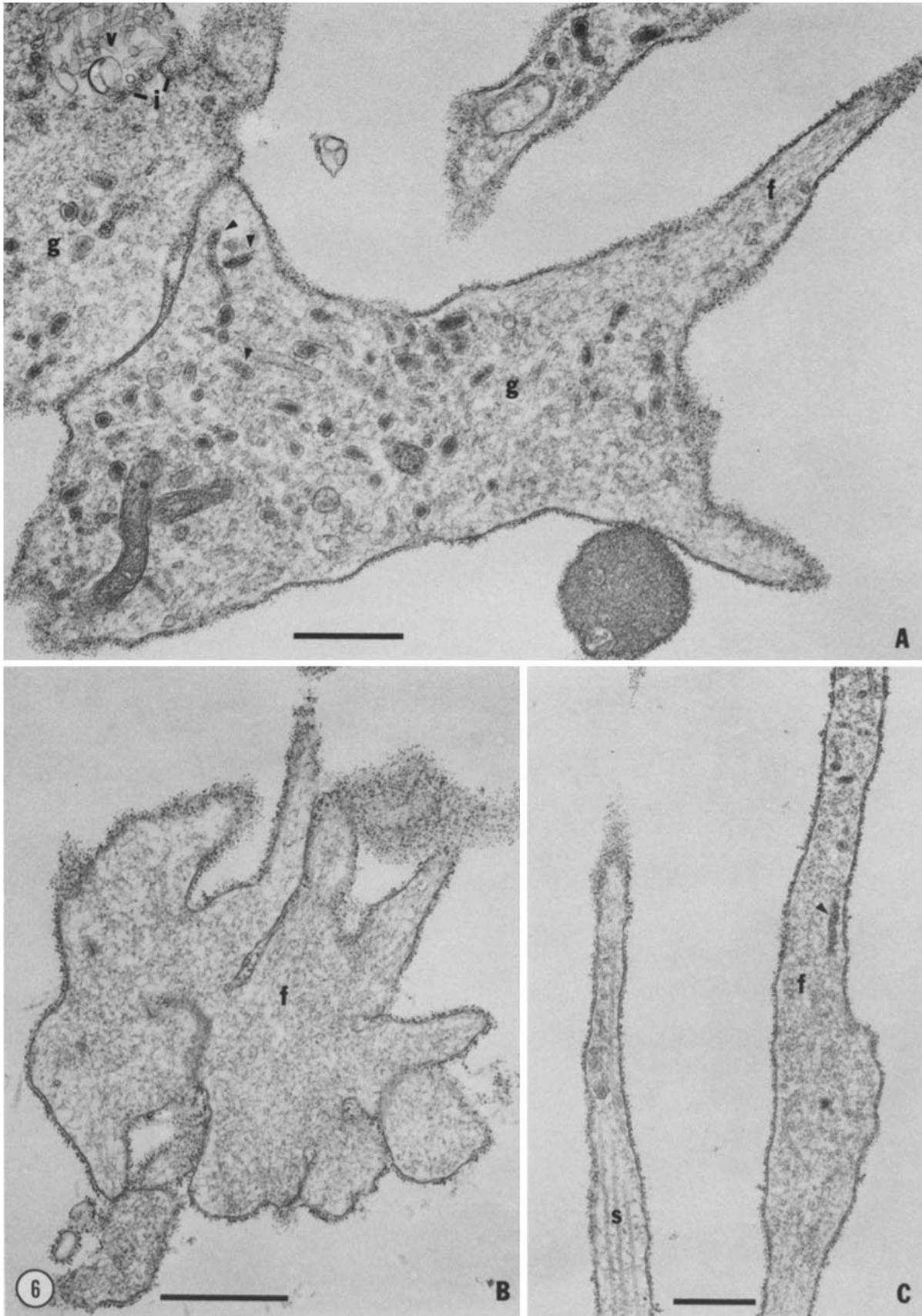


FIGURE 6 Single-label pulse-chase experiment with F-WGA, labeled. 15-min chase time. Note completely uniform label distribution including the surfaces of growth cone filopodia (*f*) and neuritic shafts (*s*). Note internalized conjugate in smooth membrane sacs (arrowheads). The vesicles of an SPV cluster (*v*) are not labeled. Its basal invaginations (*i*), however, contain the tracer. Bars, 0.5  $\mu$ m. A,  $\times 38,000$ ; B,  $\times 44,100$ ; and C,  $\times 28,800$ .

