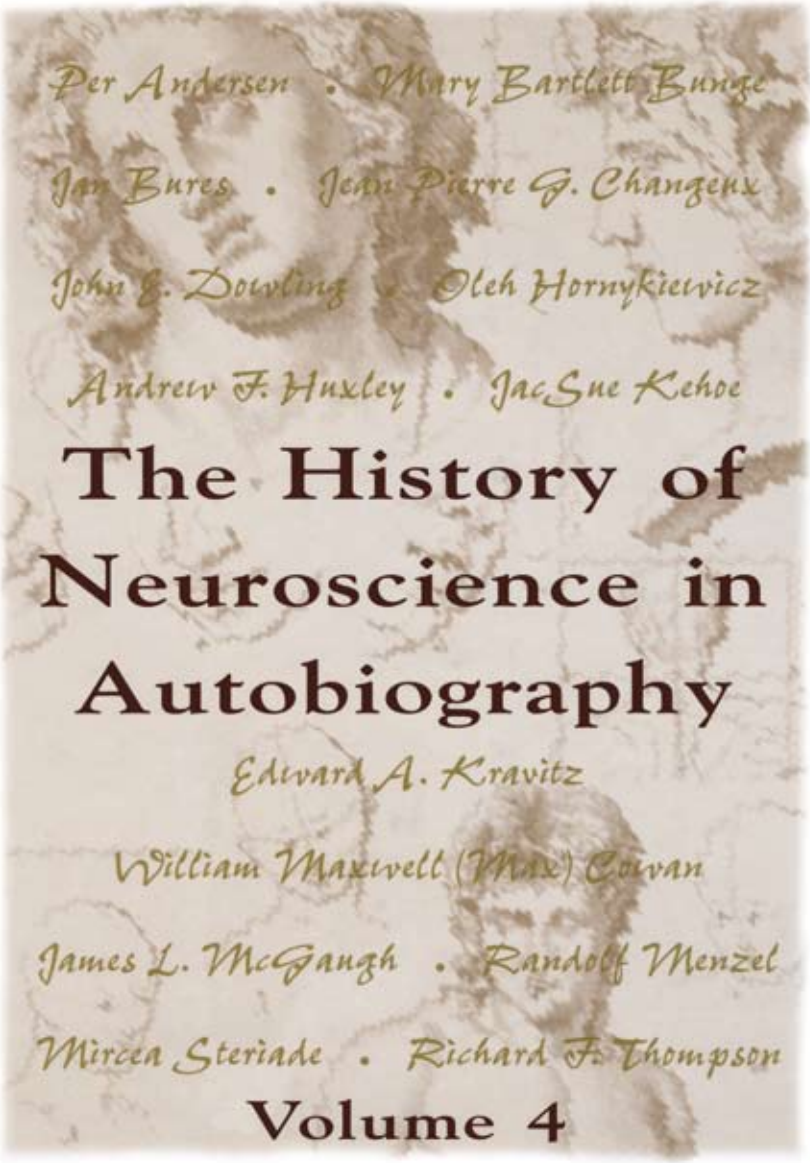

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The History of Neuroscience in Autobiography

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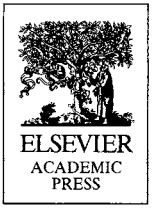

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Contents

Mary Bartlett Bunge

BORN:

New Haven, Connecticut
April 3, 1931

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Simmons College, B.S. (1953)
University of Wisconsin Medical School, M.S. (1955)
University of Wisconsin, Ph.D. (1960)

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Harvard Medical School, Boston (Sabbatical, 1968)
Washington University School of Medicine, St. Louis (1970)
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University of Miami School of Medicine, Miami (1989)
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HONORS AND AWARDS (SELECTED):

Chair, Development of Women's Careers in Neuroscience Committee, Society for Neuroscience (1994–2002)
Wakeman Award, Spinal Cord Repair (1996)
Dana Alliance for Brain Initiatives (1999)
Dean's Research Award, University of Miami School of Medicine (1999)
Vice President, National Neurotrauma Society (2001–2002)
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Christopher Reeve Research Medal for Spinal Cord Repair (2001)
Florida Woman of Achievement (2002)

Early work by Richard and Mary Bunge demonstrated that demyelination and remyelination occur in the adult mammalian spinal cord. This and other work led to a better understanding of myelin formation in the central and peripheral nervous systems. Mary Bunge has contributed information on the mechanisms of axonal growth in culture. Both Bunges have been pioneers in the area of neural cell-extracellular matrix interactions and spinal cord injury and repair.

Mary Bartlett Bunge[1]

As an only child growing up in a small Connecticut River harbor town, I was fortunate to have the woods at my back door and, down a long steep incline, a small stream at the front of the house. Spending much time alone, the woods and the stream provided hours of exploration and enjoyment. Steering my rowboat slowly near the banks overhung with elderberry bushes, the tadpoles and frogs were endlessly intriguing. This led to a developing interest in biology and to borrowing books from the library about Marie Curie. How romantic it seemed to be stirring pitchblende for hours! Beyond the stream was a meadow that, when flooded and frozen in the winter, provided an opportunity to pretend I was Sonja Henie, a three-time Olympic gold medalist who brought beauty and ballet to figure skating. A mile down the road lived my ballet teacher, whose lessons inspired me to dream of being a dancer like Anna Pavlova, one of the greatest ballerinas of all time. Being chosen to represent the Girl Scouts in that area of the Connecticut River valley brought me into contact with my fourth heroine, Mrs. Eleanor Roosevelt. The prowess of my maternal grandmother in sewing led to my ability to create my own clothes and, thus, to dream of being a fashion designer in New York City.

A profession involving artistic expression vied heavily with one in science. Both my parents were artistic. My mother, Margaret Elizabeth Reynolds Bartlett, was descended from the renowned British painter and founder of the Royal Academy, Sir Joshua Reynolds. She herself painted and was a decorator with no college education; her father thought college education was useless for women. My father, George Chapman Bartlett, spurned education after high school to study the violin intensely. Despite having a very gifted student who gave a recital in New York, he tired of teaching and turned to creating new houses and renovating very old ones, such as a 1785 house built by Captain Ebenezer Williams where we lived for a few years.

Being different from my classmates in that I was actually interested in learning, it was a relief to migrate to a more northern area of the Connecticut River valley to the Northfield School for Girls for the last two years of high school. My education was greatly improved and led to matriculation

[1]With partial commentary on Richard P. Bunge.

at Simmons College in Boston. This college was chosen because interests in biology had finally surpassed the others. I reasoned that the artistic interests could be pursued as hobbies, but it would be impossible to do laboratory work without training. My goal was to be a laboratory technician, and Simmons offered a course in laboratory technology. It was the vision of businessman John Simmons that women should be given both a liberal arts and a practical education. Upon graduating from college, I would be prepared for a job. This goal was to change, however.

At the end of my junior year in college, I was accepted into the well-known college program at the Jackson Memorial Laboratory in Bar Harbor, ME. For the first time I was in a community of 18 highly motivated students pursuing medical and/or research professions. Not only did we attend classes and participate in research, but we also were responsible for preparing our meals and ate in a common dining room. It was a dream summer socially and scientifically, including socializing with the senior scientists. Though renowned for cancer research in animals, I was placed with Dr. Philip R. White, who, in order to study crown gall tumors, had devised plant tissue culture techniques, including the formulation of a completely synthetic medium for plant cells. After being there for a week, he informed me that he was going on a trip and requested that I change the medium of his 20-year-old tomato root cultures. Because prepared culture medium was not available at that time, I nervously weighed each component to prepare the medium from scratch and was enormously relieved to find that the cultures survived uninfected. Dr. White occasionally grew animal tissue in culture, and after viewing contracting heart muscle one day, I was hooked on research. This extraordinary summer experience changed the course of my life and attending graduate school suddenly became compelling.

To Madison, Wisconsin: Fall of 1953

Though scheduled to start a teaching career and a Master's program at Vassar, a telegram arrived from Dr. Robert F. Schilling offering me a Research Assistantship that would be funded by the Wisconsin Alumni Research Foundation while I became a graduate student in the Department of Medical Physiology at the University of Wisconsin Medical School. Dr. Schilling had become renowned for developing a diagnostic test for pernicious anemia. He was studying a substance called intrinsic factor, which is lacking in this condition. Would I be interested in working in his laboratory for my thesis work? The Vassar plan was dumped forthwith, and my parents started to plan the car trip that would take me to Madison in the autumn of 1953.

