Other amino acids, however, bind manganese (*16*). That L-dopa might conform to this precedent is suggested by the increased concentration of ^{54}Mn (*17*) and of ^{55}Mn (*2*) in livers of mice consuming L-dopa.

Such a connection was also implied in man by a drop of blood manganese (^{55}Mn) while the patients' parkinsonism was improved with dopa (*18*) (Fig. 2). This was, however, not an indicator of responsiveness to the drug (*19*). When ranking patients as to their responses to L-dopa, one might rank them also with some simplified version of a technique utilizing ^{54}Mn (*3, 12*) and seek correlations between the two. The individual variance is so great among patients with parkinsonism and chronic manganese poisoning (*1*) that the genetic differences encountered by others (*20*) might indicate divergent genetic susceptibilities among members of these populations. One cannot test healthy blood relatives with chronically administered drugs. It remains to be determined, however, whether a genetic marker can be developed by scanning these relatives for absorption or distribution of a harmless isotope (*3, 12*).

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Branching of Central Neurons: Intracellular Cobalt Injection for Light and Electron Microscopy

Abstract. *Cobalt chloride can be injected into an identified nerve cell body in an insect ganglion and reacted with ammonium sulfide to stain the soma and its branches with a black precipitate. The stained cell body and its branches throughout the neuropil are visible in both the light and electron microscope. In whole mount preparations, the resolution of neurites within the neuropil is of a quality that permits the comparison of branching patterns between cells and during various functional states.*

The specific form of a behavioral act generated by a population of neurons may be critically determined by the pattern of connections between units in the system. It is therefore essential in analyzing the cellular basis of behavior to be able to identify the individual neurons involved and to visualize the geometry of their interacting, branched processes (neurites). One of the most useful techniques for observing individual neurons has been the Golgi method and its variations. This has been a powerful tool because a small proportion of a neuronal population is stained and those neurons that have been stained are filled entirely. Although this method gives much cellular detail and has been extended from the light microscopic to the ultrastructural level (*1*), it has the major disadvantage that neurons are stained more or less randomly and that the quality of the results can be capricious.

The correlation of neural structure and function was advanced with the technique of intracellular injection of the fluorescent dye Procion Yellow (*2*). With this and other related dyes, neurites deep within the neuropil were stained in cells whose function had been determined by intracellular stimulation and recording. There are, however, some disadvantages with the method. The Procion dye is not electron-opaque, a fact that precludes observations at the ultrastructural level. Therefore, the precise determination of synaptic contacts on the neurites of the identified cells has not been possible. We have also found it difficult to observe the detailed branching pattern of nerve processes in whole mounts of the insect central nervous system with the use of Procion Yellow. Indeed, it has been necessary in the insect, as well as in most other invertebrate preparations, to reconstruct the branching pattern of neurites from serial sections. Even in this situation, we feel that only the major branches are clearly seen and the finer processes are not adequately resolved. We have developed an intracellular injection technique for neurons which permits the direct visualization of finely branched neurites in whole mounts viewed with bright-field illumination. Processes approaching 1 μm diameter are resolved. The dye is also electron-opaque. This allows ultrastructural observation of the injected cell and its relation to other elements of the neuropil.

Our method uses the reaction of cobaltous chloride with ammonium sulfide to form a black precipitate within the neuron. It should also be possible to use the chlorides of copper, iron, or nickel, all of which are soluble and are precipitated by ammonium sulfide. Neurons were filled with the cobalt solution by either the pressure injection technique of Remler *et al.* (*3*) or by iontophoresis. For pressure injection, glass micropipettes (tip diameter, 1 to 2 μm) were filled with a 1*M* solution of cobalt chloride containing 0.4 percent Procion Navy Blue H3RS. The Procion dye was added so that filling of the neuron could be observed as cobalt chloride is not sufficiently colored to be visible when the dye is entering the cell body. When the cell body was injected iontophoretically a 1 to 50 m*M* solution of cobalt chloride was used. The Procion dye is not re-

quired when the cell is injected by iontophoresis because the cell body becomes somewhat opaque with this procedure.