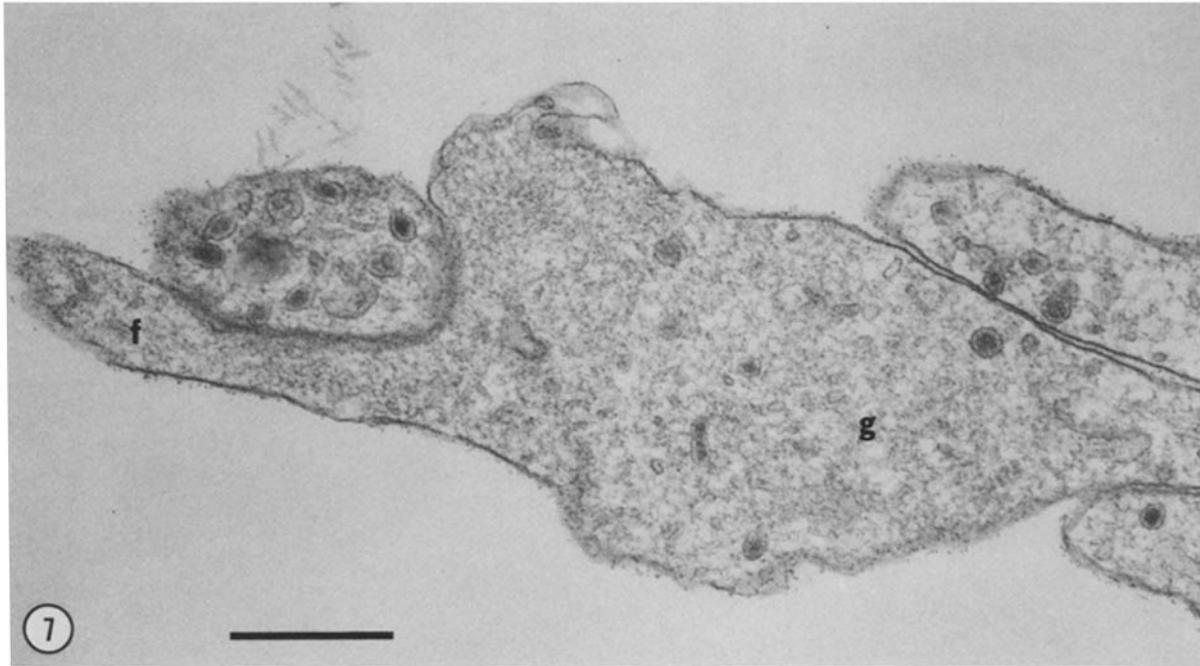


FIGURE 7 Double-label pulse-chase experiment with WGA and F-WGA. Control, carried out on ice, 9-min chase time. Note the virtual absence of the ferritin conjugate from the various growth cone ( *g* ) areas shown here. *f*, filopodium. Bar, 0.5  $\mu\text{m}$ .  $\times 43,000$ .

lectin receptors is likely to occur (e.g., references 17 and 18). Thus, these experiments can be successful only if their time-course is sufficiently fast to capture and localize the lectin receptors before lateral diffusion has randomized preexisting and newly inserted glycoconjugates. With WGA and RCA I, pulse-chase experiments can be carried out without obvious toxic effects on the cells. Con A may induce patching of lectin receptors within the short period of observation and, therefore, does not seem to be suitable. It should be emphasized that the lectins are likely to have a variety of receptors on the neuritic surface, including both glycoproteins and glycolipids. At present, we are unable to distinguish between these two major classes of glycoconjugates, so that the results shown here may pertain to the dynamics of either or both classes of lectin receptors. It should also be stressed that the distribution of lectin receptors does not parallel the distribution of intramembranous particles in the growing neuron: although intramembranous particles decrease in density from the perikaryon to the growth cone by about one order of magnitude, lectin receptors may only slightly decrease, remain constant, or even increase in density as one moves distally to the growth cone (cf. references 35, 39, 45, and 46).