Intrinsic factor in normal human gastric juice enhances the absorption of vitamin B_{12} from the gut. In the mid-1950s, the mechanism by which this occurs had not yet been elucidated, but it was known that binding of the vitamin to the factor was necessary, although not sufficient. Pseudovitamin

B_{12}, in which the 5,6-dimethylbenzimidazole (DMBI) moiety is replaced by adenine, was considered not to substitute for vitamin B_{12}. We investigated whether the pseudovitamin or the DMBI moiety alone competed with vitamin B_{12} for the process that leads to intestinal absorption of the vitamin and found that the two compounds were not effective competitors *in vivo* and did not bind to gastric juice *in vitro*. Thus, we determined that the binding of B_{12} by normal gastric juice is a highly selective process. This was the subject of my first publication in 1956. A second publication in 1957 reported the results of testing the effect of ten vitamin B_{12} analogs on vitamin B_{12} binding in gastric juice compared with certain other biologic substances (serum, saliva, colostrum). Gastric juice exhibited the greatest preference for vitamin B_{12} in the presence of excess analog. Occurrence of a sulfate, nitrate, or chloride ion in lieu of the cyanide ion of the vitamin did not diminish competition for vitamin B_{12} binding sites by gastric juice. This work also was the basis of a thesis, enabling receipt of an M.S. degree in medical physiology in 1955. Following this, during the first year of marriage, I worked full time in Dr. Schilling's laboratory while deciding about Ph.D. work.

Thus, this was a productive time and an exciting introduction to basic research with clinical relevance, an introduction that was to influence future research decisions. Dr. Schilling was an outstanding mentor who set high standards and viewed data very critically. His input into my first manuscript writing established a valuable foundation for years to come. As a beginning graduate student enjoying some of the many benefits of Madison and Lake Mendota and the unique and wonderful Student Union, a slight hint from him over lunch to work harder, including evenings, was sufficient to change my work habits to this day.

But the research experience was only part of the gain from being in Dr. Schilling's laboratory. Every summer Dr. Schilling took on medical students, and in the summer of 1954, one of those medical students was Richard P. Bunge. Although Dick spent most of the summer in the cold room working on a Raynaud's disease project, and though he vanished at 5:00 PM to wash dishes in the hospital cafeteria to help fund medical school, we became acquainted in a sailboat. Because I thought that he needed some fresh air, I invited him to go sailing and the wind often died down when we were in the middle of Lake Mendota. I had found a gem. In time I was to realize how much he personified midwestern values: no airs, no nonsense, plain speaking, integrity, modesty, and consideration that every person has value. These traits undoubtedly also resulted from having grown up in a home with a strong Christian influence.

Just three years earlier, the tall, lanky young man with a crewcut had arrived in Madison to enter the University of Wisconsin as an undergraduate. This was a big change from the rural Midwest settings where the Bunge home was often situated in an expanse of corn fields and next to a Lutheran church where his father was pastor. He had attended a one-room

schoolhouse in early years; a short period at a high school in Albany, MN, where he was a basketball star, awakened his intellect. This intellect flourished in the presence of great minds he encountered at the University and led to entry into medical school.

To pay for his education, in addition to the cafeteria job, he decided to split a year, working half of the time in research and attending medical school part time. A faculty member in the Department of Anatomy, Dr. Paul Settlage, was studying the administration of anaesthesia via the cisterna magna in adult cats. The cerebrospinal fluid was drawn into a syringe, the anaesthetic was added, and the fluid was returned; this was repeated several times. Because these animals exhibited a temporary paralysis, he considered it important to test this procedure of cerebrospinal fluid exchange without the anaesthetic. The person he chose to study this was Dick, who prepared the spinal cord tissue, stained it for myelin, and observed a non-staining rim around the lower medulla and upper spinal cord periphery. Then, one month later, he observed thin circles of new myelin in the area earlier bereft of myelin, at a time when the animals showed neurological improvement. Though the mechanism was not understood, this was the first sighting of demyelination and remyelination in the adult mammalian spinal cord, at that time a revolutionary finding. This led to a lifelong career dedicated to research, preferring that to the practice of medicine. Following medical school, he chose to enter a postdoctoral fellowship rather than to proceed to further medical training.

During my coursework for my M.S., I took a cytology course from a noted cell biologist, Dr. Hans Ris. Known for his work on the configuration of DNA in chromosomes, he was an electron microscopist. His course was fascinating, starting with descriptions of early cytologists Schleiden, Schwann, Boveri, Brown, etc. and providing captivating laboratory periods that included preparations of chromosome squashes and cell fractions and tissue sections. But, most importantly for me, he introduced us to the electron microscope. Even using the first electron microscope at the University of Wisconsin (in the Biochemistry Department), when we often had to hammer the lenses into appropriate positions and the resolution was inferior by today's standards, the images were enthralling, and I saw the opportunity to combine the acquisition of scientific data with artistic expression. The decision to work with Dr. Ris for my Ph.D. in the Zoology Department came easily.

What project to pursue? Several questions had arisen from Dick's discovery. Was demyelination partial or complete? What cells reformed the myelin? How was the myelin laid down? The spiraling mechanism for peripheral myelination had been reported in 1954. Was the same mechanism utilized by central glia? Why was the central myelinating cell not obvious as it was in the periphery? Clearly, what was needed was an electron microscopic characterization of the lesion that Dick had studied at the light microscope

level. Despite Dr. Ris' lack of experience with nervous tissue, he took me on. He was an outstanding mentor, committed to the highest standards of excellence and willing not only to mentor a now committed developing neuroscientist but also a young woman. The availability of these two mentors most certainly helped shape my future in a beneficial way. Both, to their credit, were gender blind, and I never thought of myself as a female graduate student, but as a graduate student period. He was an effective teacher as well, and I learned the preparative techniques from Dr. Ris, including thin sectioning, very quickly. He was a master of interpretation of electron micrographs.

And so Dick and I set out on our new research journey. But first I had to meet my teaching requirement for my Ph.D., teaching Zoology I laboratory at 7:30 AM. We had found an apartment close to the University, not far from the bottom of the steep hill that led to some of the main campus buildings, including the zoology building. On bitter cold, dark mornings in the heart of winter, Dick pushed me up the hill. When the teaching commitment was finished months later, we started the work in earnest. We were to have our own laboratory for a time in a brand new medical school research building due to the unfortunate death of Dr. Settlage in a drowning incident. A substitute for teaching Gross Anatomy was needed, and, when this opportunity was offered to Dick, he decided to take a year off from medical school to teach as well as do research. Thus began a love for teaching this subject (exceptionally well) for many, many years, which provided valuable background for leading The Miami Project some 30 years later. This one-year instructorship also enabled us to stay out of debt for our remaining graduate education.

Adequate preservation of central nervous tissue was difficult to achieve. Glutaraldehyde had not yet been discovered as an effective preservative. We heard tales of enormous quantities of expensive osmium tetroxide being used for whole animal perfusion in a Boston laboratory. But we were lucky because the area to be studied was at the rim of the spinal cord. Consequently, we simply withdrew the cerebrospinal fluid from the cisterna magna and replaced it with buffered osmium tetroxide. This was repeated twice to facilitate circulation throughout the intrathecal space. Only a very shallow area of tissue was preserved, but it was within this subpial circumference that the lesion was located. Small wedges of tissue were cut from the rim for dehydration and embedding. We were among the first in the United States to find that araldite was far superior to methacrylate for embedment for electron microscopy.

