

Fluorescent Carbocyanine Dyes Allow Living Neurons of Identified Origin to Be Studied in Long-Term Cultures

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Abstract. A prerequisite for many studies of neurons in culture is a means of determining their original identity. We needed such a technique to study the interactions in vitro between a class of spinal cord neurons, sympathetic preganglionic neurons, and their normal target, neurons from the sympathetic chain. Here, we describe how we use two highly fluorescent carbocyanine dyes, which differ in color but are otherwise similar, to identify neurons in culture.

The long carbon chain carbocyanine dyes we use are lipid-soluble and so become incorporated into the plasma membrane. Neurons can be labeled either retrogradely or during dissociation. Some of the labeled membrane gradually becomes internalized and retains its fluorescence, allowing identification of cells for several weeks in culture. These dyes do not affect the survival, development, or basic physiological prop-

erties of neurons and do not spread detectably from labeled to unlabeled neurons.

It seems likely that cells become retrogradely labeled mainly by lateral diffusion of dye in the plane of the membrane. If so, carbocyanine dyes may be most useful for retrograde labeling over relatively short distances. An additional feature of carbocyanine labeling is that neuronal processes are brightly fluorescent for the first few days in culture, presumably because dye rapidly diffuses into newly inserted membrane.

We have used carbocyanine dyes to identify sympathetic preganglionic neurons in culture. Our results indicate that preganglionic neurons can survive in the absence of their target cells and that several aspects of their differentiation in the absence of target appear normal.

CULTURES of dissociated neurons allow unrivaled access to cells for biophysical, developmental, and cell biological investigation. Neurons from peripheral ganglia have been most extensively studied since they can be dissected out of the embryo as relatively homogeneous populations. In contrast, it is rarely possible to dissect out a particular class of central neurons because many different cell types are present within any small area of central nervous system. Consequently, cultures made from the brain or spinal cord contain many different kinds of neurons. In vivo, a neuron can be identified as belonging to a particular cell class by its axonal projections, its position, its morphology, and its transmitter biochemistry. None of these features is a reliable means of identifying cells in vitro, since the process of dissociation strips neurons of their axonal projections and cytoarchitectonic features, and numerous studies have shown that the morphological (Denis-Donini et al., 1984; Mudge, 1984) and biochemical (Patterson, 1978) properties of at least some neurons in vivo can be influenced by culture conditions. Even many peripheral ganglia contain several subpopulations of neurons that differ with respect to their synaptic connections but are not otherwise easily distinguishable. Methods that allow for the definitive determination of the original identity of neurons in culture are therefore clearly a prerequisite for studies of the properties of many types of

neurons, as well as for studies of the interactions between neurons.

Several variants of a general scheme for identifying the origin of neurons in culture have recently been described (Bennett et al., 1980; McPheeters and Okun, 1980; Okun, 1981; Tosney and Landmesser, 1981; O'Brien et al., 1982; Schaffner et al., 1983; Tanaka and Obata, 1983; Calof and Reichardt, 1984; Leifer et al., 1984; see also Suzuki, 1984). They take advantage of the ability of neurons to transport material from their terminals back to their cell bodies. A marker is injected into the terminal field, time is allowed for retrograde transport to the cell body, and then the appropriate region is dissociated and placed into cell culture. The markers used to this point have major limitations. Horseradish peroxidase (HRP)¹ can be visualized only by histochemical reactions that kill cells, so it is not possible to know the identity of cells while they are alive. Fluorescent conjugates of wheat germ agglutinin (WGA) allow living cells to be identified, but the fluorescence disappears rapidly from the cells such

1. *Abbreviations used in this paper:* diI, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; diO, 3,3'-dioctadecyloxycarbocyanine perchlorate; EPSPs, excitatory postsynaptic potentials. FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; LS, lumbosacral spinal cord segment; RITC, tetraethylrhodamine isothiocyanate; T, thoracic spinal cord segment; TRITC, tetramethylrhodamine isothiocyanate; WGA, wheat germ agglutinin.

that cells can be identified for only a few days (Okun, 1981; O'Brien et al., 1982).

In the particular case we introduce here, we are studying the interactions *in vitro* between a class of spinal cord neurons, sympathetic preganglionic neurons, and their normal target, neurons from the sympathetic chain. Our studies require that we be able to identify each of these cell types and also to distinguish them from the other spinal cord neurons that are present in our cultures. In this paper we describe the use of two related molecules that allow us to identify neurons for several weeks in culture. These dyes also label growing nerve fibers, and thus will allow for the study of the initial interactions between the processes of different cells.

A preliminary account of some of these results has appeared elsewhere (Honig and Hume, 1985).

Materials and Methods

Retrograde Labeling

For experiments involving retrograde transport, we used the isolated spinal cord-hindlimb preparation originally described by Landmesser (1978a, b) and used previously for HRP transport and electrophysiological studies by one of us (Honig, 1982). This preparation can be maintained in a bath for many hours and allows access to the tissues of interest. In brief, White Leghorn chick embryos were removed from the egg, placed into a bath containing Tyrode solution, and staged according to Hamburger and Hamilton (1951). They were decapitated, eviscerated, and a ventral laminectomy was performed. For labeling of sympathetic preganglionic neurons, care was taken to try to keep the sympathetic chain intact from the first thoracic segment to the last lumbosacral segment.

After dye injection (see below), embryos were kept in an oxygenated Tyrode solution for 5–18 h at 24–28°C except after WGA-fluorescein isothiocyanate (FITC) injections for which the embryos were incubated at 32–34°C. Penicillin–streptomycin (22 U/ml–22 µg/ml) was added to the Tyrode, and sterile technique was used when cultures were to be made. The bath was oxygenated directly, and in addition, for overnight incubations, oxygenated Tyrode solution superfused the bath at ~100 ml/h.

To screen fluorescent dyes, we looked for retrograde transport by lumbosacral motoneurons, since we were more familiar with that system. Dye was pressure-injected from a broken-off micropipette inserted into the sciatic nerve and/or sartorius muscle of stage 29–33 chick embryos and generally 15 h was allowed for retrograde transport. Embryos were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), washed in buffer, and brought through graded sucrose solutions up to 30%. They were then sectioned frozen, transverse to the spinal cord at 16–32 µm. Sections were mounted directly onto gelatin-coated slides and were examined uncoverslipped for all dyes except WGA-FITC or coverslipped in 90% glycerol in carbonate buffer, for WGA-FITC. Sections were observed with a Zeiss IM 35 microscope, using the appropriate filter sets for each dye. For red fluorescence we used a standard rhodamine filter set, 48 77 15, for green fluorescence we used a standard FITC filter set, 48 77 09, and for blue or yellow fluorescence we used ultraviolet illumination, filter set 48 77 02. Standard Leitz and Nikon filter sets give a similar appearance.

For labeling of sympathetic preganglionic neurons, dye was injected into the sympathetic ganglia in stage 30–32 embryos. In the chick, preganglionic neurons are situated in the seven thoracic (T) and two most rostral lumbosacral segments (LS1 and LS2) (T1 = segment 16; LS1 = segment 23). Preganglionic axons exit the spinal cord through the adjacent ventral roots and project rostrally and caudally in the sympathetic chain to innervate sympathetic neurons in the adjacent ganglion and at more rostral and caudal levels. For most of these experiments, we wanted to label large numbers of preganglionic neurons for subsequent culturing. Therefore, we made injections into each sympathetic ganglion from T1 to LS2 on both sides of the embryo and frequently also into one segment more rostral and more caudal than this to label axons projecting to more distant ganglia. For convenience and to maximize the intensity of neuronal labeling, 12–16 h were generally allowed for retrograde transport.

Embryos were transferred through four changes of sterile Puck's saline. To increase the proportion of labeled cells and to eliminate motoneurons from our cultures (see Results), the medial part of spinal cord in segments

T1–LS2, which contain the preganglionic cell column, was dissected free from the lateral spinal cord using sharpened tungsten needles. The medial spinal cords were then incubated in 0.02% trypsin (Sigma type XII-S) in Ca⁺⁺, Mg⁺⁺-free Puck's for 17–20 min at 37°C, resuspended in muscle medium, and triturated. In most experiments the cell suspension was diluted with our normal medium and underlaid with medium containing 4% bovine serum albumin (BSA) and then spun at low speed as in Calof and Reichardt (1984) to remove debris. Cells were resuspended and this process was repeated. Cells were resuspended a final time in conditioned muscle medium with 5% chick embryo extract and plated onto dishes treated as described below at a density of two to three hemisegments of medial spinal cord per dish. This procedure was followed because otherwise a large amount of fluorescent debris was present in our cultures. It seemed possible that some cells might take up this fluorescent debris and subsequently be misidentified as to their origin.

1,1'-Dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (diI) and 3,3'-dioctadecyloxycarbocyanine perchlorate (diO) were obtained from Molecular Probes (Junction City, OR). The solution of diI used for injecting was made by dissolving 2.5 mg of dye in 1 ml of 100% ethanol. The solution of diO used was 3.0 mg of dye dissolved in 0.1 ml of dimethylsulfoxide and 0.9 ml of 100% ethanol. These stock solutions were sonicated to facilitate solution and then filtered through 5-µm pores to remove remaining large crystals that otherwise would clog the micropipette tip. Other dyes we tested were used at the following concentrations: True blue, 2.5–5.0% in water; granular blue, 2.5% in water; diamidino yellow, 2% in 0.2 M phosphate buffer; WGA-FITC, 0.83% in Hepes (Vector Laboratories, Inc., Burlingame, CA); tetramethylrhodamine isothiocyanate (TRITC), crystals or 0.2% in ethanol or 3.6 mg in 20 µl dimethylsulfoxide and diluted further in 1 ml of water; Lucifer yellow VS, 5% in water; tetraethylrhodamine isothiocyanate (RITC), crystals.

Dissociation Labeling

All the cells within a discrete tissue, such as a peripheral ganglion, can be labeled by dissociating that tissue and then incubating the isolated cells in the presence of a dye.

The lumbosacral sympathetic chain was removed from 8–9-d-old chick embryos and incubated in 0.01% trypsin in Ca⁺⁺, Mg⁺⁺-free Puck's for 20–25 min at 37°C. The ganglia were then triturated and incubated in the presence of dye at 37°C, usually for 1 h. To achieve a suitable level of labeling, we diluted our stock solution of diI (2.5 mg/ml) in serum-containing medium so that the final concentration of dye when added to the cells was roughly 40 µg/ml. This concentration of dye labeled cells very brightly so sometimes, to label cells less brightly, we used a concentration as low as 12 µg/ml or reduced the incubation time to as short as one-half hour. The stock solution of diO (3.0 mg/ml) was similarly diluted to give a final concentration of ~170 µg/ml. Although concentrations of diO as low as 100 µg/ml could label cells, we generally used the higher concentration and an incubation time of 1½ h to increase the intensity of labeling. The diluted dye solutions were usually filtered through 5-µm pores before being added to the cells. This was done since it was otherwise difficult to remove remaining dye crystals from the cell suspension during subsequent washes (see below), and label might then spread to other (unlabeled) cells *in vitro*. However, dissociation in the presence of dye crystals sometimes resulted in labeling of cells and of fine filopodial extensions that was much brighter than that shown in Fig. 5 b.

After incubation cells were washed three to five times in serum-containing medium to remove excess unbound dye. Cells were plated at a density of ~1/8 chain per dish (roughly 0.5–1.5 × 10⁴ cells per dish). In some experiments, ciliary ganglia from 7–10-d-old embryos or lumbosacral dorsal root ganglia from 7–8-d-old embryos were labeled in this way.

