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Axoplasmic Transport

Edited by
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With affection to my children
Jameel and Shareen

PREFACE

Axoplasmic transport is the intracellular movement of cellular components required for the maintenance and normal physiological functioning of neuronal cells. This book provides an up-to-date reference for both established investigators as well as for those entering in the field.

The chapters contributed by the acknowledged experts in the area of axoplasmic transport discuss state of the art information about the subject. The first six chapters describe the history and methods of the study of transport and the involvement of energy, ions, calmodulin, microtubules and other cellular components in transport. In the next five chapters the transport of polypeptides, lipids, nucleic acids, neurotransmitter containing components and various other particles in nerve fibers, is discussed. A significant portion of this book is devoted to axoplasmic transport studies related to metabolic and toxic neuropathies. Areas dealing with retrograde transport, regeneration and the relevance of transport in neurotropic functions are described in the last four chapters, followed by a discussion on the mechanism of axoplasmic transport.

As editor, I found that the collaborative efforts of my fellow neuroscientists gave me a renewed education in the field of axoplasmic transport. I am thankful to all the contributors for their cooperation in completing this book. I am especially grateful to Drs. S. Ochs, M. I. Sabri, and R. Hammerschlag for their advice and help. I am also indebted to my father, Professor S. A. Sandilvi and to my late mother, Saleha, who have been a constant inspiration to me.

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Chapter 1

A BRIEF HISTORICAL INTRODUCTION TO AXOPLASMIC TRANSPORT

Sidney Ochs

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I. DEVELOPMENT OF THE CONCEPT OF TRANSPORT IN NERVE

A. The Older Conception of Nerve Function

The idea of a spirit-like influence flowing in nerve can be traced back to ancient Greek physiological speculation. As later formulated by Galen in the 2nd century, a spirit-like principle is inhaled during inspiration and carried by the blood to the brain where an animal (animating) spirit is abstracted into the ventricles of the brain. The animal spirit then moves down within the hollow nerves to effect motion and sensation. The hollow nerve concept was an extrapolation from what appeared to be the case in the bovine optic nerve.[1] This was an extraordinarily long-lasting concept with traces of it seen lingering even into the 19th century.[2] In the mechanistic twist given to Galen's theory by Descartes in the 17th century,[3] the animal spirits, were viewed as the "liveliest and subtlest" of particles. To explain reflex action the animal spirits are able to leave the ventricles when flaps within the individual nerve fibers are pulled on by sensory stimulation. Under control of the pineal, the animal spirits then flow from the ventricles of the brain down within the opened individual hollow nerve fiber into their muscles to inflate them and thus cause the body to move in an appropriate fashion.

An inflation of muscle by animal spirits or a nerve fluid was disproved by the plethysmographic studies of Swammerdam soon after Descartes' formulation. Using the isolated frog muscle preparation, Swammerdam in the 17th century found on stimulation of its nerve that there was no increase in muscle volume during contraction. Similar plethysmographic studies made by Steno and Glisson at this time using the human arm also showed the lack of an increased muscle volume on contraction.[4,5]

In the 17th and 18th centuries various theories were advanced to account for nerve and muscle action with most retaining the concept of nerves consisting of hollow tubes with a nerve fluid of some kind moving down inside them. In one theory the nerve substance was considered to bring about an explosive reaction in the muscle to account for its contraction. There were also more physical conceptions of nerve action, with the nerve fibers transmitting some kind of a vibratory influence, either like sound waves or as a vibration of ether within the fibers.[6]

When electricity came under renewed scientific study in the late 18th century, it was usually dealt with as a flow of one or more fluids. Galvani[7] conceived of the animal electricity produced in the body as a fluid of a lively and peculiar kind which he assimilated to the old idea of a nerve spirit or fluid moving down within hollow nerve tubes. He wrote that, "the electric fluid is produced by the activity of the cerebrum, that it is extracted in all probability from the blood and that it enters the nerves and circulates within them in the event that they are hollow and empty or, as seems more likely, they are carriers for a very fine lymph or other similarly subtle fluid which is secreted from the cortical substance of the brain, as many believe. If this be the case, perhaps at last the nature of animal spirits, which has been hidden and vainly sought after for so long will be brought to light with clarity".

B. Nerve Form and Material Transport

The concept of a nerve fluid had been given substantiation by the early microscopic studies of Van Leeuwenhoek[8] as described in a letter written in 1717. When he placed freshly cut cross-sections of bovine optic nerve under his simple microscopes the nerve fibers were seen to be circular. Within a minute or so, a pearly fluid exudate appeared over the center of each of the circular cross-sections to form a little mound. This soon passed off as a vapor with the fibers collapsing to form flattened bands. Van Leeuwenhoek concluded that normally the nerve fluid within the fibers distends the walls of the nerve tubes, causing them to assume a cylindrical form and on cutting the nerves the escape of the nerve fluid allows the pressure on the walls to decrease and the fibers to collapse.

Chromatic and other optical distortions inherent in the lenses then used for microscopy made studies at higher magnification difficult until the achromatic compound lens microscope became available in the early part of the 19th century. With such a microscope Ehrenberg[9] in the 1830s clearly pictured the individual fibers. However, because he separated nerve fibers by compressing the entire nerve under microscopic control,[10] the fibers assumed an abnormal beaded shape which led to their confusion with nerve cell bodies. Purkinje and Valentin soon thereafter identified the nerve cell body by the presence of the nucleus.[11,12] Unfortunately, from their microscopical studies, these pioneers considered the nerve cell bodies and fibers to be two separate and distinct neural entities.[2,13] The cell body was considered to be the active element which in some fashion causes the nerve fluid contained in the apposed nerve fibers, the passive element, to circulate.[14,15] The fibers were viewed as closed loops with their central ends apposed to the cell bodies in the ganglia or in the central nervous system, their peripheral ends acting as sensory endings or contacting muscles to cause them to contract. This concept of a circulation of nerve fluid in looped fibers could have been influenced by some earlier hypotheses made of a circulation of nerve fluid in nerve fibers[16] inspired by Harvey's demonstration of the circulation of blood.