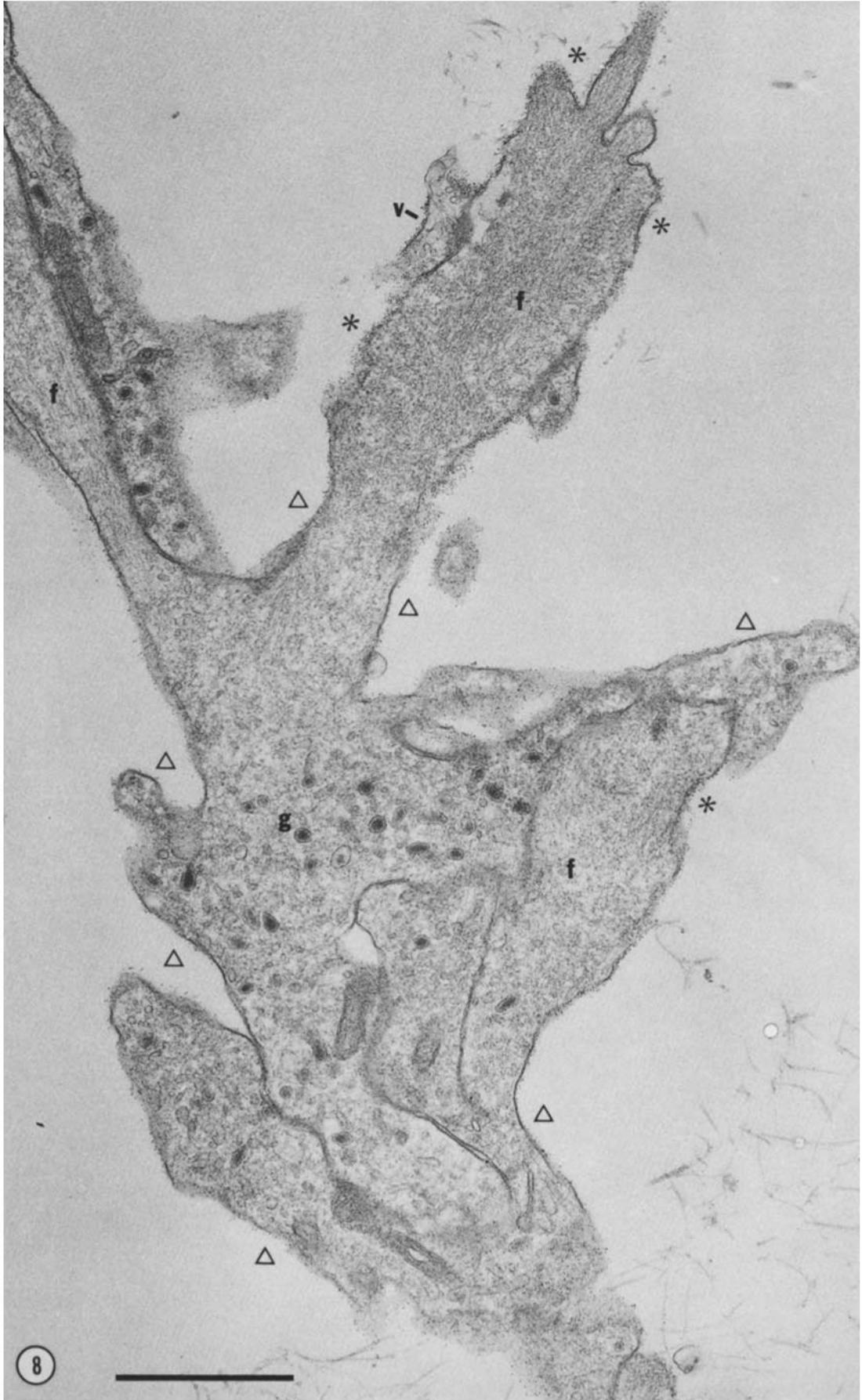
#### Relative Movement of Neuritic Lectin Receptors during Plasmalemmal Growth

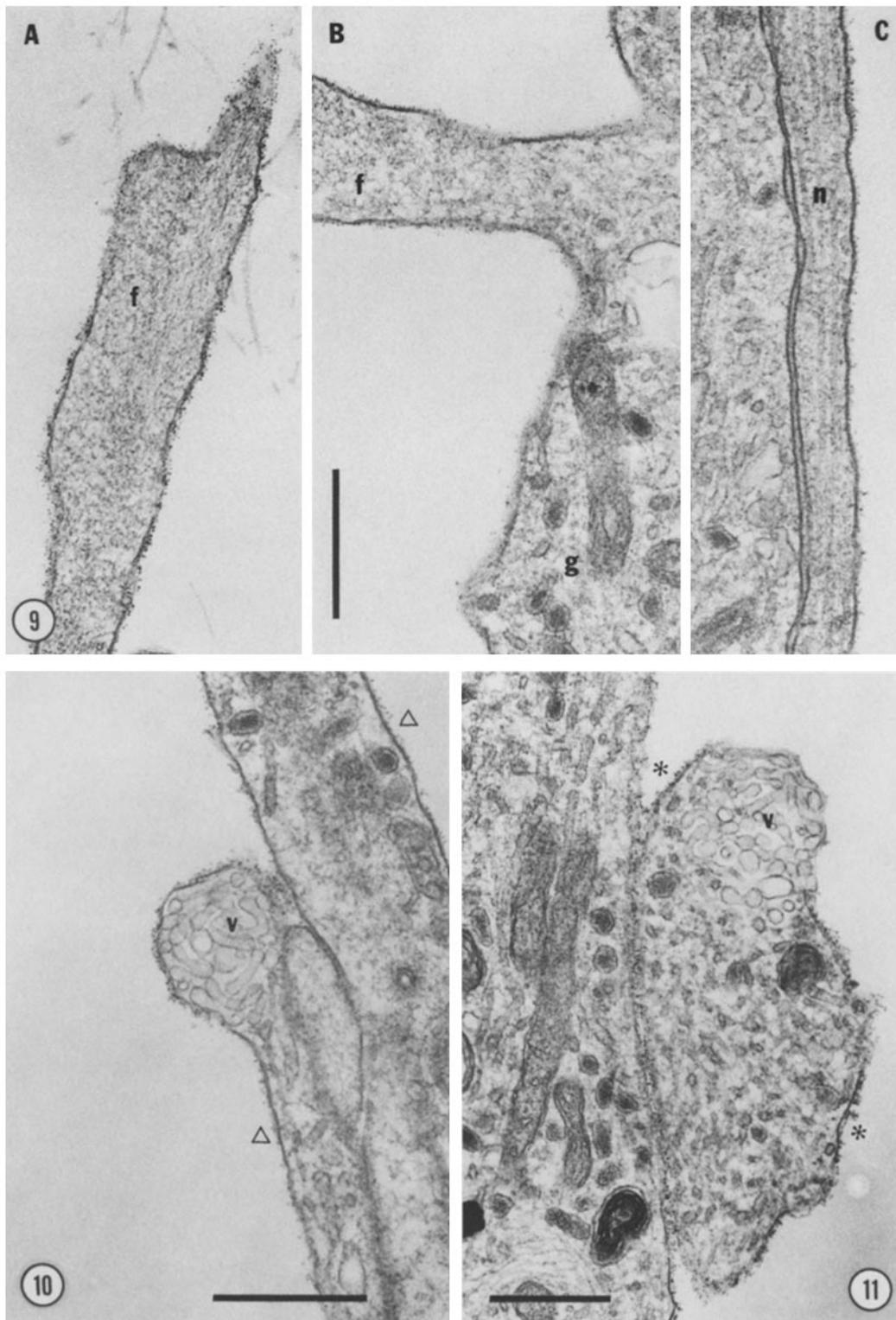
The numerous single-label pulse-chase experiments with various chase and growth periods demonstrate that, for times of 3–20 min, changes in the distribution of labeled lectin