General Culture Methods

To optimize the visualization of fluorescently labeled neurons, we plated neurons onto glass coverslips. A hole (18-mm diam) was cut in the bottom of a 35-mm plastic petri dish, and a cleaned coverslip was glued to the bottom of the dish. This created a well in the center of the dish with a volume of 100–125 µl. To facilitate adhesion of the neurons to the glass, the dishes were then treated as follows. They were coated overnight with polyornithine (0.1 mg/ml in borate buffer), washed in water, sterilized with ultraviolet irradiation, coated with a thick layer of gelatin (0.4–0.5 ml of solution containing 0.5 mg/ml in water), and allowed to air dry overnight. Fibroblasts from the skin of 8–9-d-old chick embryos were then plated onto the dishes and grown to confluence before they were lysed by replacing the medium with sterile double-distilled water.

Neurons were plated onto the lysed fibroblasts by adding a 100–125- μ l aliquot of cell suspension directly to the central well which had been rinsed with medium. The dishes were placed in a humidified chamber in a 5% CO₂ incubator at 37°C. After the neurons had adhered to the dish, an additional 1½–2 ml of medium was added to each dish and the cultures returned to the incubator.

In some experiments, neurons were added to previously established cultures of neurons or muscle. In these cases, most of the medium was removed from the dish and a small volume of cell suspension was added to the central well as just described. Muscle cells from 11-d-old embryos were themselves plated onto lysed fibroblasts. Pectoral muscle was dissociated and grown as previously described in Eagle's minimum essential medium with Earle's salts (Gibco, Grand Island, NY), 10% heat-inactivated horse serum, 40 μ g/ml conalbumin, 50 U/ml penicillin, and 50 μ g/ml streptomycin (Hume and Honig, 1986). After 3–4 d, 10 μ M cytosine arabinoside was added for 2–3 d to reduce the numbers of fibroblasts. Neurons were added to 6–14-d-old muscle cultures after these had been treated with cytosine arabinoside. Muscle was grown under similar conditions on gelatin-coated plastic tissue culture dishes to provide conditioned muscle medium.

Medium used for sympathetic ganglion cell cultures (and occasionally for preganglionic neurons) had the following composition: Coons modified F12 (Gibco), 2% heat-inactivated horse serum, 2 mM glutamine, 8 μ g/ml insulin, 40 μ g/ml conalbumin, 12 μ g/ml ascorbic acid, 50 U/ml penicillin, 50 μ g/ml streptomycin, and 50 ng/ml 7S nerve growth factor. While this concentration of nerve growth factor is sufficient for the survival of sympathetic neurons (Greene, 1977; Wakade et al., 1983), more recently we have used higher concentrations (1 μ g/ml), which allow for maximum levels of protein synthesis by similar neurons from the rat (Chun and Patterson, 1977). More typically, the medium used for sympathetic preganglionic neurons (and for ciliary ganglion neurons) was conditioned muscle medium to which 5% chick embryo extract was added. Co-cultures of preganglionic neurons and sympathetic ganglion neurons were grown in either a 1:1 mixture of these two media or in the medium used for sympathetic neurons. Cultures of sympathetic neurons alone and preganglionic neurons alone were also sometimes grown in the mixture of the two media. Co-cultures of neurons and muscle contained a 1:1 mixture of the conditioned muscle medium with 5% chick embryo extract and fresh muscle medium. Fibroblasts were grown in fresh muscle medium to which 5% chick embryo extract was added.

Electrophysiology

Recordings were done using standard techniques and recording solutions as previously described (Hume and Honig, 1986). Conventional microelectrodes were filled with 3 M KCl for myotube recordings and were filled with intracellular solution for recording from neurons in older cultures (>5 d). Lucifer yellow (4% in distilled water) was used for intracellular dye injection. For neurons in younger cultures, it was generally necessary to use the whole cell recording technique with fire-polished patch electrodes filled with an intracellular solution (Hamill et al., 1981). Acetylcholine was applied by iontophoresis from microelectrodes (1 M) or by pressure ejection from 5- μ m diam pipettes (10 μ M). In some experiments a solution containing high potassium (140 mM) was applied from puffer pipettes to depolarize neurons.

Results

To distinguish sympathetic preganglionic neurons from other spinal cord neurons and from sympathetic ganglion neurons, it was necessary to find a fluorescent dye that retrogradely labels neurons *in vivo*, that is retained when the neurons are then placed in dissociated cell culture, and that remains visible in cultured cells for several weeks. We initially tried using WGA-FITC and found, as had others who had used this dye or similar derivatives (WGA-Lucifer yellow, succinylated WGA-FITC), that labeled neurons could not be distinguished after a few days in culture (Okun, 1981; O'Brien et al., 1982). We then screened a number of fluorescent dyes that had been used by other investigators for retrograde transport studies primarily in adult animals (Bentivoglio et al., 1979; Keizer et al., 1983; see also Skirboll et al., 1984). All were retrogradely transported to the spinal cord from injection sites in the sciatic nerve or limb muscle.

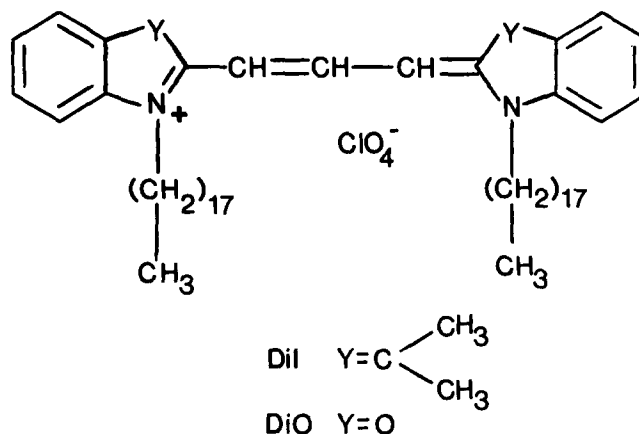


Figure 1. Chemical structure of carbocyanine dyes. Only the functional group at position Y differs between diI and diO.

However, we found that suspensions of these dyes (granular blue, True blue, diamidino yellow) were difficult to inject into the small target structures of 7-d-old chick embryos. Since the intensity of labeling was also not as bright as we had hoped to achieve, we did not pursue the use of these dyes further. We also tried RITC and TRITC, which had been used previously by Bonhoeffer (Bonhoeffer and Huf, 1980, 1985; Thanos and Bonhoeffer, 1983) to study embryonic chick retinal ganglion neurons *in vivo* and *in vitro*. Here again, we could not distinguish labeled neurons for as long as we needed in culture. The fact that TRITC could be used as a retrograde marker, however, suggested to us that other lipid-soluble dyes might be useful for this purpose. Fortunately, one of the first dyes that we then tested, 1,1'-dioc-tadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate, which is commonly referred to as diI-C₁₈(3) and which we will refer to as diI, has properties that are close to ideal. We were immediately struck by how brightly it labeled cells and by how little it faded (see also Sims et al., 1974). However, because diI had not previously been used for retrogradely labeling neurons, it was necessary to demonstrate that it is transported specifically *in vivo* and that it is not transferred to other cells during dissociation or in culture.

Properties of Carbocyanine Dyes

diI is one of a family of cyanine dyes that have been used to investigate the structure and dynamics of cell membranes and artificial lipid bilayers. The structure of this molecule is illustrated in Fig. 1. It is bilaterally symmetric, with each half consisting of a positively charged conjugated ring and an attached long hydrocarbon chain. Carbocyanine dyes have a bridge of three methine groups joining the two rings; in related cyanine, dicarbocyanine, and tricarbocyanine dyes, this bridge contains 1, 5, and 7 methine groups, respectively. Related dyes can have minor alterations in their ring structure with either an oxygen ("diO") or a sulfur ("diS") instead of an isopropyl group (which yields an indole ring and hence the abbreviation "diI"). Together the particular ring structure and number of methine groups determine the fluorescence characteristics of the molecule (Sims et al., 1974). These dyes can also vary in the length of their hydrocarbon chains, which alter their partitioning into the bilayer (Sims et al., 1974; Klausner and Wolf, 1980). Dyes with short chains (less than seven carbons long) have been used extensively as mem-

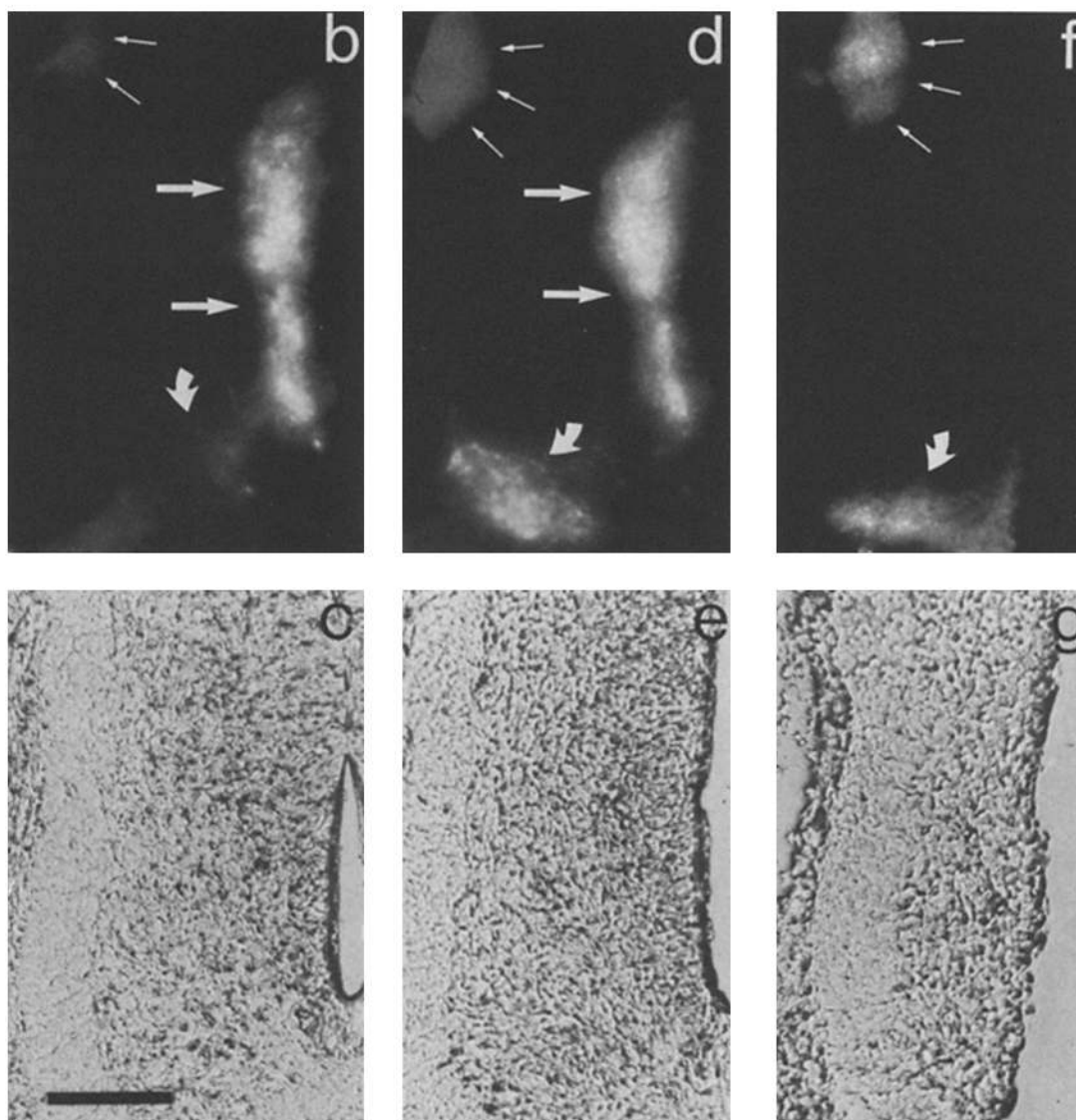
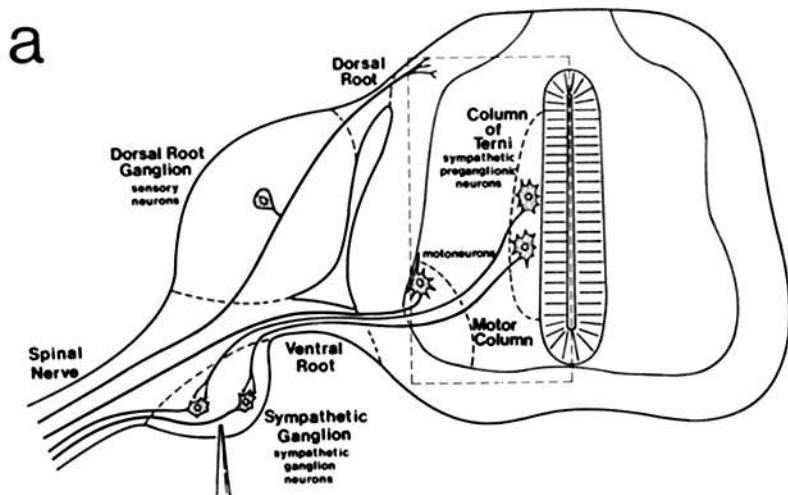


