(4 percent, weight to volume) is advanced until it penetrates the cell. Resting membrane potentials of the order of 60 to 70 mv (inside negative) are normally recorded. The cell is injected by passing hyperpolarizing pulses (approximately 10^{-8} ampere) 500 msec in duration at a frequency of 1 per second until many of the fine processes of the cell can be observed. The time for injection is variable (10 to 30 minutes) and depends mainly on the inside diameter of the electrode tip. To date, 12 lamprey neurons, including both giant interneurons and dorsal cells, have been injected by this method. All cells successfully penetrated, as evidenced by a maintained resting membrane potential, were adequately injected for EM. After injection the tissue containing the cell is removed and prepared for sectioning by using a modified method described by Smith *et al.* (*7*). The tissue is fixed in a 2.5 percent glutaraldehyde solution in 0.05*M* cacodylate buffer (*p*H 7.4) and 1 percent sucrose for ½ hour, and postfixed in 1 percent osmium tetroxide in 0.05*M* cacodylate buffer and 2 percent sucrose for 1 to 2 minutes. Cell membrane definition is not compromised by this short osmium fixation time and the tissue is quite transparent; the injected cell and its processes are visualized much like neurons impregnated by the Golgi method. In one whole mount a process 2 to 5 μm in diameter was followed 250 μm from the cell. The tissue is dehydrated in a series of alcohol solutions of increasing concentration. A 50 percent mixture of Epon 812 and Araldite is used for infiltration and embedding of the tissue. After the plastic is cured, 5-μm sections are cut on an LKB Ultratome III and mounted on clean glass slides until the cell is completely sectioned through.

Figure 1A is a light micrograph of an unstained 5-μm plastic cross section of one-half of a larval lamprey spinal cord. After identification of the injected cell with the light microscope, thin sections for EM are cut from the same thick section according to the method described by Campbell and Hermans (*8*). Figure 1B is a montage made from several electron micrographs (taken on a JEOL 100B microscope) of one of the thin sections cut from the section shown in Fig. 1A. The increased density of the giant interneuron (*gi*) and the dorsally projecting processes distinguishes them from the surrounding tissue. A direct correlation of the injected processes can be made between the light and electron micrographs so that there can be no error as to the cellular profiles containing the dye. To test the possibility that the cellular staining was an artifact or a consequence of osmium fixation, one cell was processed for EM without the osmium fixation. In the EM the density of the injected cell cytoplasm appeared no different from that of the tissue treated with short osmium fixation times. If anything, the contrast between injected cell cytoplasm and the surrounding neuropil appears to decrease if long osmium fixation times (15 minutes) are used.

Higher magnifications of portions of the injected processes shown in the left- and right-hand boxes of Fig. 1B are shown in Fig. 2, A and B. The cytoplasm of the injected processes (*gi*) is denser than the surrounding tissue and the dye appears to be contained within the plasmalemma. Mitochondria appear somewhat swollen in Fig. 2A, although the mitochondrial membrane is intact since no dye appears within. Mitochondria in Fig. 2B appear more normal, possibly because this area is farther from the injection site. Presynaptic profiles (*ps*) are numerous and appear to make normal contact with the injected processes. The presynaptic and postsynaptic membrane thickenings can be seen and do not appear to be altered by the technique.

Procion brown combines the advantages of Procion yellow and cobalt for light and electron microscopy without incorporating their disadvantages. The dye is visible in the light microscope, remains in the cell after injection, and requires no further reaction to make it visible in the EM. Injected processes are identified by using combined light and electron microscopy applied to the same section. In preparations such as the lamprey, where both presynaptic and postsynaptic cellular elements are large enough for microelectrode penetration, this technique can be used to identify them in the EM, and a study can be made of the synaptic contact about which some of the physiology is known.