receptors occur predominantly within the growth cone region, on the surface of its filopodia and numerous SPV clusters, but not along the neuritic shafts—except, again, on the surface of SPV clusters, which can be observed on the shafts occasionally. The findings are the same for WGA concentrations from 50 to 500  $\mu\text{g}/\text{ml}$  and for the saturating amount of RCA I. Retrograde transport of lectin as observed by Koda and Partlow (23), who used Con A linked to large particles, is not evident in this material. The nature and selective location of changes in lectin receptor distribution reported here point to the growth cone as the main site of membrane dynamics. The critical question is whether the (relative) movements of membrane components described here are related to the growth process, i.e., to plasmalemmal expansion by the insertion of new materials, or to some other cellular function.

One cannot immediately rule out the possibility that the bare areas observed in the single-label pulse-chase experiments are the result of localized clustering—patching or capping—of cross-linked lectin receptors (16, 31, 48, 49, 51) because of the divalent (RCA I) and tetravalent (WGA) nature of the lectins used (26). However, the transition from bare to labeled cell surface as one moves from the tip of a filopodium to more proximal neuritic regions (or from an SPV cluster to the plasmalemma surrounding it) is always gradual, never formed by a zone of particularly densely packed ferritin particles. Furthermore, we have not found dense patches of surface label within bare membrane regions, nor is there much internalization occurring in the growth cone periphery (39). Thus, the observed labeling pattern is not consistent with the occurrence

FIGURE 8 Double-label pulse-chase experiment with WGA and F-WGA, 3-min chase time. A small group of growth cones ( *g* ) is shown. Note the abundance of ferritin label on filopodia ( *f* ), the most distal, microfilament-filled regions of the growth cones (asterisks), while the more proximal growth cone areas, as recognized by their content of membrane-bounded organelles, exhibit minimal amounts of the marker (triangles). A heavily labeled SPV cluster ( *v* ) is also visible on this section. Bar, 1  $\mu\text{m}$ .  $\times 30,000$ .





FIGURES 9-11 Double-label pulse-chase experiments with WGA and F-WGA (Figs. 9 and 10), 3-min (Fig. 9 A-C) and 15-min (Fig. 10) chase times, and, for comparison, a single-label pulse-chase experiment with the same lectin, 15-min chase time (Fig. 11). The high-power views allow for a more detailed examination of lectin labeling patterns. Note the high density of ferritin particles on filopodia (*f* in Fig. 9 A and B) and on an SPV cluster (*v* in Fig. 10) while more proximal regions, growth cone body (*g* in Fig. 9 B) and neuritic shaft (*n* in Fig. 9 C), are only very sparsely labeled. The appearance of SPV clusters in the double label experiment (Fig. 10) is strikingly complementary to that in the single-label experiment (Fig. 11): note the reversal of heavily labeled (asterisks) and marker-free (triangles) membrane areas. Bars, 0.5  $\mu$ m. Fig. 9 A-C,  $\times 46,000$ ; Fig. 10,  $\times 46,800$ ; and Fig. 11,  $\times 37,000$ .