Figure 2. (a) Schematic view of the organization of the sympathetic nervous system in the chick. Sympathetic preganglionic cell bodies are situated in the column of Terni which occupies a medial position in the spinal cord, adjacent to the ependymal layer. Each preganglionic axon leaves the spinal cord in the ventral root at the same level as its parent cell body and then projects rostrally and caudally within the sympathetic chain to innervate sympathetic ganglion neurons. diI is injected into the sympathetic ganglia which, at thoracic levels, are

brane potential-sensitive dyes and pass readily in and out of cells (Sims et al., 1974; Waggoner, 1976). When cells are incubated in dilute dye solutions, dyes with longer carbon chains, such as the 18 carbon diI we use, become inserted into cell membranes with the hydrocarbon chains embedded in the lipid bilayer parallel to the phospholipid acyl chains and their conjugated bridge chromophore parallel to the surface of the cell (Axelrod, 1979). diI has been widely used to measure lipid mobility in membranes (e.g., Schlessinger et al., 1977; see Discussion for additional references) and has also been used to assess cell fusion (Neff et al., 1984; see also Sowers, 1984).

diI (in ethanol) absorbs maximally at 546 nm and has its maximum emission at 563 (Sims et al., 1974). Cells labeled with diI and excited and viewed with filters appropriate for rhodamine fluoresce bright orange-red. When viewed through FITC filters, diI-labeled cells are much dimmer and appear goldish-yellow. diI fluorescence is still visible when viewed with filter sets that use shorter wavelength excitation, that is, violet or ultraviolet illumination.

1,1'-Dioctadecyloxycarbocyanine, commonly referred to as diO-C₁₈-(3) and which we will refer to as diO, is closely related to diI but has been used less frequently. diO (in ethanol) absorbs maximally at 489 nm and its peak emission is at 499 (Sims et al., 1974). Cells labeled with diO give off a bright green fluorescence when excited and viewed with filters appropriate for FITC. diO fluorescence is somewhat greener and less yellow than that of FITC. diO-labeled cells were not visible when rhodamine optics were used.

Retrograde Labeling with Carbocyanine Dyes

Sympathetic preganglionic neurons in the chick are situated in segments T1-LS2 in a medial position near the central canal (Fig. 2 a). This preganglionic nucleus, which is identifiable with conventional histological stains, is referred to as the column of Terni (Terni, 1924; Levi-Montalcini, 1950; Oppenheim et al., 1982). Preganglionic neurons originate from the ventral part of the ependymal layer, migrate laterally toward the ventral horn, and then from 4½ to 8 d of incubation undergo a secondary migration dorsomedially (Levi-Montalcini, 1950; Oppenheim et al., 1982). For these studies, we wanted to use fairly young embryos, since the viability of cultured spinal cord neurons is often greater in younger embryos (Fischbach and Nelson, 1977). At the same time, however, we wanted to be able to clearly distinguish preganglionic neurons from motoneurons on the basis of

their position. Therefore, in these experiments, we used embryos only at stages 30-32 (day 6½ to day 7½) when the secondary migration is virtually complete. Fig. 2 b shows that injection of diI into sympathetic ganglia resulted in bright labeling of cells in the column of Terni. Occasionally, in stage 30 embryos, a few neurons were labeled which were situated between the ventral horn and the column of Terni, and therefore were probably preganglionic neurons still in the process of migration. When a single thoracic ganglion was injected ($n = 7$), most labeled cells were found at the same segmental level as the injected ganglion. Some labeled neurons were found in the adjacent segment in both the rostral and caudal directions but none were found in more distant segments. This segmental distribution is similar to, but slightly less extensive than, that found after retrograde and orthograde transport of HRP (Yip, 1985 and personal communication). The HRP labeling shows that up to five segments can project to a single ganglion.

To more carefully test if labeling with diI was specific, we took advantage of the extensive information available on the projections of motoneurons in chick embryos. Landmesser (1978a, b) has shown that motoneurons innervating specific muscles in the hindlimb are located in discrete columns within the ventral horn, with each motoneuron pool having a characteristic position in the rostral-caudal and medial-lateral axes. We therefore compared the positions of labeled motoneurons after injection of a discrete limb muscle with diI with the position of that motoneuron pool as shown by retrograde transport of HRP (Landmesser, 1978a, b; Honig, 1982 and unpublished observations). After injection of five sartorius muscles and two adductor muscles at stage 32-34, labeled cells were found in only the appropriate segments and in the appropriate medial-lateral position for that motoneuron pool. Fig. 3 shows that most cells labeled by sartorius injections were in LS1 and were situated laterally in the ventral horn, while most cells labeled by adductor injections were in LS2 and were situated medially. Thus, labeling with diI seems to be restricted to neurons projecting to the injection site. It therefore is very unlikely that diI spreads significantly from the injection site or is transferred from labeled axons to other axons coursing within the same spinal nerve. Further, labeling was not obvious in the dorsal part of the spinal cord. Thus, at least within the 16-h maximum transport times used here, diI does not appear to be transported transneuronally as can be the case with some other markers (see Trojanowski, 1983; Sawchenko and Gerfen, 1985 for recent reviews).

closely apposed to the spinal nerves. The box indicates the approximate regions shown in b-g. (b-g) Cryostat sections of chick spinal cords. In each case, diI was injected into the sympathetic ganglia. The embryos were fixed 16-18 h later and then sectioned. (b and c) Fluorescence and phase-contrast views, respectively, of a section at a level where a small injection that was confined to the sympathetic ganglion was made in this stage 30 embryo. Cell bodies (*large arrows*) in the column of Terni are brightly labeled. The axons (*curved arrow*) of preganglionic neurons coursing toward the ventral root are also fluorescent. Small arrows indicate the central processes of dorsal root ganglion neurons, which were labeled as a result of spread of dye to the spinal nerves in adjacent segments. At this stage of development, the central processes ascend and descend for several segments in the dorsal column, but do not penetrate far into the gray matter (Honig, M. G., unpublished observations). (d and e) Fluorescence and phase-contrast views, respectively, of a section from the same embryo as in b and c, at a level where a large amount of diI was injected into the sympathetic ganglion. Dye spread to the proximal part of the spinal nerve and consequently labeled not only preganglionic neurons (*large arrows*), but also motoneurons (*curved arrow*), dorsal root ganglion neurons (not shown), and their central processes (*small arrows*). (f and g) Fluorescence and phase-contrast views, respectively, of a section from a stage 31 embryo similar to that in b-e. The medial part of the spinal cord was removed and cultured. Shown is the remaining piece of lateral spinal cord which contains the motor column. The cell bodies of the labeled preganglionic neurons are absent, but their axons coursing through the motor column (*curved arrow*) and toward the ventral root are present. Bar, 100 µm for b-g.

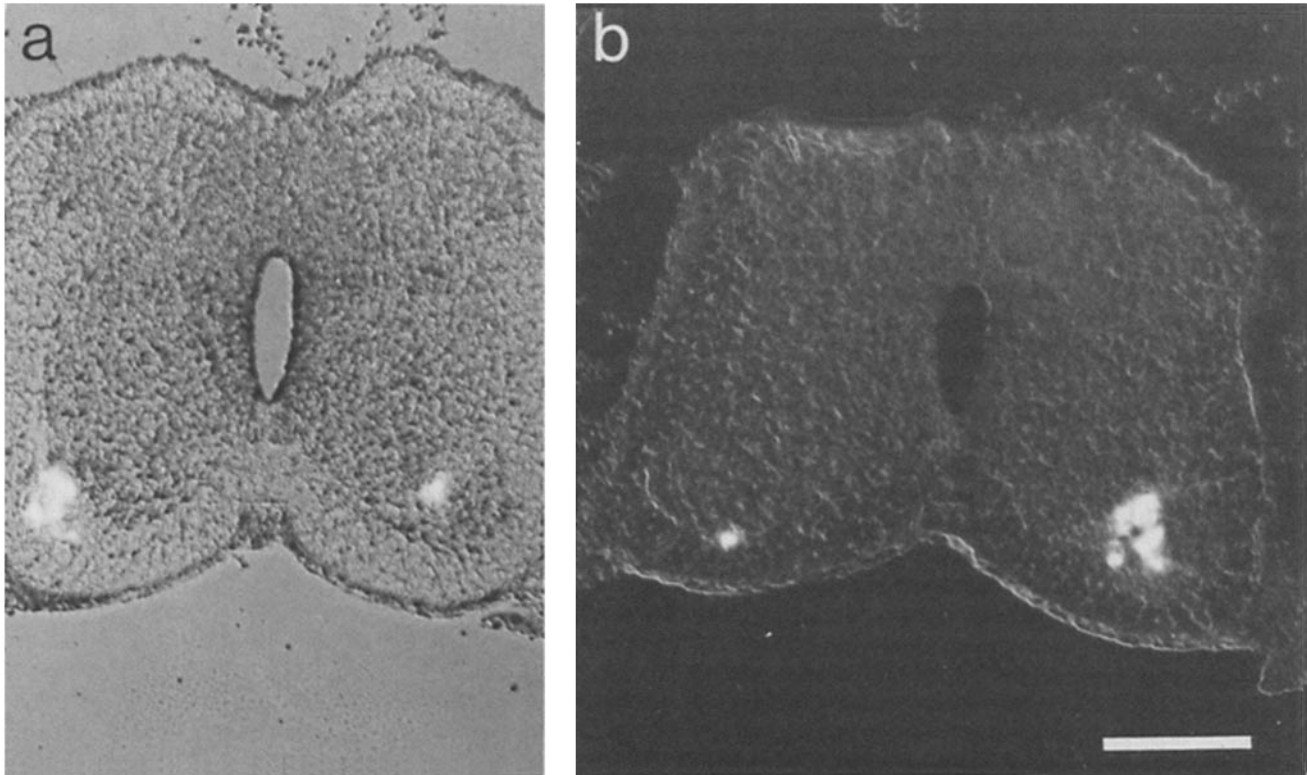


Figure 3. Retrograde labeling of motoneurons with diI. These two photomicrographs are from a stage 34 embryo that had received injections of diI into the sartorius muscle on the left and the adductor on the right. These photomicrographs were exposed and printed so as to aid in the visualization of the positions of the labeled motoneurons in the spinal cord. The actual intensity of labeling and the extent of dendritic labeling were much greater and can be better seen in the fluorescence only photograph showing labeled motoneurons (Fig. 2 *d*). Labeling was found only in the lateral motor column and not in other regions of the spinal cord even when serial sections through the spinal cord were viewed solely for fluorescence. (a) A combined phase-contrast-fluorescence view of a section at the caudal end of LS1. Many labeled sartorius motoneurons are visible at the lateral edge of the ventral horn. Fewer motoneurons labeled by adductor injections are present and these are situated in a medial position characteristic of adductor motoneurons. (b) A combined pseudo-darkfield-fluorescence view of a section at the border of LS2 and LS3. While only one labeled sartorius motoneuron is present and is situated ventrally, as is characteristic of sartorius motoneurons at this level, many labeled adductor motoneurons are present in this section and are situated medially. In this particular embryo, most sartorius motoneurons were located in LS1 and none were found caudal to the section shown in *b*. The adductor motoneuron pool extended from the caudal half of LS1 to the anterior part of LS3. Bar, 200 μ m.