After the solution was infused through the soma for 1 to 2 hours the preparation was immersed for 20 to 30 minutes in 10 ml of insect saline containing approximately 0.05 to 0.1 ml of 100 percent ammonium sulfide solution. The preparation was then washed in saline and fixed for 1 hour in 3.5 percent glutaraldehyde that was brought to *p*H 7.2 with cacodylate buffer. Although this proved suitable for whole mount preparations, the best preservation for electron microscopy was obtained by the following procedure, modified from Karnovsky (*4*): fixation for 2 hours in a mixture of 4.5 percent paraformaldehyde, 1.1 percent glutaraldehyde, and 4.5 percent of 0.15*M* phosphate buffer. After fixation the material was dehydrated and embedded in Epon 812 of Spurr low-viscosity resin (*5*). The preparation could then be viewed directly as a whole mount in the plastic block or sectioned for light or electron microscopy.

The best observations with whole mount preparations were obtained by clearing the tissue in creosote and viewing it mounted in creosote in a shallow depression slide. The dehydrating alcohols were buffered with 0.15*M* phosphate to prevent loss of the stain precipitate, which is soluble in acid. No postosmication procedure was used, so that the stained cell could be directly observed while thick and thin sections were cut. This insured that the dense profile observed in the sections came from a neurite of the stained cell.

The preparation used in developing this technique was the metathoracic ganglion of the cockroach *Periplaneta americana*. The somata of the larger motor neurons have been mapped with respect to the nerve trunk through which their axons exit from the ganglion (*6*). Most studies were done on cell 28 from this map. When a desheathed ganglion was viewed with properly angled and focused illumination, the soma of cell 28 was readily identified by its size and consistent relation to tracheal landmarks.

We stimulated this cell by passing depolarizing current through a microelectrode placed in the soma and observed twitches in coxal muscles that depress the leg. Figure 1 shows an intracellular recording obtained from muscle 179 (*7*) and evoked by intracellular stimulation of cell 28. The muscle response is that typical for an insect "fast" muscle (*8*).

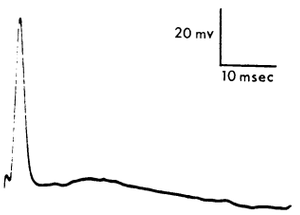

Fig. 1. An intracellular recording from a "fast" muscle fiber in the coxal depressor muscle 179 (*7*). The response was evoked by a 3-msec intracellular depolarizing pulse applied to the soma of cell 28 in the metathoracic ganglion.

The cell body and neurites of cell 28 stain black when the cobalt salt is injected; this is seen in the stereoscopic pictures of whole mounts (Fig. 2). The branches extend throughout the 1 mm depth of the ganglion, from the cell body on the ventral surface to very near the dorsal surface. The axon is seen leaving the ganglion through nerve trunk 5. There is remarkable similarity in the branching pattern of this cell from one preparation to the next.

It is difficult to record consistent electrical activity from the cell body of most insect motor neurons. However, one group of central neuron somata, located in the dorsal medial region of all thoracic and abdominal ganglia of the cockroach, show large overshooting spikes when a microelectrode is placed in the cell body (*9*). We therefore used these dorsal cells for examining the optimum concentration of cobalt chloride to be used where intracellular stimulation and recording was followed by dye injection into the cell. With cobalt chloride at concentrations ranging from 1 to 50 m*M*, it is possible to record overshooting action potentials from the cell bodies of the dorsal neurons. The potentials were similar to those described by Kerkut *et al.* (*9*) for these cells and could be recorded for more than 1 hour from an impaled soma. After the electrical recording, we were able to stain the cell successfully by iontophoretic ejection of the cobalt salt into the cell body. Positive pulses of 5×10^{-8} amp, 0.5 second in duration, were delivered one per second for a 1-hour period.