of patching or capping. However, the critical result ruling for or against patching- or capping-like redistribution of lectin receptors comes from the experiment in which pulsed and chased material is relabeled after aldehyde fixation. The ab-

sence of bare membrane patches in these relabeling experiments suggests that new lectin receptors appear in specific cellular regions as previously labeled lectin receptors are moved away from them. However, we have to consider the possibility

that the newly labeled lectin receptors have already been present on the cell surface, sterically inaccessible to the marker but uncovered by the lateral displacement of labeled glycoconjugates during the chase period. This explanation of our findings does not seem likely for the following reasons: (a) The phenomenon of cocapping (52) speaks against the presence of two independently mobile lectin receptor classes with the same specificity. (b) Identical results can be produced with two different lectins, WGA and RCA I (steric hindrance and independence of mobility are unlikely to apply to two different lectins in the same way). (c) Most importantly, even though the WGA and RCA I receptors appear closely spaced in cross sections, they are, in reality, quite far apart from each other, as can be seen in tangential sections through the membrane (Fig. 6 B) and can be calculated from the lectin receptor densities;<sup>2</sup> this renders very unlikely the existence of a second, independent population of receptors for WGA and RCA I masked by the glycoconjugates labeled during the first step of the experiment. In conclusion, the movement of lectin receptors observed in the growing neurite cannot be explained by capping or patching. In fact, patching of WGA or RCA I receptors does not seem to occur at all on the growing neurite within the first 30 min of observation, under the present experimental conditions.

This conclusion is in apparent conflict with the findings of Letourneau (24) and of Wessells et al. (50). However, the disagreement may be explained as follows: Because of only minimal rinsing of the cultures before the experiments, contamination of cell surfaces with medium glycoproteins may cause large glycoprotein-lectin or (glyco)protein-cationized ferritin aggregates and, thus, patchy appearance of the label in both cases. Chase with complete medium is likely to further enhance the formation and eventual shedding from the cell surface of (glyco)protein-cationized ferritin aggregates in the experiments of Wessells et al. (50), even in the cold. Con A, especially if used at low concentration as in Letourneau's experiments (24), may indeed cause patching of membrane components as we suspect from our own observations and, therefore, may not be comparable to WGA and RCA I.

In agreement with other investigators who have observed internalization of compartment tracers (6, 12, 15), we find that membrane markers also are internalized by coated vesicles as well as smooth membrane sacs, both of which are abundant in distal neuritic regions. After longer periods of exposure (15 min), considerable amounts of surface label are found in multivesicular bodies. However, our results differ significantly from those of the other authors on one point: after chase periods of 3–20 min, we do not find the lectin label in SPVs. This rules out the possibility of their being pinocytotic in nature. This problem will be dealt with in a forthcoming communication.

### *Appearance of New Lectin Receptors during Plasmalemmal Growth*

The double-label pulse-chase experiments are designed to demonstrate selectively those cell surface glycoconjugates that become accessible to the marker during the chase period (cf.

reference 10). As explained above, it is very likely that these glycoconjugates become selectively labeled because they are newly inserted into the plasmalemma, and not because they have been unmasked by the patching of other lectin receptors. Two further arguments can be added: (a) In the double-label experiment, the initial labeling step is carried out with the unconjugated lectin, which is both much smaller than its ferritin conjugate<sup>3</sup> and is applied at twice the concentration of the conjugate. Therefore, initial labeling is done under conditions that favor access to, and saturation of, existing lectin receptors. (b) The result of the control experiments carried out on ice—the virtual absence of ferritin marker on the growth cone (except for a low, random “background”)—excludes the possibility of fixation-induced exposure of otherwise hidden lectin receptors on filopodia and SPV clusters. These control experiments, as well as the controls with hapten sugar (39), also exclude the possibility of regional nonspecific binding. We can thus conclude that the demonstration of lectin receptors in the double-label pulse-chase experiments is selective for glycoconjugates that have been inserted into the plasma membrane during the chase period, i.e., during neuritic growth.

The short time-scale of our experiments does not allow one to investigate the fate of membrane components after incorporation into the plasmalemma, especially their spreading proximally on the neuritic shaft. Nevertheless, it appears to be possible to localize membrane glycoconjugates before complete mixing with existing components has taken place. It is possible that cross-linking of existing glycoconjugates by saturating concentrations of the divalent or tetravalent lectins slows down lateral mobility of membrane components (16, 51) and favors the study of insertion of new lectin receptors described here. Complete randomization of existing and newly inserted membrane components, however, is expected to occur eventually. Nothing is known about the intrinsic fluidity of nerve growth cone plasmalemma, nor do we have information on cytoplasmic anchoring of its integral membrane proteins. Therefore, it is impossible at present even to estimate diffusion rates of membrane components and to compare them with the rate of plasmalemmal expansion and the speed of dislocation of labeled membrane components seen in these experiments. However, plasmalemmal growth and label dislocation rates seem quite compatible. The calculated rate of membrane expansion is  $\sim 0.5 \mu\text{m}^2/\text{min}$ , and the selectively labeled membrane areas found after 3–15 min of chase are estimated at a few to several square microns.

The low density of ferritin-labeled lectin receptors on the proximal growth cone and neuritic shaft in the double-labeling experiments raises the question of whether these are also newly inserted glycoconjugates. Although we cannot answer this question fully, some of this more proximal, random label may be explained by diffusion from sites of insertion. On the other hand, the control material double-labeled in the cold also exhibits random “background label,” suggesting that this is attributable to dissociation of unconjugated lectin during the chase and/or exchange with the ferritin conjugate during the second labeling step. However, random insertion into the plasmalemma of a small number of these lectin receptors cannot be excluded at present.