From our sectioned material, it was not possible to ascertain that diI was not transferred to other cells (perhaps glia) in the same region as the retrogradely labeled cell bodies. When sections were viewed immediately after sectioning, the label appeared to be restricted to neurons, but within a few minutes the labeling became less discrete. The spread of dye resulted because diI was not fixed in place by the paraformaldehyde that we used to preserve the tissue. Labeling remained discrete when cells were kept intact in whole mount preparations (Landmesser and Honig, 1986 and unpublished observations). Further, the results of the cell culture experiments described below make it unlikely that diI was transferred to other cells in vivo. One possible exception to this was that part of the ependyma appeared fluorescent in some sectioned material (see Fig. 2, *b* and *d*). This might have resulted from the spread of the unfixed dye after sectioning. Alternatively, some ependymal cells might actually have been labeled, having taken up dye that leaked out of the injected sympathetic chain into the bath or as a result of some dye transfer in vivo from the adjacent preganglionic neurons.

We were also able to label preganglionic neurons with diO but the appearance of these cells was not as bright as with

diI. Motoneurons could also be labeled after injections of diO into the sciatic nerve, but again, the labeling was dim. This dim labeling undoubtedly reflects the use of a filter set that blocked a considerable part of diO's emission and possibly also some actual differences in the properties of diO as compared with diI, although their rates of transport should be similar (Derzko and Jacobson, 1980). Since diO seemed less suited for retrograde transport, at least under the conditions we describe here, we always use diI for retrograde labeling. However, diO is well suited for dissociation labeling, as will be described later.

Retention of Specific Cell Labeling In Vitro

Having shown that diI is transported specifically in the embryo and labels neurons brightly in sectioned material, we next wanted to know how useful it would be for in vitro studies. For our cell culture experiments, we needed to obtain fairly large numbers of labeled preganglionic neurons. To maximize labeling, we therefore routinely made rather massive injections of diI into the sympathetic ganglia at each segmental level from T1-LS2 (see Materials and Methods).

Since the sympathetic ganglia lie closely apposed to the proximal part of the spinal nerves (Fig. 2 *a*), these injections sometimes spread to the spinal nerves and resulted in labeling of motoneurons and of dorsal root ganglion neurons (Fig. 2 *d*). Cultures made from the spinal cord of such an embryo would therefore contain labeled motoneurons as well as labeled preganglionic neurons. Rather than restrict the injection site and reduce our yield of labeled preganglionic neurons, we decided instead to make large injections and to then use only the medial part of the spinal cord for cultures (see Materials and Methods). The remaining lateral portion of the spinal cord, which was left in the embryo, was fixed and sectioned to verify that it contained most of the labeled motoneurons (Fig. 2 *f*) and that the cultured piece of spinal cord therefore did not contain a substantial number of labeled motoneurons.

The appearance of labeled neurons in cultures at early times was striking (Fig. 4, *b–e*). Dye was present throughout the plasma membrane as well as in small cytoplasmic granules. As processes grew out during the first day in culture, they were labeled, and frequently growth cones could be visualized with fluorescence (see, especially, Fig. 4 *c*). With increasing time in culture, the labeling in growth cones and in processes diminished. By 3 d, only dim labeling remained in the cell membrane, but relatively bright punctate cytoplasmic labeling could be seen in cell bodies and in proximal neurites (Fig. 4 *i*). Many neurons remained visibly labeled for up to 2½ wk in culture which is the longest we have looked. The properties of retrogradely labeled sympathetic preganglionic neurons in culture are described later in this paper.

In cultures examined at 1 d after plating, nearly all of the labeled cells appeared to be neuronal in that they were round and phase-bright; most had extended processes (Fig. 4, *a–e*). Non-neuronal cells usually were not labeled; it is therefore unlikely that dye was transferred to them *in vivo*, as is the case with some fluorescent dyes such as Nuclear yellow (see Skirboll et al., 1984), or during dissociation. In some cultures, a few cells were lightly labeled and were columnar in shape, and therefore probably were ependymal cells (Fig. 4, *f* and *g*). These cells might have been labeled *in vivo* (see above) or they might have taken up some fluorescent debris during the dissociation process or in culture. In any case, only rarely were such cells observed, and they could readily be distinguished from neurons.

The percentage of the cells that were visibly labeled was low (compare Fig. 4 *a* with 4 *b*). Preliminary estimates of the proportion of labeled cells in the initial cell suspension before plating, obtained with a fluorescence-activated cell-sorter, ranged from 4 to 9% in three different experiments. This is consistent with the slightly lower proportion (2–6%) of labeled cells counted in seven sets of cultures at 23–30 h after plating, when some proliferation of non-neuronal cells would have occurred. The actual number of labeled preganglionic neurons was roughly 1,500–3,000 per dish. The percentage of cells labeled after injections into the limb buds of stage 27 embryos to obtain cultures of motoneurons was also low, ~5%, and was comparable to the percentages found by O'Brien et al. (1982) and Calof and Reichardt (1984) using WGA-Lucifer yellow. Estimates based on the yield of cells in spinal cord cultures (Fischbach and Nelson, 1977; Tanaka, 1983) and on the numbers of preganglionic neurons (Oppen-

heim et al., 1982) and motoneurons (Hamburger, 1975) suggest that each of these cell classes would have comprised roughly 5% of the cells placed into their respective cultures. This evidence, combined with the lack of non-neuronal cell labeling, lead us to conclude that dye is not transferred to an appreciable degree during the process of dissociation.

It seemed possible, from examination of the sectioned material, that, despite careful dissection, the cultured piece of medial spinal cord might still contain some labeled motoneurons in addition to the labeled preganglionic neurons. To estimate how many labeled motoneurons might be present in our cultures, in two experiments diI was injected into the sympathetic chain, and the lateral spinal cord was dissociated and cultured, rather than sectioned. In these cultures the number of labeled neurons that seemed to be viable based on their appearance with phase microscopy was only 1–5% of the number of viable, labeled neurons found in cultures from the medial spinal cord of the same animal. The low yield of labeled motoneurons reflects in part the fact that the injections were not intended to label motoneurons, and therefore many cells in the motor column were never labeled. However, many labeled cells were obviously dead by 1 d after plating. The extensive death of motoneurons means that the cultures of medial spinal cord probably contain virtually no viable motoneurons, even if the cuts in the cord were not made particularly accurately. If we estimate from sectioned material that roughly 10% of the motor column was placed into the medial spinal cord cultures, then only 0.1–0.5% of the labeled neurons should be motoneurons and over 99% should be preganglionic neurons.

Dissociation Labeling of Neurons

Peripheral ganglia, such as the sympathetic chain ganglia, can be dissected from the embryo as a relatively homogeneous population and placed into culture. However, in the co-cultures that we prepare, a marker is necessary to distinguish ganglion cells from unlabeled spinal cord cells. This can readily be accomplished by incubating the ganglion cells in the presence of a dilute solution of dye before plating. This method has worked with a variety of substances that others have used (e.g., FITC and RITC, Burt and Gierer, 1979; Bonhoeffer and Huf, 1980) and all that we have tested, including Lucifer yellow VS and CH and TRITC. However, as with retrograde labeling, the carbocyanine dyes were retained by living cells for longer periods of time in culture than are most other fluorescent markers (see below).

The appearance of neurons labeled by dissociation in the presence of either diI or diO was very similar to that seen after retrograde labeling with diI. As shown in Fig. 5, *a* and *b*, cells labeled with diO were initially labeled in their entirety. For the first day or two in culture many neuronal processes were well labeled; it was often possible to see the fine features of growth cones (Fig. 5, *c* and *d*). At later times, labeling in the membranes of the cell bodies and processes diminished and only punctate cytoplasmic labeling remained (Fig. 5, *e* and *f*). All ganglionic non-neuronal cells were also labeled initially but only some of them were labeled at later times, presumably because cell division leads to a dilution of the label. Neurons labeled by incubation retained the label for many days. In six sets of cultures of either sympathetic or of ciliary ganglion neurons, we determined the percentage