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29 August 1973

Neuron Geometry and Circuitry via the Electron Microscope: Intracellular Staining with Osmiophilic Polymer

Abstract. *Intracellularly injected cobalt catalyzes the generation of an osmium-binding polymer from 3,3'-diaminobenzidine in neurons of* Aplysia californica. *Excellent structural preservation of nervous tissue is coupled with easy recognition of fine branches of the injected neurons in the electron microscope. This permits detailed structural analysis of synaptology.*

Morphological analysis of cell geometry and of neural networks is increasingly important in neurophysiological, genetic, developmental, and behavioral studies in the neurosciences (*1–5*). Recently introduced techniques of intracellular injection of diffusible fluorescent dyes (*2*) and metal ions (*3*) to display neuron geometry are now widely used in investigations of neural circuitry and the relations of neuronal form with function (*4*). However, dye and metal ion injection techniques have been used with good results only at the light microscope level; attempts to adapt them to use with the electron microscope have produced unsatisfactory results largely because of the compromises necessary in tissue fixation to preserve the intracellular marker. The use of Procion yellow dye to mark leech ganglion cells (*5*) in thick sections for light microscopy, which were subsequently sectioned for electron microscopy allowed only 3 minutes fixation in OsO_4. Longer fixation, necessary to preserve neuronal membranes, destroyed the fluorescent properties of the dye. Similarly, the use of cobalt precipitated as a black sulfide for an electron

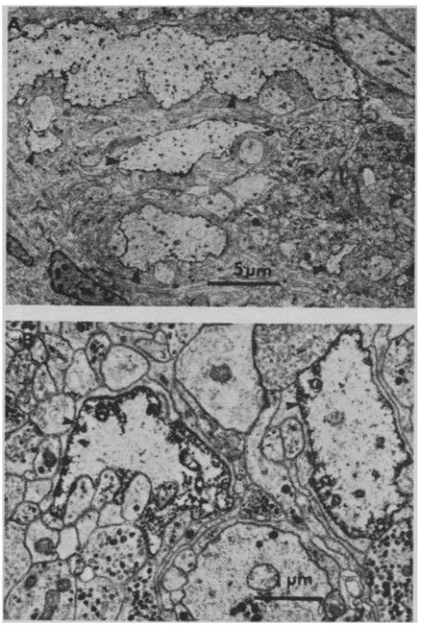

Fig. 1. Processes of the major interneuron L10 in the neuropil of the abdominal ganglion of *Aplysia* labeled with the osmiophilic polymer of DAB, which is catalytically generated by intracellularly injected cobalt. The polymer appears as amorphous clumps within the axoplasm and as a fuzzy layer at the inside of the cell membrane. (A) Fine branches of L10 (arrows) near a region of small vesicle-filled processes of other cells. (B) Two similar processes of L10 at higher magnification (arrows).

opaque marker has allowed only minimal osmium fixation (*3*).

We report here a new intracellular staining technique based on intracellular cobalt filling and subsequent catalytic generation of a nondiffusible osmiophilic polymer. The technique allows superior preservation of neural ultrastructure, fair preservation of the filled cell, and identification of fine branches of the injected cell less than 0.5 μm in diameter for distances of hundreds of micrometers. The technique is very simple to apply; its elements are routine neurophysiological and ultrastructural methods.

Abdominal ganglia were dissected from specimens of the gastropod mollusk *Aplysia californica* and pinned under *Aplysia* saline (*6*) at room temperature. Glass microelectrodes filled with 1*M* $CoCl_2$ and broken to resistances of 3 to 5 megohms were used for electrical recording and pressure injection. The somata of the major interneuron L10 and an adjacent follower cell were impaled. The interneuron was identified by recording hyperpolarizing postsynaptic potentials in the follower simultaneously with action potentials in L10 (*7*). It was then filled with cobalt until there was a 30 to 50 percent drop in input resistance, all spontaneous electrical activity (including postsynaptic potentials) disappeared, and the cell body appeared distinctly grayish when seen through a dissecting microscope. In two ganglia the follower cell was also filled with cobalt.

