Labeling of cat spinal cord neurons with horseradish peroxidase

by

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A Thesis Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

Department: Veterinary Physiology and Pharmacology
Major: Physiology (Pharmacology)

Iowa State University
Ames, Iowa

1981

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INTRODUCTION

Literature Review

The early major published anatomical work on the spinal cord was done in the second half of the nineteenth century. Clarke, Golgi, Cajal and others (cited by Rexed, 1954) used various techniques in their investigations of spinal cord neuronal morphology. The principal methods used were the Golgi method introduced by Camillo Golgi in 1873 (cited by Ramon-Moliner, 1970) and methylene blue staining introduced by Ehrlich in 1886 (cited by Bracegirdle, 1978). Although both methods have been used for selective and complete staining of single neurons, the results obtained by using these methods were inconsistent and they required considerable experience to perform. A third major experimental technique, the degeneration method of Nauta (Nauta and Gygax, 1951), was also introduced at a later date. It was used to provide information about axonal trajectories. In 1952, Rexed proposed a cytoarchitectonical classification of the spinal cord grey matter into ten laminae. Since that time, much work has been done on the spinal cord. Such morphological aspects as neuronal size and population density of lumbosacral spinal cord neurons in the cat (Aitken and Bridger, 1961), neuronal volume and surface area (Schadé, 1964), and dendritic patterns of spinal neurons (Ramon-Moliner, 1962; Matsushita, 1970) have been investigated. Various techniques have been used to study the morphology of the spinal cord reticular formation (Leontovich and Zhukova, 1963), brachial cord (Sterling and Kuypers, 1968), Clarke's column (Szentagothai and Albert, 1954; Réthelyi, 1968; Loewy, 1970), motor

neurons (Nyberg-Hansen, 1965; Prestige, 1966; Scheibel and Scheibel, 1966), interneurons and Renshaw cells (Scheibel and Scheibel, 1966) and ventral horn neurons (Testa, 1964).

The disadvantage of the techniques used in these early spinal cord studies was that anatomists could not determine the physiological properties of neurons that they observed. Similarly, physiologists had no direct method for determining the morphology of neurons that they analyzed with electrophysiological techniques. A partial solution to this problem was found when Kerkut and Walker (1962) used a microelectrode to deposit potassium ferocyanide intracellularly in single neurons while making electrophysiological recordings. Thus, the neuron recorded from could be identified following the use of specific histochemical procedures. Subsequently, several dyes including aniline blue, orange G, fast green, and niagara blue were used for intracellular staining (Kater and Nicholson, 1973). The main problem with all of these dyes was that they stayed where they were injected and did not migrate sufficiently along the neuronal processes; the result was that the complete cell was not well-stained.

To resolve this problem, Stretton and Kravitz (1973) introduced the use of procion dyes for direct visualization of physiologically identified neurons. Following their introduction, procion dyes, especially procion yellow, were used with satisfactory results by a number of investigators (Bryan et al., 1972; Brown et al., 1974, 1975). However the use of procion dyes did have some limitations: (1) Stretton and Kravitz (1973) reported that procion did not always pass out of the micropipette during iontophoresis; (2) procion did not stain axons and axon collaterals well

(Jankowska, 1975; Brown et al., 1977a); (3) Light and Durkovic (1976) reported that procion was cytotoxic; (4) procion yellow was not electron dense; therefore, it was not readily used for electron microscopy (Jankowska et al., 1976).

Injection of radiolabelled amino acids intracellularly, introduced by Globus et al. (1968), also provided a method for morphological determination of electrophysiologically characterized neurons. The main drawback of this technique was that the autoradiographic procedure was technically complex and it required 5-6 weeks to obtain results (Cowan and Cuenod, 1975; Kater and Nicholson, 1973; Lynch et al., 1974b).

The advent of intracellular neuronal staining with horseradish peroxidase (HRP) has introduced a new method which allows the determination of the morphological characteristics of electrophysiologically identified neurons. Horseradish peroxidase, an enzyme (mol. wt. 40,000), was initially used in the study of kidney function (Straus, 1957). The histochemical technique of localizing HRP deposits by processing tissue with 3,3' diaminobenzidine was introduced by Graham and Karnovsky (1966) in their studies of kidney physiology. HRP was first used to study the peripheral nervous system by Kristensson and Olson (1971) and was applied to the study of the central nervous system by LaVail and LaVail (1972) shortly thereafter. Both of these early studies involved depositing HRP outside of a neuron or neurons and observing the HRP after it had been taken up by the axon terminals and transported preferentially in the retrograde direction toward the neuronal cell bodies. The neurohistochemical basis for localization of HRP is that following a suitable sur-

vival period, the tissues at the desired locations are cut and sections of tissue are incubated in appropriate media in order to produce a colored reaction-product at sites containing HRP activity. The incubation medium contains hydrogen peroxide (H_2O_2) and a chromogen (Chr), which assumes a densely colored form only in the oxidized state. At sites containing HRP activity, the general reaction $(Chr)H_2 + (HRP \cdot H_2O_2) \rightarrow (Chr) + HRP + 2H_2O$ takes place and the color of the oxidized chromogen becomes visible (Mesulam, 1976). Until recently, DAB has been used almost exclusively as the chromogen in central nervous system research. In the past few years, tetramethyl benzidine (Mesulam, 1978) and a mixture of p-phenylenediamine and pyrocatechol (Hanker-Yates reagent; Hanker et al., 1977) have also been introduced as chromogens.

While in early studies HRP was injected extracellularly by positive pressure, it was later applied by microelectrophoresis (Graybiel and Devor, 1974). Several excellent reviews exist which cover in detail the advantages and limitations of the retrograde transport method using HRP (Cowan and Cuenod, 1975; Adams, 1977; Winer, 1977).

The fact that HRP in membrane bound organelles appears to move in the anterograde as well as retrograde directions was realized in early studies (Hansson, 1973). Lynch et al. (1973) utilized this fact to study the dentate mossy fiber system by injecting HRP extracellularly near the cell soma and identifying HRP that was picked up at the soma and transported somatofugally. Lynch also used extracellular recording electrodes to characterize neurons before labeling them with HRP (Lynch et al., 1974a, b). Brown and his co-workers (Snow et al., 1975) were the first to intro-

duce intracellular injection of HRP through a recording glass microelectrode in their study of spinal neurons. This work was quickly followed by other intracellular studies. Thus, HRP was used to study motoneurons (Cullheim and Kellerth, 1976), interneurons (Czarkowska et al., 1976), spinocervical tract cells (Jankowska et al., 1976; Brown et al., 1977a), cortical neurons (Kitai et al., 1976), cerebellar neurons (McCrea et al., 1976), and hair follicle afferents (Brown et al., 1977b). Recently, HRP in conjunction with electrophysiological recording has been used to investigate dorsal horn neurons (Light et al., 1979), motoneurons (Cullheim and Kellerth, 1978; Cullheim and Ulfhake, 1979), cultured mouse spinal cord neurons (Neale et al., 1978), spinal axon collaterals (Futami et al., 1979), and spinocervical tract neurons (Brown et al., 1980). These studies have confirmed the usefulness of HRP over other techniques:

(1) HRP stains axons, axon collaterals and dendrites better than procion yellow (Brown et al., 1977b);

(2) Axons stained with HRP can be traced farther than procion stained axons. Whereas maximum length of axons stained with procion yellow is 2-3 mm (Jankowska and Lindstrom, 1972), intracellularly injected HRP has been traced over 25 mm (Brown et al., 1977a);

(3) The HRP processing technique is relatively easy to perform;

(4) HRP is sufficiently electron dense and therefore can be used for electron microscopy without additional modifications;

(5) The enzyme filled micropipettes have acceptable recording properties;

(6) The enzyme appears to be transported somatofugally in the axon, and since the injected neuron is the only stained element in the tissue, the course of the axon can be followed in serial sections to its termination (McCrea et al., 1976).

Most investigators agree that the general picture presented by a HRP stained neuron is quite similar to the best examples of Golgi stained material with the advantage of singling out just one neuron (Light et al., 1979). Only HRP has been used to trace the axon of a single neuron to its termination (Bishop, 1977).

During our initial work with HRP, we also attempted to demonstrate retrograde transport of HRP from the cerebellum to the cells of Clarke's nucleus. The dorsal spinocerebellar tract (DSCT) is probably the most thoroughly studied tract among the spinocerebellar pathways (Mann, 1973). Most of the cells of origin of this tract are found in Rexed's lamina VII as the nucleus dorsalis or the dorsal nucleus of Clarke. It has generally been accepted that Clarke's nucleus extends from the first thoracic to the third or fourth lumbar spinal cord segment. Afferents from Clarke's nucleus travel via the DSCT to terminate ipsilaterally as climbing fibers primarily in the anterior lobe of the cerebellum in lobules I to V (Grant, 1962). Three cell types, classes A, B and C, have been identified in Clarke's column on the basis of their size (Loewy, 1970). HRP studies have shown that types B and C send their projections to the cerebellum (Petras and Cummings, 1977), while type A probably represents a local circuit neuron.

Objectives

The purpose of this work was to develop the technique for intracellular injection of neurons in the spinal cord of the cat with the enzyme horseradish perioxidase (HRP). The project was conceived as the preliminary work which would provide the basis for later study of the morphology of individual spinal neurons following their electrophysiological characterization. The more specific aims of this investigation were to determine: (1) the amount of current necessary to iontophoretically deliver enough HRP into a cell so that it would be adequately labeled; (2) the relationship between the survival time of the cat following the intracellular injection of HRP and the degree of labeling of a cell; (3) the lengths of axons and dendrites stained in relation to the amount of HRP delivered. In addition, a number of qualitative problems needed solving: (1) developing good perfusion methods, (2) learning the specific histochemical techniques for processing the spinal cord, (3) developing electrophysiological techniques for intracellular recording from a single neuron, (4) finding the optimum characteristics of microelectrodes to be used.

MATERIALS AND METHODS

General Procedure

Thirty-eight adult cats weighing 2-4 kg were used. Each cat was deprived of food but allowed water for 12 hours prior to the experiment. The cat was anesthetized with sodium pentobarbitone (Nembutal, Abbott) given intraperitoneally (40 mg/kg). To maintain a stable level of anesthesia, additional quantities of Nembutal (3-6 mg/hr) were administered through a cannula in the left cephalic vein.

Following induction of anesthesia, hair was removed from the cat's neck, chest, forearm and back. An intravenous cannula was then inserted into the left cephalic vein. A glass "Y" cannula was inserted into the trachea and tied to it just below the larynx for the administration of artificial respiration. Arterial pressure was measured from a catheter introduced into the left common carotid artery by a strain-gauge transducer connected to a blood pressure monitor (Statham model SP1400). Mean arterial pressure was usually about 100 mm Hg. In a few experiments, an i.v. drip (5% dextrose in 0.45% saline) was administered to prevent a fall in blood pressure. The cat's head was fixed in a special holder and its pelvis was immobilized by two pins of the stereotaxic frame (David Kopf Instruments model 1780). The base of the stereotaxic frame, a small plate heater, a fan forced auxiliary heater and an infrared lamp were all used to maintain the cat's temperature at 37±2°C. A thermistor probe monitored rectal body temperature continuously and controlled the temperature of the small plate heater and the stereotaxic frame by a negative feedback cir-

cuit. Prior to the onset of intracellular recording, the cat was connected to a respirator (Harvard Apparatus) and ventilated ten breaths per minute with a tidal volume of 60 ml for an average (2.5 kg) cat. All animals were immobilized with gallamine triethiodide (Flaxedil, 4 mg/kg/hr i.v.). Tidal volume and respiratory rate were adjusted to provide the end-tidal CO_2 concentration of 3.5-4% (monitored with an infrared detector: Beckman Medical Gas Analyzer model LB1).

In three cats initially anesthetized by sodium pentobarbitone, the brain was anemically destroyed by bilateral occlusion of the common carotid and vertebral arteries. The spinal cord was then transected at the first cervical level and no further anesthesia was administered.

A lumbar laminectomy was performed exposing the spinal cord from the last thoracic to the fourth lumbar vertebrae. The vertebral column was rigidly fixed with two vertebral clamps (David Kopf Instruments); a pool was formed from skin flaps; and the spinal cord was covered with warm mineral oil (37°C). The dura mater was cut via a midline incision and retracted. Movements of the spinal cord associated with respiration were minimized by performing a bilateral pneumothorax and by covering the exposed cord with 3% agar (Difco) dissolved in lactated Ringers solution (Hartman's solution, Abbott) after the warm mineral oil was removed. A small patch of pia mater was removed on the dorsal surface of the spinal cord prior to the insertion of an electrode.