It was essential to determine whether

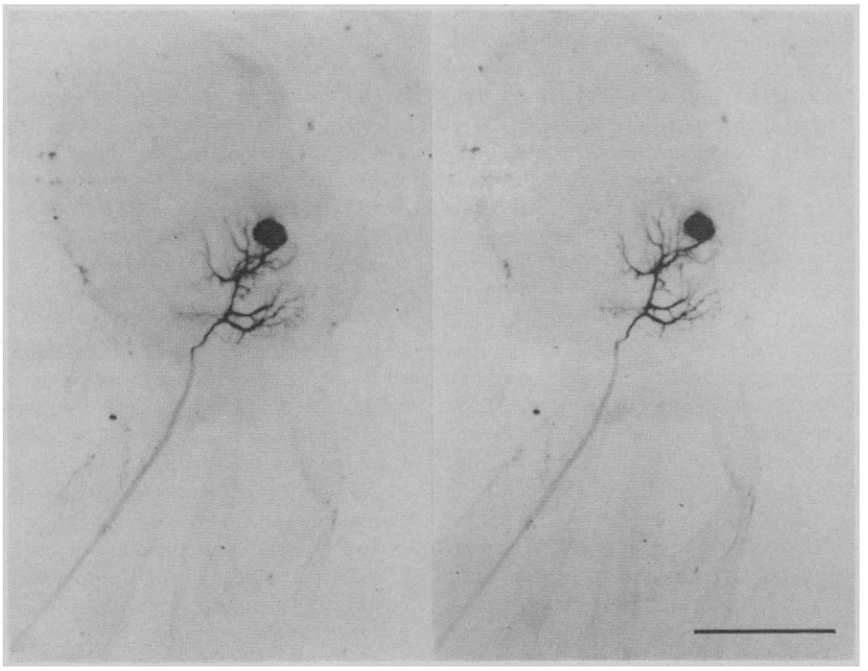

Fig. 2. Stereo photographs of the metathoracic ganglion. Cell 28, stained with intracellular injection of cobalt chloride, is shown. The difference between the viewing angles for the photographs is approximately 9°. Observe these pictures through standard stereoscopic viewing glasses (*11*). This is a dorsal view of a whole mount preparation. The entire ganglion was immersed in creosote on a depression slide. The cell body is seen through the depth of the ganglion, with the initial process running toward the dorsal surface. The characteristic medial and lateral branching pattern is apparent throughout the depth of the ganglion. The axon traverses the dorsolateral region to exit through right nerve 5. The posterior edge of the ganglion is to the left. The calibration is 0.5 mm.