C. Cell Body-Fiber Dependence

Remak in an early paper in 1838 and definitively in his thesis 2 years later,[17] was the first to advance the idea that the nerve fibers and cell bodies constitute a single neural entity, i.e., to espouse an "early neuron doctrine".[2,18] Remak wrote that, "the organic fibers originate from the substance of the nucleated globules itself. In spite of the fact that this observation is very difficult and requires great dexterity in preparation as well as in observation, it is so well founded that it already would not be possible to doubt (it)".[19] This early conception of the neuron as an entity was in direct opposition to the dual neural element concept of Valentin and Purkinje and led to a long and dogged dispute between Remak and Valentin.[19] Only late in the 19th century, with the work of His,[20] Forel,[21] and Ramon y Cajal[22,23] among others, did the evidence for a single neuron element become massive and convincing and in Waldeyer's review,[24] the concept became formally baptized as the neuron doctrine. Finally, the outgrowth of fibers from cells shown directly in the tissue culture studies of Harrison[25] served to remove lingering reservations even as late as 1910 concerning the neuron doctrine.[26]

In spite of the uncertain status of the true nature of the neuron in the mid-19th century, Waller[27] inferred from his experiments on nerve degeneration that the viability of the nerve fiber depends on its connection with the cell body. He considered that some trophic influence passes from the cell body into the fiber to maintain its viability. This was clearly indicated in a classic study where the roots and nerves of the 2nd cervical ganglia of kittens were separately transected and those portions of the fibers of the nerves or roots connected to the dorsal root ganglion were seen to retain their normal form, while the part of the fiber separated from the ganglion underwent degeneration. Waller[28] concluded that, "the ganglion corpuscles (cell bodies) present in the dorsal root ganglion exert a trophic influence necessary to maintain the form and function of the fibers ascending in the dorsal root fibers as well as on the sensory fibers descending in the peripheral nerve". And, "as long as the influence of the ganglion over the nerve fiber occurs, this equilibrium (forces of renewal as opposed to those of degeneration) is maintained, but as soon as the connection of the ganglion corpuscle with the nerve fiber is destroyed, its peripheral (severed) end ... is subjected to forces of destruction (degeneration)". Waller[29] later figuratively put it that "a ganglion therefore was to the fibers connected to it what a river was to the rivulet that trickled from it, a source of nutritive energy".

D. Fibrillary Elements Within the Fibers — Cytoskeleton

The presence of a nerve fluid capable of moving in hollow nerve fibers appeared to conflict

with another of the pioneer observations made by Remak,[30] namely that a fibrillary, somewhat solid material, was contained in the primitive nerve fiber, or nerve band, as he called it. This view of the axon was verified by Purkinje[31] who renamed it the nerve axis or axon, the term by which it is referred to today. There was some doubt as to the reality of the presence of fibrillary material in nerve fibers. Schultze[32] later in the 19th century using more advanced staining methods than previously available, clearly established its presence in the nerve fiber. He considered that the function of the neurofibrils was to conduct the nerve impulse.

By the early part of this century it was generally recognized that the axon membrane is the seat of the nerve action potential[33] and that some other role had to be assigned to the neurofibrils. Parker[34] proposed that the fibrillary elements are the means by which the cell body exerts a metabolic control over its fiber. Considering the T-shaped neurons of the dorsal root ganglion earlier demonstrated by Ranvier, Parker wrote that,

> "in the ordinary sensory neurons, nerve impulses originate at the peripheral end, make their way centrally over the neurite, and without entering the body of the cell, pass on to discharge at the central end of the neuron. The metabolic influences on the other hand originate in the region of the nucleus of the cell body, pass down its neck to the tract of nervous transmission where they separate into two streams, one flowing peripherally over the neurite and the other centrally over the central nerve fiber process. And thus, the course of the neurofibrils does not follow that of the nerve impulses but does duplicate exactly that of the metabolic influences. I conclude therefore that the neurofibrillar system in the neuron is concerned specifically with the distribution of the metabolic influences and not with the conduction of nerve impulses. These influences start in the region of the neuronic nucleus and spread over the lines of neurofibrils throughout the whole neurone."

Parker's view was indeed very much in line with our modern one of transport, but he could not say what the nature of the materials transported might be. Gerard,[35] summarizing the evidence that the nerve impulse depends on oxidative metabolism, proposed that the enzymes necessary to maintain the metabolism of the fibers were being continually transported down within the nerve fibers from their source in the nerve cell bodies. This concept was similar to one earlier expressed by Goldscheider, who suggested that a transport of ferment-like substances from the cell body moves down along the whole course of the axon to their extremities as is required for the nutrition of the axons.[36]

Scott,[37] struck by the similarity of the staining of the neurosomes in the neuron cells to zymogen granules in secretory cells, considered that "nerve cells are true secretory cells". He also wrote that, "in the body of the nerve cell a substance is formed from the nucleus and Nissl bodies which gradually passes into the nerve fibers; and also that (the) stimulation of other cells by a nerve fiber is brought about by the passage of some of this substance into the cells on which the fiber acts".[38] He could thus be considered one of the forerunners of the concept of transport and neurotransmission as well as noting the similarity of the neuron to that of a secretory cell.

Torrey,[39] a student of Parker, proposed that the substance supplied by the cell body to its nerve fibers was a hormone-like material necessary for the maintenance of the nerve fiber and he also considered that "the substance involved may be enzymatic in character, produced by the nucleus of the neuron, and transported peripherally by the way of the neurofibrils." Again, we are close to the modern concept based on molecular biology showing the role of DNA and RNA to synthesize proteins in the cell body and the revelations of fine structure

in the fibers by the electron microscope, with a differentiation of the neurofilaments, the cytoskeleton, into microtubules, neurofilaments, and microfilaments.