Microelectrodes

Microelectrodes were drawn from 1 mm o.d. microfilament filled capillary glass tubing (A-M Systems Inc. model GCF-100-4) on a vertical microelectrode puller (David Kopf Instruments model 700C). While the electrode tips were observed directly under a microscope, they were broken to overall outside diameters of 1.4-2.8 μm. Electrodes were stored in a microelectrode jar above the level of a solution containing Tris buffer (pH 8.6). Several hours before use, HRP (Boehringer-Mannheim) was dissolved in a 0.05M Tris buffer solution (pH 8.6) containing 0.5M KCl. The KCl was used to lower the impedance of the electrodes. The solution was buffered to pH 8.6 because the transported HRP isozymes are positively charged at this pH (Bunt et al., 1976). A basic pH was maintained because HRP is not stable in acid solutions (Straus, 1962). The concentration of HRP used in our experiments ranged from 10-25%. Electrodes were back-filled with this HRP solution using a 100 μl microsyringe (Hamilton) and a 32 gauge dental needle. Electrodes were selected for use on the basis of their impedances, which ranged from 6-45 MΩ (measured on an electrode impedance meter at 5 hz).

Electrophysiological Recording and HRP Iontophoresis

Figure 1 shows schematically the experimental setup used. In the search for cells to label, an electrode was lowered manually with a micro-manipulator (David Kopf Instruments model 1761) until it penetrated the surface of the spinal cord. A dual beam oscilloscope (Tektronix RM 565), a millivoltmeter (Keithley Instruments model 610C) and an audio monitor

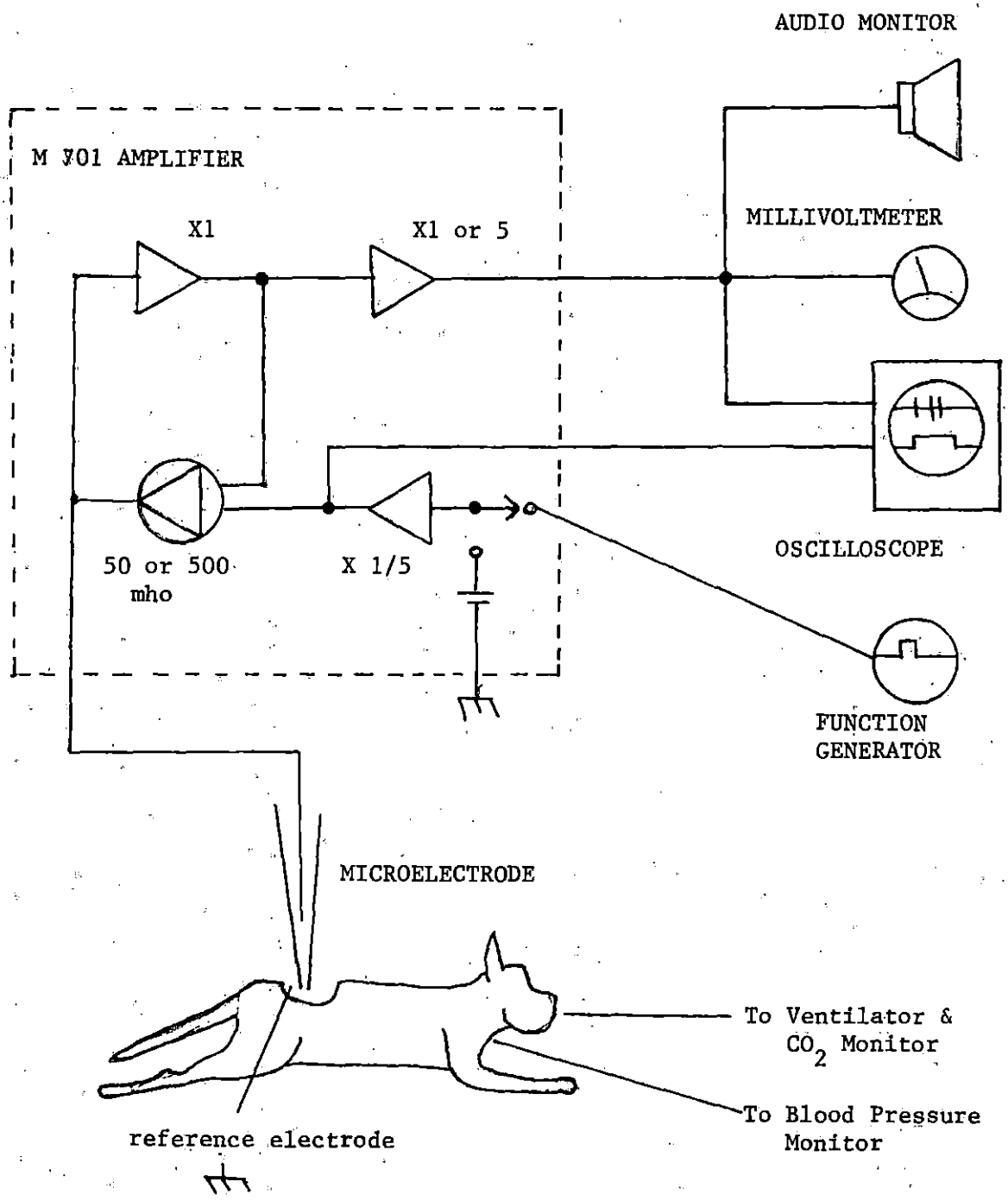


Figure 1. Experimental setup

(Grass Instruments, AM8) all received amplified signals from the microelectrode (amplifier: WPI model 701). Voltage measurements of the resting membrane potentials of spinal neurons were made with the microelectrode in reference to a silver silver-chloride reference electrode (WPI model EP2) which was attached to the exposed epaxial musculature. This electrode served as a ground. Once the spinal cord was penetrated, the electrode was lowered manually 200 μm into the cord and the resistance was measured. This measurement was made by passing a current of one nanoampere through the microelectrode to ground. One millivolt baseline shift with respect to ground seen on the oscilloscope screen corresponded to one megohm of electrode resistance. Resistance of electrodes measured in our experiments ranged from 6-60 MΩ. The intensity of current delivered through the microelectrode during HRP electrophoresis was adjusted to a maximum value of 15-20 nA by adjusting the voltage of the function generator (Grass Instruments S88 stimulator) while observing the output of the current monitor on the oscilloscope. The electrode was lowered gradually in steps of 2-5 μm into the spinal grey matter by a remotely controlled miniature hydraulic microdrive (David Kopf Instruments model 607). Spontaneously firing cells could be detected by recordings of their extracellular action potentials. These action potentials grew larger as cells were approached by the microelectrode. Intracellular penetration was determined by a sudden dc potential shift indicating a negative transmembrane potential. Successfully labelled cells had initial resting potentials of 20-60 mV. Once penetration occurred, the resting potential was observed for about 10 seconds, and if it remained stable, HRP was

ejected from the microelectrode either by using constant positive current (15-20 nA) or 500 msec pulses of 15-20 nA depolarizing current at a frequency of one pulse/sec. The amount of HRP delivered was calculated by multiplying the driving current by the time of delivery in minutes to give nanoamp-minutes. This was then converted to microcoulombs (μC) by multiplying by 0.06. For the purpose of this calculation, we assumed that the driving current was constant during the HRP iontophoresis. A minimum amount of 3.4 μC (18 nA for 4 min) was used for all successfully labeled cells. In most cases, current pulses were used and membrane resting potential was monitored in the intervals between pulses. If continuous current was used, the electrophoresis was repeatedly interrupted (about once/min for 5 sec) to monitor the resting membrane potential of a cell. Resting potentials usually became less negative during HRP administration. Cell depth within the spinal cord was determined from the digital monitor of the microdrive unit. Once a cell was labeled, the electrode was withdrawn and the approximate site of HRP injection was marked by inserting a pin in the agar near the injection site. A new site 1-5 mm away was chosen for the next penetration. A map of injection sites with approximate distances between labeled cells was kept for each experiment. All injections were made in spinal segments L_1-L_3. In order to facilitate location, tracing, and reconstruction of HRP stained neurons, the number of injected cells was limited to 4-6 in each experiment. Animals were allowed to survive 10 min to 6 hr after HRP iontophoresis.

Perfusion and Processing

Prior to the onset of perfusion, most animals were given 10,000 units of heparin (USP) i.v. The cats were sacrificed by perfusion through the left ventricle or the descending aorta (the latter proved more satisfactory). The animals were perfused first with 500 ml of 0.9% sodium chloride and then with one liter of a mixture of 2.5% paraformaldehyde and 1% glutaraldehyde in 0.1M phosphate buffer at a pH of 7.4 (Graham and Karnovsky, 1966). Both solutions were kept at room temperature. At the start of the saline perfusion, an outlet for flow was made by incising the right atrium. Perfusion pressure was slightly greater than 75 mm Hg (determined by the height of the perfusion solution containers above the preparation). Following perfusion, the spinal cord (segments L_1-L_4) was removed and placed in a post-fix solution (15% sucrose in a solution of equal parts fixative and 0.1M phosphate buffer, pH 7.4) overnight at 4°C. The following day, the tissue was placed in a Tris buffer solution (pH 7.6) containing 30% sucrose and stored at 4°C for at least 24 hr. Serial horizontal or transverse sections (75-100 μm thick) were cut on a freezing microtome (Reichert) and collected in a cold (10°C) solution of 5% sucrose in Tris buffer (pH 7.6). The sections were processed for peroxidase for 30 minutes at 37°C in a solution of 0.05% 3,3' diaminobenzidine tetrahydrochloride (DAB, Baker) containing 5% sucrose in Tris buffer (pH 7.6). After this incubation period, 0.05% H_2O_2 was added (12 ml of 0.06% H_2O_2 per 300 ml DAB solution) and the sections were shaken intermittently for 30 min at room temperature. The sections were washed in two changes of Tris buffer (pH 7.6), rinsed with distilled water and

then mounted conventionally on chrome alum gelatin slides (Pappas, 1971). After allowing the sections to air dry, they were processed through the following solutions in sequence:

0.1% toluidine blue (counterstain)	30 sec
50% EtOH	2 min
70% EtOH	2 min
80% EtOH	2 min
95% EtOH	2 min
Abs EtOH	2 min
Abs EtOH	2 min
Xylene	5 min
Xylene	5 min

Slides were coverslipped using Permount mounting medium (Fischer).

In one experiment, sections were processed with 0.15% Hanker-Yates reagent (p-phenylenediamine and pyrocatechol mixed in a ratio of 1:2) in 0.05M Tris buffer (pH 7.6) plus 1 ml of 1% H_2O_2 instead of incubating in DAB and then adding H_2O_2 (Hanker et al., 1977). The sections were incubated for 15 min. The remainder of the processing was similar to that of the other sections.

Cell Reconstruction and Analysis

Cellular morphology was analyzed by viewing the sections under the light microscope. The location of each cell was determined by measuring the dorsal or ventral distance of the cell from the central canal and the medial distance from the lateral boundary between grey and white matter. These measurements were used to make the composite picture seen in Figure 1. Neuronal reproductions were made using a Zeiss drawing apparatus (camera lucida). Cell drawings were photographically reduced. Stain

neurons were not corrected for shrinkage which, in this material, may amount to 30%.

Labeling of Clarke's Column Neurons Using the Method of Retrograde Transport of HRP

Five cats were prepared in the same manner as those previously described except that only the head was shaved and a cephalic vein cannula inserted. A midline incision was made between the temporal crests of the skull, and the temporalis muscle was retracted on the left side exposing the skull. A dental drill (Emesco Dental Co. model 90N) was used to remove a part of the parietal bone (2×2 cm) over the occipital lobe of the cerebrum. The cerebrum was exposed, dura mater was incised, and the portion of the cerebrum over the tentorium was removed by aspiration. A small bone rongeur was used to remove the tentorium overlying the left anterior cerebellum exposing lobules IV and V of the cerebellar vermis (Larsell, 1953). A microelectrode having a tip diameter of 45-60 μm and filled with 25% HRP in Tris buffer (pH 8.6) was held in a micromanipulator (Narishige model MM3) and inserted one mm deep into the cerebellar cortex of lobules IV and V. HRP was iontophoretically delivered with an initial positive continuous current of 5-6 μA for several minutes; however, due to an increase in the electrode resistance, current fell to 1-2 μA by the end of the 10 min of injection time. After electrophoresis, the electrode was left in situ for 5 min before it was withdrawn. The procedure was repeated at three adjacent sites in the cerebellar cortex within a nine square millimeter area.