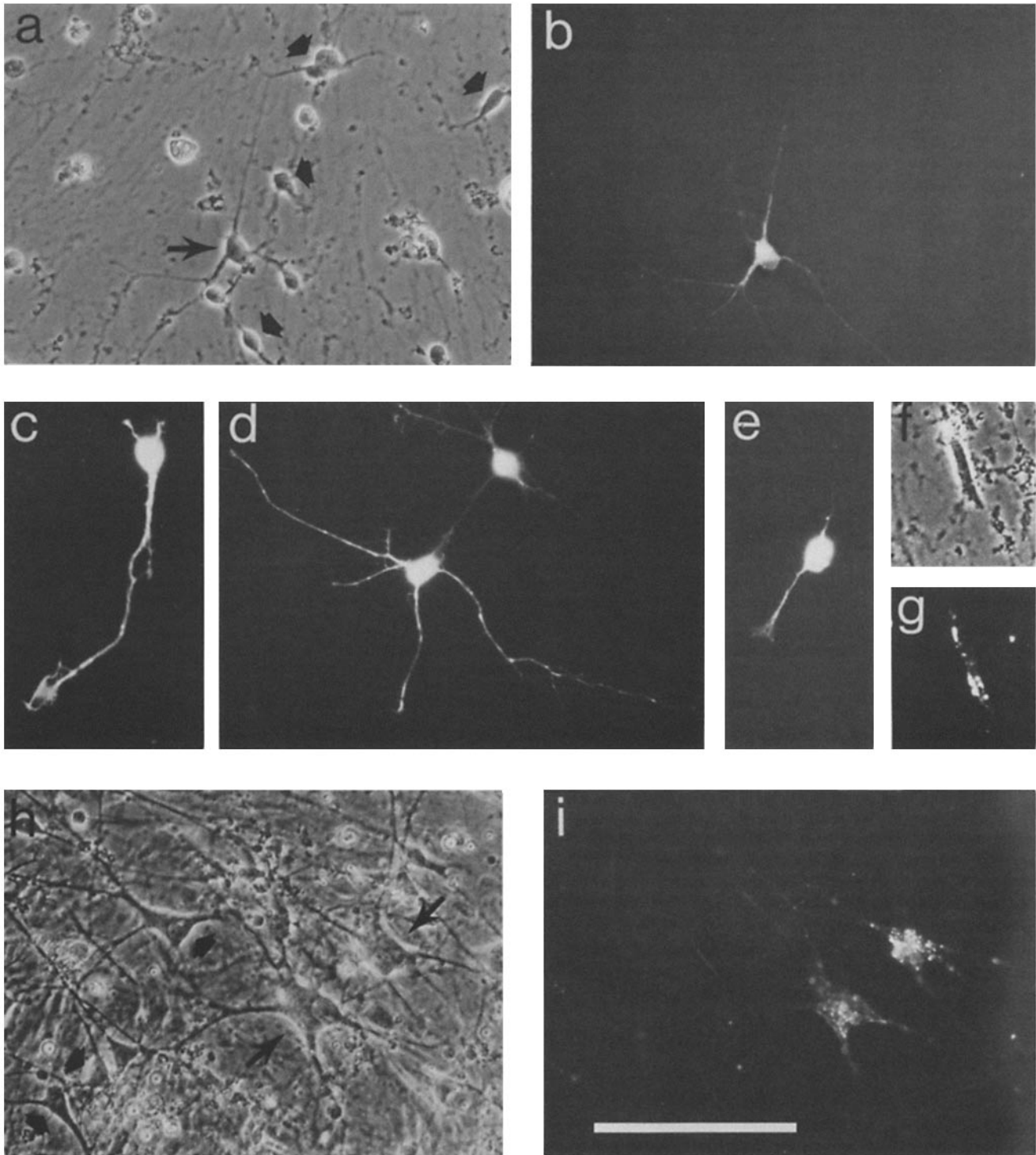


Figure 4. Preganglionic neurons retrogradely labeled with diI and placed into culture. Sympathetic ganglia were injected with diI, and the medial part of the spinal cord was dissociated and cultured. (*a* and *b*) Phase-contrast and fluorescence views, respectively, of the same field from a culture 1 d after plating. Several neurons (some indicated by *short arrows* in *a*) which have extended processes are present, but only one neuron is labeled (*long arrow* in *a*). Typically only 5% of the cells in such cultures were labeled, preganglionic neurons. (*c*, *d*, and *e*) Additional examples of preganglionic neurons labeled with diI and viewed with fluorescence at 1 d in culture. *b–e* illustrate that at 1 d cells are labeled in their entirety. Dye appears to be in the plasma membrane and in small cytoplasmic granules. Distinct granules are not discernable in these photographs because the exposure used was intended to show labeling in processes. The variability in neuronal shape, in neurite lengths, and in the morphology of growth cones is typical of preganglionic neurons at this time in culture. (*f* and *g*) Phase-contrast and fluorescence views, respectively, of a dimly fluorescent non-neuronal cell, presumably an ependymal cell, in the same culture dish as the preganglionic neurons shown in *b* and *c*. Non-neuronal cells with detectable fluorescence were extremely rare at 1 d after plating and their fluorescence was very dim compared with retrogradely labeled neurons from the same embryo. (*h* and *i*) Phase-contrast and fluorescence views, respectively, of a field from a culture 6 d after plating. Two labeled preganglionic neurons are present (*long arrows* in *h*). Labeling now seems to be restricted to the cell bodies and the proximal parts of neurites and is punctate and cytoplasmic. Note that these cell bodies are two to three times larger than those of preganglionic neurons at 1 d in culture. Other neurons (some indicated by *short arrows* in *h*) are present and are unlabeled, as is the dense mat of non-neuronal cells. Bar, 100 μm .

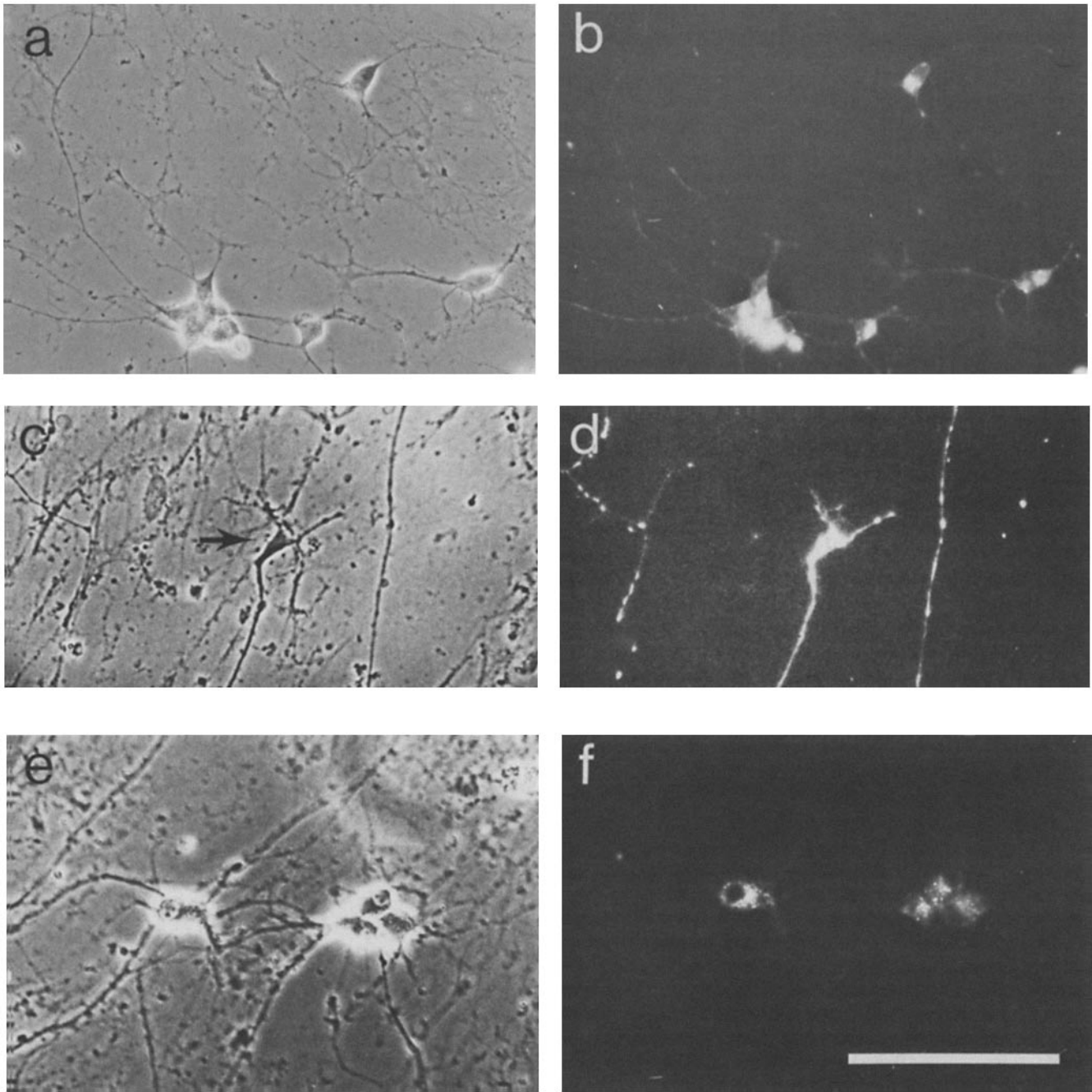


Figure 5. Sympathetic neurons in culture labeled by dissociation in diO. The lumbosacral sympathetic chain was removed from stage 34–35 embryos, dissociated in the presence of diO, and placed into culture. (*a* and *b*) Phase-contrast and fluorescence views, respectively, of a field from a culture 1 d after plating. All cells visible in the phase-contrast view and all others (including supporting cells) present in this dish were from the sympathetic chain and fluoresced brightly. As with retrogradely labeled neurons, there is both punctate labeling in the cytoplasm (individual granules not discernable with this exposure) and diffuse labeling in the membranes of cell bodies and neuronal processes. (*c* and *d*) Phase-contrast and fluorescence views, respectively, from a culture similar to that in *a* and *b*, but at a higher magnification, showing that growth cones (*arrow* in *c*) and processes of diO-labeled neurons appear fluorescent at 1 d. This growth cone was particularly large and elaborate. (*e* and *f*) Phase-contrast and fluorescence views, respectively, of a field of diO-labeled sympathetic neurons at 7 d in culture. Labeling is now punctate and is primarily in the cell bodies. Note that all neurons are still labeled. Bar, 100 μm for *a*, *b*, *e*, and *f* and 63.5 μm for *c* and *d*.

of neurons visibly labeled with diO at various times after plating. Virtually all neurons (>95%) were labeled for up to 12 d, and 70–90% were still detectably labeled at 2–3½ wk. Similar results were seen with diI. The intensity of labeling clearly decreased in many cells with time in culture (com-

pare Fig. 5 *f* with 5 *b*). It is therefore likely that some of the neurons that appeared unlabeled with the objective we used (40 \times , 0.75 numerical aperture, water immersion) would have been detectably labeled had we used an objective with a greater light gathering ability.

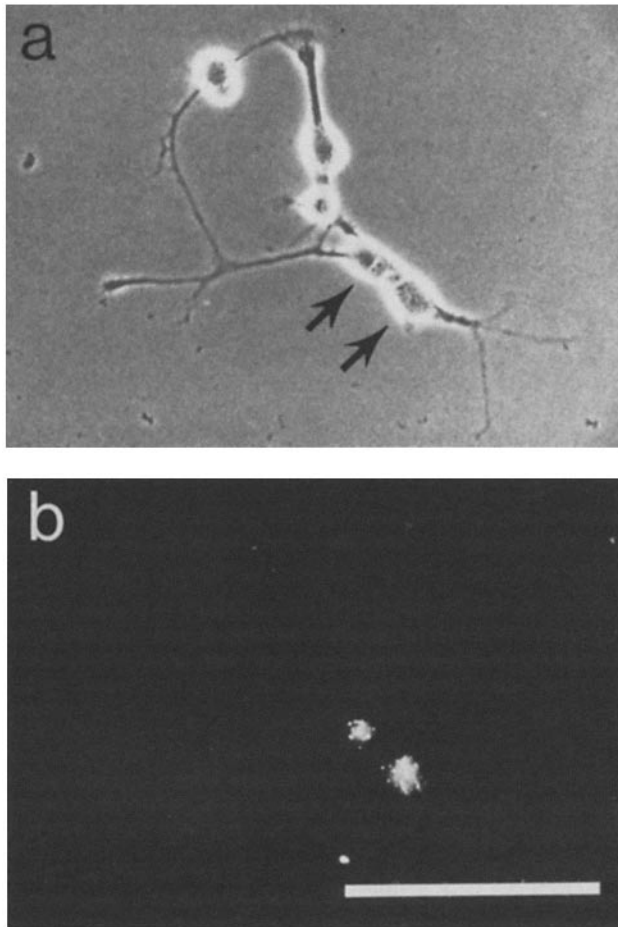


Figure 6. A mixed culture of unlabeled and dissociation-labeled neurons. Ciliary ganglion neurons were dissociated, incubated in diI, washed to remove excess, unbound dye, and plated in the same dish and at the same time as other ciliary ganglion neurons that had not been labeled. (a) Phase-contrast and (b) fluorescence views of a field from a 1-d-old culture showing five neurons, two of which (arrows in a) are labeled. These photomicrographs and other experiments (see text) demonstrate that dye does not spread from labeled to unlabeled neurons. Bar, 100 μm .

We wanted to be certain that when unlabeled cells were grown with labeled cells, they would not become labeled by dye released into the culture medium or by dye exchange between adjacent cells. To examine these possibilities, we cultured ciliary ganglion neurons labeled with diI in the same dish with a roughly equal number of ciliary ganglion neurons

labeled with diO. We used ciliary ganglion neurons because their large size makes them easier to count than the smaller sympathetic ganglion neurons. All neurons examined subsequently were labeled either red or green ($n = 100$ and $n = 43$ at 1 and 6 d, respectively); none were double-labeled, even if a single cell of one color was situated amidst a large clump of cells of the other color. In contrast, when cells were initially incubated in the presence of both dyes, then all neurons examined ($n = 40$ at 2 d, $n = 15$ at 6 d) were obviously double-labeled. These experiments suggest that dyes do not spread appreciably from labeled cells to other cells.