E. Damming and Fluid Outflow as Evidence of Transport

Throughout most of the 19th century the dominant belief was not only that nerve fibers and nerve cell bodies were separate elements, but that a divided nerve could heal by a reunion rather than by a regeneration of new fibers from the proximal part of the divided fibers. This was the case in spite of the early evidence of Waller[27] showing regeneration to be due to an outgrowth of new fibers. Only with the acceptance of the neuron doctrine could the concept of regeneration as an outgrowth of neurites from the proximal stump become established.[26,40,41]

The effect of a partial constriction of nerve is of interest in that in some sense the old concept of a fluid flow in nerve fibers was carried over in explaining the phenomena seen. Ramon y Cajal[41] in his studies of regeneration using partially compressed nerves described a series of bulges and narrowings in the fibers above the compressed region which he interpreted as due to a starting and stopping of regenerative nerve fiber growth. A new growth was identified by the fine neurites seen in and beyond the region of partial constriction with growth cones at their distal ends. Weiss and Hiscoe[42] who studied the effects of partial compressions of nerve trunks by means of contracted arterial segments, saw a somewhat similar picture. However, the bulges and other form changes in the fibers above the constriction were differently interpreted. They considered the axonal contents to be flowing or constantly growing down within their sheaths at a rate of 1 to 3 mm/day as a column of semisolid axoplasm. On meeting with the obstruction due to the partial constriction, the column becomes dammed up, throwing the fibers into the beads and the other tortuosities seen.

A somewhat similar concept of a continual outflow of axoplasm within the nerve fibers had also been advanced by Young[43] at about the same time. In an amputated portion of nerve the fibers appeared to contract to form folds and spirals while "the central portion swells as if under the influence of an increased turgor pressure". The normally oriented molecules in the fiber (the neurofibrils of Parker) were considered to become disorganized in the amputated nerve stumps. On the second day after cutting, the central ends of the nerve fibers are swollen and "during the succeeding days the material of the central end flows out from the tube, forming one or several regenerating strands". Young[44] had earlier observed an outflow of axoplasm from the cut end of squid giant axons and from this he had inferred that the continued production of axoplasm by the cell body acts to force axoplasm down within the nerve fibers. The pressure exerted by the downflowing axoplasm is opposed by the lateral pressure in the walls of the fibers. Those concepts were carried over by Young to the myelinated nerve fibers.[45] The classical beads and ovoids, and other form changes of Wallerian degeneration which appear in amputated nerves were explained by Young as due to the unopposed surface tension of the myelin wall giving rise to the similar appearing beads shown in model experiments and attributed to surface tension by Plateau.[46] The oozing of axoplasm from the cut ends of myelinated nerve fibers described by Lubińska[47] seemed to be in accord with Young's concept. However, the assumption of a surface tension produced by a fluid-like myelin sheath giving rise to Plateau figures was not reconcilable with the laminar nature of the myelin sheath deduced by Schmitt from X-ray diffraction studies and later shown by electron microscopy to be produced by a wrapping of Schwann cell membranes around the axons.[48]

F. Tracer Techniques, Enzymes, and Optical Methods

While the morphological studies of Weiss and Young published in the 1940s suggesting a fluid downflow in nerve fibers did much to stimulate further study of the phenomenon of

material transport in nerve, the concept that a semisolid fluid slowly moves down the axons at a rate of several millimeter per day was soon to undergo revision with the introduction of new techniques. Samuels et al.[49] in 1951 first reported isotopes as tracers to the study of downflow using ^{32}P-orthophosphate as a precursor. After its uptake by spinal cord motoneurons from the blood there was a differential outflow of various labeled phosphorus components in the motor fibers, a finding which suggested a more complex outflow of axonal contents. Lubińska[47] in the 1960s discovered, after crushing nerves, that the acetylcholinesterase present in motor fibers became redistributed with time. The enzyme accumulated just above a distal crush as expected of an anterograde transport and to accumulate also just below an upper crush indicating a retrograde transport as well. This result was clearly inconsistent with a simple downflow of the axoplasm as a whole. Dahlström[50] later in 1969 found that amines in adrenergic nerve fibers accumulated at nerve crushes or ligations at a fast rate. The use of isotopes of amino acids as precursors injected near cell bodies for selective uptake revealed, after its incorporation, a still faster and regular transport of labeled proteins at a rate of 410 mm/day in addition to a slow transport.[51] Optical studies also revealed particles moving within nerve fibers at a fast rate in both the anterograde and retrograde directions.[51]

The modern era of investigation of material transport has established the cell body as the site of the synthesis of a wide range of materials supplied to the axon, components which include proteins and polypeptides, lipids and other substances which turn over in the membrane and the cytoskeletal elements and within the various other organelles of the fiber. Also synthesized are neurotransmitter and neurotransmitter components supplied to the nerve terminals, the vesicle membrane and those enzymes needed for local synthesis and turnover of neurotransmitter within the terminals. Additionally, there is a transport of trophic substances destined to be carried from the nerve terminals into post-synaptic cells to regulate some of its functions.

The mechanism of transport in the axon for anterograde transport materials is not as yet definitively established but it is recognized to be a local process within the nerve fiber, one which requires a supply of ATP and, in many nerves at least, depends on calcium.[51] The retrograde transport of materials has similar requirements. A number of models have been proposed to account for the transport mechanism[51] but here we are dealing with current problems. For their further explication we turn to the following chapters of this book.

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Chapter 2

ANALYTICAL TECHNIQUES FOR THE STUDY OF AXOPLASMIC TRANSPORT

S. Y. Chan and Z. Iqbal

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I. INTRODUCTION

Since the discovery of the movement of intracellular materials in neuronal processes, as described in the previous chapter, a large number of experimental procedures has been employed in the study of axoplasmic transport. In this chapter, we will describe, in brief, some of the most commonly used methods. Details of some of these procedures are described in various chapters of this volume and recent reviews.[1-6]

The existing approaches for the study of the transport can arbitrarily be divided into physiological/biochemical and physical methods. There is a great deal of overlapping in these categories and very often, an investigator has to employ a combination of methodology to achieve the desired goals.