After the HRP injection, the dura was pulled back over the cerebellum and gelfoam (Upjohn) was used to fill the cerebral cavity. Muscle and skin were sutured into place using silk thread (Ethicon size 0) and simple interrupted sutures. The animal was given antibiotics (50,000 units of procaine penicillin plus dihydrostreptomycin sulfate: Combiotic, Pfizer) and allowed to recover. On the third postoperative day, the cat was anesthetized with sodium pentobarbitone and perfused transcardially. The spinal cord and cerebellum were removed and processed as previously detailed. A block of the cerebellum containing the injection sites was sectioned sagittally into 75 μm thick sections. The spinal cord from T_2 to L_4 was sectioned transversely. Only every third 75 μm spinal cord section was saved for analysis. After all sections were observed under a light microscope, photographs of retrogradely labeled cells were made on a Leitz photomicroscope using photomicrography color film (Kodak, PCF).

RESULTS

General

Attempts were made to stain 77 spinal neurons in lumbar segments L_2-L_3 with intracellularly injected HRP. In 38 cases, stained neurons were recovered at the expected level in the histological material. Of these, 12 units were well-stained and 26 were partially stained. Well-stained cells had distinctly brown stained somas and dendritic processes which were clearly demarcated from any of the surrounding unlabeled cells. Partially stained cells were usually stained cell processes. They consisted of either an axon or one to several dendrites. In this category also were units where staining was weak or projections of neuronal processes could not be followed with certainty. Neurons with cell bodies obscured by extracellular HRP or somehow lost or damaged in processing also were classified as partially stained. All cells were located in spinal cord segments L_2-L_3.

Microelectrodes

We were most successful in labeling neurons when electrodes with tip sizes of 1.4-2.8 μm were used. Filled with a solution of 10-25% HRP, these electrodes had resistances of 6-60 MΩ if measured in the spinal cord. The measured resistance value frequently rose as the electrode moved through the spinal cord tissue due to partial blockage of the tip with tissue particles. If the electrode resistance rose to over 100 MΩ, it could generally be reduced to near its original resistance value by

either backing up the electrode or by ejecting brief pulses (1-2 sec) of positive current (15-20 nA).

Resting Membrane Potentials

The resting membrane potentials of our well-stained cells ranged from 20 to 60 mV (all potentials were negative with respect to ground). If we attempted to stain cells which had initial resting potentials of less than 20 mV (and therefore were probably injured), the potential rapidly declined to zero and the HRP injection was aborted.

HRP Transport and the Minimum Amount of HRP

In both dendrites and axons, the transport rate of HRP was calculated by dividing the longest length of labeled process by the time taken for HRP transportation. The latter was the period between the time of cell injection with HRP and the time the animal was sacrificed. Dendritic transport rates ranged from 1-58 mm/day, although, in most cases, the rate was 1-12 mm/day. Axonal transport rates were 6-23 mm/day.

The minimum amount of HRP injected necessary to obtain a well-stained neuron was 3.4 microcoulombs (μC). One cell (63/3; Figure 8A) stained with 3.5 μC distinctly shows the neuron cell body and seven primary dendrites.

Characteristics of Well-Stained Cells

In 12 cases, HRP staining clearly delineated the geometry of the cell. A number of details were usually distinguishable including soma

shape, orientation of the perikaryal region in the spinal cord and dendritic branching patterns. Salient characteristics of these twelve well-stained cells are summarized in Table 1. Figure 2 illustrates the location of the well-stained cells within the spinal cord grey matter. As can be seen in Figure 2, six cells were located in the intermediate grey, five in the ventral horn and one in the dorsal horn.

Two of the well-stained cells were located at the border of the dorsal horn and lamina VII, two (cells 45/2 and 58/4) were located in lamina VII and two (cells 43/5 and 75/3) were located in Clarke's column. Cells found in the intermediate grey had initial resting potentials of 20 to 55 mV. They were injected with 3.4-8.4 μC of HRP. Soma sizes of horizontally sectioned cells ranged from 7×20 μm to 23×50 μm (mediolateral × cranio-caudal). Each cell body was generally elliptical in shape with the long axis parallel to the long axis of the spinal cord. Cell bodies were stained dark brown, and though dendritic staining appeared lighter as fiber diameter decreased, dendrites were evenly filled with HRP down to their finely tapering processes. Five to seven primary dendrites were observed for each cell ranging from 2-8 μm in diameter. Dendritic branching was seen starting 10-40 μm from the cell body. All cells had their longest dendritic extent along the cranio-caudal axis of the spinal cord. Although organized primarily parallel to the long axis of the spinal cord, dendritic branching patterns varied considerably. Cell 45/2 (Figure 3A) shows a pattern of few dendrites in any but the cranio-caudal direction, whereas cells 58/4 (Figure 3B) and 52/1 (Figure 4) show dendritic spread in the mediolateral direction as well. The dendritic projections of all

Table 1. Characteristics of well-stained spinal neurons

Cell No.	43/5	45/2	52/1	58/4	67/2	75/3
Location	IG[a]	IG	IG	IG	IG	IG
Rexed's lamina	Clarke's column	VII	V	VII	V	Clarke's column
Resting potential (mV)	30	55	45	40	25	20
Amount of HRP injected (μC)	5.4	6.1	7.1	3.4	6.0	8.4
Soma size[d]						
mediolateral	40	7	15	15	23	12
cranio-caudal (μm)	25[e]	20	45	30	50	25[e]
No. of primary dendrites	6	7	6	5	5	-
Dendritic extent (μm)						
cranial	1050	325	1270	400	500	300
caudal	800	315	545	270	560	300
dorsoventral	220	80	400	75	400	150
mediolateral	250	40	300	300	150	50
Axon length (μm)	1050		725			
direction	to DLF		toward lateral funiculus			

[a]Intermediate grey.

[b]Ventral horn.

[c]Dorsal horn.

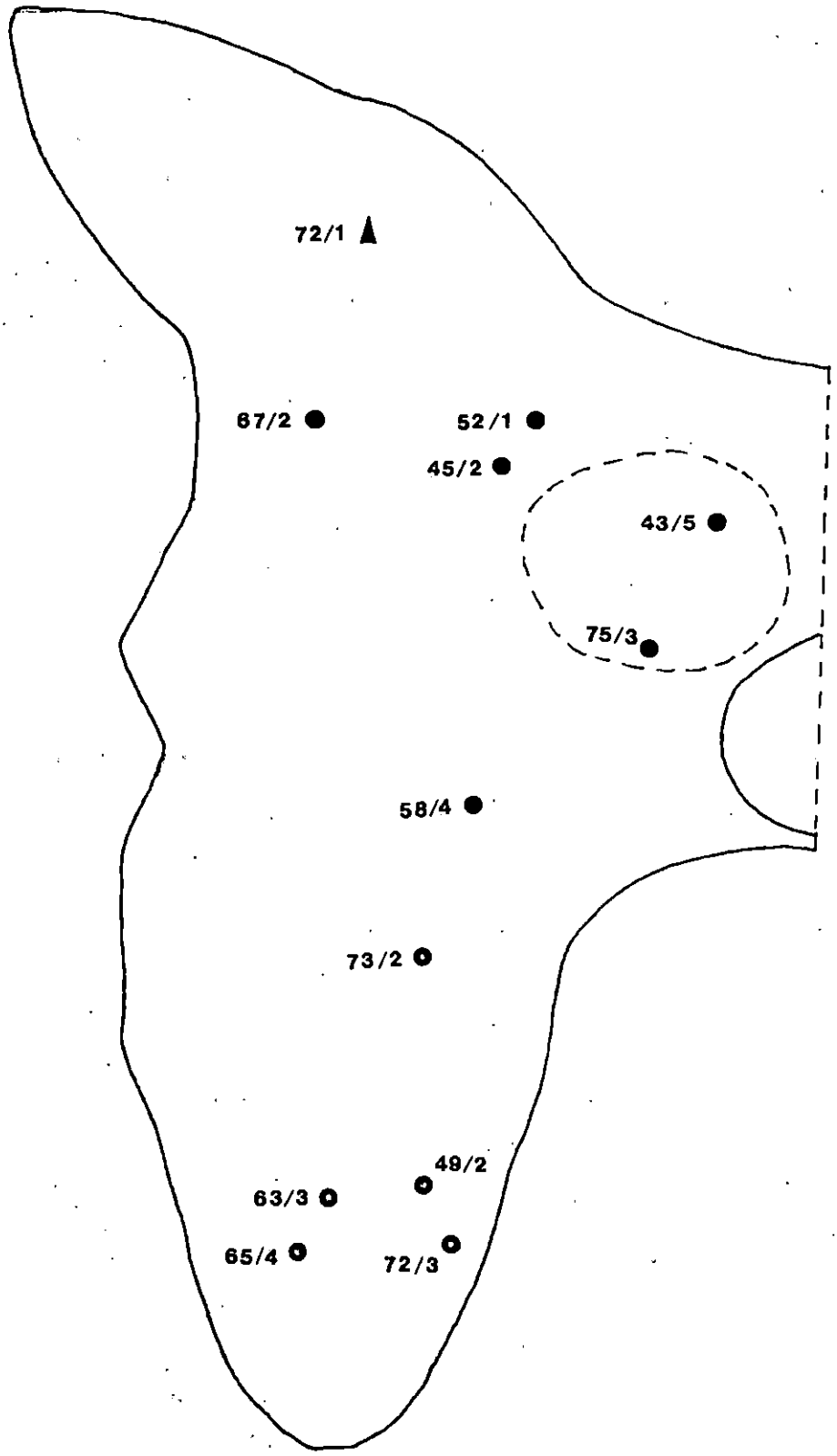
[d]In a horizontal plane.

[e]Cells sectioned transversely; this dimension is dorsoventral.

49/2	63/3	65/4	72/3	73/2	72/1
VH[b] IX	VH IX	VH IX	VH IX	VH VIII	DH[c] III
–	40	40	60	20	50
6.1	3.5	7.2	8.6	10.3	9.6
37	25	25	25	25	13
50[e]	55	50	95	37	63
7	8	7	11	6	6
640	410	400	1190	350	190
480	400	1200	860	400	250
400	300	200	500	300	100
340	380	160	500	500	50
–	–	–	–	–	–

Figure 2. Distribution of well-stained neurons. Solid line depicts the boundary between grey and white matter. Dotted line is the outline of Clarke's column