We studied tissue changes from 29 hr to 460 days after cerebrospinal fluid exchange in a total of 19 cats. Dick's brother, Walter, also a student at the University of Wisconsin, procured the cats for us from local farmers. Twenty-nine hours after lesioning, most myelin sheaths were deteriorating and typical macroglia (fibrous astrocytes and oligodendrocytes) were no longer visible. Myelin breakdown presented as layers of varying thickness split apart, with the edges of these layers yielding

subsequently to a honeycomb- or alveolar-like pattern of dispersion. These configurations were clearly different from inadequate preservation. Phagocytosis of the myelin debris had begun. The phagocytes typically displayed long, sheet-like processes that embraced the axon and its myelin sheath or invaded the deteriorating sheath itself. In three-day lesions, despite the extensive myelin breakdown, axons were intact. Axons, completely demyelinated by six days, were ensconced in expanses of greatly swollen debris-laden macrophages. They were denuded only very briefly, however, for by six days macroglia appeared in the lesion area and started to invest some of the axons. These glial processes resembled neither fibrous astrocytes nor oligodendrocytes; their cytoplasm was dense with closely packed organelles and numerous filaments that were not observed in normal astrocytes.

An occasional myelin sheath was first seen at 19 days; by 64 days all axons were at least thinly myelinated. The cytoplasm of the myelin-forming cells was, as above, unlike that of either the oligodendrocyte or fibrous astrocyte that is observed in normal cord. These macroglia were large, and one cell dispatched many processes that embraced many axons. A spectrum of intermediate types between these reactive macroglia and highly filament-laden glia (fibrous astrocytes) became evident after remyelination had begun. Many of the myelinating cells became scarring astrocytes. We proposed that these macroglia were identical to the hypertrophic or swollen astrocytes common to many neuropathological processes and were considered to be progenitors of the scarring fibrous astrocytes. Consequently, we raised the possibility that the presence of hypertrophic astrocytes in multiple sclerosis plaques may be responsible for remyelinating fibers that could then lead to the characteristic clinical remissions.

Recognizable oligodendrocytes appeared in the lesion at 45 days, well after remyelination had begun. Phagocytes disappeared gradually; seen only rarely at 220 days, they were not found in the lesion thereafter. At 460 days, slender processes filled with large compact bundles of filaments dominated the tissue. Oligodendrocytes were more numerous at this time.

We observed that the myelin sheath was formed by spiral wrapping of a sheet-like glial process around the axon. When the first turn of the spiral was completed, a mesaxon was formed. As cytoplasm was lost from this process, the plasma membrane came together along its outer and cytoplasmic surfaces to form compact myelin. Only a small amount of cytoplasm was retained, confined to the paramesaxonal region inside the sheath and, on the sheath exterior, to an external mesaxon or a longitudinal ridge which appeared in cross-section as a small loop. This outer ridge or loop resulted from the loss of glial cytoplasm on one side of the external mesaxon. The orientation of the outer loop compared with that of the inner mesaxon implied a spiral. These vestiges of spiral membrane wrapping were also found in normal adult spinal cord. This mechanism was basically similar to that proposed for the peripheral nerve. It did not agree with the current view on the

mechanism of central myelination. In fact, even the electron microscopic identification of central glia was very controversial at that time. In our exceptionally well-preserved control cord, fibrous astrocytes and oligodendrocytes could be identified and distinguished easily.

An obvious question was whether this spiraling mechanism of a glial cell process that we observed during remyelination was the same as the mechanism of first myelination during development. This led us to studies of kitten spinal cord, first in Madison and later in New York. Initially, we observed the same inner mesaxonal and outer loop configurations, suggestive of a similar mechanism. The five-day kitten spinal cord was the best time to observe the continuity between the glial cell perikaryon and its extended processes, at the ends of which myelination occurred. My first glimpse in the electron microscope of an area from the five-day cord was one of my most exciting research moments. As I increased the electron beam to view the tissue, there in the center of the field was a configuration not unlike old-fashioned ice tongs: an oligodendrocyte nucleus and surrounding cytoplasm (lacking the filaments seen during remyelination), from which extended two processes tipped by axons being myelinated. This configuration appears in the 1962 reference. It must be admitted that the rather poor preservation, which led to substantial space in this immature tissue, eased the detection of such configurations. In this way, we discovered that in the central nervous system myelination occurred at some distance from the glial perikaryon, in contrast to Schwann cell myelination in the peripheral nervous system. With maturation, these processes lose much of the cytoplasm, and the resulting thin process is not detectable in the thicket of myelinated axons. This explained why we and others had not yet observed the cytoplasmic life lines to myelin in the remyelinated or normal adult cord. Our observations were incorporated into a cartoon showing the relationship between an oligodendrocyte and the myelin sheaths it formed; this figure continues to appear in textbooks.

It was an intense and exciting time, arriving home between 1:00 and 2:00 AM and then rising a few hours later to resume our work. We worked together extremely well, each bringing different strengths to the project. The electron microscopic images were not only revelatory, but also satisfied my artistic bent as anticipated; I loved creating the most handsome micrographs possible. I have to the present remained intrigued with the developments in electron microscopy in the 1950s and 1960s. One moment of panic occurred when our longest term animal, 460 days following lesioning, escaped from his cage and was seen heading for remote parts of the campus. With a stroke of luck, we somehow managed to catch him. Madison was an idyllic location for graduate students, not only for sailing in the summer, but for swimming at lunch breaks as well. The observations made in Madison led to our first European trip to present at the IVth International Congress of Neuropathology in 1961. We did not realize at the time that this was a harbinger of much

travel to come. We managed to be awarded our M.D. and Ph.D. degrees, respectively, at the same graduation ceremony in 1960.

To New York City: Summer of 1960

We set out for Columbia University College of Physicians and Surgeons where we were to be postdoctoral fellows with Drs. Margaret R. Murray (Laboratory of Cell Physiology, Department of Surgery) and George D. Pappas (Department of Anatomy). Dr. Pappas, an electron microscopist, had trained at the Rockefeller Institute with Dr. Keith R. Porter, who along with Drs. George Palade and Albert Claude had pioneered the development of a new body of knowledge that could only have arisen by using the electron microscope. Dr. Margaret R. Murray was herself a pioneer in developing the organotypic explant culture system for both peripheral and central nervous tissues. Dick was to learn these nerve tissue culture techniques so as to be able to ask biological questions without the complexity of performing studies in the whole animal. I was to serve as a bridge between the Pappas and Murray laboratories to initiate electron microscopic characterization of the sensory ganglion and spinal cord culture systems. We were funded by fellowships from the NIH (MBB) and from the National Multiple Sclerosis Society (RPB). We set up a household in the shadow of the medical school on 163rd Street, so that we could walk to work. This northern area of Manhattan was at that time an old and stable neighborhood beside the Hudson River.

Margaret Murray and I had much in common: she, too, was an only child who spent time near the water in a family home on Put-in Creek off Chesapeake Bay; both our precedent families had arrived in the 1600s; she was visually oriented; and she had spent time at Washington University in St. Louis. Her career started in 1929 when she was invited to Columbia University College of Physicians and Surgeons to help establish a research program in surgical pathology, the aim of which was to study human tumors grown *in vitro*, in part to reveal their cellular origins. The nomenclature that she and Dr. Arthur Purdy Stout developed for tumors stands today. She received more notoriety when in 1939 she reported that peripheral nerve sheath tumors originated from Schwann cells. She and her long-term and gifted collaborator, Mrs. Edith Peterson, reported another milestone in 1955: that myelination occurred in culture, proof that a remarkable degree of differentiation could be obtained in the organotypic explant culture system. Her efforts led to the formation of the Tissue Culture Association, which helped devise standardized nerve tissue culture techniques that, among other developments, led to the production of commercial culture media. She opened her laboratory to trainees and investigators from all over the world to learn the techniques that she had developed; in 1970 it could be said that about 75% of the worldwide research effort in nerve tissue culture was carried out by scientists (and their students) trained in her laboratory.

In contrast to what would be future directions in nerve tissue culture techniques, the goal was to try to mimic the *in vivo* environment as closely as possible. Consequently, the culture medium was complex due to inclusion of human placental serum and embryo extract, and the pieces of tissue were cultured intact in the complex Maximow double coverslip assembly. The tissue culture room was basically a surgical suite, whose walls were scrubbed down once a week and in which full surgical regalia was worn. The talents of Mrs. Peterson enabled the cultures to be grown for months or more, during which differentiation of the initially embryonic tissue occurred. The nests of healthy sensory ganglion somata were particularly beautiful and easily seen. Because myelin also was visible in living cultures, the somata and sheaths could be followed continuously for weeks or months to better understand neuronal reactions and causes of myelin breakdown after various treatments. The gradual replacement of culture medium with fixative and the lack of mechanical handling of the tissue enabled outstanding preservation for electron microscopy.