II. PHYSIOLOGICAL/BIOCHEMICAL METHODS

The majority of axoplasmic studies have been performed by utilizing a class of materials in a particular nervous system, either endogenous, such as enzymes[7] and neurotransmitters[8] or exogenous, such as horseradish peroxidase[9,10] and radiolabeled precursors for proteins,[11,12] lipids,[13] and nucleic acids.[14] The two general methods to estimate the velocity of anterograde as well as retrograde transport are the so-called segmental analysis and the accumulation or ligation technique, both of which originated from physiological procedures. However, the identification of transported materials often requires biochemical techniques such as enzyme assays, SDS gel electrophoresis, subcellular fractionation, chromatography etc. In recent years, more and more biochemical techniques have been incorporated into the traditional methods and have become a powerful tool to study material transport in nerve processes.

A. Segmental Analysis

This procedure[11,15] entails the injection of a small amount of radiolabeled precursors into the proximity of well-defined neuron cell bodies. After the uptake and incorporation of these precursors into the cellular machinery, the outflow pattern of labeled activity moving down the nerve fibers can be analyzed by cutting the nerve into small segments and counting the radioactivity in each segment. The velocity of the transported materials is determined by the downflow of radioactivity at various time intervals after incorporation. Figure 1 depicts the experimental approach used for this method. Typically, 5 $\mu\ell$ of [^{3}H]-amino acid having high specific activity is injected into the L7 dorsal root ganglion of anaesthetized cat over a period of several minutes using a micropipet which is left in place for a short time after injection to prevent leakage of the tracer through the injection site. After a period of [^{3}H]-precursor incorporation into proteins and downflow, the sciatic nerves are taken out, sliced into 2 to 5 mm segments, and the radioactivity determined. The rate of advancement of the crest of radioactivity gives the velocity of the most rapidly transported material. A number of amino acids such as [^{3}H]-leucine,[11] [^{3}H]-proline[12] and [^{35}S]-methionine[16] have been used as precursors for the study of transport of different proteins.

Besides amino acids, other precursors can be used for the analysis of specific components. As described in Chapter 9, radiolabeled fucose and glucosamine are used for the analysis of glycoproteins and glycolipids. Likewise, glycerol and choline are used to study the transport of phospholipids.[17] For the investigation of nucleic acid transport, labeled nucleosides are employed, as detailed in Chapters 8 and 17. The study of neurotransmitter transport is carried out either by injecting radiolabeled transmitter such as [^{14}C]-norepinephrine[18] or radiolabeled transmitter precursors such as [^{3}H]-5-hydroxytrytophan[19] and [^{14}C]-DOPA[20] for the study of serotonin and dopamine, respectively.

Incorporation of labeled amino acids into protein is usually rapid, generally 5 to 20 min.[21,22] However, incorporation of other precursors such as [^{32}P]-phosphate,[23,24] may require longer periods, up to 3 to 4 hr.

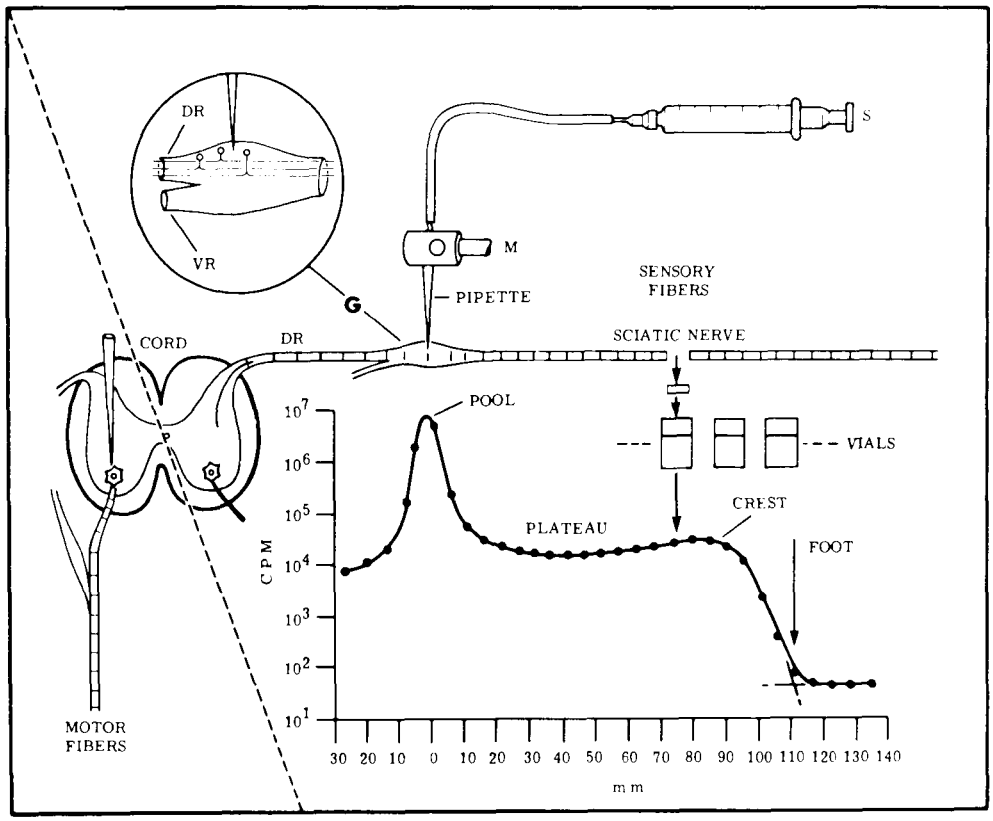

FIGURE 1. Injection and sampling technique showing transport. The L7 ganglion shown in the inset contains T-shaped neurons with one branch ascending in the dorsal root, the other descending in the sciatic nerve. A pipette containing ^{3}H-leucine is passed into the ganglion; and after its injection and the incorporation of precursor, the downflow of labeled components in the fibers is sampled at various times by sacrificing the animal and sectioning the nerve. Each segment is placed in a vial, solubilized, scintillation fluid added, and the activity counted. The outflow pattern is displayed on the ordinate log scale; the abscissa is in millimeters, taking the distance from the center of the ganglion as zero. The fastest moving activity is a crest falling abruptly to baseline levels. Behind it is a plateau with a high level of activity remaining in the ganglion. The left side of the cord shows motoneurons injected in the region of the L7 cell body. Removal and sampling of the ventral root and sciatic nerve at a later time gives a similar outflow pattern. (From Ochs, S., in *Hypothalamic Peptide Hormones and Pituitary Regulation*, Porter, J. C., Ed., Plenum Press, New York, 1977, chap. 2. With permission.)

