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23. Recovery of the DPN internal standard ranged from 85 to 90 percent, all NMOR values were corrected for the DPN recovery. The NMOR and DPN values from 8- to 16-g aliquots of powder, varied less than 5 percent from the average, on a per-gram weight basis. Low concentrations of DMN (2 to 3 ng per mouse) were occasionally found in untreated controls or controls exposed to NO_2 or MOR, or both.
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Intracellularly Injected Cobaltous Ions Accumulate at Synaptic Densities

Abstract. *Physiologically identified neurons in the locust were iontophoretically injected with a mixture of cobaltous and potassium ions. After being fixed for electron microscopy, 2.5-micrometer sections of the epoxy-embedded ganglia were intensified with silver. The intensified material was resectioned and examined in the electron microscope. The cobalt-silver precipitate appeared as discrete densities. Localized accumulations of the precipitate were seen within the injected cell along the neuronal membranes and especially at synapses. Location and recognition of the stained neuron in the electron microscope was facilitated by the tendency of the cobaltous ions to aggregate at the synaptic sites.*

The need for an approach combining anatomy with physiology in the study of neuronal circuitry has been recognized for more than a decade (*1*). The introduction of dyes that could also be used as recording solutions in microelectrodes (*2, 3*) has made such combined studies feasible. Although a variety of dyes can provide excellent results for light microscopy, all of the previously reported techniques have had limited usefulness for the electron microscope (EM). Earlier methods with cobalt resulted in poor fixation, thought to be caused by the high concentration of cobaltous ions required for them to be seen in the EM (*3–5*). Procion dyes also disrupt organelles and may trigger phagocytosis by surrounding glia (*6, 7*). The diffuse, electron-opaque horseradish peroxidase reaction product obscures the ultrastructure of the injected cell and greatly increases electrode resistance, making recording more difficult (*7, 8*). Here I report a technique for silver intensification of intracellularly injected cobaltous ions which overcomes these problems.

Neurons were physiologically identified and filled by cobalt-potassium-filled electrodes (*9*), which have both good recording and good staining characteristics. This technique allows unambiguous identification of the stained neuron, including its synaptic sites, without the necessity of EM serial reconstruction. The cobalt-silver precipitate is discrete and highly electron-opaque making it easily seen in the EM. The results presented here are from three fast extensor tibiae motoneurons stained in one mesothoracic and two metathoracic ganglia of the locust *Schistocerca americana gregaria*. Successful results with this technique have also been obtained for 15 other neurons in the locust, including local nonspiking interneurons (*10*). A preliminary report of the technique has been made elsewhere (*11*).

Neurons were identified in the meso- and metathoracic ganglia by standard techniques (*12*). An isotonic saline was used (*13*). After physiological identification of the motoneuron, cobaltous ions were injected into the soma with 10- to 20-nA current pulses of 50-msec duration and 10-Hz frequency for 40 minutes. The ganglion was then excised, transferred to a Vaseline well on a glass slide, and flooded with fixative (2.5 percent glutaraldehyde, 0.05*M* sodium phosphate buffer, 0.2*M* sucrose, *p*H 7.2). After 15 minutes, the extreme edge of the ganglion opposite to the soma of the filled neuron was cut away to ensure that the solution would penetrate. The solution in the well was changed to 0.5 to 1.0 percent ammonium sulfide in buffer (0.05*M* sodium

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phosphate, 0.4*M* sucrose, *p*H 7.2) for 10 minutes to precipitate the cobaltous ions as cobaltous sulfide. After two or three rinses in fresh fixative, the ganglion was left in fixative overnight (14 to 20 hours). The next day, after a buffer rinse, the ganglion was then fixed in osmium (1 percent in 0.05*M* sodium phosphate buffer, 0.2*M* sucrose, *p*H 7.2) for 1 hour. This was followed by en bloc staining with 2 percent aqueous uranyl acetate for another hour and dehydration through a series of ethanols to propylene oxide. The procedures were carried out at 4°C. The ganglion was embedded in Epon 812.

The ganglia were serially sectioned at 2.5 μm, and the thick sections were dried onto glass slides. At this point the unintensified cobaltous sulfide was not visible in the light microscope. Silver intensification of the cobaltous sulfide was carried out on the sections (*14*). Sections were then mounted in Epon, and selected sections were photographed for later reference. In the primary neurite shown in Fig. 1A, there was very little background reaction in the surrounding tissue. The lighter ring around the cell body is not a staining artifact, but is due to the many glial fingers, or trophospongia, invading the somata of insect neurons, but remaining unstained by this technique.

The serial thick sections were reembedded (*15*) and resectioned at 50 to 80 nm with a diamond knife. The sections were picked up on 75-mesh collodion-coated grids and stained with uranyl acetate and lead citrate (Fig. 1B). The cobalt-silver was also found in the nucleus of the injected cell. The ultrastructural preservation of organelles in the stained neuron (such as the mitochondria, Golgi, and endoplasmic reticulum) is comparable to that of the uninjected neighboring cells, even though in these experiments the soma was the site of dye injection.