Long-term cultures (up to 76 days) of 17- to 18-day fetal rat spinal cord were characterized electron microscopically. It was known that myelination occurred and that the cultures were capable of complex bioelectric activity, due to the efforts of Dr. Stanley Crain and Mrs. Peterson. Neurons were positioned in several strata beneath a region of neuropil. The general ultrastructural characteristics of *in vivo* mammalian central nervous system (CNS) tissue were found in culture, including well-developed Nissl bodies and an extensive system of microtubules and filaments in the neuronal cytoplasm; close packing of cells and processes with little intercellular space; typical oligodendrocytes and fibrous astrocytes as well as glia intermediate in morphology, the characteristic pattern of CNS myelin, including nodes of Ranvier and typical axosomatic and axodendritic synapses (including Type 1 and Type 2 synapses of Gray). A basis for the complex synaptic networks detected by electrophysiology had been found by electron microscopy.

When the fetal tissue was examined at the time of explantation, it was observed to be very immature, indicating that most of the differentiated features had developed *in vitro*. For example, whereas in the mature cultures synapses were found in nearly every field, a recognizable synapse was extremely rare in the new explant. This offered an opportunity to study for the first time the morphological development of mammalian synapses *in vitro* and to see if there was a close correlation between the initiation of synapse formation and the onset of functional synaptic networks.

Synapses first appeared consistently in 70-hr cultures of 14-day fetal rat cord. These initial profiles covered only a small area, displayed only small clusters of vesicles, contained little cleft material, lacked mitochondria, and were exclusively axodendritic. The time at which these immature synapses were first seen was the time that bioelectric evidence of functioning

synaptic networks was found. As the synapses increased dramatically at 78–100 hr, the response of the explant to electrical stimuli became more easily elicited and more complex. Some synapses displayed more associated vesicles and covered a larger area, although mitochondria were still seldom seen. The gradual accumulation of vesicles and, later, mitochondria mirrored results from *in vivo* studies. Synapses on dendritic spines and axosomatic terminals were now observed. It was at this time, after 93–100 hr, that strychnine sensitivity was first detectable, suggesting that inhibitory networks were starting to function. The close correlation of morphology and activity was thus observed despite isolation of the tissue from normal afferent and efferent connections.

Long-term organotypic cultures (up to three months) of fetal ($16\frac{1}{2}$–19 days) rat dorsal root ganglia were characterized as well. The neurons resembled their *in vivo* counterparts in nuclear and cytoplasmic content and organization. The larger neuronal somata exhibited "cytoplasmic roads" composed of neurofilaments and microtubules. Nissl bodies were prominent in the larger perikarya. Satellite cells formed a complete investment around the neuronal perikarya, and their exterior was covered by basal lamina. From the central cluster of neuronal cell bodies, neurites emerged to form a rich network of organized fascicles that often reached the edge of the coverslip (10 mm). This outgrowth, of course, was formed completely in culture. Unmyelinated and myelinated fibers were in the typical relationship with Schwann cells, with only one Schwann cell being related to one myelinated axon, but one Schwann cell being related to multiple unmyelinated fibers. The abaxonal Schwann cell surface was covered by basal lamina. Collagen fibrils and *in situ*-like perineurial ensheathment were also components of the fascicles. The myelin sheaths exhibited their *in vivo* attributes: mesaxons, Schmidt-Lanterman clefts, and nodes of Ranvier. Despite the overall cytological fidelity to similar tissue *in vivo*, perikaryal size, myelinated fiber diameter, and myelin internode length did not reach the larger dimensions observed *in situ*. Ganglion cultures, in which neuronal perikarya and Schwann cells and their myelin sheaths could be well visualized in the living state, were to be the objects of many future studies in numerous laboratories.

When Dick became an Assistant Professor in the Anatomy Department at Columbia in 1962, he set up a tissue culture laboratory. Once again, training in the Murray laboratory had led to the birth of a new nerve tissue culture facility and more third generation culturists began to be trained. Dick was successful in acquiring grants from both the NIH and the National Multiple Sclerosis Society. I became a Research Associate to be supported by the grants that Dick obtained, spurning the goal to be a tenure-track faculty member. By that time our first son, Jonathan Bartlett Bunge, had joined our family. I could not envision lecture preparation, teaching, grant writing, and committee membership added to my research schedule and new motherhood responsibilities. I worked part-time for the ensuing eight years

(with the exception of a sabbatical period). A second son, Peter Taeuber Bunge, was born in 1964. I felt more and more like a symphony conductor, trying to keep all the instruments playing in harmony.

Among the studies that we started in our own laboratory was a project investigating the cytological outcome of X-irradiation of the organotypic rat dorsal root ganglion cultures over a two-week period. Investigators continued to ask whether degenerative changes observed after ionizing radiation to the animal were primarily the result of direct radiation injury to the neural elements, and thus independent of secondary effects from a radiation-damaged vascular supply, or whether they resulted from other host-associated humoral or inflammatory responses. The well differentiated, two- to three-month-old organotypic ganglion cultures would be an appropriate tool to address this question. A trainee, Dr. Edmund B. Masurovsky, who worked with us to correlate light and electron microscopic images, saw a striking spectrum of neuronal changes that started as a chromatolytic pattern and evolved into vacuolar or granular degeneration over a two-week period. Interestingly, degenerative changes were not observed in the occasional binucleate neuron. The neurons were more radiation resistant than the satellite cells, which were decimated acutely; this satellite cell loss left only basal lamina coverage of the neuronal cell body. When this occurred, the small evaginations that normally arose from the perikarya and were surrounded by satellite cell cytoplasm were abnormally deeply invaginated into the neuronal perikaryon. A corresponding wave of acute degeneration also was evident in Schwann cells ensheathing unmyelinated fibers; breakdown of myelin (beginning at the node) and their associated Schwann cells followed a few days later. This selective radiation sensitivity of nonmyelinating Schwann cells had not been noted before. Axonal changes were not apparent until 14 days. The demise of the cellular elements took place within the basal lamina tubes. Our observations demonstrated that X-irradiation produced striking cytopathological changes in nervous tissue that was isolated from the host and that many of these changes resembled the effects of radiation *in vivo*.

Dick pursued additional studies utilizing the organotypic ganglion culture system with new trainees. Also, he and Dr. Masurovsky determined that fluoroplastic (Aclar) coverslips could replace glass for long-term culture; differentiation of the cultures was the same as on glass, and the plastic was highly inert, unbreakable, and easily separated from polymerized epoxy resin when cultures were embedded for electron microscopy. The plastic was to be important in devising new culture strategies later in St. Louis. I collaborated with Dr. Murray and one of her trainees, Dr. Celia F. Brosnan, to study the effect of adding chlorpromazine to sensory ganglion cultures. Its mode of action was unknown, and, because it is a fluorochrome, its destination could be followed in the highly visible neurons. The initial diffuse fluorescence seen within a few minutes in the cell soma became abnormally granular, starting

at 4 hr. These chlorpromazine-induced granules were enlarged lysosomes, as determined by electron microscopy and by the Gomori technique for detecting acid phosphatase. Cultures recovered within a few days. This reaction to the tranquilizer was considered to be a general cytological reaction by which the cytoplasm is cleared of an abnormal substance. There was the possibility, however, also suggested in the work of others, that the lysosomal response was the primary mechanism by which chlorpromazine exerts its pharmacological action. Work in our Columbia laboratory was to be interrupted by a sabbatical period, and, upon our return for our last year at Columbia, much time was taken to update the 16th edition of Bailey's *Textbook of Histology*, a venerated textbook started in 1904. Dr. Copenhaver, Chairman of the Anatomy Department who was involved in more recent editions, requested our participation. An exciting artistic-related endeavor in the mid-1960s was working with the Director of Photography at the Museum of Modern Art in Manhattan to choose electron micrographs for an exhibit.