Our conclusion that the appearance of certain new lectin

<sup>2</sup> On SCG growth cones, both WGA and RCA I receptors occur at an average distance of  $\sim 22$  nm from each other ( $\sim 2,300$  binding sites/ $\mu\text{m}^2$  for both lectins, assuming even distribution in a single plane [39]), whereas the diameter of the ferritin conjugate is only  $\sim 12$  nm (20).

<sup>3</sup> The native lectins have molecular weights of 37,500 (WGA) and 120,000 (RCA I) as compared with ferritin-conjugated lectins whose molecular weight and diameter are  $\sim 1,000,000$  and 12 nm, respectively (20).

receptors is a localized phenomenon occurring predominantly within the growth cone region, i.e., on the surface of filopodia and SPV clusters (and only to a limited degree on neuritic shafts), is consistent with the predictions of distal growth made by Hughes (22) and by Bray (8, 9). Furthermore, in consonance with the lectin binding results, radioautographic and biochemical studies on phospholipids (34) suggest that these membrane components are synthesized in the neuronal perikaryon and are shuttled primarily to distal, neuritic SPV cluster areas where exocytosis-like fusion of the vesicles may lead to the expansion of the plasmalemma. The various results obtained by lectin labeling, radioautography, and also by freeze-fracture (35) leave little doubt that SPV clusters are regions of special functional significance in the growing cell. However, their structural configuration and even their existence are still subject to debate (21, 28, 33). This problem, as well as a more detailed description of the lectin labeling of SPV areas, will be dealt with in a forthcoming paper.

Regardless of the SPV question, selective appearance of new lectin receptors primarily at the level of the growth cone establishes in the sprouting neuron polarized—rather than random—export of newly synthesized membrane components from the sites of synthesis to the cell periphery (cf. reference 34). Most probably, the new plasmalemmal constituents are transported in the form of preassembled membrane. This phenomenon, for which there is increasing evidence in the retinal rod as well (e.g., references 5 and 54), may be a major mechanism for the establishment of specialized membrane regions in polarized cells. Such a mechanism may readily explain the biochemical (lectin receptors), functional ( $\text{Na}^+$  channels), and morphological (intramembranous particles) differences observed when the plasmalemma of growth cone and perikaryon in the sprouting neuron are compared (35, 39, 45, 47). However, the time-dependent, gradual increase in the density of intramembranous particles in the axolemma of differentiating neurons, a process of membrane maturation (35), indicates that the newly inserted membrane, albeit preassembled, is incomplete. This implies that membrane biogenesis is a stepwise phenomenon, involving the later insertion of additional components. Incorporation into the plasmalemma of additional membrane constituents may occur by polarized insertion, possibly combined with subsequent redistribution, or by a random mechanism. The work of Carbonetto and Fambrough (14) indicates that insertion of  $\alpha$ -bungarotoxin binding sites into the plasmalemma of the sprouting neuron is likely to be generalized but predominant at the perikaryon. Perikaryal insertion would also explain the distribution of certain classes of intramembranous particles in the growing axon (45). The appearance of patches of new, immature membrane at the growth cone amalgamates two seemingly opposite views of membrane biogenesis, polarized insertion of preassembled membrane and stepwise membrane biogenesis.

This work was started in the Section of Cell Biology at Yale University, School of Medicine, and the authors are very grateful to Dr. George E. Palade for his many helpful suggestions and constructive criticisms regarding the experiments as well as the preparation of this manuscript. During the authors' tenure at Yale University this work also profited from the excellent technical assistance by Hans Stukenbrok and Mark Tramo. At Columbia University, Marian Johnson and Linda Friedman greatly contributed to these studies with their expert technical assistance. Christine Wade offered skillful secretarial help with the completion of this manuscript. The assistance of all these collaborators is herewith gratefully acknowledged.

This work was supported by U. S. Public Health Service grant NS34166 and an Irma T. Hirsch Career Scientist Award to K. H. Pfenninger.

Received for publication 22 August 1980, and in revised form 27 January 1981.