In addition to the injection of precursors into cell body area and the determination of the transported material *in situ*, the outlined procedure can also be used to study the uptake and transport of material in vitro. Peripheral nerves together with its attached cell bodies (located in ganglion) are isolated and placed into a multicompartmental tray containing appropriate incubation media in each compartment.[25,26] Labeled precursors are then applied to cell bodies (sealed off from axons with grease) and after a predetermined time period, nerves are segmented and analyzed for the transported materials. For the studies of the effect of pharmacological agents, the in vitro chamber system is very useful. The drugs can be added into the area containing cell bodies to see their effect on the uptake of a precursor and/or the synthesis of components which are destined for the transport in the fibers[26-28] while the exposure of the nerve fibers to the compound of interest can be useful in determining its effect on the movement of materials within fibers.[29] Although the in vitro system has proved to be valuable in dissecting the overall transport process into uptake, synthesis and movement along nerve fibers, it can be applied only for the studies aimed at the components which

are transported at a fast rate, as isolated nerves cannot be kept functional for a long period of time in vitro.

We have studied the effect of ions and various pharmacological agents on transport using segmental procedure by a combination of both *in situ* and in vitro systems. In performing such studies, attention should be given to the perineurial sheath of peripheral nerves since it is a permeability barrier to certain drugs and ions.[30,31] As in our studies, the cat sciatic nerves had to be desheathed before exposing to suitable incubation media for the desired effect.[31,32] The procedure for such a preparation is depicted in Figure 2. The precursor, in this case [^{3}H]-leucine, is injected into the L7 dorsal root ganglion as described and about 2 hr of *in situ* incorporation and transport is allowed. The sciatic nerve, together with the ganglion is then removed from the animal and placed into a special chamber filled with buffer. The peroneal branch of the sciatic nerve is desheathed since it has less branching, whereas the tibial branch is left intact as control since it is smaller, more branched, and harder to desheath. After desheathing, the whole nerve is then placed in temperature controlled (37°C) and oxygenated test medium for a predetermined period before segmental analysis. It should be noted that the two branches should be adjusted to the same length before desheathing since the desheathed nerve is often longer than the intact nerve, which will complicate the analysis of transport. A method of adjusting the nerve length, taking advantage of the band of Fontana,[33] is described in Chan et al.[32] By utilizing this procedure, we have shown that the transport of labeled proteins was dependent on an appropriate concentration of Ca^{2+}.[32] The fact that transport requires microtubular integrity was also supported by experiments involving the exposure of desheathed nerve fibers to a class of antimicrotubular agents, vinca alkaloids.[34] Recently, we have also shown that anticalmodulin drug trifluoperazine (TFP) blocks the transport, thus suggesting an important role for calmodulin in the transport process (see Chapter 5 for details).

The desheathing procedure may be physically damaging to axons in some cases and is not applicable to small and greatly branched nerves. An alternate procedure for permeabilization of nerve has been recently devised. The procedure requires the incubation of nerve with a mild concentration of detergents such as soponin.[35] Such permeabilized nerves which are viable physiologically have been used to study the effect of various drugs on transport.

The studies mentioned above utilizing the tracer technique, *in situ,* in chamber or a combination of both have mainly been conducted in the sciatic nerves of cat,[11] rat,[26] frog,[27,28] or chicken[36] for the ease of access and the fact that sciatic nerve offers a longer length for transport studies. The system also offers the possibility to study both the sensory axons[11] (by injection of tracer into the ganglion) and motor axons[37] (by injection into the ventral horn).

The study of axoplasmic transport by segmental analysis has also utilized a number of other nervous systems such as optic, olfactory, or vagus. The optic system entails the injection of radioactive tracer into the eye and the labeled components are subsequently analyzed by autoradiography in the optic axons.[38] The procedure, first employed in mice by Taylor and Weiss,[39] has been extended to goldfish,[38] rabbit,[14,40,41] and other optic systems.[42] The study of slower transported components has been performed mainly through the use of segmental analysis procedure in optic systems.[41,42] The vagus nerve has been popular because of long unbranched nerve and both the motoneurons and sensory fibers can be studied in this system.[43,44,45] The olfactory nerve system is suitable for the study of transport rate due to its long length, especially in garfish[15,46] and pike.[47]

Although the segmental analysis is probably the most commonly used method in the transport studies, it is not free of limitations. These include the loss of precursor from the injection site,[48] possibility of precursor diffusion down the axons,[40] and blood-borne label reaching the nerve and getting incorporated locally.[48] The system also poses a problem when the investigator wishes to identify a specific component being transported in the nerve fiber.