To Boston for a Sabbatical: 1968–1969

An opportunity arose for Dick to initiate culture of lobster ganglia with Dr. Edward Kravitz and for me to explore the motile leading tips of lengthening nerve fibers in the laboratories of Drs. Edwin Furshpan and David Potter, all in the Department of Neurobiology in the quadrangle at Harvard Medical School. We moved children and cats to a wonderful town-house on Griggs Park in Brookline. It was an exciting year, in part due to the discussions and frequent seminars at lunchtime when so many of the faculty and trainees convened, often to listen to a visitor. It was a neurobiological feast! Dick grew impatient with the slower development of lobster ganglia compared with those from the mammal, but, nevertheless, he developed the culture system that remains an important part of current research in the Kravitz laboratory (Ganter et al., 1999).

Working with Drs. Furshpan and Potter was a trainee named Dr. Dennis Bray. He had begun novel studies on dissociated sympathetic neurons. Their growth cones were remarkably visible in the culture dish and enticing enough that I began a study of their fine structure. Also, it was our aim to have recorded the recent history (in life and during fixation) of the growth cone that was subjected to electron microscopy. Because the growth cones were flattened on the dish, they were serially thin sectioned in a plane parallel to the dish, not for the faint-hearted because their thickness occupied only a few thin sections at the surface of the dish. The moving parts of the cone, the leading flange and/or filopodia, contained only a filamentous network and occasional membranous structures. Additional organelles, agranular endoplasmic reticulum, vacuoles, vesicles (including dense-cored and coated), mitochondria, microtubules, some neurofilaments, polysomes, and lysosomes, were clustered in the central core of the cone. We were particularly

interested in the structural basis for the movement of the growth cone and for the addition of surface membrane. The feltwork of microfilaments (actin) appeared to provide the structure for movement. Dennis had already acquired data to support the idea that the growth cone was the site of surface membrane addition. The plethora of agranular membranous components in the cone and their occasional continuity with the plasma membrane that we observed was consistent with this idea. Our evidence for autophagic vacuole formation in the cone suggested recycling of membrane. A detailed investigation of membrane uptake would be accomplished later.

To St. Louis: Summer of 1970

We received an invitation from Dr. W. Maxwell Cowan to consider faculty positions in the Department of Anatomy (and later, also Neurobiology) at Washington University School of Medicine. He was building an outstanding department that would be among the best in neurobiology in the country. It was a great honor to be asked to join his department, and I will be forever grateful for the exceptional quality of the environment that I greatly valued and benefitted from for the next 19 years. Again, I chose to be a Research Assistant Professor rather than be on the tenure track for reasons given earlier, but was on a full-time schedule. By 1974, I had started to teach and was promoted to Associate Professor with tenure; I became Professor in 1978. We purchased a home in University City, close to the Washington University campus and Forest Park, still preferring to live close to work; the medical center was at the other end of the park.

We were sitting on unpacked boxes, awaiting completion of our new laboratory, when Dr. Patrick M. Wood sauntered in to inquire about job opportunities. An Assistant Professor of Botany, he had been informed that the Botany Department was to be abolished. Fortunately, we said yes to his query, one of the very best decisions that we ever made, because in time he would become one of the most gifted nerve tissue culturists in the world.

The spinal cord, cerebellum, and ganglion culture systems were soon replicated in St. Louis, with laminar flow hoods instead of a designated Columbia-type culture room. Another holdover from Columbia was the Maximow double coverslip assembly, which was too cumbersome and too time-consuming for the feeding regimen and was inadequate to support pieces of tissue large enough for the transplantation work that Dick envisioned. Pat took on the task of modifying the culture chamber. He prepared a mold that could be heated to shape the Aclar coverslip into a bottle cap configuration which could hold more tissue and culture medium. Three of these could simply be placed in a petri dish for long-term culture. One of the first experiments with myelinated sensory ganglion cultures in these Aclar "hats" was conducted with Dr. William Blank, Jr., who found that the Schwann cell-axolemmal junction at the paranode was sensitive to lowered calcium levels

in the medium; fluid accumulated between the axon and the myelin sheath due to loss of adhesion of the cytoplasmic loops to the axolemma.

When Dr. Karl Pfenninger came to our laboratory to initiate freeze-fracture studies of axonal growth cones in culture, he introduced the use of fluorodeoxyuridine to create an outgrowth free of non-neuronal cells. This anti-mitotic agent was next used by an M.D./Ph.D. student, Mitchael Estridge, to remove non-neuronal cells from sympathetic and sensory ganglion cultures in order to compare the surface membrane content of those two types of neurons. About that time, James Salzer, in the laboratory for a rotation, treated sensory ganglion cultures with this agent. He could remove Schwann cells temporarily, but they kept coming back (without fibroblasts). When Dick inspected Jim's cultures, he immediately grasped the potential to obtain purified populations of Schwann cells for transplantation. He mentioned this insight in print in 1975. Simply by cutting out the explant, where the neuronal somata were located, the axons quickly degenerated and left only Schwann cells behind. This was the preparative method for Schwann cells for many years. With additional anti-mitotic treatment, the sensory neurons could be rid of Schwann cells. The ability to prepare these purified populations and then add them in different combinations gave us the opportunity to study interactions between neurons, Schwann cells, and fibroblasts *in vitro*. This became a major focus of work in our laboratory.

Pat observed that the purified populations of Schwann cells did not divide after neurons were removed. He then added ganglion neurons (bereft of Schwann cells) to beds of Schwann cells and found that where the outgrowing axons contacted Schwann cells, they started to divide. There was a mitogenic signal from the axon that caused Schwann cells to proliferate. This was the first interaction discovered using the new ganglion culture system and led to a series of experiments to define the signal; this effort was headed by Dick and, as I was not involved, will not be further detailed here.

While these culture systems were being perfected, I continued studying growth cones, again taking advantage of their superior visibility in culture. Rosemary Rees, Dick, and I characterized morphological changes in the pre- and postsynaptic elements during their initial contact and maturation in sympathetic neuron/spinal cord explant cultures. Upon contact, the growth cone filopodia of the cord neuron became extensively applied to the sympathetic neuron plasmalemma, and numerous punctate regions developed in which the membranes were closer together than normal. The Golgi apparatus of the target neuron exhibited increased numbers of coated vesicles, which also were seen in continuity with the close contacts; it appeared that they contributed undercoated postsynaptic membrane, the first definitive sign of synapse formation. Tracer work confirmed that the coated vesicle traffic was from Golgi to surface. Following the appearance of postsynaptic densities, filopodia disappeared, synaptic vesicles gradually accumulated,

cleft width and content increased, presynaptic density appeared, and membranous structures and lysosomal structures vanished.

In a solo study I found that protein tracer uptake occurred within minutes, not at the base of the cone, but at the leading edge into a variety of membranous structures, some of which became related to the lysosomal system. More endocytosis occurred at the neurite tip than along the shaft. Vincent Argiro, Dr. Mary Johnson, and I observed that neurites of sympathetic neurons from embryonic, perinatal, and adult rats extended at different rates in culture. We made the novel finding that this resulted from variations in individual growth cone behavior, i.e., differences in growth cone form and pattern of translocation. Growth cones of younger neuronal origin moved at high peak rates of advance and exhibited filopodial and lamellipodial excrescences in contrast to those from adult neurons, which exhbited scant cytoplasm, short filopodia, lack of lamellipodia, and more stationary phases. When the younger cones advanced at their fastest rates, the conformation was predominantly lamellipodial rather than filopodial. Filopodia of growth cones arising from embryonic neurons exhibited higher initial extension rates than did those of postnatal neurons.

Growth cone activity will determine the final geometry of the nerve cell; those that migrate more rapidly and branch with a higher frequency lead to the formation of a larger and more complex geometry. Dr. Dennis Bray, Kevin Chapman, and I (while on sabbatical; see below) found that differences in this neuronal geometry depended upon both extrinsic (the culture substratum) and intrinsic (stage of development of the neuron) factors. Other studies, showing that neurite outgrowth patterns on varying substrata reflect not only differences in neuronal age, but also variation in the behavior of accompanying non-neuronal cells, will not be described here. Dr. March Ard, working with Dick and me, observed in culture that either the Schwann cell surface or the extracellular matrix produced and assembled by Schwann cells (the bands of Bungner) promoted and guided neurite outgrowth from several types of peripheral and CNS neurons.