The double label experiments might fail to detect a slight transfer of dye, so we performed an additional type of experiment. We made cultures in which half the neurons were labeled with dye and half were unlabeled. We then counted the number of labeled and unlabeled neurons at several times. If neurons could become labeled by dye released into the medium or directly transferred from cell to cell, then the percentage of labeled neurons should increase with time in culture. Such an increase was not observed. At 1, 3, and 9 d in culture the percentage of labeled neurons was 48 ± 6 , 46 ± 8 , and 41 ± 8 , respectively (mean \pm standard deviation, >100 cells counted in a total of four dishes at each time point). Further, unlabeled cells often were found adjacent to labeled cells (Fig. 6). These results support the contention that there is no significant spread of dye from labeled to unlabeled neurons in culture (see also Neff et al., 1984 for the absence of spread from labeled to unlabeled myoblasts over short time periods). Given that the dyes are not transferred between cells, these experiments also suggest that the dyes are relatively nontoxic. The nearly constant proportion of labeled cells over time in culture suggests that labeled cells are not more likely to die than unlabeled cells.

Physiological Properties of Dye-labeled Neurons

Visual observations indicate that carbocyanine dye labeling is compatible with cell survival, growth, and process extension, but gives no indication whether the dyes might have some more subtle physiological effect on neurons. We were particularly concerned about this possibility since some cyanine dyes affect ion transport (Simons, 1979; but see also Yoshikami and Okun, 1984). To address this issue, we recorded intracellularly from labeled ciliary ganglion neurons and sympathetic ganglion neurons. At 6–14 d in culture the resting potentials and action potential amplitudes of cells labeled with either dye appeared normal (Fig. 7 a). To determine whether the dyes had any effect during the first few days in culture, when much more of the labeling was in the plasma

Table 1. Properties of Sympathetic Ganglion Neurons Labeled with Carbocyanine Dyes

Label	Resting potential	Input resistance	Input capacitance	Action potential amplitude	Action potential width at 1/2 peak amplitude
	<i>mV</i>	<i>MΩ</i>	<i>pF</i>	<i>mV</i>	<i>ms</i>
None	-45.1 ± 2.6	611 ± 86	18.6 ± 2.8	85.8 ± 6.1	$4.6 \pm .43$
diO	-42.5 ± 2.0	648 ± 77	15.6 ± 2.5	90.0 ± 4.9	$4.0 \pm .31$
diI	-39.6 ± 2.6	759 ± 120	16.2 ± 4.1	86.1 ± 5.2	$5.5 \pm .63$

Data were obtained by making whole cell intracellular recordings from neurons in culture for 2–3 d. Labeled and unlabeled neurons were grown in the same dishes to minimize variation. All values mean \pm SEM.

membrane, we studied a series of labeled sympathetic ganglion neurons at 2–3 d in culture with whole cell intracellular recording. Cells labeled with diI, cells labeled with diO, and unlabeled cells were plated in the same dish to minimize culture to culture variation. Table I illustrates that even at these early times no significant differences were found between labeled and unlabeled cells. The basic physiological properties of these cells were similar to those reported by Chalazonitis et al. (1974).

The acetylcholine sensitivity of labeled sympathetic ganglion neurons was also normal. Fig. 7 *b* illustrates the currents recorded in whole cell voltage clamp mode from a 12 d diO-labeled sympathetic neuron. In a series of neurons studied at 3 d in culture, the peak currents at a holding potential of -50 mV were -36.5 ± 9.0 , -39.0 ± 11.6 , and -49.8 ± 13.2 pA (mean \pm SEM) for unlabeled, diO-labeled, and diI-labeled neurons, respectively. These values for acetylcholine sensitivity are similar to those found by Role (1985) under similar conditions.

Although the dyes have no discernable effect on the basic physiological properties of neurons, we were concerned that they might interfere with some aspect of synaptic transmission. We therefore examined the effect of dyes in several systems in which synapses normally form. We first demonstrated that diO-labeled sympathetic ganglion cells could receive synapses. In this series of experiments we recorded from labeled sympathetic neurons that were close to small explants of medial spinal cord. To stimulate these explants, which contained preganglionic neurons retrogradely labeled with diI, we puffed a high potassium solution onto their centers. Synaptic potentials in response to puffs were observed in 26/66 neurons examined in this manner. One example is shown in Fig. 8 *a*. The proportion of innervated cells we detected is comparable to that found by Role (1985) using unlabeled sympathetic neurons and dorsal spinal cord explants and also by Ko et al. (1976) in co-cultures of rat superior cervical ganglia and spinal cord explants under slightly different conditions.

Several different types of experiment demonstrated that cells labeled with diI could release neurotransmitter. In one series of experiments, retrogradely labeled preganglionic neurons were cultured with myotubes. Muscle was selected for these preliminary experiments because the large size of myotubes increased the likelihood that a preganglionic axon

would encounter the target. Although skeletal muscle is not a normal target for preganglionic axons, these neurons can innervate skeletal muscle *in vivo* after various surgical manipulations (Langley and Anderson, 1904; Landmesser, 1971; Wigston and Sanes, 1985). Fig. 8 *b* shows a synaptic potential recorded from a cultured myotube in response to stimulation of a retrogradely labeled preganglionic neuron. We found that both preganglionic neurons and motoneurons (obtained by culturing the lateral part of the spinal cord which normally would be fixed and sectioned) could form neuromuscular synapses. However, in both cases, only a small proportion of the pairs tested (10.1%, $n = 138$ and 7.5%, $n = 53$, respectively) appeared to be connected.

To make a more detailed study of the effects of dye on transmitter release, we examined synaptic transmission between ciliary ganglion neurons and myotubes from skeletal muscle. This combination of cells forms synapses with a reasonably high probability (Betz, 1976; Nishi and Berg, 1977). To directly compare the release properties of labeled and unlabeled neurons while minimizing differences in culture conditions, we placed neurons labeled by dissociation in diI and unlabeled neurons in the same culture dish. 15 pairs of labeled neurons and muscle and 15 pairs of unlabeled neurons and muscle were tested at 2 or 3 d of co-culture. Of these four, and three pairs, respectively, showed excitatory postsynaptic potentials (EPSPs) at short, fixed latencies. For all seven connections the EPSP amplitude exceeded 10 mV, and four of the connections (two labeled, two unlabeled) were suprathreshold. Thus, diI-labeled neurons can form synapses with about the same frequency and efficacy as unlabeled neurons.

In summary, we have not found any physiological property that is affected by labeling cells retrogradely or by dissociation in carbocyanine dyes.

Properties of Preganglionic Neurons in Culture

For the first few days in culture we had difficulty in making stable microelectrode penetrations of preganglionic neurons; however, it was easy to make tight seal intracellular recordings (Hamill et al., 1981) at this time. Preganglionic neurons had resting potentials in the range of -40 to -50 mV and many had action potentials that exceeded 80 mV. Standard intracellular recordings from neurons in culture for more than

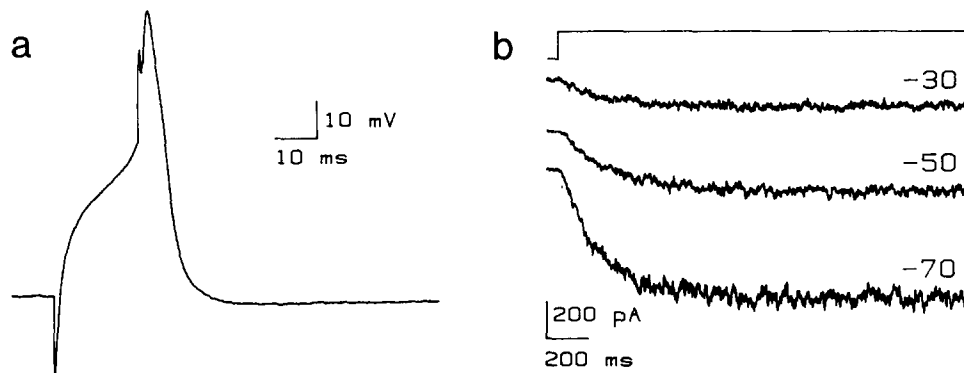


Figure 7. Physiological properties of neurons labeled with diO. (*a*) An action potential recorded using intracellular recording from a sympathetic ganglion neuron in culture for 7 d. Resting potential, -50 mV. Stimulating current, $+0.1$ nA. (*b*) Acetylcholine sensitivity of a sympathetic ganglion neuron in culture for 12 d. This panel illustrates currents recorded in whole cell voltage clamp mode, in response to puffs

from a pipette containing $10 \mu\text{M}$ acetylcholine. The puffs began at the time indicated by the step shown in the top trace. The numbers above each current trace indicate the holding potential in millivolts. For this cell the estimated peak conductance was 12.5 nS, and the reversal potential was -12 mV.

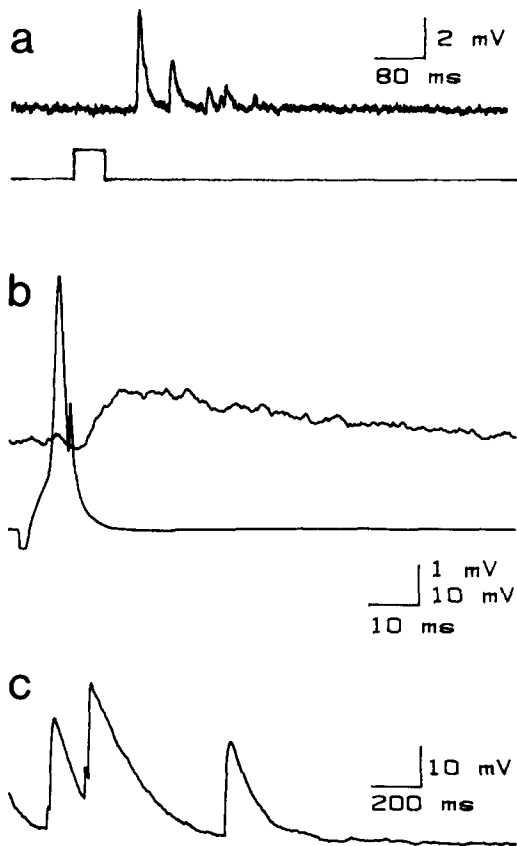


Figure 8. Synaptic connections formed by carbocyanine-labeled neurons. (a) diO-labeled neurons can receive synaptic input as illustrated by the top trace showing synaptic potentials recorded intracellularly from a diO-labeled sympathetic ganglion neuron at 5 d in culture. These were evoked by stimulation of a spinal cord explant containing preganglionic neurons with a puff of a high potassium solution. The explant had also been in culture for 5 d. The timing of the puff is indicated by the bottom trace. Resting potential, -60 mV. (b) diI-labeled preganglionic neurons have action potentials and can release neurotransmitter. These recordings were made from a medial spinal cord-muscle co-culture. The spike in the preganglionic neuron, shown in the bottom trace, was evoked by passing depolarizing current through the intracellular recording electrode. An intracellular recording from a nearby muscle fiber (top trace) shows an excitatory postsynaptic potential at short latency. The muscle resting potential was -70 mV. The muscle cells had been in culture for 12 d, and the preganglionic neurons for 6 d. (c) diI-labeled preganglionic neurons can receive synaptic contacts. This intracellular recording was made from a preganglionic neuron in a 13-d culture containing both the medial part of the spinal cord and sympathetic ganglion neurons. These synaptic potentials occurred spontaneously. In this trace, the cell was held at -90 mV by passing current through the recording electrode. At the resting potential of -60 mV, many of the synaptic potentials were suprathreshold.

1 wk gave similar results. The majority of preganglionic neurons had stable resting potentials and showed no sign of synaptic input. However, occasional cells received EPSPs at a frequency of several per second (Fig. 8 c). Spontaneous inhibitory potentials were not noticed, although an extensive search was not made. In addition, as noted above, preganglionic neurons could release transmitter.