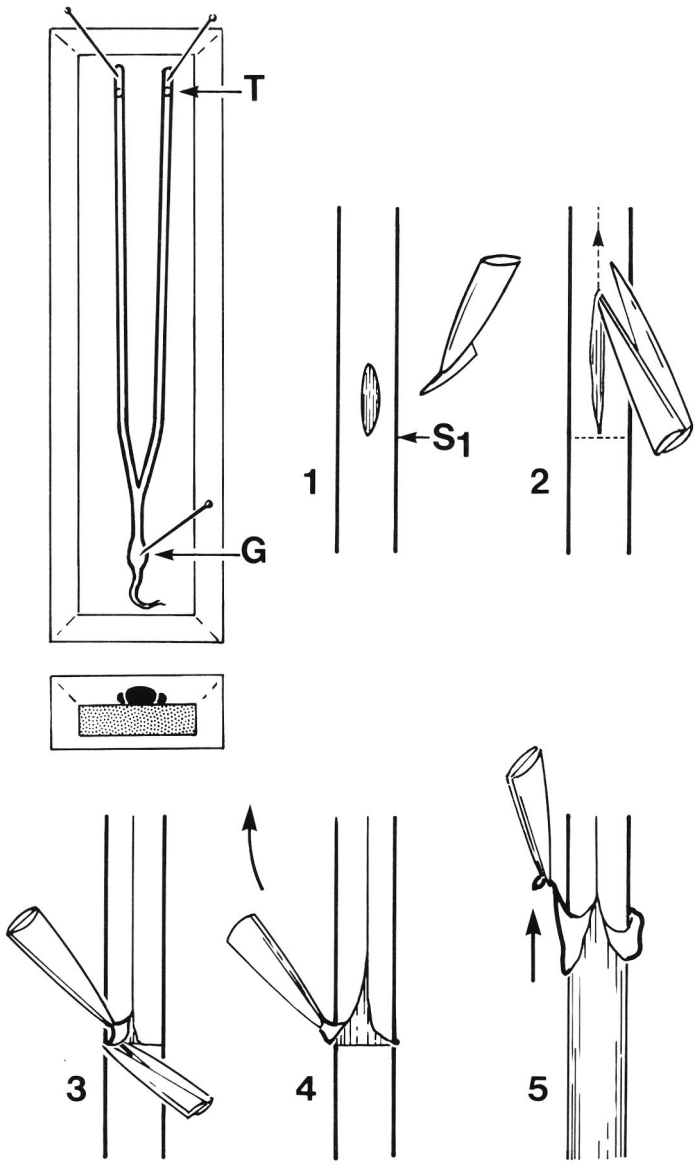

FIGURE 2. Desheathing procedure in cat sciatic nerves. The nerve was pinned down on the chamber on the ganglion (G) and the ends. A tie (T) was placed on the ends of each branch to serve as a marker after they were stretched. Step 1: a slit was made on the peroneal branch approximately 35 mm distal to the ganglion by means of a knife made from a sliver of razor blade. Step 2: the blade of a pair of iris scissors was used to slit the perineural sheath distally. Step 3: the sheath was then cut circumferentially at the proximal end of the slit. Steps 4 and 5: the perineurium was peeled back down the nerve to the point where branching occurs, usually 135 to 150 mm distal to the ganglion. (From Chan, S., et al., *J. Physiol.*, 301, 477, 1980. With permission.)

Some of the above mentioned shortcomings can be avoided by the use of single neurons. Microquantities of tracers can be injected directly into the cell body of the giant cerebral neurons of Aplysia[19,49] and the rate of transport of labeled components observed. In this system, the radioactivity remains confined to the axon of the neuron along the entire length of the nerve.

Proteins can also be labeled, in vivo, covalently by *N*-succinimidyl [2,3-^{3}H]-propionate (NSP) which can penetrate cell membranes and bind to intraneuronal proteins.[50,51] No significant change has been found for the electrophoretic mobilities in NSP labeled proteins either in SDS-PAGE or isoelectro-focusing gels. The NSP labeling was originally thought to be nonselective and could be applied to any part of the neuron as no protein synthesis was required. However, a recent report[52] indicates that the [^{3}H]-NSP has a tendency to prominantly bind to a small number of proteins, which may limit its usage as a probe to study axoplasmic transport.

B. Ligation and Accumulation Technique

The transport of endogenous components such as structural proteins, enzymes, neurotransmitters etc. have been successfully studied by the accumulation technique with which the transported components are disrupted either by ligating the nerve fibers with a fine thread[53] or by exposing a region of the nerve to cold block.[54] The accumulation or depletion of those components are determined by sectioning out a small segment at the disruption point and analyzing the accumulated material(s) with a variety of biochemical, histochemical, or electron microscopic methods.

The bidirectional movement of the materials in nerve fibers can be determined by double-ligation technique. In this procedure, initially used for the study of acetycholinesterase (AChE)[55,56] transport (also see review[57]), the materials accumulating on both the proximal and distal sides of a nerve ligation are detected. Usually, the accumulation is confined to a narrow region extending a few millimeters from the point of ligation on either side and the amount of enzyme accumulated is due to the redistribution, caused by the intra-axonal transport of the enzyme being analyzed. Such conclusion is derived from the observations that the amount of enzyme damming at both ends is balanced by a depletion of the enzyme in the middle segment of the nerve, thus the total amount of enzyme remains unchanged[57] and the inhibition of protein synthesis has no significant effect on the amount of enzyme accumulated.[58]

The cold block technique,[54,59-61] a modification of the ligation procedure requires the exposure of a region of nerve to cold temperature (4°C) at which the transport is blocked locally, causing the damming of transported material at the cooling region. Rewarming to body temperature allows the accumulated material to move along the nerve beyond the block. This technique has an advantage over the conventional ligation methods as it allows the resumption of original conditions by rewarming and thus avoids damage to the nerve fibers by ligation or crushing. Additionally, injury to nerves caused by the ligation could induce a response in local synthesis[62,63] or degradation of certain materials under investigation.

The above mentioned procedures have been used extensively in the study of transport of enzymes[53,54,56] and neurotransmitters,[63,64] as well as the transport of mitochondria.[65] Accumulation techniques offer certain advantages over the segmental analysis, as they allow the determination of bidirectional flow of endogenous materials. Further, the procedure can be used for measuring transport in short axons where profiles of activity along the nerves are difficult to obtain.

The investigators using the accumulation technique should also be aware of the few pitfalls in the method. Besides the artifacts encountered due to injury in the ligation procedure, the rate of transport obtained from the accumulated samples is a weighed average since the materials accumulated at the disruption point are also subjected to turnaround or retrograde transport.[63] For those components which are transported in small amounts, the available analysis techniques may not be sensitive enough for detection.

C. Combination of Tracer and Accumulation Technique

There are a number of intraneuronal components which are transported in small amounts,

thus making their detection difficult by either the tracer or ligation method. By combining both techniques, we have been able to detect the fast transport of calmodulin and microtubular proteins.[66] The radiolabeled precursor is injected into the ganglion of cat sciatic nerve, as described in Section II.A. After 1 to 2 hr of uptake and transport of labeled components from the cell body, a ligation is placed distal to the ganglion to stop further transport of labeled components. A second ligation is placed a few centimeter distal to the first ligation down the axon. After a predetermined time interval, the labeled material associated with the fast moving crest is analyzed for calmodulin activity. This procedure is potentially valuable for the analysis of fast-transported components which are transported in small amount.