My major focus, however, was on characterizing the culture requirements for Schwann cell function. One of my favorite experiments was to demonstrate that Schwann cells required contact with matrix in order to differentiate. Dick and I had noticed that when Schwann cells in differentiation-supporting medium did not contact the collagen substratum, but were present on guy-roping fascicles of sensory neurites that extended from the explant to a distance beyond in the outgrowth, they lacked basal lamina and also were abnormally clumped and rounded rather than regularly spaced and aligned on the neurites. We placed small pieces of plastic coated with collagen onto regions of the cellular clumps. Only those Schwann cells that were able to contact this collagen rapidly began to align along the neurites and then to ensheathe and form myelin, a very striking result.

This demonstration of a requirement for connective tissue contact for normal Schwann cell function led us to question whether an extracellular matrix deficiency explained the abnormalities that were observed in nerve roots and peripheral nerves in the dystrophic (*dy*) mouse. The Schwann cells perched on unensheathed axons in the *dy* root were reminiscent of the clumped cells we observed on the suspended fascicles in culture. Working with Dr. Eiko Okada, we were able to mimic in cultures of *dy* sensory ganglion the abnormalities found in *dy* nerves *in situ*: deficient Schwann cell basal lamina, elongated nodes of Ranvier, short myelin internodes, abnormal positioning of the Schwann cell nucleus, and occasional incomplete ensheathment of unmyelinated axons. Co-cultures of *dy* Schwann cells and normal axons showed these abnormalities, whereas normal Schwann cells combined with *dy* axons did not. When fibroblasts were omitted from the cultures, the Schwann cell basal lamina defect was more pronounced than when they were present. Also, when fibroblasts from a normal mouse were added to *dy* neuron and Schwann cell cultures, the basal lamina and ensheathment defects were corrected. We speculated that fibroblasts had contributed matrix because more matrix was in evidence in the corrected cultures. We concluded that an abnormality of contact between Schwann cells and a matrix component could underlie the abnormal Schwann cell behavior in the *dy* mouse nerve.

The possibility that fibroblasts and extracellular matrix components promote Schwann cell differentiation was further investigated in later experiments conducted by a graduate student, Valerie Obremski, working with Dr. Mary Johnson, Pat, and myself. It was learned from work done by Dr. Dikla Roufa in our laboratory that when Schwann cells were cultured with *sympathetic* neurons (without fibroblasts), and in medium that promoted differentiation of Schwann cells associated with *sensory* neurons, ensheathment and basal lamina and collagen assembly were all deficient. Valerie found that adding fibroblasts to the sympathetic neuron/Schwann cell cultures corrected these deficiencies. The addition of purified basal lamina components (laminin, Type IV collagen, and heparan sulfate proteoglycan) partially mimicked the effect of adding fibroblasts. We speculated that superior cervical ganglion neurons were unable to stimulate full Schwann cell extracellular matrix expression and that this led to a basal lamina deficiency which prevents ensheathment from occurring. It will be mentioned later that Schwann cells require neurons to generate basal lamina. Valerie discovered that Schwann cells organized basal lamina in the presence of fibroblasts or in fibroblast-conditioned medium without neurons. Even without neurons, the generation of matrix led to dramatic changes in Schwann cell morphology: the cells elongated and aligned with each other.

The availability of purified populations of sensory neurons and Schwann cells lacking fibroblasts provided an opportunity to determine the endoneurial and perineurial constituents that are contributed by Schwann

cells. Axons of neurons cultured alone were unensheathed and devoid of basal lamina and extracellular banded collagen fibrils. When Schwann cells were added to cultures of neurons, Type IV collagen-containing basal lamina and fibrils were formed. Two matrix experts, Drs. J. Uitto and J. Jeffrey in the Division of Dermatology, aided Pat Wood, Ann Williams, and me in also detecting Types I, III, and A-B collagens in the cultures. That Schwann cells produced this variety of matrix components had not been hitherto recognized. When fibroblasts were added to neuron/Schwann cell cultures, the collagen fibrils were larger in diameter, and typical perineurium formed. We laid to rest a long-standing question: Is perineurium formed from Schwann cells or fibroblasts? With the help of Dr. Joshua Sanes in our department, we infected Schwann cells or fibroblasts with a retrovirus carrying the gene for β-galactosidase. We discovered that the perineurium formed in culture expressed this enzyme when fibroblasts were lac-Z positive, but not when Schwann cells were lac-Z positive. Pat, Ann, and I found that neurons were required for the generation of basal lamina (but not its persistence) on the Schwann cell. Further work with Dr. M. Blair Clark revealed that substantial contact between axon and Schwann cell was necessary for the assembly of normal-appearing basal lamina; neither released diffusible neuronal factors nor products released from adjacent basal lamina-assembling Schwann cells were sufficient. The outstanding quality of electron micrographs of these and other cultures was due, in large part, to Ann Williams and later to Margaret Bates, both long-term assistants in our laboratory.

The finding by Dr. Fernando Moya working with Dick and me that a defined medium (N2) supported Schwann cell proliferation but not differentiation enabled us to further explore Schwann cell–neuron interactions, specifically ensheathment and myelination. In N2 medium only, Schwann cells neither ensheathed and myelinated axons nor formed basal lamina and collagen fibrils, even after many weeks in culture. When serum and embryo extract were added to N2, however, within a few days ensheathment, myelination, and basal lamina and collagen formation were evident. This contrast was particularly striking in electron micrographs; in N2, the Schwann cell processes were long and meandering and only adjacent to axons rather than in an encircling mode. Charles Eldridge, a Ph.D. candidate, determined that when embryo extract was eliminated from serum-containing culture medium, Schwann cell differentiation was arrested; the substitution of ascorbic acid was sufficient to promote myelination and basal lamina assembly. Further work demonstrated that in a number of culture conditions it was the level of ascorbic acid in the medium that was critical for myelination and that myelination and Schwann cell basal lamina assembly were paired. Our thinking was that the ascorbic acid in embryo extract was required for Type IV collagen assembly, which is an important component of basal lamina formation, and that this assembly was required for myelination and ensheathment to proceed. The hypothesis that this assembly was

required was supported by finding that provision of exogenous basal lamina matrix to cultures grown in defined medium without ascorbic acid promoted these Schwann cell functions. In this way (in part), we discovered that acquisition of basal lamina (or binding to laminin receptors) was a critical prefatory step for Schwann cell differentiation. We developed the concept that Schwann cell surface molecules required polarization (as in epithelia) prior to undergoing the shape changes involved in ensheathment and myelination. The importance of the collagen substratum as well has been noted earlier.

Our laboratory in St. Louis had been not only a productive, but also a happy place. Dick and I were guided by the golden rule: treat others as we would want to be treated. We also allowed ourselves to be "vulnerable" in the sense that we welcomed criticism and would act on it. In the early years in St. Louis, when the group was smaller, all birthdays were celebrated in the laboratory at a sit-down lunch with candles, tablecloth, casserole, and salad brought from home and a birthday cake. A weekly tea, at which the focus was on the goodies brought by everyone to accompany the tea, enabled all to be informed of latest developments, be they scientific advances or personal milestones. Telling jokes was as important as the pastries, and Dick liked nothing better than to embellish them for greatest impact. The laboratory had a "family" feel. Many of our trainees reflected happily on this atmosphere and carried it with them to their new destinations. A glass sculpture by Dale Chihuly, commissioned by me to remember a graduate student, remains in the foyer of the medical school.

To London for a Sabbatical: 1984

We chose to spend nearly half a year in Dr. Dennis Bray's laboratory in the MRC Cell Biophysics Unit, King's College, on Drury Lane in London. A site of historical significance, the seminar room still held some of Wilkin's DNA models. We had always admired Dennis's ability to devise simple and direct experiments to answer basic questions about axonal growth. He had worked in our laboratory in 1978–1979 when he did seminal, often cited work on the avoidance of retinal and sympathetic fibers when they confronted one another in culture and other work on the sorting out of nerve fibers to form fascicles that contained only one type of fiber. He was a conscientious host and a valued discussant. The sabbatical was a time not only of scientific gain, but also of cultural enrichment, because the laboratory was ensconced in the heart of the Covent Garden district. Influences on the formation of outgrowth from a single neuron were studied, as described above. Here we started one of my favorite experiments as well.