▲ dorsal horn cell
● intermediate grey matter cells
● ventral horn cells


72/1
67/2
52/1
45/2
43/5
75/3
58/4
73/2
49/2
63/3
65/4
72/3

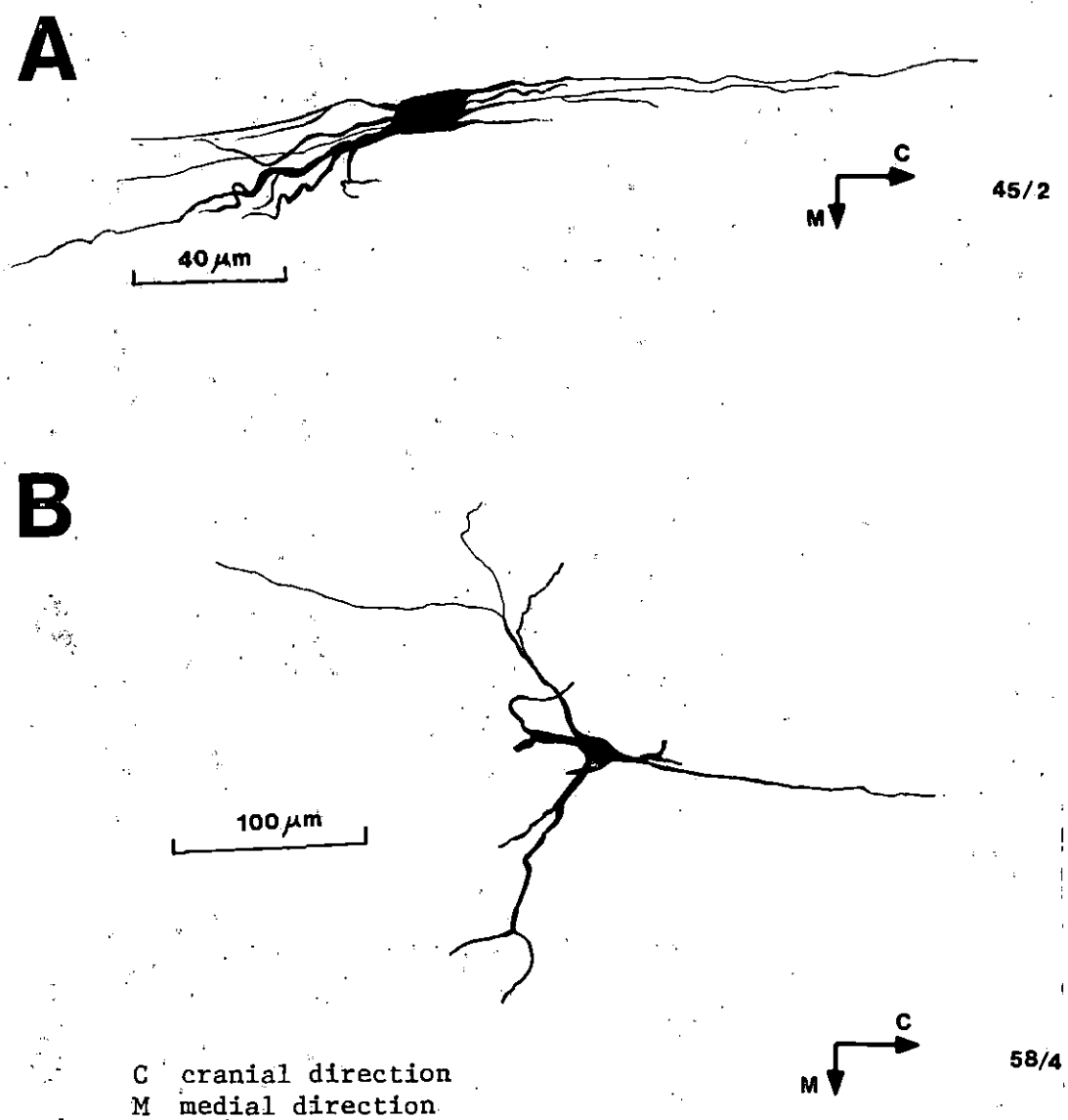
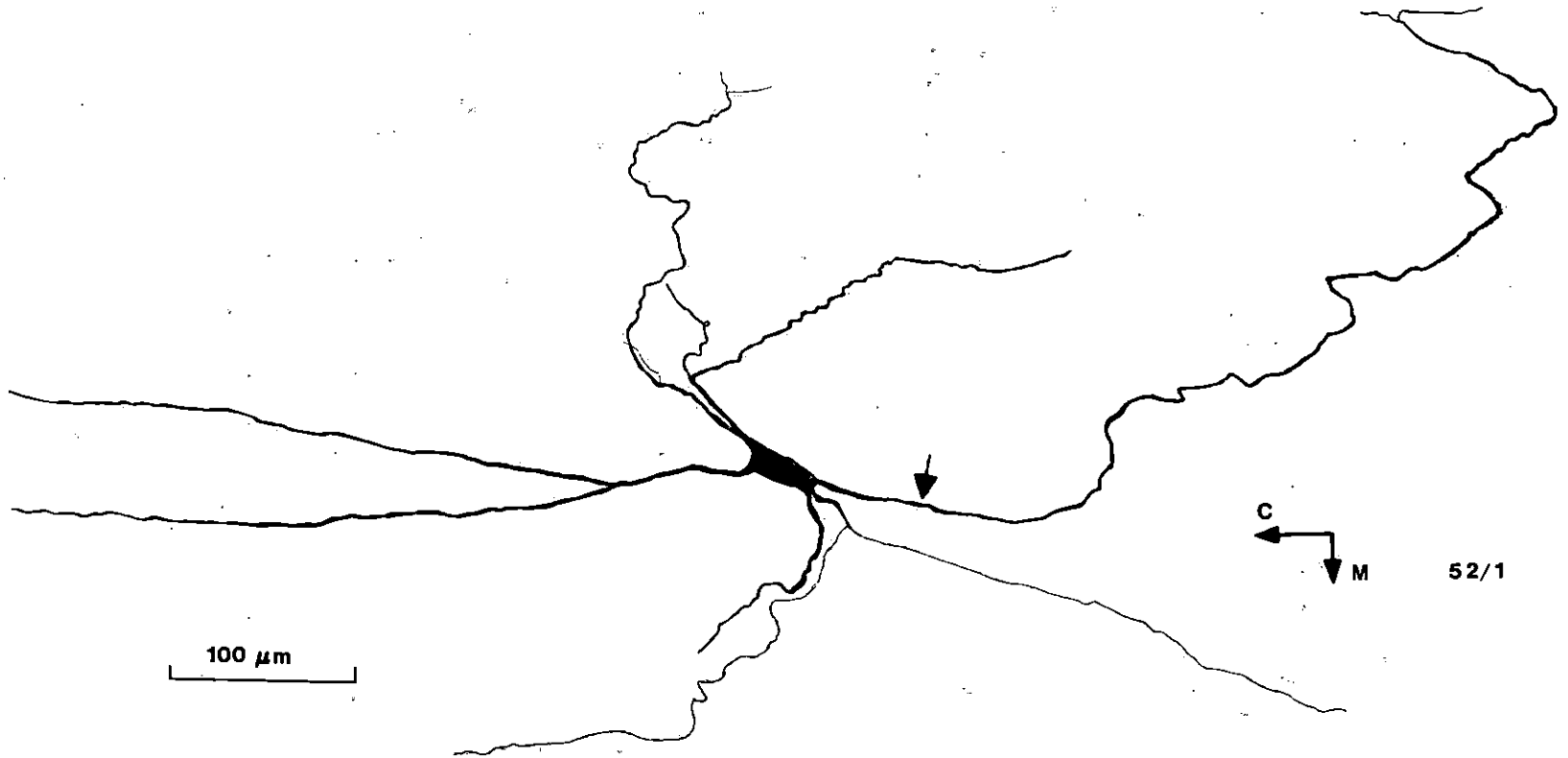


Figure 3. Camera lucida drawings of two HRP stained spinal neurons (cell 45/2 in A; cell 58/4 in B) located in the intermediate grey matter. Sections were cut in the horizontal plane

Figure 4. Camera lucida drawing of an HRP stained neuron located in the intermediate grey. The axon (arrow) was stained for 725 µm as it proceeded toward the lateral funiculus. Sections were cut horizontally

C cranial direction
M medial direction


C
M
52/1
100 µm

the intermediate grey cells remained within the intermediate grey area and were not seen to enter either the dorsal or ventral horns. Dendrites extending in a cranial direction were stained for 300-1270 µm, while those extending caudally were stained 270-800 µm dorsoventrally and 40-300 µm mediolaterally. Two cells had stained axons. Figure 4 shows the axon arising from the cell body of unit 52/1 which could be followed for 725 µm as it extended toward the lateral funiculus. The other stained axon was from a cell found in Clarke's column and is described below.

Two cells were from spinal cord material which was sectioned transversely. They were found to lie in the area of Clarke's nucleus. Their soma sizes were 12×20 µm and 40×25 µm (mediolateral × dorsoventral). Both cells had dendrites which did not extend outside of the boundaries of Clarke's column. Because these cells could not be adequately reconstructed, it is not possible to comment on their dendritic branching patterns in the transverse plane. Figure 5 shows the location of one of these cells (43/5) in the spinal cord and the cell's HRP filled axon which could be followed for 1050 µm as it proceeded into the dorsolateral funiculus. No stained axon collaterals were seen.

Of the five stained cells located in the ventral horn, one was from a cord which was sectioned transversely and could be seen lying in Rexed's lamina IX. Three cells from horizontally sectioned spinal cords were located in the tip of the ventral horn and also were probably located in Rexed's lamina IX. Cell 73/2 was located at the base of the ventral horn, probably in lamina VIII. The initial resting potentials of these cells ranged from 20 to 60 mV. They were injected with 3.5-10.3 µC of HRP. In

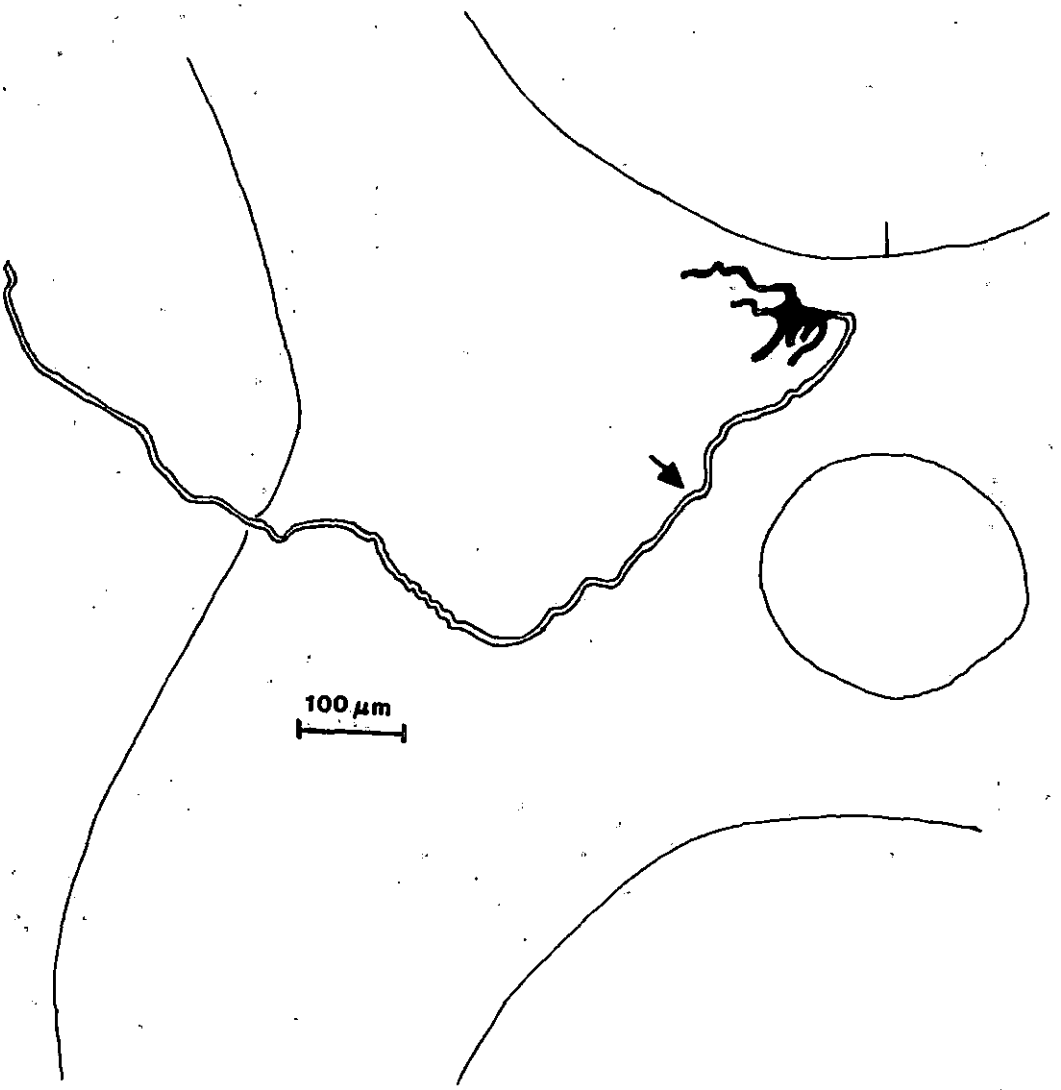


Figure 5. Camera lucida drawing of a partially reconstructed HRP stained spinal neuron (cell 43/5) located in Clarke's column. Section was cut in the transverse plane. The axon (arrow) is shown as it proceeds into the DLF. No axon collateral was seen

horizontally sectioned cells, soma sizes ranged from 25×37 μm to 25×95 μm (mediolateral × cranio-caudal). In each case, the longer axis of the elliptical cell body was aligned parallel to the long axis of the spinal cord. There were generally six to eight primary dendrites seen per cell; however, one well-stained cell (72/3, Figure 6) had 11 primary dendrites. In some cases, cell 73/2 (Figure 7B), for example, dendrites came off around the cell body at almost regular intervals and dendritic processes were easily distinguished from one another. In other cells (72/3, Figure 6 and 63/3, Figure 8A), primary dendrites were clustered more along the cranio-caudal poles of the soma and branched a short distance from the cell body making it difficult to determine the number of primary dendrites accurately. The diameter of the primary dendrites was generally 5-7 μm. Dendrites of all cells had their longer extensions organized cranio-caudally parallel to the axis of the spinal cord. Dendrites were stained 350-1190 μm in the cranial direction and 400-1200 μm caudally from the soma. Stained dendrites extended 200-500 μm dorsoventrally and 160-500 μm mediolaterally. The secondary dendritic branches originated 10-40 μm from the cell body. In cell 49/2 (Figure 7A), which was from transversely sectioned material, and cell 73/2, which was at the base of the ventral horn, dendrites were seen extending into lamina VII. Although dendritic processes generally were seen only in the grey matter, dendrites from two of the cells could be seen extending into the ventral funiculus (cells 72/3 and 49/2, Figures 6 and 7A, respectively). Stained dendrites from cell 72/3 also were seen in the lateral funiculus. No definitely stained axons were identified.