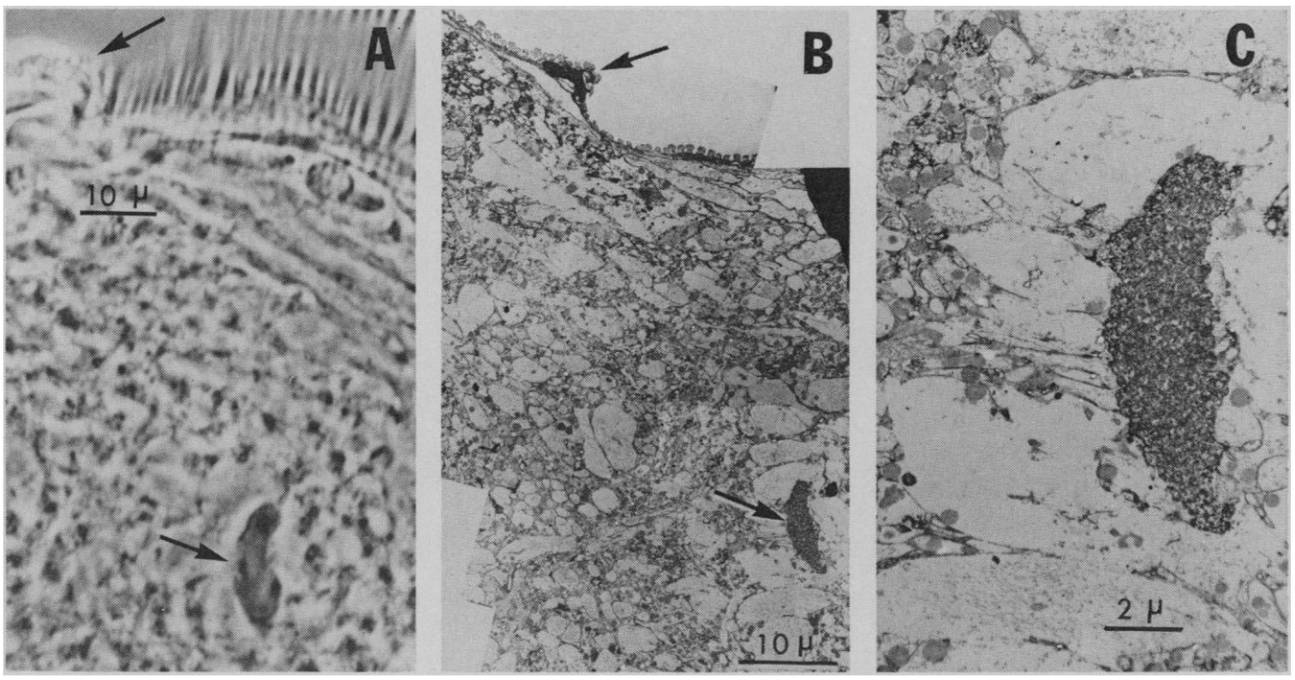

Fig. 3. (A) Phase micrograph of a 4-μm plastic section of part of the metathoracic ganglion. The lower arrow shows a stained nerve process from a cobalt-injected cell, and the upper arrow indicates a fold in a large tracheole. (B) A montage made from electron micrographs of a thin section that was cut immediately adjacent to the thick section in A. The lower arrow points to the same stained profile shown in A. Note the electron opacity of the nerve process in a cell injected with cobalt. The upper arrow indicates the same tracheole fold visible in A. (C) A higher-magnification electron micrograph of the same cobalt-stained profile in A and B. Note the electron-opaque, granular appearance of the cytoplasm.

or not cobalt chloride was able to freely cross electrotonic synapses or was confined primarily to the boundary of the injected cell. To test this, we injected the lateral giant fiber of the crayfish. These giant fibers have a series of segmentally arranged septa along their length which are points of electrotonic coupling. Procion Yellow can cross these synapses, but its movement is more restricted across this junction than within the axoplasm (*10*). After cobalt was injected into the lateral giant fibers, its movement was primarily restricted to a single segment.

In order to determine whether or not the cobalt precipitate seen with the light microscope was electron-opaque, we cut alternate thick (4 μm) and thin sections through neurites of the stained cell embedded in plastic. This enabled us to compare successive sections of a stained neurite, one observed by phase microscopy and the next, by electron microscopy. When viewed by phase microscopy, the stained neurite appears as a dense structure, clearly delineated from other profiles of the neuropil (Fig. 3A). An adjacent thin section shows the ultrastructural appearance of the same stained neurite (Fig. 3B). The stained profile has a dense granular appearance when viewed at high magnification in the electron microscope (Fig. 3C). The electron-opaque material appears confined to a discrete profile and is not dispersed into adjacent areas.

The improved resolution of neural branching patterns which is possible with this technique has permitted us to obtain initial results on the effect of partial deafferentation and axotomy on the geometry of neurites from cell 28. A metathoracic leg was amputated near the coxal-body junction. The neurons were injected with cobalt 10 days to 5 weeks after the operation. In the majority of cells examined there was a marked reduction in the branches stained within the neuropil. The soma and the initial process leaving it stained a dense black in these experimental cells. The characteristic extensive lateral and medial neuropil branches, so evident in the normal cell 28 (Fig. 2), were not visible in the experimental cells when they were viewed in whole mount. The absence of staining in the major neuropil branches of cell 28 under these conditions could be due to a breakdown or retraction of these processes. Another possibility is a change in the internal transport mechanisms that might affect the distribution of the injected cobalt chloride. This technique should be useful for investigating the degree to which neuronal geometry is open to modification under different functional conditions.

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