## REFERENCES

- Abercrombie, M., J. E. M. Heaysman, and S. M. Pegrum. 1972. Locomotion of fibroblasts in culture. V. Surface marking with concanavalin A. *Expt. Cell Res.* 73:536-539.
- Arnold, J. M. 1976. Cytokinesis in animal cells: new answers to old questions. In *The Cell Surface in Animal Embryogenesis and Development*. G. Poste and G. L. Nicolson, editors. North Holland Biomedical Press, Amsterdam. 55-80.
- Atkinson, P. H., S. A. Moyer, and D. F. Summers. 1976. Assembly of vesicular stomatitis virus glycoprotein and matrix protein into HeLa cell plasma membranes. *J. Mol. Biol.* 102:613-631.
- Besharse, J. C., and K. H. Pfenninger. 1978. Freeze-fracture of rod and cone photoreceptors in relationship to membrane assembly and degradation. *J. Cell Biol.* 79 (2, Pt. 2):97a (Abstr.).
- Besharse, J. C., and K. H. Pfenninger. 1980. Membrane assembly in retinal photoreceptors. I. Freeze-fracture analysis of cytoplasmic vesicles in relationship to disc assembly. *J. Cell Biol.* 87:451-463.
- Birks, R. I., M. C. Mackey, and P. R. Weldon. 1972. Organelle formation from pinocytotic elements in neurites of cultured sympathetic ganglia. *J. Neurocytol.* 1:311-340.
- Bluemink, J. G., and S. W. DeLaat. 1973. New membrane formation during cytokinesis in normal and cytochalasin B treated eggs of *Xenopus laevis*. I. Electron microscopic observations. *J. Cell Biol.* 59:89-108.
- Bray, D. 1970. Surface movements during the growth of single explanted neurons. *Proc. Natl. Acad. Sci. U. S. A.* 65:905-910.
- Bray, D. 1973. Branching patterns of isolated sympathetic neurons. *J. Cell Biol.* 56:702-712.
- Brown, S. S., and J.-P. Revel. 1976. Reversibility of cell surface label rearrangement. *J. Cell Biol.* 68:629-641.
- Bunge, M. B. 1973. Fine structure of nerve fibers and growth cones of isolated sympathetic neurons in culture. *J. Cell Biol.* 56:713-735.
- Bunge, M. B. 1977. Initial endocytosis of peroxidase or ferritin by growth cones of cultured nerve cells. *J. Neurocytol.* 6:407-439.
- Bunge, R. P., and P. Wood. 1973. Studies on the transplantation of spinal cord tissue in the rat. I. The development of a culture system for hemisections of embryonic spinal cord. *Brain Res.* 57:261-276.
- Carbonetto, S., and D. M. Fambrough. 1979. Synthesis, insertion into the plasma membrane, and turnover of  $\alpha$ -bungarotoxin receptors in chick sympathetic neurons. *J. Cell Biol.* 81:555-569.
- del Cerro, M. P. 1974. Uptake of tracer proteins in the developing cerebellum particularly by the growth cones and blood vessels. *J. Comp. Neurol.* 157:245-280.
- Edelman, G. M., I. Yahara, and J. L. Wang. 1973. Receptor mobility and receptor-cytoplasmic interactions in lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* 70:1442-1446.
- Edidin, M. 1974. Rotational and translational diffusion in membranes. *Annu. Rev. Biophys. Bioeng.* 3:179-201.
- Edidin, M., and D. Fambrough. 1973. Fluidity of the surface of cultured muscle fibers. Rapid lateral diffusion of marked surface antigens. *J. Cell Biol.* 57:27-37.
- Grove, S. N., and C. E. Bracker. 1970. Protoplasmic organization of hyphal tips among fungi: vesicles and spitzkörper. *J. Bacteriol.* 104:989-1009.
- Haggis, G. H. 1965. The iron oxide core of the ferritin molecule. *J. Mol. Biol.* 14:598-602.
- Hasty, D. L., and E. D. Hay. 1978. Freeze-fracture studies of the developing cell surface. II. Particle-free membrane blisters on glutaraldehyde-fixed corneal fibroblasts are artefacts. *J. Cell Biol.* 78:756-768.
- Hughes, A. F. 1953. The growth of embryonic neurites. A study of cultures of chick neural tissue. *J. Anat.* 87:150-162.
- Koda, L. Y., and L. M. Partlow. 1976. Membrane marker movement on sympathetic axons in tissue culture. *J. Neurobiol.* 7:157-172.
- Letourneau, P. C. 1979. Inhibition of intercellular adhesion by concanavalin A is associated with concanavalin A-mediated redistribution of surface receptors. *J. Cell Biol.* 80:128-140.
- Letourneau, P. C., and N. K. Wessells. 1974. Migratory cell locomotion versus nerve axon elongation. Differences based on the effects of lanthanum ion. *J. Cell Biol.* 61:56-69.
- Lis, H., and N. Sharon. 1977. Lectins: their chemistry and application to immunology. In *The Antigens*. M. Sela, editor. Academic Press, Inc., New York. 4:429-529.
- Maylié-Pfenninger, M.-F., and J. D. Jamieson. 1979. Distribution of cell surface saccharides on pancreatic cells. I. General method for preparation and purification of lectins and lectin-ferritin conjugates. *J. Cell Biol.* 80:69-76.
- Nuttall, R. P., and N. K. Wessells. 1979. Veils, mounds and vesicle aggregates in neurons elongating in vitro. *Exp. Cell Res.* 119:163-174.
- Palade, G. E. 1975. Intracellular aspects of the process of protein secretion. *Science (Wash. D. C.)* 189:347-358.
- Partlow, L. M., and M. G. Larrabee. 1971. Effects of nerve-growth factor, embryo age and metabolic inhibitors on growth of fibers and on synthesis of ribonucleic acid and protein in embryonic sympathetic ganglia. *J. Neurochem.* 18:2101-2118.
- Pernis, B., L. Forni, and L. Amante. 1970. Immunoglobulin spots on the surface of rabbit lymphocytes. *J. Exp. Med.* 132:1001-1018.
- Pfenninger, K. H. 1979. Synaptic membrane differentiation. In *The Neurosciences: Fourth Study Program*. F. O. Schmitt, editor. Massachusetts Institute of Technology Press, Cambridge, Mass. 779-795.
- Pfenninger, K. H. 1979. Subplasmalemmal vesicle clusters: real or artifact? In *Freeze-fracture: Methods, Artifacts, and Interpretations*. J. E. Rash and C. S. Hudson, editors. Raven Press, New York. 71-80.
- Pfenninger, K. H. 1980. Mechanism of membrane expansion in the growing neuron. *Soc. Neurosci. Symp.* 6:661.
- Pfenninger, K. H., and R. P. Bunge. 1974. Freeze-fracturing of nerve growth cones and young fibers. A study of developing plasma membrane. *J. Cell Biol.* 63:180-196.
- Pfenninger, K. H., and M.-F. Maylié-Pfenninger. 1975. Distribution and fate of lectin binding sites on the surface of growing neuronal processes. *J. Cell Biol.* 67 (2, Pt. 2):332a (Abstr.).