Figure 6. Camera lucida drawing of an HRP stained cell located in the ventral horn. Dotted line is the border of the ventral funiculus and the grey matter. Cell was sectioned horizontally

C cranial direction
M medial direction

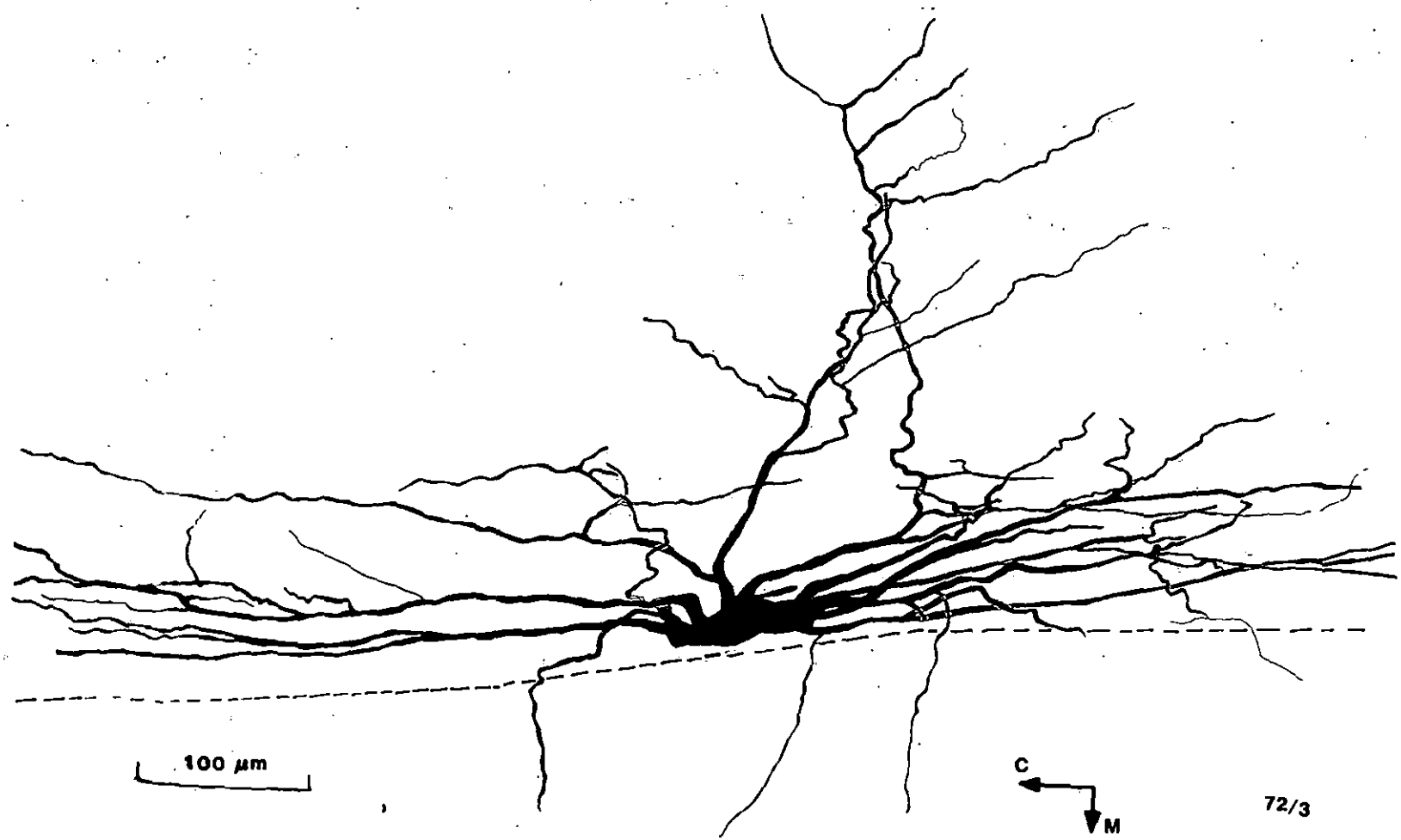

100 µm
C
M
72/3

Figure 7. Camera lucida drawings of two cells located in the ventral horn of the spinal cord. Cell 49/2 (A) was sectioned in the transverse plane (dotted line is the border of the ventral horn and the ventral funiculus). Cell 73/2 was sectioned horizontally

C cranial direction
L lateral direction
M medial direction
V ventral direction

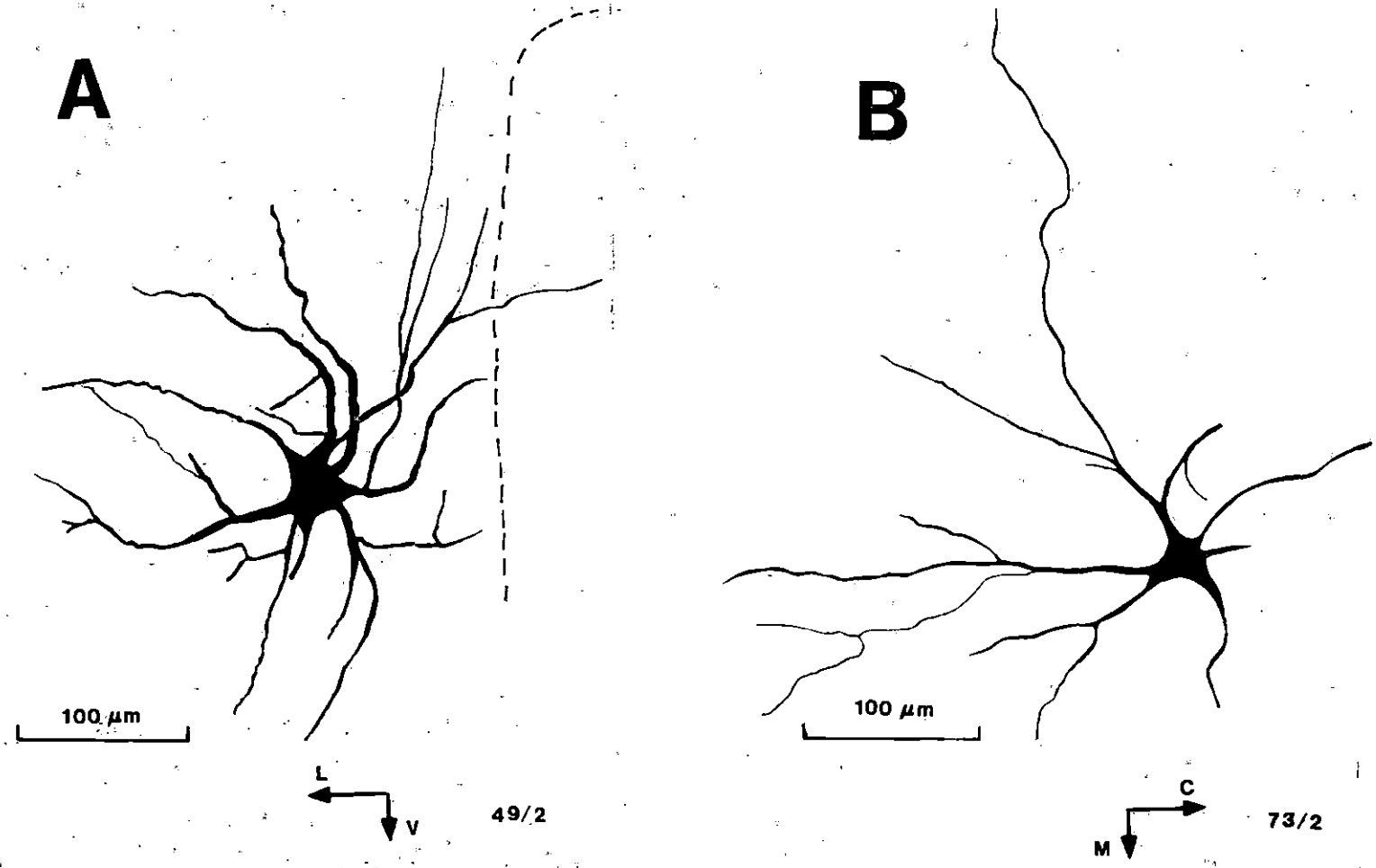
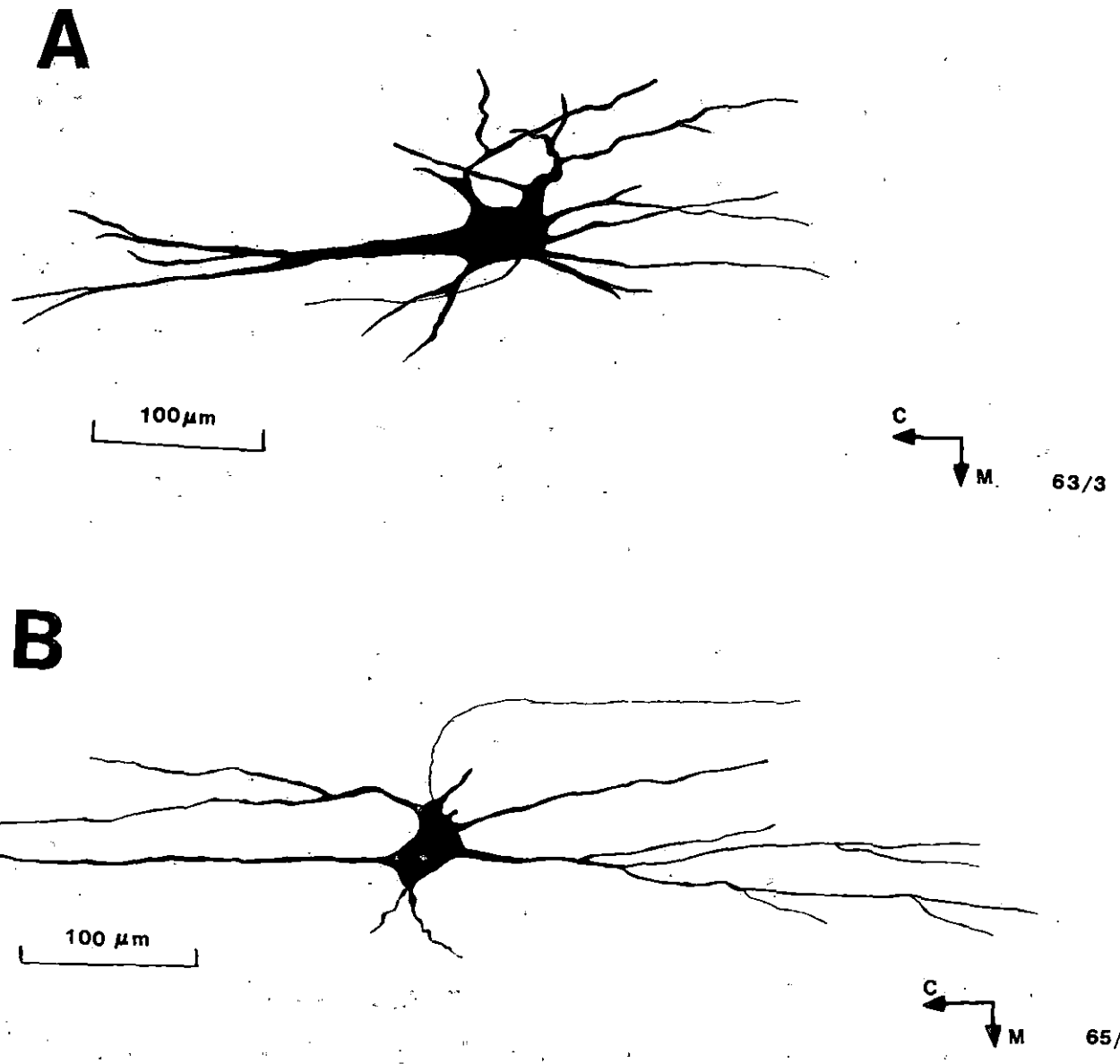

A
100 µm
L
V
49/2
B
100 µm
C
M
73/2

Figure 8. Camera lucida drawings of two HRP stained spinal neurons (cell 63/3 in A; cell 65/4 in B) ventral horn. Both cells were sectioned horizontally

C cranial direction
M medial direction


A
100µm
C
M
63/3
B
100 µm
C
M
65/4

One cell was recovered from the dorsal horn in the area of Rexed's lamina III. Its initial resting potential was 50 mV. Despite having a calculated amount of injected HRP of 9.6 μC, the cell was relatively lightly stained and the cell nucleus could be seen as an unstained pale area within the cell body. The cell soma measured 13×63 μm (mediolateral × cranio-caudal). There were six primary dendrites which appeared to radiate primarily from the cranial and caudal ends of the cell soma. Stained dendrites extended cranially for 190 μm and caudally for 250 μm. The dendrites could be seen projecting 100 μm dorsoventrally and 50 μm mediolaterally. Few secondary dendritic branches were seen probably due to the light staining. No axon was seen.