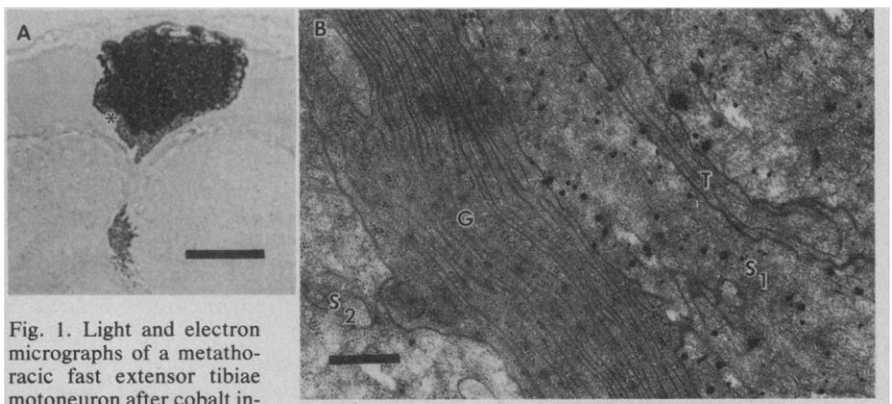

Fig. 1. Light and electron micrographs of a metathoracic fast extensor tibiae motoneuron after cobalt injection and silver intensification. The first 0.2 μm of the thick section are not used for ultrastructural analysis, because the silver precipitates heavily on the surface of the injected neuron and also because of some nonspecific scattering. In the remaining 2.3 μm of the section, the cobalt-silver is confined to the injected neuron, where it appears as very electron-opaque spheres between 15 and 40 nm in diameter. (A) Light micrograph of a 2.5-μm-thick section through the cell body and a portion of the primary neurite. The cobalt-silver appears to be confined to the injected neuron only. (B) Electron micrograph of the area marked by the asterisk in (A); the very dark cobalt-silver particles are confined to the injected cell body (S_1) and is not found in the neighboring soma (S_2), the glia (*G*), or in the trophospongium (*T*) that invades the labeled cell body. Scale bars: (A) 50 μm; (B) 0.5 μm.

Examination of the primary neurite and branches reveals equally good localization of the cobalt-silver precipitate. Both pre- and postsynaptic endings are seen in stained locust motoneurons (Fig. 2). The synaptic vesicles appear normal and show no disruption in the injected neurons. Synaptic densities are retained, and in favorable section T-bar capping structures over the presynaptic dense projection (*16*) are evident. Identification of synapses was based on standard structural criteria (*16*).

The accumulation of the cobalt-silver at specific loci within the neurons was an unexpected finding. The concentration was highest in the soma and appeared to be fairly evenly distributed there. In the rest of the neuron the highest concentrations were localized in three areas. In the primary neurite, most of the cobalt-silver was aggregated at or near the neuronal membrane. In the finer neurite branches, it was seen on or near microtubules and within the synaptic densities. Aggregation was most marked at presynaptic densities, where the precipitate lined up among the vesicles (Fig. 2A). Cobalt-silver was associated with 44 of 55 synapses made by and onto the three dye-labeled neurons.

There are two possible explanations for the observed association of the cobalt-silver with neuronal membranes. (i) The cobaltous ions may be attracted to charged amino acid groups in the synaptic densities. Cytochemical studies of these paramembranous densities have shown them to be rich in polar substances and that the less dense internal "fuzz" coat of neuronal membranes has a composition similar to that of the densities at synaptic regions (*17*). This then might explain the attraction of the cobalt for the neuronal membranes. (ii) Since cobaltous ions are competitive blockers of calcium channels in nerve and muscle (*18*), the cobaltous ions could be associated with calcium binding sites in or near the membranes. Calcium-binding proteins such as calmodulin have been isolated from a variety of vertebrate and invertebrate tissues, and this calcium-dependent regulator protein is required for protein kinase activity and neurotransmitter release from synaptosome fractions (*19*). In vertebrate tissue, calmodulin is associated with postsynaptic