37. Pfenninger, K. H., and M.-F. Maylié-Pfenninger. 1977. Localized appearance of new lectin receptors on the surface of growing neurites. *J. Cell Biol.* 75 (2, Pt. 2):54a (Abstr.).
38. Pfenninger, K. H., and M.-F. Maylié-Pfenninger. 1978. Characterization, distribution, and appearance of surface carbohydrates on growing neurites. In *Neuronal Information Transfer*. A. Karlin, V. M. Tennyson, and H. J. Vogel, editors. Academic Press, Inc., New York. 373-386.
39. Pfenninger, K. H., and M.-F. Maylié-Pfenninger. 1981. Lectin labeling of sprouting neurons. I. Regional distribution of surface glycoconjugates. *J. Cell Biol.* 89:536-546.
40. Rothman, J. E., H. Bursztyjn-Pettegrew, and R. E. Fine.: Transport of the membrane glycoprotein of vesicular stomatitis virus to the cell surface in two stages by clathrin-coated vesicles. *J. Cell Biol.* 86:162-172.
41. Rothman, J. E., and H. F. Lodisch. 1977. Synchronised transmembrane insertion and glycosylation of a nascent membrane protein. *Nature (Lond.)*. 269:775-780.
42. Sabatini, D. D., and G. Kreibich. 1976. Functional specialization of membrane-bound ribosomes in eukaryotic cells. In *The Enzymes of Biological Membranes*. A. Martonosi, editor. Plenum Publishing Co., New York. 2:531-579.
43. Sidman, R. L. 1974. Contact interaction among developing mammalian brain cells. In *The Cell Surface in Development*. A. A. Moscona, editor. J. Wiley & Sons, New York. 221-253.
44. Siekevitz, P. 1972. Biological membranes: the dynamics of their organization. *Annu. Rev. Physiol.* 34:117-140.
45. Small, R., and K. H. Pfenninger. 1978. Properties and maturation of axolemma in growing neurons. *Soc. Neurosci. Symp.* 6:661.
46. Small, R., G. R. Strichartz, and K. H. Pfenninger. 1979. Membrane properties of the growing axon: intramembranous particles and saxitoxin binding sites. *J. Cell Biol.* 83 (2, Pt. 2):279a (Abstr.).
47. Strichartz, G., R. Small, C. Nicholson, K. H. Pfenninger, and R. Llinas. 1980. Ionic mechanisms for impulse propagation in growing nonmyelinated axons: saxitoxin binding and electrophysiology. *Soc. Neurosci. Symp.* 6:660.
48. Taylor, R. B., P. H. Duffus, M. C. Raff, and S. DePetris. 1971. Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by anti-immunoglobulin antibody. *Nature New Biol.* 233:225-229.
49. Unanue, E. R., W. D. Perkins, and M. J. Karnovsky. 1972. Ligand-induced movement of lymphocyte membrane macromolecules. I. Analysis by immunofluorescence and ultrastructural radioautography. *J. Exp. Med.* 136:885-906.
50. Wessells, N. K., R. P. Nuttall, J. T. Wrenn, and S. Johnson. 1976. Differential labeling of the cell surface of single ciliary ganglion neurons in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 73:4100-4104.
51. Yahara, I., and G. M. Edelman. 1972. Restriction of the mobility of lymphocyte immunoglobulin receptors by concanavalin A. *Proc. Natl. Acad. Sci. U. S. A.* 69:608-612.
52. Yahara, I., and G. M. Edelman. 1975. Modulation of lymphocyte receptor mobility by locally bound concanavalin A. *Proc. Natl. Acad. Sci. U. S. A.* 72:1579-1583.
53. Yamada, K. M., B. S. Spooner, and N. K. Wessells. 1971. Ultrastructure and function of growth cones and axons of cultured nerve cells. *J. Cell Biol.* 49:614-635.
54. Young, R. W. 1974. Biogenesis and renewal of visual cell outer segment membranes. *Exp. Eye Res.* 18:215-223