the average number of colors sufficient to color various collections of graphs. This problem, although tied to the four-color conjecture, is not solved by the resolution of the four-color conjecture.

Since the proof by Appel and Haken required that so many logical decisions be executed by a computer, it raises the question of whether there is some simpler way to obtain the result and, if not, whether the computer proof can be trusted. Such dilemmas arising from the increasing likelihood that computers will be used to yield otherwise unobtainable results in mathematics are a subject of heated debate among mathematicians and computer scientists (*Science*, 4 June 1976, p. 989). Some graph theorists believe that their colleagues will continue to search for a proof of the four-color conjecture that does not rely on a computer. On the other hand, even those who believe in the method used by Appel and Haken are bound to check their calculations. In fact, Frank Allaire of the University of Manitoba in Canada and Edward Swart of the University of Rhodesia are attempting to prove the four-color conjecture by a method analogous to that of Appel and Haken but with different computer algorithms.

The proof of the four-color conjecture, then, does not put an end to anything in mathematics except perhaps the speculation that this problem could not be solved. Research on problems derived from the four-color conjecture continues unchanged, and the search for a simpler proof of this conjecture is not likely to be abandoned. Some mathematicians, though, believe that the requisite use of a computer to solve this problem may herald a new era in mathematics research in which human theoreticians find themselves increasingly relying on computers for unavoidable calculations that will prove necessary to solve difficult problems.—GINA BARI KOLATA

Computers: Helping to Study Nerve Cell Structure

As neurobiologists probe more deeply into the function of the nervous system their need for quantitative data about nerve cell structure increases. They want to know, for example, about the branching patterns of neurons, about the number of connections that one neuron makes with others, and about how these properties are altered by genetic and environmental changes that affect behavior. The idea is to correlate structure with function.

But neurons normally have many branched projections that extend in a three-dimensional network over distances that are large compared to the size of typical cells. Thus, determining complete neuronal structures by standard cytological techniques is both tedious and time-consuming. More important, the structural data so acquired are difficult to store and record in useful form. Recently, however, a number of investigators have developed computer-assisted systems that gather, store, and analyze neuroanatomical data. The systems are helping to put neuroanatomy on a quantitative basis. Moreover, many investigators think that the systems are now producing information that could not otherwise be obtained.

Reconstruction of the three-dimensional structure of neurons is one of the principal uses of the computer systems. Several investigators have developed them for this purpose. They include W. Maxwell Cowan, Donald Wann, and their colleagues at the Washington University School of Medicine, Cyrus Levinthal of Columbia University, with Randall Ware, who is now at the California Institute of Technology, Paul Coleman and William Simon of the University of Rochester Medical Center, Rudolfo Llinas and Dean Hillman of the University of Iowa, and Robert Lindsay of the University of California in Los Angeles.

One way to define the three-dimensional structure of a neuron is to determine the spatial coordinates of its anatomical features. A neuron consists of a cell body, several dendrites, and usually one axon. The axon is a long projection that carries nerve impulses away from the cell body to the target neurons or other target cells. The dendrites, which frequently have a treelike branched structure, transmit incoming impulses from other neurons toward the cell body. Knowing the x, y, and z coordinates of features such as the points of origin of the dendrites on the cell body and their branch and end points would enable the investigator—or the computer—to reconstruct the dendrite structures.

Some systems use computer-assisted microscopes to obtain the coordinates directly from preparations of stained nerve tissue. The stain most often used is the Golgi stain, which is named after Camillo Golgi, who developed the technique in 1873. It involves impregnating neurons, usually in brain or spinal cord, with silver. The silver normally stains only a small fraction of the nerve cells in the tissue, but these stand out clearly against a translucent background. Impregnation of the dendrites is virtually complete, although the axon may or may not be stained. The stained nerve tissue is then sliced into relatively thick (about 100 micrometers) sections and placed on slides for microscopic study (Fig. 1A).