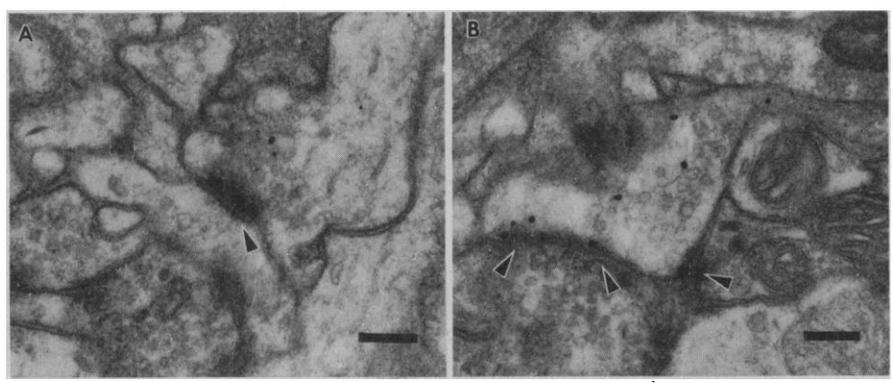

Fig. 2. Pre- and postsynaptic endings marked by the cobalt-silver precipitate. Their ultrastructure is well preserved. (A) A marked branch of the fast extensor tibiae motoneuron is presynaptic to an unidentified neuron. The cobalt-silver is lined up against the presynaptic membrane among the vesicles, within the densities. Subjunctional dense bodies are present beneath the postsynaptic membrane (arrow). (B) In another marked branch of the same neuron, precipitate is found among the paramembranous densities where the cell is postsynaptic to two unidentified neurons (arrows). The presence of the precipitate does not hinder the identification of synapses. Scale bars, 200 nm.

densities by biochemical assay, and calmodulin antibody labeling in the EM has localized the protein on postsynaptic microtubules as well as in the densities (*20*). If the cobaltous ions in the labeled neuron are bound to calcium-binding proteins, the accumulation of cobalt-silver at the synapses would indicate a higher concentration of these proteins there. That the cobalt-silver has been found in four-fifths rather than in all of the synapses is not unprecedented and may reflect on the active state of the labeled synapses at the time of staining. In the crayfish, localization of cobalt-filled synaptic specializations in the light microscope has been correlated with functional synaptic interaction (*21*), but a similar analysis has not been made at the EM level.

This method permits neurons to be physiologically characterized and subsequently stained for EM by iontophoresis. Neuronal processes are readily visible in both the light and the electron microscope. Ultrastructural preservation of the tissue, including the dye-filled cell, is excellent, allowing the study of synaptic contacts onto, as well as from the marked neuron. The aggregation of cobalt-silver at the synaptic membranes facilitates location of the marked synapses in the EM. The accumulation at synapses and on or near microtubules is in good agreement with the sites where calmodulin has been localized.

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Cytosolic and Microsomal Epoxide Hydrolases: Differential Properties in Mammalian Liver

Abstract. *The epoxide hydrolase activities of the 100,000*g *pellet (microsomal) and 100,000*g *soluble (cytosolic) fractions of mouse, rat, and guinea pig liver were measured with three closely related compounds used as substrates. Differences between the species in the distribution of the cytosolic and microsomal hydrolases and in their substrate specificities and* p*H optima demonstrate why epoxide hydrolase activity in the cytosolic fraction was not detected earlier in spite of intensive work on the microsomal epoxide hydrolase.*

Many natural and man-made compounds exist in the environment as epoxides, and epoxides may result from biological oxidation of alkenes or arenes. Since some epoxidized compounds are electrophilically reactive and are toxins, mutagens, or carcinogens, it is important to understand their routes of degradative metabolism in mammalian tissue.

In mammals, epoxides are degraded chemically and enzymatically through several pathways. Usually important is their conversion to 1,2-diols by epoxide hydrolases (*1*). These enzymes have been widely assumed to be bound to the endoplasmic reticulum or nuclear membrane (*2*); however, some studies have demonstrated that for many lipophilic substrates (such as terpenoid, steroid, and fatty ester epoxides), most of the epoxide hydrolase activity is present in the 100,000*g* soluble (cytosolic) subcellular fraction (*3, 4*).

We recently found that the cytosolic fraction of mammalian liver also hydrates a wide variety of simple aliphatic epoxides including some known mutagens and carcinogens. Since the substrate selectivity of the cytosolic fraction partially overlaps that of the microsomal fraction, it superficially appears that the results of our studies on the cytosolic epoxide hydrolases contradict those of many studies on the microsomal enzymes. However, the different properties of the cytosolic and microsomal enzymes reported here merely indicate how epoxide hydrolase activity in the cytosolic fraction was overlooked by other laboratories for nearly a decade.

Microsomal epoxide hydrolase is reported to hydrate monosubstituted and *cis*-1,2-disubstituted epoxides (*2, 5*). The cytosolic fraction hydrates the former most rapidly, but it also hydrates *trans*-1,2-disubstituted and tri- and tetrasubstituted epoxides (*3, 4*). Styrene oxide has been used by many researchers for monitoring microsomal epoxide hydrolase activity (*2, 5-10*), but minimal hydration of styrene oxide was detected in the cytosolic fraction under several conditions employed in our study (*11*). Two closely related compounds, which differ from styrene oxide by a single carbon atom (Fig. 1), are rapidly hydrated (Table 1) (*12, 13*). These compounds include *trans*-β-methylstyrene oxide, which is hydrated by the cytosolic but not the microsomal fraction, and allyl-

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