Partially Stained Cells

Among the partially stained cells, there were two structures stained which were not found in any of the well-stained cells. Figure 9 shows an axon collateral stained with HRP. The axon is located in the lateral funiculus and is shown giving off a collateral soon after entering the white matter. This lightly stained axon was located near one of the well-stained cells (67/2) and was probably filled with HRP due to injury by the microelectrode and uptake of extracellularly located HRP from the surrounding tissue. In another cell which received 11.5 μC of HRP, only the dendrites were stained. Figure 10 shows the stained dendritic spines which were located in the intermediate grey matter.

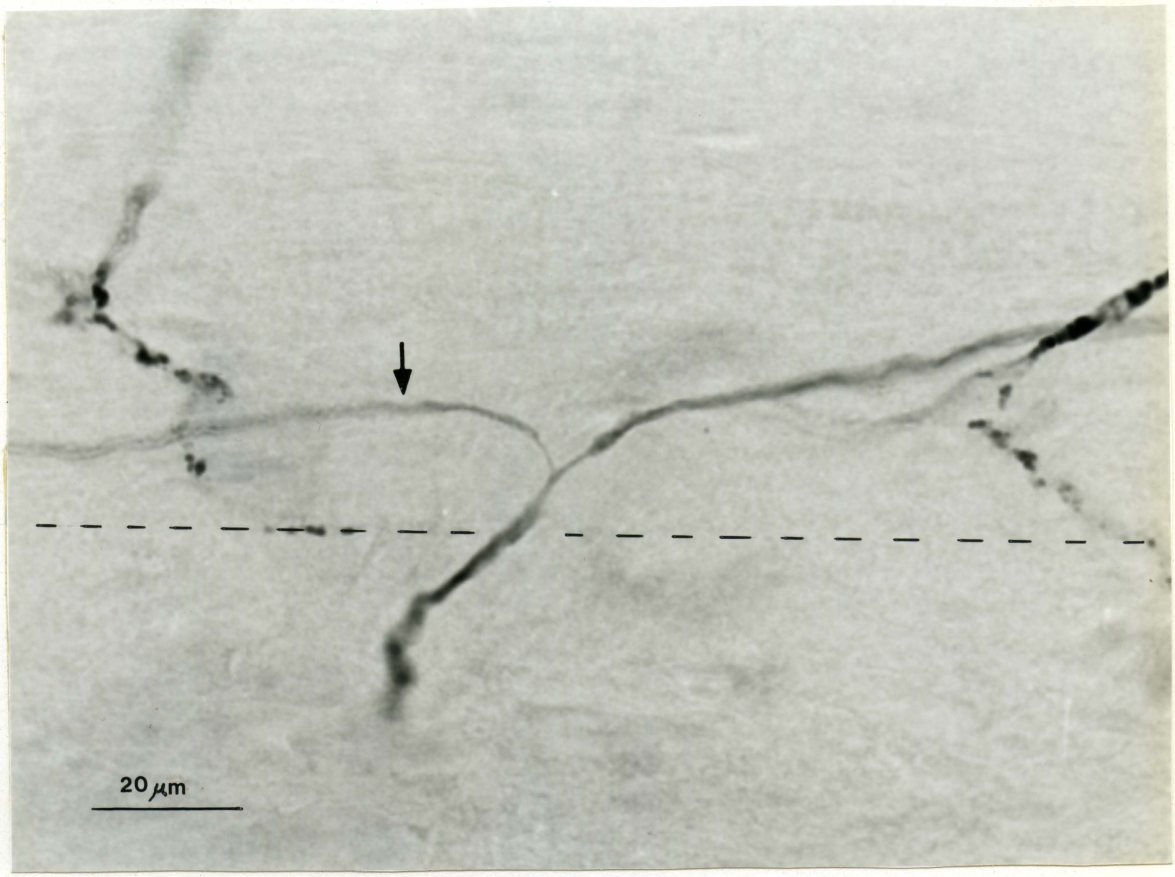


Figure 9. Lightly stained axon and axon collateral (arrow). Both were probably filled with HRP due to axonal injury. Dotted line indicates the boundary between grey and white matter. The axon lies mostly in the lateral funiculus

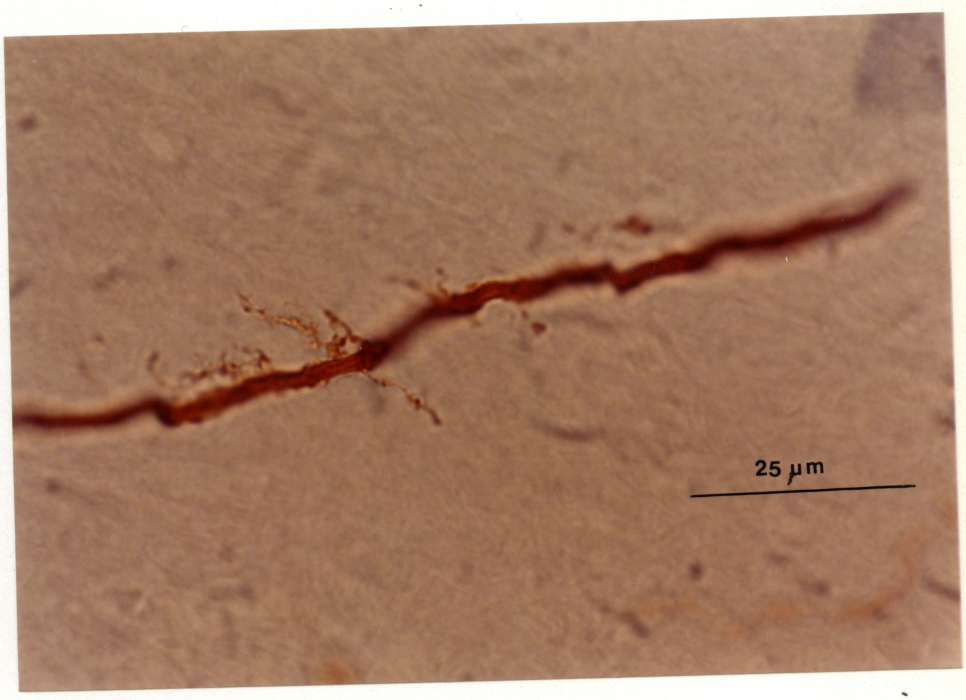


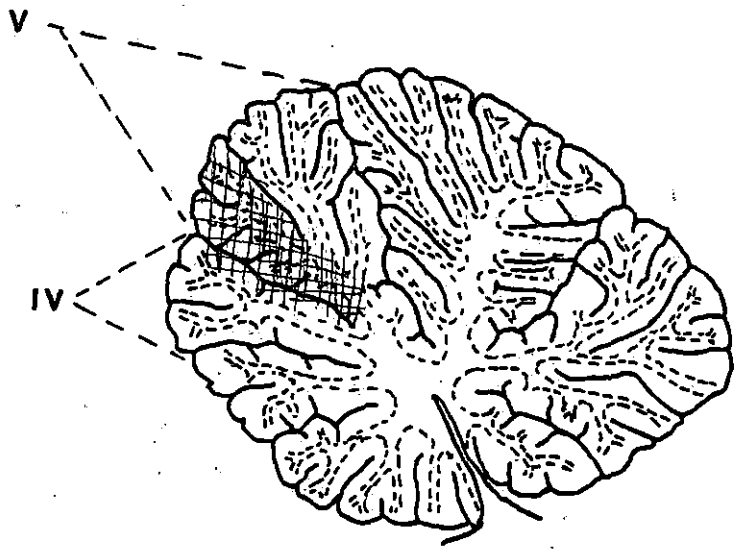
Figure 10. Dendritic spines from a partially stained cell located in the intermediate grey matter. Only the dendrites of this cell were seen stained in the histological material

Retrograde Transport of Extracellularly Injected HRP

We were successful in finding retrogradely labeled cells in only one out of five experiments. This shortcoming was probably the result of difficulties encountered with the extracellular application of sufficient amounts of HRP by iontophoresis, poor tissue perfusion and problems with the processing of tissue sections. The following results and the discussion, which appears later, deal with the successful experiment.

Sagittal cerebellar sections 75 μm thick were examined to confirm the site of HRP injection. Figure 11 shows a sagittal section of the left cerebellar vermis and indicates the site of multiple applications of HRP. Injected HRP was found mainly in the lower part of lobule V. The staining was found in 12 sections which, since every alternative section was saved, amounted to about a 2 mm wide (mediolateral) section of the cerebellum. Light microscopic examination of the spinal cord sections showed nine retrogradely labeled cells on the ipsilateral side of the spinal cord in the area of Clarke's nucleus of spinal segments T_2, T_3, T_{11}, T_{12}, T_{13} and L_4. The intensity of the HRP staining varied widely from faintly stained cells which were only obvious under high magnification (400x) to cells which were readily apparent under low power (40x). In all cases, no more than one retrogradely labeled cell was found on a single histological section. Three cells were found in the T_2 spinal cord segment, two in T_{11}, and one in each of the other mentioned spinal cord segments.

The best example of a retrogradely labeled cell, found in the T_2 segment, is seen in Figure 12. The cell body appears to be diffusely filled with dark brown granules. The brown HRP filled vesicles can be



IV vermian lobule four
V vermian lobule five

Figure 11. Schematic of a sagittal section of the cerebellum indicating the site of multiple extracellular injections of HRP. Shaded area indicates the location of injected HRP

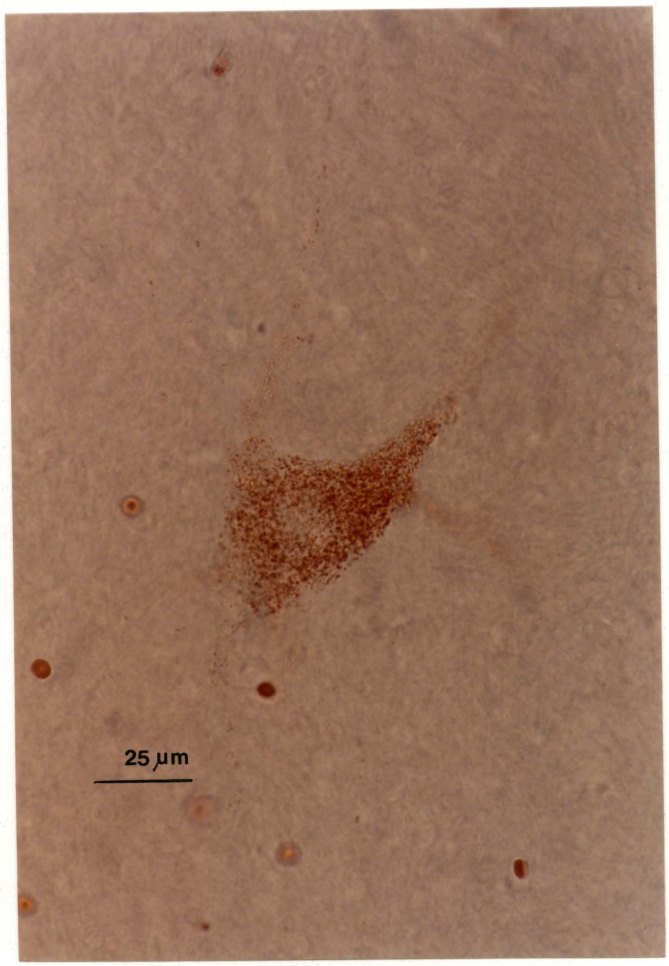


Figure 12. Spinal neuron retrogradely labeled with horseradish peroxidase. The cell was located in Clarke's column of spinal segment T_2. The spinal cord was sectioned transversely

seen extending into the bases of the dendritic processes. The size of retrogradely labeled cell bodies ranged from 18×28 μm to 37×50 μm measured along the long and short axes of the elliptically shaped cells.