Labeled preganglionic neurons could be distinguished in cultures as old as $2\frac{1}{2}$ wk, the oldest we examined. However,

the number of labeled neurons in cultures grown under several different conditions clearly declined over the first 10 d in culture. Since virtually all neurons labeled by dissociation retain their label for at least this long (see above), it seemed likely that the decrease in the number of preganglionic neurons was the result of cell death. To address this issue directly, we studied neurons in microcultures, which allowed us to reidentify individual neurons. We initially observed these cultures 1 d after plating, when cells damaged by the dissociation process would have already died, and reexamined them at 3-d intervals. Of the 11 neurons initially identified, seven were still labeled and alive at 10 d. Living, unlabeled neurons were not visible at the former locations of the four missing cells, so we assume that these cells had died, and the debris had been washed away. Thus, in these cultures, the decrease in the number of labeled cells was attributable to cell death. The decrease in the number of labeled neurons in the mass cultures was consistent with this amount of cell death.

Although after a few days in culture neuronal processes were no longer obviously labeled, we could identify the preganglionic neurons by their punctate cytoplasmic labeling (Fig. 9 b) and examine their morphology by impaling them with microelectrodes filled with Lucifer yellow. For the 44 neurons examined at 3–14 d the branching patterns were quite similar (Fig. 9 c). Each cell had multiple (3–14) primary processes. All but one (or in four cases, two) of these were similar to dendrites in vivo in that they branched extensively fairly close to the cell body and often had short spines. The remaining process appeared to be axonal in that it was much longer than the others, of even caliber, and extended for several hundred micrometers before branching. At least some “axons” then broke up into multiple fine collaterals, some bearing obvious varicosities. These arborizations were sometimes, but not always, found in the vicinity of sympathetic neurons.

In the majority of our experiments we grew preganglionic neurons in co-cultures with sympathetic ganglion cells; however, in roughly one-fourth of the cultures, no sympathetic ganglion cells were present. Preganglionic neurons grown with their normal spinal cord neighbors, but without their normal target cells, were not obviously different from those grown with the target. Some preganglionic neurons grown in cultures without target cells survived for up to 16 d (the longest time examined) and, at least for the first week in culture, had normal resting and action potentials. Their morphology also did not appear to differ (compare Fig. 9 c and d). Again, each cell had a single “axon” and multiple “dendrites” (11 cells without target, 5.7 ± 2.7 primary dendrites; 44 cells with target, 6.5 ± 1.7 primary dendrites). Surprisingly, although their normal target cells were not present, the axons of some of these preganglionic neurons (at least eight of 16) broke up into fine branches that bore obvious varicosities.

Discussion

The use of carbocyanine dyes has allowed us to unambiguously identify several classes of neurons in vitro. We label sympathetic preganglionic neurons retrogradely with diI so that they fluoresce red, and sympathetic ganglion neurons by incubation in diO so that they fluoresce green. All other spinal cord neurons remain unlabeled. These dyes have many

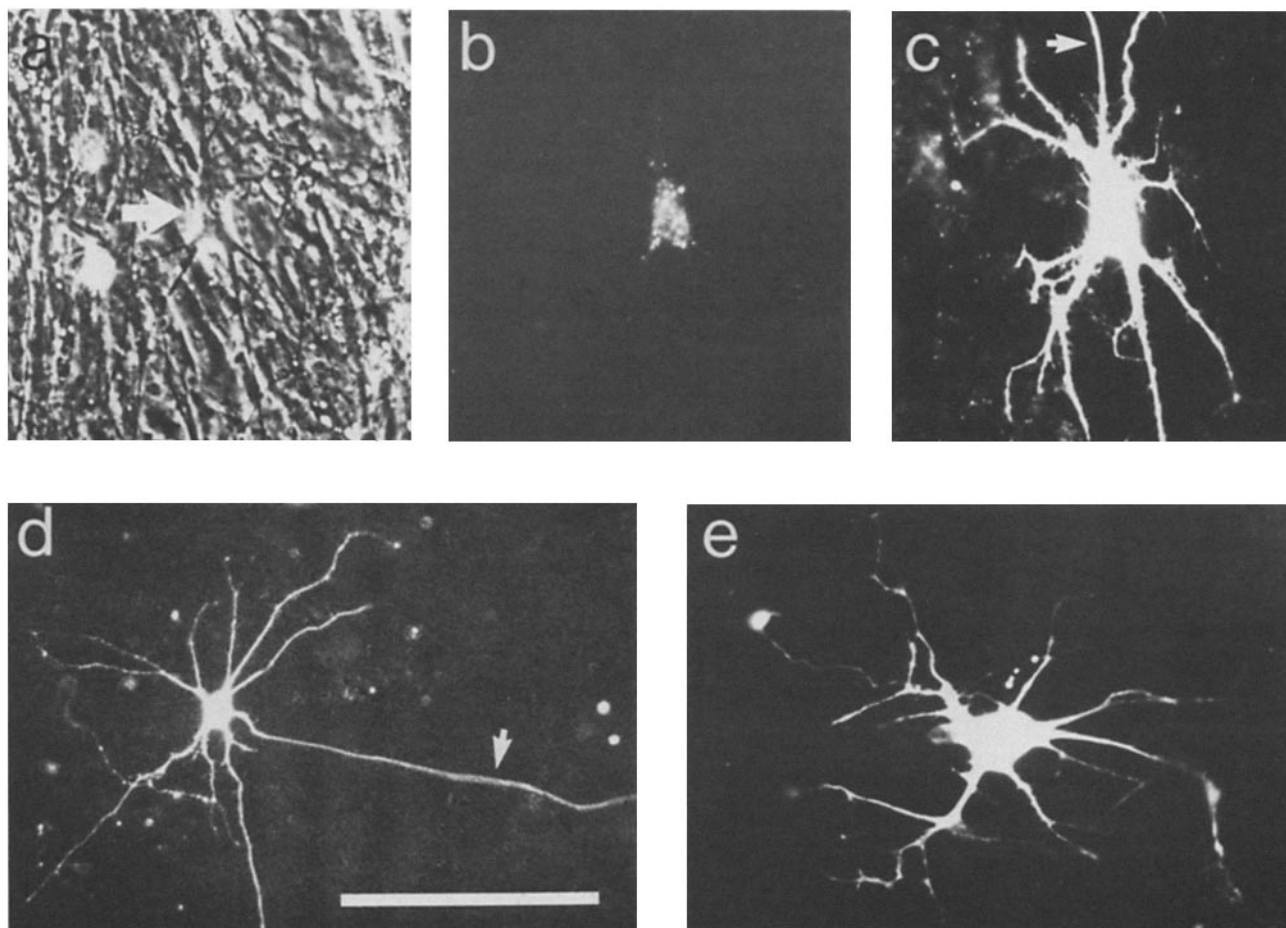


Figure 9. Lucifer yellow injections of identified neurons in culture. (a) A phase-contrast view of a culture containing diO-labeled sympathetic neurons plated 12 d previously and cells from the medial part of the spinal cord added after 6 d. One multipolar neuron (*arrow*) is present in the center of the field. (b) The same field as in a. The neuron in a fluoresced bright red, indicating that it was a preganglionic neuron. (c) The fluorescent neuron in b was injected with Lucifer yellow to see the details of its morphology. It had multiple highly branched processes which appear to be dendritic and one longer smooth process (*arrow*) which appears to be axonal and extended for several hundred microns beyond the field of view. (d) Another preganglionic neuron that had been identified with red fluorescence as in b and then injected with Lucifer yellow, at 8 d in culture. In contrast to a-c, sympathetic neurons were not present in this dish yet this neuron still had multiple "dendrites" and a single long "axon" (*arrow*). (e) A sympathetic neuron injected with Lucifer yellow and viewed with fluorescence. This neuron was from the same culture dish as the neuron shown in a-c. This and other sympathetic neurons were multipolar (see Dryer and Chiappinelli [1985] for the in vivo morphology of sympathetic ganglion neurons). Bar, 100 μ m for a-c and e; 200 μ m for d.

favorable properties. Their fluorescence is bright and relatively resistant to fading, and the dyes appear to be physiologically innocuous and relatively nontoxic (see also Montecucco et al., 1979), even when excited by light. However, the two most striking features of these dyes are that growing processes are labeled for the first few days in culture, and that detectable cell body labeling persists for several weeks. While RITC and TRITC label processes (Bonhoeffer and Huf, 1980) and rhodamine-labeled latex microspheres retrogradely transported to neuronal cell bodies remain fluorescent indefinitely (Katz et al., 1984), no single marker combines both these features. The first property means that axon guidance and neurite interactions in vitro (e.g., see Bonhoeffer and Huf, 1980, 1985; Fallon, 1985) can readily be studied, while the second property facilitates the examination of long-term interactions between neurons in vitro. These two characteristics seem likely to reflect differences between the mechanism by which carbocyanine dyes label

cells and the mechanisms responsible for labeling with other markers.

Mechanism by Which Cells Become Labeled

The mechanisms by which HRP and conjugates of WGA are retrogradely transported are well understood (see Trojanowski, 1983; Sawchenko and Gerfen, 1985 for reviews). These markers are endocytosed at nerve terminals and transported back to the cell body inside membranous compartments. The label stays within these membranous compartments (e.g., Harper et al., 1980) and, with light microscopy, its appearance is granular and is generally restricted to the cell body and proximal processes. Cytoplasmic labeling with HRP can occasionally appear diffuse but this is seen primarily in younger embryos than we have used here (<6 d) and may result from extensive damage to immature axons (Oppenheim and Heaton, 1975; Landmesser 1978b; Honig, 1982 and unpublished observations).

For cells retrogradely labeled with diI, the label initially appears to be primarily in the plasma membrane, although some label appears punctate and cytoplasmic. This latter type of labeling becomes more pronounced over the first few days in culture. Numerous studies have shown that diI and diO insert directly into membranes (e.g., Sims et al., 1974; Schlessinger et al., 1977; Axelrod, 1979), so at the injection site diI should become incorporated into the membranes of the preganglionic nerve terminals and preterminal axons. From this point, two distinct routes of transfer to the cell body must be considered. First, membrane at the injection site that becomes internalized will contain diI, so there should be a component of the label that will reach the cell body by fast axoplasmic transport, as is the case for HRP and WGA. To account for the labeling that we see, this internalized label would then somehow have to be transferred to the cell surface (e.g., Sleight and Pagano, 1985). A second possible route by which diI might reach the cell body would be by lateral diffusion in the plane of the membrane. Such a mechanism would easily explain the membrane labeling we see not only in cell bodies but also in axons labeled retrogradely (see the labeled axons of preganglionic neurons coursing toward the ventral root in Figs. 2, *b* and *f*) or orthogradely (Landmesser and Honig, 1986; Honig, unpublished observations). This mechanism can also explain why "transport" of these dyes can occur in either direction.

Is it plausible that enough diI could reach preganglionic cell bodies by lateral diffusion to label them brightly? Numerous studies have shown that diI and diO diffuse freely in the plane of the membrane with a diffusion constant of 10^{-9} to 2×10^{-8} cm²/s in various types of cellular membranes (Schlessinger et al., 1977; Jacobson et al., 1981; Zidovetzki et al., 1981) and of $\sim 10^{-7}$ cm²/s in various kinds of artificial bilayers (see Vaz et al., 1982 for a review). In 7-d chick embryos (stages 30–32) the distance from the sympathetic chain to the preganglionic column ranges from ~ 400 μ m to ~ 600 μ m. To consider first a simplified diffusion model, if dye was applied as a point source and allowed to diffuse to infinity, the relative distribution of dye at all times would have a Gaussian distribution and would be described by the equation

$$C_{(x)} = C_{(x=0)} e^{-x^2/4Dt}$$

where x is the distance from the source, D is the diffusion coefficient, t is time, $C_{(x)}$ is the concentration of dye at distance x , and $C_{(x=0)}$ is the concentration of dye at the point where dye was initially applied, but at time t . Note that this form of the diffusion equation gives only the relative distribution of dye, since $C_{(x=0)}$ will decrease as a function of time in a way that is determined by the number of directions in which dye can diffuse. If we take a diffusion coefficient in the middle of the reported range ($D = 10^{-8}$ cm²/s), at 5 h (the earliest we have looked for labeling), the concentration of diI 600 μ m from the injection site would be 1% of the amount still at the injection site, and at 15 h (the time usually allowed for "transport") the concentration at 600 μ m would be nearly 20% of that remaining at the injection site. More realistic assumptions suggest that the actual amount of dye accumulating in the cell body would be greater than that predicted by this simple case. Dye can actually diffuse only to the end of the cell (rather than to infinity) and, in addition, may become trapped at the cell body since the diffusion

coefficients of lipids in the membranes of growing neuronal processes may be higher than in the cell bodies (de Laat et al., 1979). Also, dye may remain in the extracellular space at the injection site and be available for continuous uptake rather than act as a point source. In conclusion, it seems likely that a significant amount of diI could reach the preganglionic cell body membrane by lateral diffusion.