The stage controls of any microscope move the slide in the x and y directions. Adjustment of the focus control determines the depth of the focal plane being examined; this is the z direction. The stage and focus controls of the computer-assisted microscopes are driven by stepping motors, which move the controls in small increments determined by the investigator. The motors are hooked up with the computer in such a way that the movements of the controls can be monitored and their positions recorded when the feature of interest has been brought into sharp focus.

Most of the current systems are considered semiautomatic because they require a human operator to track the neuronal projections by manipulating the motor controls and to signal the computer when to record a set of coordinates for a particular feature. The operator must first select a structure, usually the cell body, to serve as a reference point from which the coordinates are measured.

Many investigators think that fully automatic recognition of patterns as complex as those of neuronal structures are beyond current capabilities to devise programs for the computer. Although Coleman, for example, has designed a system that focuses on and tracks dendrites automatically, it still requires an operator to monitor the process and to make corrections if needed. The operator must also answer the computer's queries about identification of end and branch points. This system reduces but does not eliminate the need for an operator.

However, the computers of even the semiautomatic systems are programmed to "remember" branch point positions and to return to them automatically in order to insure that all the branches are tracked. In other words, computers have good memories but poor pattern discrimi-

nation whereas the converse is often true for humans. Nevertheless, Llinas and Hillman are developing a system that tracks the dendrites automatically once the operator has initiated the procedure.

In any event, after the spatial coordinates have been recorded in the computer memory, the appropriate program permits the reconstruction of the three-dimensional structure of the nerve cell. This can be displayed in the form of a stick figure (Fig. 1B) on an oscilloscope screen. An important feature of most of the systems is that they have the capacity to present the neuron as it would be viewed from any angle. The display program can compute from the measured set of spatial coordinates, which completely specify the three-dimensional structure of the nerve cell, a new set of coordinates corresponding to the selected view. The time required to map neurons varies, but Cowan says that with their system, which is semiautomatic, they can log the data for a nerve cell with as many as six or eight dendrites in less than 40 minutes.

Another way to reconstruct the three-dimensional structure of a neuron involves tracing the perimeter of the cell as it appears at different focal planes in a thick section of tissue. The idea is to form a series of ring contours that can be stacked together to build up an image of the cell. This can be done manually by actually tracing the contours on plastic sheets, for example, but the same kind of thing can be done with a lot less work by appropriate computer systems. The contour traces are recorded in the computer memory for image reconstruction.

The tracing of contours need not be done directly from sliced tissue specimens but can be accomplished from electron or light micrographs of a sequence of serial tissue slices. There are also systems for generating stick figure reconstructions from series of light or electron micrographs of serial tissue slices. Since these techniques can be applied to electron micrographs, they are capable of giving information about fine cell structure that could not be obtained from light microscopy. For example, electron, but not light, micrographs permit observation of synapses, which are the points where neurons make connection with each other or with their target cells. This makes it possible to reconstruct how neurons are connected together in a functional network in addition to reconstructing the structures of individual neurons.