D. Biochemical Analysis of Transported Materials

The number of components being transported in either directions at any given time is large and the composition complex. The segmental analysis, in general, demonstrates that a class of labeled components is moving at a given rate. However, the identification of the various materials being transported in nerve depends heavily on biochemical techniques designed for specific purposes. Using a subcellular fractionation procedure, it has been shown that most of the particulate proteins including some transmitter synthesizing enzymes and glycoproteins are transported at a fast rate.[67,68] On the other hand, most of the soluble proteins including cytoskeletal elements are found to be moving in nerve fibers at a slow rate.[69]

The soluble components can be further separated and characterized by gel filtration or by ion-exchange chromatography. For example, after injection of ^{45}Ca into cat dorsal root ganglion, about 25% of ^{45}Ca was found present in the soluble fraction. Using gel filtration columns containing Sephadex G-100 and Bio-Gel A-5m, the radioactivity was found to be associated with a calcium-binding protein.[70] The particulate components, on the other hand, need to be solubilized before they can be separated on the chromatographic columns. With the advent of sodium dodecyl sulfate-polyacrylamide gel electrophoresis[71] (SDS-PAGE), these insoluble particulate components could be resolved into a number of different bands, separating on the gel according to their molecular weight. A single dimensional gel separation technique could identify about a dozen of fast transported components.

With the introduction of two-dimensional gels (2DG) which separates proteins according to two independent physico-chemical criteria, namely the isoelectric point and molecular weight; it has now been possible to identify over 100 individual fast transported proteins.[72] The technique, first applied by O'Farrell[73] in 1975 in the separation of *E. coli* proteins has been modified for use with neuronal tissue.[72,74-76] Essentially, the samples are mixed with urea, SDS, nonionic detergent such as NP-40 and then separated on thin gels of acrylamide or agarose containing ampholytes of different isoelectric points, by electrophoresis. The proteins migrate to their corresponding isoelectric points and thus get separated from one another. In the second dimension, the proteins are separated according to their molecular weights using conventional slab gel SDS-PAGE technique. After second dimensional electrophoresis, the gel is dried and subjected to fluorography. An alternative method is to remove the spots from the original gel using the fluorographic pattern as a guide and process for radioactivity determination by scintillation counting.[74] With this punch and count procedure, the sensitivity of detecting the protein components is greatly enhanced. In our laboratory, we have been able to demonstrate fast transport of tubulin in rat sciatic nerve.[75]

The 2DG procedure has been very helpful in the identification and determination of proteins and glycoproteins[76,77] which can be transported anterogradely or retrogradely and at fast or slow rate. The precursors employed are either [^{3}H]-labeled or [^{35}S]-labeled. By the use of double-labeling with [^{35}S]-methionine as protein precursor, along with [^{3}H]-labeled fucose or galactose for glyco-moiety precursor, identification of transported glycoproteins has been made possible with great success (see Chapter 9 for details).

III. PHYSICAL METHODS

Physical methods as a means to study axoplasmic transport are relatively new and still under development. Besides complimenting the physiological/biochemical techniques, physical methodology opens up new frontiers in obtaining informations that are off-limit to the other approaches. If current technical problems can be resolved, this method of analysis will probably contribute much informations regarding the underlying mechanism(s) governing axoplasmic transport.

In general, physical methods of studying axoplasmic transport can be divided into two broad categories: (1) the direct-monitoring techniques[3,78] and (2) the optical or microscopical methods.[3,79,80]

A. Direct-Monitoring Techniques

By injection of a radiolabeled precursor into cell bodies and allowing downflow, labeled materials can be monitored by external radiation detectors, also called position-sensitive detectors. These devices, capable of detecting ionizing radiations such as gamma or strong beta, can be placed at desired positions along the axon in order to evaluate the labeled material(s) being transported. There are three types of external radiation detectors reported so far, namely, the gamma or scintillation camera, the silicon p-n junction diode, and the multiwire proportional chamber (MWPC) or multiple proportional counter (MPC).

The gamma camera was first used to externally monitor axoplasmic transport in cat nerve using [^{75}Se]-methionine as the labeled precursor.[81] This is the first instance reported to use an external detector successfully for such purpose. However, it was later found that the detector had rather poor spatial resolution as well as few suitable radiolabeled precursors. The use of silicon p-n junction diode was pioneered by Takenaka et al. in 1978.[82] The semiconductor radiation detector is windowless and has a diameter of just 2 mm and thickness of 1 mm. [^{14}C]- and [^{32}P]-labeled[82,83] precursors can be used as radiation source and both in vivo[83] and in vitro[82] detections have been accomplished. The semiconductor detector needs to be repositioned during the course of the experiment due to the small size of the detector, which results in variations of detection efficiency. The low level of activity detected due to self-absorption by the nerve fibers also limits its use on relatively small nerves such as frog and rat sciatic nerves. The multiple proportional counter, introduced by Snyder et al.[84,85] seems to avoid some of the above-mentioned problems. Simplistically, MPC consists of a plane of parallel anode wires with an electrically common ground. β-Particles emitted from the labeled material(s) inside the axon are detected by any of the anode wires that are above the region of the radiation source and thus, the progress of the transported material along the axon continuously monitored. It is a relatively complicated device and the mechanical and technical workings are detailed by Synder.[86]

On the whole, direct-monitoring devices have certain advantages over conventional techniques. Their ability to follow temporal changes in axoplasmic transport without injury to the axon provides a more reliable procedure to analyze transported components by biochemical techniques. The devices (aside from their limitations), once setup, are simple to use in various nervous systems. However, direct-monitoring is mostly restricted to in vitro studies so far although in vivo study has been reported.[83] If in vivo studies can be done routinely, both fast- and slow-transport can potentially be monitored in one preparation and tissue assayed for different transport components. The existing devices have very limited range of detection and have not been improved to the extent that thicker nerves such as cat sciatic nerves can be included. Thicker nerves have the advantage that more tissue is available for biochemical analyses.