We pursued a project to better understand the mechanism by which peripheral myelin is formed. Because it had been known for many years that the Schwann cell nucleus shifted during this process, we asked if following this movement and then coordinating it with the orientation of the Schwann

cell membranes (as detected electron microscopically) could contribute useful insight into the mechanism. In particularly thin areas of dorsal root ganglion neurites and Schwann cells in culture, we could identify potential sites of myelination because both the region of the axon and the relating and elongating Schwann cell tellingly increased in size. Dick and I took turns, every 4 hr, to map the position of the Schwann cell nucleus as myelination began and proceeded for one to three days. We spurned time lapse photography because we had to be there to focus the microscope to determine the exact position of the nucleus. Our histories of nuclear circumnavigation and carefully preserved and mapped cultures returned with us to St. Louis, where the samples were put into the hands of Margaret Bates, our electron microscopy assistant who possessed a special talent for finding a specific area in a culture and preparing it successfully for electron microscopic scrutiny. It was critical that, through all the preparation stages, the same orientation be maintained. We found eight consistent cases in which the direction of nuclear circumnavigation corresponded to that for the inner, not the outer, lip of Schwann cell cytoplasm, suggesting that myelin was laid down by progression of the innermost leading edge of the Schwann cell around the axon. Consistent with this finding was that the observation of basal lamina and macular adhering junctions on the outer lip of cytoplasm implied anchorage rather than movement.

To Miami: Summer of 1989

As superior as the Washington University milieu was, we nonetheless decided to take new faculty positions in The Miami Project to Cure Paralysis at the University of Miami School of Medicine. Dick was selected to be Scientific Director. With his medical education, extensive knowledge of gross anatomy as well as cell biology, breadth of understanding, and now a new and total commitment to spinal cord injury, this was an inspired decision. Whereas Dick had envisioned transplantation of tissue, particularly purified populations of Schwann cells, into injured spinal cord for over 20 years and had actually performed a number of transplantation studies, we saw this as a chance to tackle spinal cord repair anew. A part of the vision was the promise of using Schwann cells from a piece of peripheral nerve from a spinal cord-injured person for autologous transplantation. (To generate large numbers of human Schwann cells in culture was a goal sought and accomplished by Dick and his collaborators in Miami.) Schwann cells could also provide myelin for demyelinated or regenerated fibers. Going to Miami provided an opportunity to bring to the challenge of spinal cord repair our cumulative knowledge of the biology of the cell of Schwann, Dick's preferred terminology.

From work with Dr. Carlos Paino, we knew that axons would grow into a Schwann cell graft placed in lesioned spinal cord. Cultures of Schwann

cells in association with neurites were prepared to create bands of Bungner upon neurite demise by removal of neuronal somata. Transplants were prepared by loosening the supporting collagen substratum from the dish and rolling it and the bands of Bungner (or dissociated Schwann cells) into a jelly roll configuration. Twenty-eight days after positioning the transplant in a photochemically induced lesion, abundant axonal growth was observed within the roll of surviving collagen. After moving to Miami, I initiated a detailed characterization of the photochemically induced lesion up to a year and a half after induction, employing the 1 μm thick plastic sections of tissue prepared for electron microscopy for superior light microscopic histology. Despite this study and the promise of the rolled Schwann cell/collagen implants, we decided to develop a new complete transection/Schwann cell bridge model to obtain unambiguous results; complete transection would eliminate the complication of spared and sprouted fibers and enable detection of regenerated fibers with certainty. Dick termed these implants "the bridges of Dade County."

It was fortunate that a new postdoctoral fellow, Dr. Xiao Ming Xu, came to our laboratory, for it was he who was most responsible for developing this new model. Also, Dr. Veronique Guénard, with us at the time, had gained experience in using the polymer tubes when she was a Ph.D. student with Dr. Patrick Aebischer. Dr. Naomi Kleitman, in our St. Louis laboratory as a Fellow starting in 1985 and then in The Miami Project as faculty, was an important contributor to initial and subsequent bridging studies. Help from Anna Gomez was key in beginning to generate the very large numbers of rat Schwann cells that we needed for transplantation, six million per bridge. By placing a cable of Schwann cells inside a polymer (polyacrylonitrile/polyvinylchloride) channel and then inserting stumps of the transected cord into the channel, nerve fibers were coaxed to regenerate into the cable from both stumps. Careful tracing revealed that the fibers, originating from spinal cord and sensory ganglion neurons, did not exit the grafts in this complete transection paradigm. This was probably due, at least in part, to the presence of proteoglycans at the Schwann cell/host spinal cord interface. Dr. Giles Plant, a Fellow in our laboratory, was to observe their presence later in a detailed immunostaining study.

Because of the eventual possibility of autotransplantation, we desired to determine if human Schwann cells derived from adult tissue and generated in culture would support axonal regeneration as did the rat Schwann cells. Dr. James Guest, working toward a Ph.D. in our laboratory in the middle of a neurosurgery residency in Vancouver, transplanted human Schwann cell bridges into the transected spinal cord of the adult nude rat. The human cells were found to be as effective as the rat cells. Dr. Martin Oudega, a former Fellow in our laboratory and later a collaborating faculty member, began to explore another polymer, polylactic/polyglycolic acid, as a substitute for the channels we usually used. Also, Dr. Xu began to develop a lateral

hemi-section/Schwann cell/hemi-channel transplantation paradigm before leaving our laboratory. In this case, some regenerated axons exited the hemi-bridge, possibly because the spinal cord was more stable than in the complete transection model.

Studies of Schwann cell biology in cultures of purified sensory neurons had not been abandoned. Dr. Cristina Fernandez-Valle, a Fellow in our laboratory, made numerous observations. In her first investigation, she demonstrated that the addition of ascorbate, which promotes basal lamina assembly, induced expression of the protein zero gene that encodes the major structural protein of myelin. Expression of protein zero mRNA and protein occurred only in the subset of Schwann cells contacting myelin-inducing axons; Schwann cells in contact with axons that did not induce myelin did not express protein zero mRNA, although they generated basal lamina components. Because the ascorbate requirement could be bypassed by adding a purified basal lamina component, laminin, she next explored receptors for laminin, the β1 subfamily of integrins. Undifferentiated Schwann cells were observed to express large amounts of α1β1 and α1β6, whereas, when myelinating, the predominant integrin was α6β4. Function-blocking antibody to β1 inhibited attachment to laminin, and myelin formation and basal lamina assembly were deficient. This work showed that a β1 integrin bound the laminin present in the basal lamina to the Schwann cell surface and transduced signals critical for differentiation into a myelinating cell. In a third study, actin polymerization disruption was found to inhibit myelin formation and to prevent expression of mRNA encoding the myelin-specific proteins, cyclic nucleotide phosphodiesterase, myelin associated glycoprotein, and protein zero. F-actin, therefore, influenced myelin-specific gene expression in Schwann cells. The combination of purified populations of sensory neurons and Schwann cells without macrophages enabled us to address the long-standing question of the participation of Schwann cells in myelin degradation and their ability to proliferate in Wallerian degeneration. Cristina demonstrated that rat Schwann cells were capable of both substantial myelin degradation and proliferation without the assistance of macrophages.

Studies of Schwann cell bridges continued, but in combination with additional strategies. Clearly, a thoracically positioned Schwann cell bridge was an encouraging start, but the complete transection paradigm was inadequate to promote growth of regenerated fibers off the bridge or to promote regeneration of fibers from brain stem neurons. Drs. Aqing Chen and Xu tested the addition of the corticoid methylprednisolone to the bridge paradigm because this compound was in routine use after human spinal cord injury to curb secondary damage. We found that the cord stump tissue placed into the polymer channel did not break down, the interface between bridge and cord exhibited far less scar tissue, two times as many cord neurons extended axons into the bridge, three times more myelinated axons were in

the bridge, fibers from brain stem neurons grew into the bridge as well, and a modest number of regenerated fibers exited the bridge when methylprednisolone treatment was combined with bridging. The combination strategy was, thus, a substantial improvement. Because methylprednisolone has numerous effects, it is not possible to know how it acted to improve outcome in our paradigm. In a separate strategy, Dr. Martin Oudega observed that when methylprednisolone was administered at the time of transection (without a bridge in this case), at a number of time points up to eight weeks, the number of microglia/macrophages was substantially diminished in both stumps, tissue loss was lessened, and dieback of vestibulospinal fibers was reduced.