DISCUSSION

General

Results obtained with intracellular HRP staining of the spinal cord neurons clearly reveal the advantage of this technique, not only because of the possibility of visualization of a physiologically characterized neuron but also because of the quality of staining for morphological analysis. Light et al. (1979) reported that the general picture presented of an HRP-stained neuron is similar to the best examples of Golgi-stained material, with the advantage that one neuron can be singled out with the HRP technique. A consistent difference that they and others found between the two methods is that Golgi staining often shows abrupt, relatively thick dendritic terminations, whereas HRP staining shows finely tapering dendritic processes visible down to the limits of the light microscope. Although several of our camera lucida figures show some abrupt dendritic terminations, these were usually continued on subsequent sections but were not included because they could not be accurately reconstructed.

Delivery of HRP by Intracellular Iontophoresis

Microelectrodes

Our well-stained cells were injected with microelectrodes having tip diameters of 1.4-2.8 μm and *in-vivo* resistance of 6-60 MΩ. The tip sizes and resistances of the electrodes employed where cells were successfully labeled in our experiments are comparable to those used by other investigators. Cullheim and Kellerth (1976) used electrodes with tip sizes of

1.5-2.5 μm and resistances of 30-60 MΩ, whereas Jankowska et al. (1976) used microelectrodes with tip diameters of 2-3 μm having 25-35 MΩ resistances. Brown et al. (1977b) and Snow et al. (1975) reported that successful labeling of cells could be achieved with electrodes having resistances of 25-40 and 5-25 MΩ, respectively. Although no investigators reported using microelectrodes with tip sizes larger than 3 μm for intracellular labeling with HRP, a few investigators routinely used electrodes with smaller tips than we found satisfactory in our work. Thus, McCrea et al. (1976) used electrodes with tip sizes of 1-1.5 μm and 50-100 MΩ resistances to inject Purkinje cells and Light and Durkovic (1976) reported successful labeling of spinal motoneurons with microelectrodes having 0.5 μm tips and 50-80 MΩ impedances.

One of the probable factors responsible for a relatively low yield (12 well-stained cells out of 77 attempted) of successfully labeled spinal neurons in our experiments was the inability of some of the microelectrodes to inject sufficient quantities of HRP. Light and Perl (1979) reported that their low yield (22%) of successfully marked units partly reflected the fact that, in several cases, the iontophoretic current failed to eject HRP. It should be noted that although release of the iontophoresed substance from the microelectrode is linearly related to the current passed, substantial variations in amounts delivered have been reported between different micropipettes even when made of the same glass and filled with identical solutions (Kelly, 1975).

Generally, a microelectrode with a larger tip size had a lower impedance value in-vitro. However, there was no correlation between tip

size and the resistance value measured in-vivo in our experiments. This lack of correlation was probably due to partial blockage of some but not all electrodes with tissue particles during penetration of the spinal cord. In our hands, electrodes with resistances greater than 60 MΩ did not give adequate HRP staining of spinal cells because they showed a tendency for blockade of their tips. Cells penetrated with electrodes having resistances of less than 8 MΩ usually did not show stable resting potentials, very likely due to damage of the cell membrane by these larger tips.

Iontophoretic current

We calculated the amount of HRP delivered into a cell by multiplying the iontophoretic current (in nanoamps) by the total time of HRP application (in minutes) and then converting the values obtained to microcoulombs. At the present time, we think that this is the best method for expressing the relative amounts of injected HRP. The HRP was ejected from the microelectrode either by using positive current pulses (15-20 nA) or constant positive current. With these currents, satisfactory filling of cell bodies and dendrites was achieved using a minimum of 3.4 μC of HRP and a duration of intracellular application of 7.5 min. Cells 58/4 (Figure 3B) and 63/3 (Figure 8A) were stained with 3.4 and 3.5 μC of HRP, respectively. From our data, we cannot report any advantage of one method of HRP current delivery over the other. We used current pulses more often than constant current in our attempts to stain cells and the majority of our well-stained cells (eight out of twelve) were labeled with current pulses. Although most investigators have used current pulses for

intracellular HRP delivery, Cullheim and Kellerth (1978) and Cullheim and Ulfhake (1979) have reported good intracellular staining of motoneurons using constant positive current for injection of HRP. Perhaps the preponderance of the use of dc pulses is due to investigators having experiences similar to those reported in the paper introducing the technique of microelectrophoretic delivery of HRP, where Graybiel and Devor (1974) warned that the use of continuous current often caused severe instability in the impedance of HRP filled pipettes in their experiments.

Resting Potentials

In contrast to work by Brown et al. (1977b), where they reported that a resting potential greater than 40 mV correlated with better staining, we found no correlation between the initial resting potential (provided it was greater than 20 mV) and staining quality (length of longest dendrite stained).

We were unable to successfully stain spinal neurons having initial resting potentials less than 20 mV. The low resting potential was probably indicative of a damaged cell membrane which resulted in the inability of a cell to retain intracellularly injected HRP. In agreement with our findings, McCrea et al. (1976) also reported that they did not get good staining results with cells having resting potentials of less than 20 mV.

The resting potentials of our cells usually declined during the course of the injection. Several factors might have contributed to this: (1) membrane leakage caused by damage during microelectrode pentration of the cell membrane, (2) the electrode coming out of the cell, (3) the

injection of positively charged HRP ions into the cell which resulted in cell depolarization. Several of the well-stained cells had resting potentials which declined to zero by the end of HRP iontophoresis. Comparisons of these cells with cells which had resting potentials which did not decline to zero and were given approximately equal amounts of HRP, revealed no discernible difference in staining quality. Similarly, Cullheim and Kellerth (1978) found no correlation between the success of HRP iontophoresis and the electrophysiological behavior of a cell during intracellular recording. They reported that, in many instances, a cell did not show any physiological sign of injury but was nevertheless found to be badly deteriorated morphologically, while on the other hand, cells which had lost their antidromic action potentials and showed large decreases in membrane potentials during iontophoresis could appear intact as judged in the light microscope.

Plane of Section

Spinal cords were sectioned predominantly horizontally, although in the initial experiments, transverse sectioning was done because of our intent to work on Clarke's column neurons and the necessity of locating them in Rexed's lamina VII. The advantage of horizontal sections was that all of our well-stained cells had dendritic fields which were longest in the cranio-caudal direction, so in each case, a horizontal section allowed the greatest amount of dendritic extent to be viewed. The major dendritic organization of horizontally sectioned cells was therefore more readily observable.

Scheibel and Scheibel (1966) reported that dendrites of spinal motoneurons, when viewed in transverse section, appear diffuse and widely ramifying, while in sagittal sections, they appear to have a high degree of rostrocaudal organization. This point was confirmed in our experiments in a comparison of the dendritic fields of two of our well-stained cells which were recovered from the ventral horn. Cell 72/3 (Figure 6) was sectioned horizontally, whereas cell 49/2 (Figure 7A) was from transversely sectioned material. The rostrocaudal organization of cell 72/3 is readily apparent, while the transversely sectioned cell's dendrites appear to spread in all directions. In addition, the reconstruction of the horizontally sectioned cell was much easier.

Horizontal sections were much easier to process serially than transverse sections. For example, a two centimeter long piece of spinal cord sectioned transversely would give two hundred serial sections 100 µm thick, while the same piece sectioned horizontally would amount to only about 40 sections. In addition, the larger horizontal sections were easier to handle and to mount. Horizontal sections also were easier to scan since a two centimeter length of cord, at one depth, could be examined rapidly by viewing a single section. In many cases, it was only necessary to look at seven or eight sections to note all injection sites and labeled cells.

Perhaps the one drawback of this method is that it does not allow easy use of Rexed's laminar classification of the spinal cord grey matter.

Hanker-Yates Reagent Versus Diaminobenzidine

One of the HRP labeled cells (45/2, Figure 3A) was processed with Hanker-Yates reagent instead of DAB. Although the procedures are very similar in relation to the ease of processing of the histological sections, Hanker-Yates reagent has been reported to be more sensitive to and more specific for plant hydroperoxidases than is DAB (Hanker et al., 1977). The intensity of brown-black staining of neurons by the Hanker-Yates reagent was similar to that seen in sections processed with DAB. One disadvantage, however, was that the sections stained with Hanker-Yates reagent appeared to take on a diffusely brownish tinge making it harder to discriminate HRP labeled neurons. However, we initially had a similar experience with DAB which we attributed to either poor fixation or oxidation of the chromogen. Although we prefer the DAB method for development of HRP and most investigators seem to use it, our experience with the Hanker-Yates reagent was limited to one experiment. This obviously does not permit any meaningful conclusions to be drawn. It is interesting to note that Light and Perl (1979) reported using DAB treatment followed by Hanker-Yates processing to produce a darker staining of HRP injected cells.

HRP Transport Rates

Our highest rate of HRP transport in dendrites was 58 mm/day. This was seen in only one cell (58/4), located in the intermediate grey, in which the time available for HRP transport was quite short (10 min) and the entire cell was found on one histological section. This value com-

pares favorably with rates reported by Cullheim and Kellerth (1978) of 55-60 mm/day in dendrites and axons of spinal motoneurons. However, in the majority of our stained cells, dendritic transport was calculated to be only 1-12 mm/day. The probable major reasons for the low rates of HRP transport in our experiments were: (1) it was impossible to reconstruct neurons for more than a few histological sections with confidence, therefore, the distal parts of some of the dendrites were not measured; (2) the average amount of HRP that we injected was 7.2 μC. In contrast to this, Cullheim and Kellerth (1978) and Burke et al. (1979) routinely used 18-22 μC and 18-27 μC, respectively. Thus, it is reasonable to assume that our inability to detect HRP in the finer extensions of dendrites resulted in part from our application of smaller amounts of HRP.

Only two axons of well-stained cells were clearly seen and the HRP axonal transport rates were calculated to be 6 and 23 mm/day. These values are considerably smaller than the rate reported by Cullheim and Kellerth (1978) of 55-60 mm/day or that of 36-60 mm/day mentioned by Snow et al. (1975). These reported rates indicate that the anterograde axonal transport of HRP takes place not at the "fast" (100-300 mm/day) or "slow" (1-10 mm/day) transport rates but at an intermediate rate. Our inability to follow the axon for more than a few sections was probably the reason for the low rates calculated for HRP transport in the two stained axons.

Well-Stained Cells

Of the cells located in the intermediate grey matter, two had cell bodies located in Clarke's nucleus. Cell 43/5 (Figure 5) was a large cell

measuring 40×25 μm (mediolateral × dorsoventral). On the basis of soma size, this cell was identified as the large Clarke cell mentioned by Boehme (1968) or the class C cell described by Loewy (1970). The length of its stained dendrites compares favorably with lengths previously reported from Golgi studies. Boehme (1968) reported that the extent of dendrites in the cranio-caudal direction was over 500 μm for large cells, while Loewy reported several cases where dendrites of class C cells could be followed for distances of more than 1,000 μm. Also Randic et al. (1981) reported that the dendrites of their best HRP stained class C cells were filled for up to 1250 μm. The observed path of the axon from this cell into the lateral funiculus (Figure 5) is similar to the direction of the axon shown in Figure 1 of Boehme's work and the description offered by Loewy (1970). The axon arises from the medial part of the cell body, initially passes ventrally, then curves laterally toward the lateral funiculus. The axon was labeled for 1050 μm and no collaterals were seen. The latter finding is in agreement with recent data of other investigators (Loewy, 1970; Randic et al., 1981) who have not seen axon collaterals from axons of large Clarke cells.