To assure ourselves that the predictions of the simple model were plausible, we made rough estimates of relative fluorescence intensity. The relative brightness of fluorescence was determined in sectioned material by placing calibrated neutral density filters in the path of the exciting beam until the brightness of the fluorescence at the injection site (estimated by eye and by readings on the spot meter of a camera system) was attenuated to about the level seen in the preganglionic column with full illumination. For transport times of both 5 and 15 h, these estimates of relative fluorescence were fairly close to those predicted by the simple diffusion model. Furthermore, our observations suggest that our threshold for detecting fluorescence was $\sim 3\%$ of that displayed by the preganglionic column at 5 h.

It is important to note that lateral diffusion is a plausible mechanism for delivering dye to the cell body only if the distance between injection site and cell body is small, since the diffusional profile falls off steeply with distance. For example, according to the simple model presented above, at 5 h the amount of diI at 1.6 mm from the injection site will be only $\sim 10^{-16}$ as much as that at the injection site, and even at 20 h it will still be only $\sim 10^{-4}$ as much. For reasons discussed above, this example is clearly somewhat conservative in its assumptions; moreover, we have seen bright labeling of motoneuron cell bodies, 3 mm from the injection site in the limb, after 14½ h. One additional possible cause of the underestimate of the maximum distance from the injection site at which dye can be detected is that the diffusion coefficients of lipids in the membranes of growing neuronal processes may be especially high (de Laat et al., 1979). Considering the effects of finite, rather than infinite diffusion and taking the fastest diffusion coefficient that has been reported in any artificial bilayer allows diffusion for a few additional millimeters. It seems unlikely, however, allowing a day or two for labeling, that cells more than ~ 1 cm from the injection site could be detectably labeled by diffusion. In summary, carbocyanine dyes are likely to label the membranes of neurons only if the injection site is relatively close to the cell body. However, many parts of the nervous system, particularly in young embryos, are within this distance.

It was noted above that diI is also likely to reach the cell body as a constituent of retrogradely transported internalized membranous organelles. Diffusion places no limit on the distance that detectable amounts of diI might travel by this route, since the membranes of these organelles would not necessarily be diluted as they are transported. Thus diI may perhaps be a useful retrograde tracer for long distances as well, but in these cases the labeling would be expected to be exclusively cytoplasmic, and probably restricted to the cell body.

The mechanism by which neurons become labeled during the process of dissociation seems likely to be quite straightforward. Presumably, diO (or diI) is directly incorporated into the plasma membranes of neurons as has been shown previously for several other cell types including fibroblasts (e.g., Struck and Pagano, 1980; Jacobson et al., 1981), myo-

blasts (Schlessinger et al., 1977), and neuroblastoma cells (de Laat et al., 1980). Similarly, when these dyes are added to already established cultures, dye becomes incorporated into the membranes of all cells present in the dish (Honig M. G., and R. I. Hume, unpublished observations; see also Axelrod et al., 1978).

Process Labeling during Outgrowth

When neurons are placed into culture, they are round, the axons and dendrites they had in vivo having been sheared off when the tissue was dissociated. As new processes grow out, they initially are diffusely labeled throughout their plasma membranes. The diffusion coefficient of the dyes, which is rapid compared with the rate of process extension (neurites grew 50–300 μm during the first day in culture; see Fig. 4, *b–e*), ensures that they will remain in diffusional equilibrium. Thus dye that was formerly in the cell body plasma membrane (after both retrograde and dissociation labeling, see above) spreads throughout the plasma membrane of processes as well. TRITC and RITC, which are also lipid-soluble, also label growing neurites.

With time, the membrane labeling becomes dim and patchy and the processes, in particular, become difficult to visualize with fluorescence. This can be explained by two mechanisms. First, as the cell grows, primarily by adding new membrane at its growth cones (Bray, 1970; Feldman et al., 1981; Pfenninger and Maylie-Pfenninger, 1981; Pfenninger and Johnson, 1983), the dye will become increasingly diluted. Second, some of the labeled plasma membrane will become internalized as a result of the normal endocytotic activity of all cells. Thus, one can visualize punctate cytoplasmic labeling not only in the cell body (see below) but also in processes and growth cones (at 1–4 d in culture).

Persistence of Labeling

Bright grains of fluorescence remain in the cell body for up to several weeks. By focusing through the cell it is clear that these grains are intracellular (see also Axelrod et al., 1978; Jacobson et al., 1981). We assume they are in intracellular membranous organelles as has been shown to be the case for other internalized lipid probes (Sleight and Pagano, 1985; Pagano and Sleight, 1985), but the particular organelles have not yet been identified. Fluorescence presumably remains for long periods because enzymes that can rapidly degrade these dyes are not present. The gradual decrease in cell body fluorescence might then be due to a low level of metabolism or to some instability of the dyes themselves. In contrast, the fluorescent derivatives of naturally occurring phospholipids used by Pagano are degraded by cellular pathways, and have a half-life of only a few hours (Sleight and Pagano, 1985). Similarly, intracellular enzymes probably degrade lectins within a few days, thereby accounting for the short half-life of fluorescent labels attached to WGA (Okun, 1981; O'Brien et al., 1982).

Specificity of Labeling

Carbocyanine dyes will partition into any available membrane. All specificity therefore depends on the way that the dyes are applied. The two methods that we use to achieve specificity are retrograde labeling from injections into termi-

nal fields, and dissection of discrete regions of the nervous system.

Control experiments in which sectioned material was examined indicated that retrograde labeling with diI was specific. After muscle injections, the only labeling in the spinal cord was in the lateral motor column. In addition, motoneurons labeled by injections of diI into individual limb muscles were located in the appropriate position for that motoneuron pool as determined by HRP injections (Landmesser, 1978*a, b*). Similarly, small injections of diI into individual sympathetic ganglia labeled only preganglionic neurons and these were situated only in the appropriate segments (Yip, 1985 and personal communication). In most of our experiments we intentionally gave large injections that labeled motoneurons as well as preganglionic neurons. We were, however, able to obtain a nearly pure population of preganglionic neurons by using only the medial part of the spinal cord to make our cultures (see Results for further details).

Although the initial labeling of cells clearly is specific, that specificity would be lost if a significant amount of dye transfer could take place in culture. Experiments in which we mixed labeled and unlabeled cells, or labeled cells of two different colors, gave no evidence for transfer of dyes between neurons. It should be noted that all our experiments were done with an objective with a fairly low numerical aperture (0.75), and our eyes were used as the detector. It is possible that studies with high numerical aperture objectives and very sensitive detectors might show some passive transfer of dyes. However, this would not affect our ability to positively identify cells, as it would be easy to establish quantitative criteria for assigning cells to particular classes.

The absence of dye transfer in culture is somewhat surprising, since Pagano and his colleagues (Struck and Pagano, 1980; Sleight and Pagano, 1985) have shown that several fluorescent lipid analogues can be transferred relatively rapidly from the outer leaflet of the bilayer to the membranes of unlabeled liposomes. One possible explanation for the absence of transfer of carbocyanine dyes from cell to cell is that neurons may not commonly approach each other closely enough for membrane to membrane exchange to take place. However, it is clear that even cells in the midst of a clump of labeled cells can remain unlabeled. One observation that may be related to the absence of diI transfer between the membranes of adjacent cells is that over a time course of several hours diI does not even appear to move from the outer to the inner leaflet of the bilayer (Wolf, 1985; but see Dragsten et al., 1981).

Given that dye is not transferred in vitro from cells labeled by dissociation, it would seem unlikely that it would spread from cells labeled retrogradely since in the two cases the dye appears to be in the same cellular compartments. Further, in spinal cord cultures, the percentage of retrogradely labeled cells was always low and roughly corresponded to the percentage that we would expect (see Results). That labeling was virtually always confined to neurons also suggests that dye does not spread in vivo or during the dissociation process.

Properties of Preganglionic Neurons in Culture

The physiological properties of preganglionic neurons that we have examined so far appear to be unremarkable. These

cells have resting and action potentials that are similar to those of many other neurons, and they can receive synapses from other neurons. Preganglionic neurons, which are cholinergic *in vivo*, can form synapses on myotubes and probably also on ganglion cells, suggesting that they retain the ability to synthesize and release acetylcholine under our culture conditions (see also Role, 1985).

One interesting observation was that preganglionic neurons could survive for some time in the absence of their normal target. However, since some neurons clearly died both in this situation and when target was present, it remains to be determined whether there are quantitative differences in the extent of survival. Several other characteristics of preganglionic neurons also appeared to be similar in the presence or the absence of their normal target. For example, there was no significant difference between the number of axons or primary dendrites of preganglionic neurons grown with and without ganglion cells. At least with respect to having multiple dendrites and a single axon, preganglionic neurons in culture bear some resemblance to avian (Cabot et al., 1985) and mammalian (Dembowski et al., 1985; Forehand, 1985) preganglionic neurons *in vivo*. While we need to examine these characteristics in more detail, the results so far are consistent with the idea that these aspects of cell shape may be intrinsically determined and relatively independent of environmental conditions. The characteristic morphologies of some cultured neurons, for example, cortical neurons (Kreigstein and Dichter, 1983), seem to be intrinsically determined, whereas the morphologies of other cultured neurons, such as mesencephalic neurons (Denis-Donini et al., 1984) and dorsal root ganglion neurons (Mudge, 1984), clearly can reflect their interactions with other cells.

We also observed that, even when they were grown in the absence of ganglion cells, preganglionic neurons had axons that sometimes broke up into fine branches and bore boutons. Synapses made by preganglionic neurons onto other spinal cord neurons may be present *in vivo* (Bogan and Cabot, 1985), raising the possibility that other spinal cord neurons in the cultures serve as a target for the preganglionic neurons.

Another possibility, particularly with regard to the question of survival, is that the complex medium we use supplies an essential factor, one that normally would be supplied by the target. It has been shown that several types of neurons can be maintained in culture, in the absence of their normal targets, if the necessary factors are provided (see Barde et al., 1983 and Berg, 1984 for reviews). The large numbers of other cells, in particular non-neuronal cells, present in these cultures might also provide considerable trophic support.

A final possibility is that we may have placed the preganglionic neurons into culture at a time before they become target dependent. Under our culture conditions, preganglionic neurons from embryos at stages 30–32 survive quite well, while motoneurons obtained from the same embryos do not. A plausible reason for the difference is that the period of naturally occurring cell death for motoneurons has already begun in these embryos (Hamburger, 1975), whereas the period of cell death for preganglionic neurons *in vivo* would not start until stage 34 (Oppenheim et al., 1982). Perhaps, as has been suggested previously for other types of neurons (see Berg, 1984 for a review; Calof and Reichardt, 1984), the preganglionic neurons do not become dependent on their tar-

get until the period when cell death normally occurs, and this target independence is retained, at least for some time, in culture.

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