Neurotrophin administration was combined with Schwann cell bridging. Dr. Xu delivered brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) for 14 days into the channel around the Schwann cell cable and found, after an additional 14 days, that the number of myelinated axons in the bridge and the number of cord neurons growing axons into the bridge were significantly increased. Importantly, brain stem neurons responded by extending axons into the bridge, even though the bridge was far from the brain stem. Thus, regeneration of some neuronal populations distant from the injury and the transplant could be elicited by combining trophic factors with a favorable substrate. In another study, Drs. Philippe Menei and Claudia Montero-Menei came from France to initiate an investigation to transplant Schwann cells infected with a retroviral vector carrying the human gene for BDNF/cDNA. Dr. Scott Whittemore, a faculty member in The Miami Project, added his molecular expertise to the project. The Schwann cells were deposited in a 5-mm-long trail extending into the distal cord stump from the complete transection site and also injected into the transection site itself. The trails were largely intact for the month-long experiment. In a comparison of engineered versus untreated Schwann cells, more fibers from brain stem neurons were present in the trail. When no Schwann cells were transplanted, no such fibers were found beyond the transection site. Thus, the increased levels of BDNF improved the regenerative response across the transection and into the thoracic spinal cord. More recently, Dr. Bas Blits from Dr. Jost Verhaagen's laboratory and Dr. Oudega injected adeno-associated viral vectors encoding for BDNF and NT-3 directly into the cord beyond the Schwann cell bridge to transduce the cells in that area. Transgene expression was observed predominantly in neurons for at least 16 weeks. A modest but significant improvement in hindlimb function was observed, and twice as many lumbar neurons extended processes toward the thoracically positioned Schwann cell bridge with vector injection. The lack of evidence for improved growth of regenerated axons from the bridge into the cord was possibly related to inadequate diffusion of the neurotrophins to the region next to the bridge.

Another combination strategy was to inject olfactory ensheathing glia into the stumps beside a Schwann cell bridge. Earlier reports suggested their efficacy. Also, they are positioned *in situ* to escort growing fibers from the periphery into the CNS. We reasoned that these glia might promote the exit of fibers from the bridge. This they did; regenerating fibers left the graft in contrast to lack of such growth with the Schwann cell bridge alone. There was also evidence for the long-distance axonal regeneration (at least 2.5 cm) of ascending fibers that crossed both interfaces. Fibers from a brain stem nucleus, the raphe, were able to reach the distal stump and extend at least 1.5 cm into it. With the prospect of continuing olfactory ensheathing glia transplantation, Drs. Henglin Yan and Plant tested a number of mitogens in culture to find a more effective means of obtaining sufficient numbers from adult rat olfactory bulbs. Combinations of factors, such as heregulin plus fibroblast growth factor-2, were more effective than each factor tested alone in stimulating the glia to divide; forskolin potentiated their activity. In considering Schwann cell versus olfactory ensheathing glia transplantation, one important issue is the development of myelin for the newly regenerated fibers. In a number of studies by others, transplantation of the olfactory glia has appeared to lead to myelination of regenerated or demyelinated fibers. We performed a very carefully controlled study to eliminate contaminating Schwann cells from sensory neuron-ensheathing glia cultures. Under culture conditions that enabled Schwann cells to form myelin around sensory neurites, ensheathing glia did not. Also, in electron micrographs, the typical ensheathing pattern of Schwann cells was not seen in the olfactory glia-sensory neuron cultures; the olfactory glial processes only meandered through fascicles. The cells we employed, immunopanned from adult rat olfactory bulbs, may not be as plastic as those from younger animals. More work needs to be done.

Recently, we have initiated studies of transplantation into contusion injuries, due to their clinical relevance. Drs. Giles Plant and Oudega determined that transplantation of olfactory glia was preferable (based on tissue sparing and hindlimb performance) in medium rather than in fibrin and at seven days rather than at the time of injury. Drs. Toshihiro Takami and Oudega then compared transplantation of olfactory ensheathing glia with Schwann cells into contused spinal cord; some animals received both cell types. Significantly more tissue was spared in all grafted animals. All three types of grafts contained axons, but Schwann cell grafts contained the highest number. Significantly higher numbers of propriospinal and brain stem axons extended 5–6 mm beyond the Schwann cell or Schwann cell/olfactory glia graft than the olfactory glia graft. A modest but statistically significant improvement in hindlimb locomotor performance in the Schwann cell-transplanted animals was detected by two months. Thus, in this first comparison, a Schwann cell graft appeared more effective than the others, but more remains to be learned about

olfactory glia survival in the moderately contused adult rat thoracic spinal cord.

I am committed to continue investigating strategies in combination with Schwann cell bridges, not only to improve the regenerative capability of the injured spinal cord, but also to devise neuroprotective interventions to lessen secondary tissue loss. New findings in our laboratory make this even more compelling than before. Dr. Giselda Casella, a Ph.D. candidate working with Pat and me, found that even though blood vessels were destroyed in the contusion lesion epicenter by two days, at seven days new vessels had appeared to form a seemingly continuous cordon through the lesion. By 14 days, however, the number of vessels had decreased. If this demise of the new vascular bridge could be prevented, the bridge could serve as an early scaffold to hasten axonal regeneration across the injury site. A study by Drs. Takami and Oudega combined methylprednisolone and interleukin-10 (IL-10) as a potentially more effective strategy than the use of either one alone; even with the combination, gray but not white matter damage was reduced and hindlimb locomotion was not improved. We are currently testing two new neuroprotective strategies: the administration of substances to modify the inflammatory response and provision of anti-sense oligonucleotides to interfere with the production of tumor necrosis factor-α following injury. These may be more effective than methylprednisolone and IL-10 as neuroprotective agents.

Other current projects continue to test regeneration strategies and to ask what combination of strategies will lead to successful treatment of spinal cord injury. We are testing, for example, the application of chondroitinase to the Schwann cell bridge–host spinal cord interface to interfere with the accumulation of proteoglycan molecules that may inhibit, at least in part, the exit of regenerated fibers from the bridge. A multifaceted approach will be key. Possibilities for a combination strategy could include neuroprotection, bridging with genetically altered cells that secrete appropriate neurotrophic factors, treatment to overcome inhibitory molecules, and training/rehabilitation. The development of appropriate biomaterials and application of new knowledge about guidance molecules are on the horizon. I think that I shall be as busy and as challenged in the next few years as I have been in the past. I love it! It has been a great and rewarding trip, but it is not quite over yet.

Acknowledgments

This chapter is dedicated to Richard P. Bunge, M.D. (Fig. 1). Not only my husband, best friend, and collaborator for 40 years, he served as my most valued mentor for all that time. I believe that I also mentored him. We completed each other. Among Dick's most impressive traits were his generosity of spirit and the ability to think broadly; to synthesize information from varying disciplines; to envision many novel testable ideas; and to make

Fig. 1. Mary Bartlett Bunge and Richard P. Bunge.

cogent, insightful, and witty comments in public places. He could wander into the culture room, take a glance at a culture, and make an observation, missed by others, that might spawn a new project. The work reviewed herein includes projects in which we collaborated; to also review his manifold discoveries in which I was not involved is beyond the scope of this effort. Some information is available elsewhere (Salzer and Colman, 1996; Zu Rhein and Duncan, 1997; Vikhanski, 2001). A glass sculpture by Jon Kuhn, commissioned by me to honor Dick, now resides in our new building.

I also dedicate this chapter to sons Jonathan and Peter, two highly exceptional persons. They have enriched and broadened my life experience immeasurably and have taught me much that I otherwise would not have learned. Their support in recent years has been most caring and generous and is greatly appreciated.

Enormous gratitude is due Patrick M. Wood, by far our most important collaborator. Dick and I were honored to be associated with such a clear and logical thinker, gifted experimentalist, and keen observer. In Miami, Dr. Naomi Kleitman added important strengths to our team due to her prowess in culturing Schwann cells, planning experiments, and critically evaluating (including statistically) experimental data.

To all the talented students and fellows and loyal assistants who cast their lot in the Bunge/Wood laboratory, a most appreciative thank you!

Since Dick died of esophageal cancer in 1996, I have greatly appreciated and benefitted from being in the company of outstanding scientists as a member of the Christopher Reeve Paralysis Foundation Research Consortium.

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