Cell 75/2 was a small (12×25 μm) elliptically shaped cell located at the ventral border of Clarke's nucleus. On the basis of the size, shape and location of the cell body, it appears to be what Boehme (1968) called a small-bodied cell and Réthelyi (1968) classified as a border cell. The total cranio-caudal span of stained dendrites was found to be 600 μm, which is between values of 400 μm and 1,000 μm reported by Réthelyi (1968) and Boehme (1968), respectively. Golgi studies by Boehme (1968), Réthelyi

(1968) and Loewy (1970) revealed that most neurons in Clarke's nucleus have their dendrites oriented in a cranio-caudal direction and dendrites usually leave cell bodies at the cranial and caudal poles. Recent work by Randic et al. (1981) using intracellular injection of HRP also supports these observations. Because of this dendritic organization, transverse sectioning of these two cells made it impossible to reconstruct their dendritic branching patterns with confidence. In agreement with most investigators, however, was our observation that most of the dendrites of these cells remained within the area of Clarke's nucleus.

Four of the five ventral horn cells stained in our experiments were located within the motoneuron regions of the spinal cord. However, due to the lack of electrophysiological data, we could not classify them conclusively as motoneurons. The fifth cell (73/2) was located at the base of the ventral horn out of the areas normally considered as motoneuron loci and thus it probably is not a motoneuron. Of the other four cells, one cell (72/3) had dendrites stained over 1100 μm in the cranial direction. This finding agrees with the findings obtained from Golgi-stained preparations of motoneurons by Scheibel and Scheibel (1966). Investigators (Sprague, 1948; Romanes, 1951; Scheibel and Scheibel, 1966) agree that the wide range in shape and size of ventral horn cells precludes identification on the basis of cellular morphology alone. Scheibel and Scheibel (1966) state that the only significant criterion for morphological identification of motoneurons is the nature of the axons. Unfortunately, no axons could be positively identified in our ventral horn cells.

The Amount of HRP and the Extent of Labeled Processes

Soma and dendritic staining

There appears to be some variation in reported results regarding the amount of HRP which gives the best results. We found that intracellular injection of 3.4 μC of HRP was the minimal amount of the enzyme giving good cell body delineation. This amount is not far from minimal value of 2.4 μC (given as 40 nanoamp-minutes) reported as necessary for light microscopic identification by Jankowska et al. (1976). Our results also confirm their observation that the nucleus is still visible in cells injected with less than 4.8 μC of HRP. Whereas Neale et al. (1978), in their work delineating labeling of cultured spinal cord neurons, reported best results when HRP amounts were in the range of 1.2-3.0 μC, McCrea et al. (1976) reported that the most satisfactorily stained cells were those which were injected with more than 24 μC of HRP.

McCrea also drew attention to other factors which appeared to significantly affect staining of the terminal axonal arborizations of Purkinje cells. He pointed out that cells with shorter axons, those injected with greater amounts of HRP and animals where survival times were longer to allow more time for HRP transport, had more satisfactory staining of their terminal arborizations. While our experiments did not include study of axon terminals, data from our well-stained cells showed no positive correlation between either the time allowed for transport of HRP (survival time) or the amount of HRP injected and the length of the longest processes stained. The length of dendritic processes stained had little relation to the animal's survival time. Even a short survival time

of 10 min (cell 58/4) resulted in a good demarcation of dendrites which were stained over lengths equal to the lengths of dendrites from a cell with a survival time exceeding 5 hrs (cell 75/3). Light and Perl (1979) also reported that, in their experiments, the length of fiber stained bore no relation to how much time passed between iontophoresis of HRP and fixation of the tissue. It should also be noted that Brown et al. (1977a) reported that, for extended survival times (16-26 hrs), axonal staining of spinocervical tract neurons was comparable to staining obtained with shorter survival times (4-9 hrs) although, in the former case, soma and dendritic HRP staining was lost. Also Cullheim and Kellerth (1978) observed an increased risk for morphological deterioration of cells with longer survival times (4-8 hrs). Burke et al. (1979) found that limiting of the diffusion time to less than two hours seemed to minimize neuron injury.

Although it appears from some of the studies discussed above that both survival time (provided that it is not too long) and the amount of HRP injected would affect the quality of neuronal staining (in this case, the length of dendrites stained), there are several reasons perhaps why this was not shown in our experiments. In some cases, it was impossible to reconstruct stained dendrites over their maximum lengths with confidence. This was due to the problems encountered with reassembling dendrites over several histological sections. Also, in some cells, not all HRP was deposited intracellularly, some being seen extracellularly. For those cells, the total amount of HRP recorded would be an overestimate of the quantity of HRP being transported within the cell. Perhaps more

important is the fact that we were dealing with a heterogeneous population of spinal neurons. No attempt was made to calculate the relative volume of a neuron filled per injection. Since the cells varied in their size and morphology, a more analytical approach is necessary to properly evaluate effects of survival time, amount of HRP delivered and other parameters discussed on the quality of neuronal staining. A study on a morphologically more homogeneous group of neurons varying either the amount of HRP or survival time and using length of longest dendrites stained as a measure of the quality of staining has yet to be done by any investigator.

Axonal staining

In our group of twelve well-stained neurons, only two axons were positively identified. Studies by other investigators reveal variable success with axonal staining. Czarkowska et al. (1976) reported that the 44 recovered cells out of 55 which they attempted to label all had stained axons. Light and Durkovic (1976) reported stained axons for all spinal interneurons labeled. Brown et al. (1977a) reported that, of a selected population of well-stained spinocervical tract cells, all had stained axons. On the other hand, in a study of smaller cells located in the substantia gelatinosa, Light et al. (1979) reported only three positively identified axons in seventeen well-stained cells. Randic et al. (1981), in a study of DSCT neurons, could identify only eight stained axons in a population on nineteen well-stained cells. Comparisons of the relative amounts of HRP used by different investigators suggests that the percentage of successfully stained axons is higher where greater amounts of HRP

are used. Cullheim and Kellerth (1978) reported that although 6 μC was adequate for main motoneuron axon staining, 18–22 μC of HRP was routinely used to label axons and collaterals. They also reported that, in many cases, they could only see the part of the axon passing close to the surface of their 30 μm sections. They attributed the fluctuating intensity of axonal staining to poor penetration of DAB through the myelin sheath. Our inability to identify stained axons on our stained cells could be attributed to: (1) injection of relatively low amounts of HRP; (2) inadequate reconstruction of stained cells which might have allowed for identification of an axon on the basis of its morphology; (3) poor penetration of the chromogen, DAB, coupled with the fact that we had relatively thick (75–100 μm) histological sections; (4) capricious staining of the axons of some cells.

Extracellular HRP

In several experiments, extracellular HRP was present in the tissue near the labeled cell. The source of this HRP was probably threefold: (1) small amounts of HRP may have leaked from the electrode tip as it approached the cell; (2) HRP leaked out through the injured cell membrane during labeling; (3) if the electrode came out of the cell during the labeling procedure, HRP was deposited extracellularly. Regarding the last possibility, one would expect to see extracellular HRP deposits when electrophysiological records register zero membrane potential, indicating that the electrode had come out of the cell. However, from an examination of the experimental records of resting potentials, it was quite impossible

to predict when large quantities of HRP would be present extracellularly. In some instances, when experimental records showed a membrane potential of zero at the end of labeling, little or no extracellular HRP was seen. In other cases where records indicate reasonable membrane potentials throughout the injection procedure, a substantial amount of HRP was seen extracellularly. This is further evidence that monitored resting potential is not a reliable indication of the success of HRP injection into a cell.

A practical consequence of extracellular HRP deposits is that the enzyme can be taken up by cell bodies or injured processes and, in several cases, more than one cell appeared labeled at a single injection site. Although most of the time this additional labeling was slight, in at least one instance two neurons were well-labeled for some distance. In this case it was not clear whether the second cell was labeled by extracellular HRP taken up through an injured process or if the microelectrode came out of the first cell during the iontophoresis and penetrated the second cell depositing HRP intracellularly. Constant monitoring of the cell membrane potentials and recording of the functional characteristics of the labeled cell at the start and end of the labeling procedure would have been helpful in this case to distinguish between the possibilities discussed. In those cases where doubt existed as to which cell was intracellularly labeled, the cell was not included in the data. McCrea et al. (1976) also reported staining of neuronal elements adjacent to cells primarily stained with HRP.

Retrograde HRP

After depositing HRP extracellularly in the lower part of lobule V of the left cerebellar vermis, HRP stained cells were found in parts of the ipsilateral spinal cord extending from T_2 to L_4 spinal segments. These results support Grant's report (1962) that afferents from Clarke's nucleus travel via the DSCT to terminate ipsilaterally in the anterior lobe of the cerebellum primarily in lobules I to V. The results are also in accordance with the generally accepted view that Clarke's column in the adult cat extends from spinal segment T_1 to L_4. Recovery of a relatively small number (nine in toto) of retrogradely labeled cells was not unexpected since the injection site was relatively discrete, the amount of HRP injected was quite small and not all sections were processed. Petras and Cummings (1977) reported quite a large number of retrogradely labeled cells in Clarke's column, but their study involved multiple injections of 0.5-0.7 µl of 20-25% HRP into the entire anterior lobe of the cerebellum, whereas our experiment used small iontophoretic injections of HRP into a relatively discrete site (a 2 mm wide section of the lower part of lobule V of the left cerebellar vermis).

Eight of the retrogradely labeled cells were of the class C (Clarke cell) type (Loewy, 1970). Their mean diameters were 30-43 µm, which is comparable to diameters of 32-40 µm reported by Boehme (1968) for transversely sectioned cords. One cell had a mean diameter of 23 µm. It may represent a smaller class C cell or a class B cell. Due to the lack of morphological detail, the cell cannot be classified with certainty.

SUMMARY

1. The enzyme horseradish peroxidase (HRP) was injected intracellularly into single neurons located in L_2-L_3 of the cat spinal cord.
2. The HRP was injected by passing positive current through a HRP filled microelectrode which was impaled in the neuron.
3. We were most successful in labeling neurons when microelectrodes with tip sizes 1.4-2.8 µm were used.
4. Filled with a solution of 10-25% HRP, the microelectrodes had in-vivo resistances of 6-60 MΩ.
5. Attempts were made to stain 77 spinal neurons with intracellularly injected HRP. In 38 cases, stained neurons were recovered in the histological material at the expected level. Of these, 12 units were well-stained and 26 were partially stained.
6. Seven of the well-stained cells were located in the dorsal horn and the intermediate grey matter and five cells were located in the ventral horn.
7. The resting potentials of the well-stained cells ranged from -20 to -60 mV.
8. The rate of anterograde transport of HRP in dendrites ranged from 1 to 58 mm/day. In axons, the transport rate was 6-23 mm/day.
9. Two of the well-stained cells had axons which were labeled for 725 µm and 1050 µm.
10. The minimum amount of HRP injected into a well-stained neuron was 3.4 µC.

11. We concluded that the relatively new technique of iontophoretic injection of HRP allows morphological characterization of a single neuron following electrophysiological recording.
12. HRP was also microiontophoretically injected extracellularly into lobules IV and V of the cerebellar vermis and the presence of retrogradely labeled neurons in Clarke's nucleus of the spinal cord was revealed by subsequent histochemistry.
13. Nine retrogradely labeled neurons were recovered from the ipsilateral side of the spinal cord in the area of Clarke's nucleus of spinal segments T_2-L_4.
14. Based on the uptake and retrograde transport of extracellularly injected HRP, cells of origin of afferent projections to the cerebellar vermis were identified as Clarke's column neurons.

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ACKNOWLEDGMENTS

I would like to thank my major professor, Dr. M. J. Randić, for her help and guidance throughout this graduate endeavor. I also would like to thank Dr. H. D. Dellmann for his timely advice and Dr. C. D. Drewes for his criticism and comments during the preparation of this thesis. In addition, thanks to my wife, Kathy, for lending an ear and a shoulder during experimentation and thesis writing. I gratefully acknowledge the assistance of three other friends: thanks to Vjeko Miletić for his help and advice from start to finish; thanks to Mrs. Jenna McCarley for all the technical and not so technical bits and pieces I couldn't have done without; and thanks to Frank Richardson for being the extra energy needed to complete my research. And finally, migozared (everything passes).