After the filling, the ganglia were left at 15°C for 45 to 60 minutes. A solution of 3,3′-diaminobenzidine tetrahydrochloride (DAB) (*8*) was prepared by dissolving 10 mg of DAB in 10 ml of saline with gentle heating. The DAB-saline was adjusted to *p*H 7.4 to 7.6 with NaOH. Ganglia were incubated in DAB-saline for 25 to 30 minutes, during which time the cobalt-filled cell bodies turned dark blue. The ganglia were rinsed for 20 minutes in several changes of saline alone and fixed for 15 minutes in a cold mixture of 2 percent formaldehyde, 1 percent glutaraldehyde, 4 percent sucrose, and 3 percent NaCl in 0.1*M* sodium phosphate buffer, *p*H 7.4. The left caudal quarter ganglion containing the injected cells was cut out with a razor blade and left in the fixative at 4°C for 12 to 36 hours. Tissue was subsequently rinsed for 1 hour in buffer containing 8 percent sucrose, and was then fixed for 1 hour in 1 percent OsO_4 in buffer containing 4 percent sucrose (*9*). After dehydration in ethanol the tissues were embedded in Spurr's resin (*10*) and mounted for sectioning. Thick sections (1 to 2 μm) were cut for light microscopy and stained with methylene blue. Ultrathin silver sections were mounted on Formvar-coated 50-mesh grids for electron microscopy and stained with uranyl acetate and lead citrate.

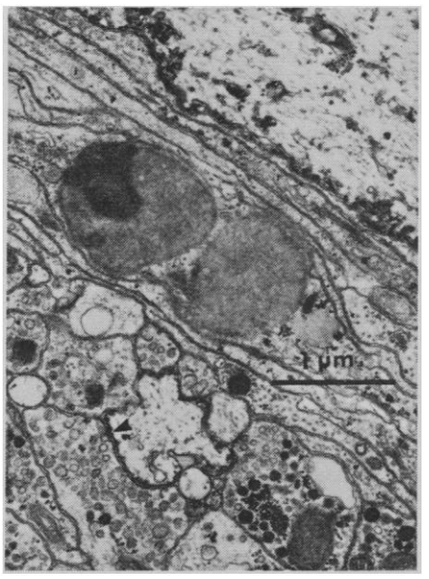

Fig. 2. A small process of L10 receives a synaptic contact (arrow). A portion of the main axon of L10 is at the upper right.

To date, eight cells in six ganglia have been successfully stained. In the light microscope with a dark-field condenser the larger processes of the injected cells appear yellow-orange, somewhat brighter than those of uninjected neurons, providing a guide for trimming the block for ultrathin sectioning. In electron micrographs the large major axons and the finer branches of injected cells are clearly distinguished from those of uninjected cells by the presence of the polymer (Fig. 1). The darkly staining polymer accumulates at the inside of the neuron membrane to give the cell processes a distinctively contrasting outline when viewed in the electron microscope screen. The course of the main axon of L10 and the distribution of its fine branches were seen to be qualitatively similar to our descriptions of the cell stained with Procion yellow (*11*).

This method provides an excellent marker for localizing presynaptic structures impinging on the injected neuron (Fig. 2) since the ultrastructure of uninjected cells survives incubation in DAB very well, allowing a detailed study of synaptic types, frequencies, and distributions (*12*). Thus, the technique of intracellular introduction of an agent catalyzing the formation of an osmiophilic polymer is a powerful tool for showing neuron geometry and circuitry of *Aplysia* with the electron microscope (*13*). Preliminary results indicate success in staining neurons of crayfish abdominal ganglion backfilled with cobalt through the axons of the third root (*14*). We expect that the method will be of great value in analyses of other neural systems.

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12. Organelles of the injected cell, although recognizable, are abnormal. From control studies we conclude that the high concentration of cobalt used for staining causes mitochondrial distortion, disruption of somatic endoplasmic reticulum, and possible artifacts in distribution of synaptic vesicles in the filled cell. It is likely that slight alterations in the method can improve the ultrastructure of the injected cell. Modification of the technique, especially for more delicate material, should allow DAB incubation after an initial aldehyde fixation. The possible use of iron and copper ions or their compounds such as Alcian Blue, or the metal-containing Procion dyes, is of interest: they might catalyze DAB polymerization effectively at low concentrations which do not damage presynaptic terminals.
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Diffusion of Weak Acids across Lipid Bilayer Membranes: Effects of Chemical Reactions in the Unstirred Layers