B. Optical Methods

Saltatory organelle movements have been observed to travel at fast rates in living axons

of a variety of animals by light microscopy.[87] This new method for studying axoplasmic transport, though still under development, has had major advances recently due to the introduction of advanced optics and new microscopic techniques to the field.[79]

The most common optical method for structural visualization inside living cells is the phase contrast microscopy. It has been used to observe organelle movements in nonmyelinated crab axons[88] and nonneuronal cells.[89] However, phase optics are only useful for very thin tissues and give poor resolution of myelinated axons due to interference of out-of-focus haloes. Darkfield microscopy has been shown to be useful in the visualization of organelle movement, especially in myelinated axons,[90] however, problems related to background light scattering hinder the detection of small particles in those axons. Differential interference-contrast (DIC) or Nomarski microscopy, coupled with video-recording, can provide high-resolution optical image of relatively thicker specimens.[79] However, DIC images are difficult to record since the highest resolution can be obtained only when the light intensity is lowest. However, a new method, named Video-Enhanced Contrast, Differential Interference Contrast (AVEC-DIC) microscopy[91] has overcome this limitation and provides very high resolution of imaging. The AVEC-DIC may be capable of visualizing single microtubules[91] and it represents an exciting new way of investigating intracellular movement. Recently, Hodge and Adelman[92] reported the technique of pseudostereoscopic viewing of image pairs as an efficient means to evaluate particle movements inside living axons.

In general, light microscopic methods offer the advantage of greater spatial and temporal resolution than any other available methods and very often, reveal details that are not accessible to others. It also allows the continuous monitoring of a particular organelle movement, instead of the average velocity estimated by other methods. However, there are still many difficulties since it requires rather restricted conditions for the system to work. Besides the requirement of high resolution and magnification, the axon preparation under light microscopic investigation need to be thin so that light can penetrate.[93] In vivo study is often impossible due to light penetration and scattering problems. Rather intense illumination is also required in Darkfield and Nomarski optics and the axons under investigation may be damaged by the heat emitted. In addition, organelles have to be large and refractile enough in order to be seen in the light microscope. These problems limit a great deal the usefulness of the light microscopy techniques in the evaluation of transport. However, as these limitations are gradually overcome through innovations and advanced technology, optical techniques will emerge as an important tool in the study of axoplasmic transport.

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Chapter 3

PRELUDE TO FAST AXONAL TRANSPORT: SEQUENCE OF EVENTS IN THE CELL BODY

Richard Hammerschlag and George C. Stone

TABLE OF CONTENTS

I. STRATEGY FOR IDENTIFYING INTRASOMAL PATHWAY

The fast anterograde transport of newly synthesized proteins can be conveniently subdivided into three phases. *Initiation* comprises the recognition, sorting, and routing to proximal regions of axon (or dendrites) of a select population of proteins. *Translocation* involves the longitudinal migration of these proteins within the axon. *Termination* is the arrival of proteins at their distal sites of function. Our studies have focused on defining the initiation phase — the events that interface synthesis in the soma with the onset of transport in the axon.

Since fast axonal transport is predominantly a process for delivering materials to the neuronal membrane, a fruitful approach has been to compare it to schemes employed by nonneuronal cells for transporting membrane and secretory proteins to their cell surface.[1] The well-characterized intracellular pathways of such proteins as digestive enzymes in pancreas,[2] acetylcholinesterase and acetylcholine receptor in muscle,[3] and immunoglobulins in plasma cells,[4] have served as models for examining the initiation or somal phase of fast axonal transport.

Our main strategy for probing these events has involved in vitro pharmacological manipulations of dorsal root ganglion neurons from bullfrog.[5,6] Preparations are incubated with a test drug after cell bodies within the ganglia have been exposed to a radioactive precursor of either protein, glycoprotein, or lipid. After various time periods, the profile of labeled fast-transported material within the axon is determined by assessing the acid-insoluble radioactivity in successive segments of the nerve trunk. A decreased amount of transport is taken to indicate that a step in the soma has been affected and that less material was exported to the axon. A somal location of drug action is verified if after exposing only the desheathed nerve trunk to the drug, the amount of transported material passing the exposed region is unaffected. Indications of the subcellular site of blockade in the cell bodies have been achieved by ultrastructural examination, and by utilizing different radioactive precursors known to be incorporated into fast-transported proteins at distinct regions of the soma.

This general approach, in combination with subcellular fractionation studies, has brought the outlines of the intrasomal pathway into focus (Figure 1).[1] Newly synthesized proteins destined for fast axonal transport are rapidly assembled into, or sequestered within, membrane. They are then sorted into subpopulations and routed through the Golgi apparatus where many acquire carbohydrate side chains and/or sulfate residues. Their exit from the Golgi occurs in association with coated vesicles and may involve entry into a storage pool from which they are delivered to the fast transport system and routed to distinct membrane domains. The evidence for each step of this itinerary will be presented in the body of this review.

II. ASSOCIATION WITH *DE NOVO* SYNTHESIZED MEMBRANE

Numerous studies have demonstrated an interrelation between fast-transported protein and lipid. Phospholipids,[7,8] cholesterol,[9] and gangliosides[10] undergo transport at rates comparable to the fast rate of protein transport. In addition, labels associated with fast-transported lipid and protein show parallel subcellular fractionation patterns, and local injection of colchicine blocks transport of both labels.[7] Observations that inhibition of protein synthesis reduces the amount of fast-transported lipid as well as protein exported from the soma[11,12] first suggested that fast-transport occurs as a concerted movement of assembled membrane rather than as an assembly line movement of membrane components.

Complementary support for this concept has come from our findings that inhibiting the synthesis of either phospholipid or cholesterol in the cell body leads to a proportional decrease in the amount of fast-transported protein exported to the axon.[13] The drug fenfluramine affects phospholipid biosynthesis by inhibiting the enzyme phosphatidic acid phosphohy-