Abstract. *Chemical reactions in the aqueous unstirred layers of solution adjacent to a membrane can have dramatic effects on the diffusion of solutes across that membrane. This is demonstrated by the diffusion of labeled salicylate and salicylic acid across a phospholipid bilayer membrane. Two types of chemical reactions are considered. The first is an isotopic exchange reaction between the ionic and nonionic forms of a weak acid,* $HA + {}^*A^- \rightleftarrows H{}^*A + A^-$. *This reaction provides a way of estimating the true membrane permeability to a highly permeant weak acid and also a way of estimating the thickness of the unstirred layers. The second chemical reaction, the dissociation of a weak acid,* $HA \rightleftarrows H^+ + A^-$, *can be used to show how the presence or absence of buffers in the unstirred layers controls the net transport of permeant weak acids across a membrane. In principle, the addition of appropriate "antacid" buffers to salicylates can increase their rate of absorption from the stomach.*

Unstirred layers of solution adjacent to a biological membrane often constitute the rate-limiting barrier for the diffusion of highly permeant substances, for example, water, alcohols, fatty acids, anesthetics, bile acids, and various uncouplers of oxidative phosphorylation (*1–3*). If the permeability of the unstirred layers to a particular substance is much less than the permeability of the membrane, then it is impossible to measure directly the true membrane permeability to that species (*4*). However, by taking advantage of a chemical reaction within the unstirred layers, we demonstrate here a way of estimating the true permeability of a membrane to a highly permeant substance. We also show how the presence or absence of buffers in the unstirred layers can control the net transport of permeant weak acids across a membrane.

Salicylic acid was chosen for these experiments because the nonionic form of this weak acid is a highly permeant, lipid-soluble species with an appropriate *pK* (negative logarithm of the dissociation constant) of about 3.0. Furthermore, recent reports in *Science* and elsewhere (*5*) have described dramatic effects of salicylates on the permeability of both biological membranes and lipid bilayer membranes. Finally, the controversy concerning the relative rates of absorption of buffered versus unbuffered salicylates from the stomach is still unresolved (*6*). Thus, we wished to find out whether chemical reactions in the unstirred layers might be involved in the absorption process.

Lipid bilayer (optically black) membranes were made by the brush technique of Mueller *et al.* (*7*). The membranes were formed from a mixture of egg lecithin and decane on a spherical hole in a polyethylene partition. The partition separated two electrolyte solutions which were stirred magnetically (*8*). The solutions contained sodium chloride, sodium salicylate, and salicylic acid, buffered with sodium phosphate, sodium acetate, or sodium citrate. We manipulated the salicylic acid concentration by varying the *p*H, as described by the Henderson-Hasselbalch equation (*9*).

After a stable membrane had been formed, 2 to 5 μc of [^{14}C]salicylate were injected into the rear compartment. Then the rate of appearance of radioactivity in the front compartment was measured by continuous perfusion (1 to 2 ml min^{-1}) and collection of samples at 5-minute intervals. The rear compartment was sampled with a microsyringe. The samples were dried, the radioactivity was counted in a low-background planchet counter, and the one-way fluxes of solute were calculated (*10*).

We calculated the membrane resistance, using Ohm's law, from the membrane potential produced by applying a known voltage pulse across the mem-

Table 1. Effects of *p*H and an "antacid" buffer on the net flux of salicylic acid across a lipid bilayer membrane. Results in the last column are given in the sequence: mean ± standard error and, in parentheses, the number of membranes. The *p*H adjustments were made with hydrochloric acid. The membrane voltage was clamped at 0 mv, and the zero-potential current was always less than 0.1 percent of the net flux, when expressed as ionic current.

Rear solution					Front solution		Net flux ($\times 10^{-9}$ mole cm^{-2} sec^{-1})
*p*H	Sodium salicylate (m*M*)	Salicylic acid (m*M*)	Sodium chloride (m*M*)	Sodium citrate (m*M*)	*p*H	Sodium phosphate (m*M*)	
3.9	35.7	4.3	150		7.4	150	7.9 ± 0.3 (3)
4.9	39.5	0.5	150		7.4	150	0.9 ± 0.1 (3)
4.9	39.5	0.5		150	7.4	150	39 ± 4 (3)