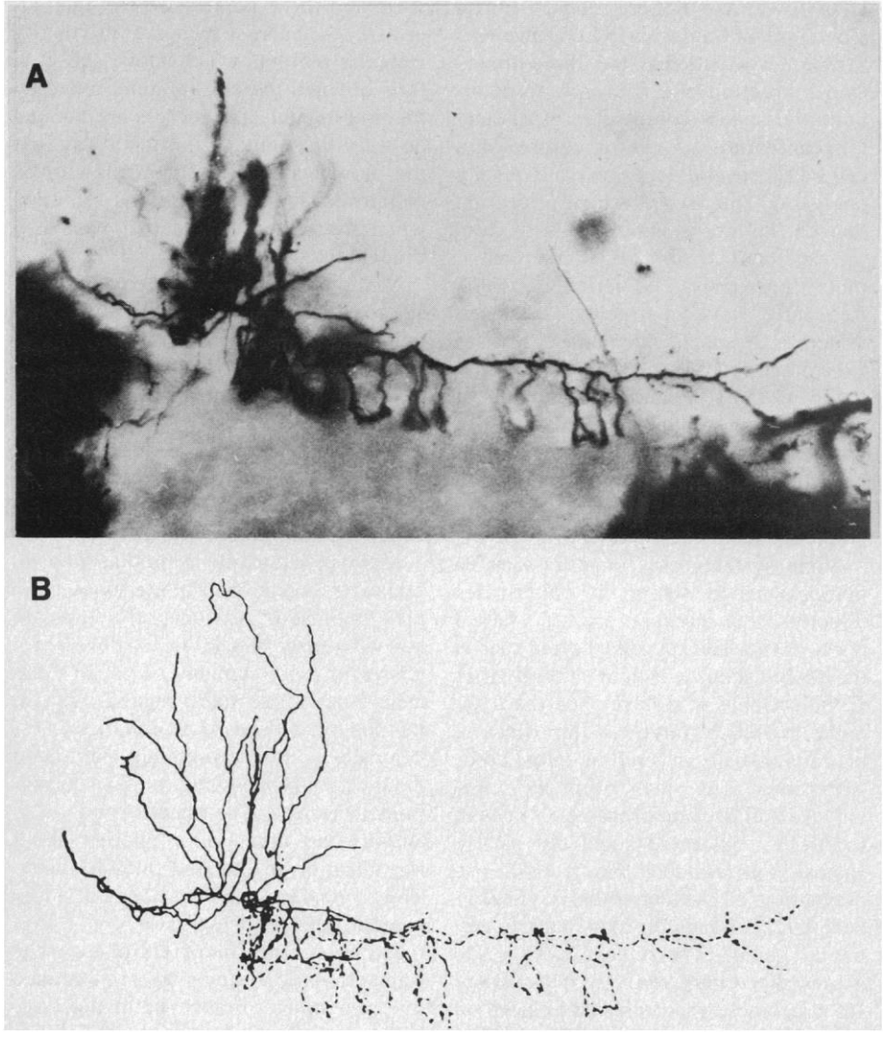

Fig. 1. (A) Light micrograph of a neuron stained by the Golgi technique. Because of the thickness of the slide, parts of the structure are out of focus and poorly defined. (B) Computer reconstruction of the same neuron. The dendrites are represented by solid lines and the axon by dashed lines. [Source: Rudolfo Llinas and Dean Hillman, University of Iowa]

Levinthal and Eduardo Macagno of Columbia University have used these techniques to analyze changes in neuronal structure during development. To do this, they first make motion picture film strips of micrographs of serial slices of the nerve tissue. Each frame is a picture of a single section, and the pictures must be aligned so that a structure that appears in a certain place in one frame appears in a corresponding position in the next, and so on. According to Levinthal, when the film strips are well made one gets the illusion of three dimensions and the effect that one is "walking through the structure" when the film is projected at near movie speeds. Each frame of the film is then analyzed by the computer and either contour or stick figure reconstructions are generated.

Levinthal and Macagno are studying the simple optic system of the water flea *Daphnia magna*. The compound eye of this organism consists of 22 simple eyes, each of which contains eight light-sensitive cells that send nerve axons to the optic ganglion (a group of nerve cell bodies) where the fibers make contact with a set of 110 neurons. Because of the simplicity of this system, corresponding fibers and cells can be identified in different animals and even at various stages during embryonic development. *Daphnia* has another advantage as an experimental animal in that under appropriate conditions, the eggs will divide without having been fertilized. This means that it is possible to generate and study clones of genetically identical individuals.

When Levinthal, Macagno, and Vincent LoPresti, also at Columbia University, compared three-dimensional reconstructions of the developing nerve fibers, they observed that one of the eight fibers originating in a simple eye grows ahead of the other seven. Only this fiber has a growth cone, that is, an enlarged area with fingerlike projections thought to be characterisitic of the ends of growing nerve fibers. And only this fiber contacts the undifferentiated neurons (neuroblasts) with which the fibers will eventually form connnections. At the time of

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exist and are always passive (that is, down the electrochemical gradient). If there is no electrochemical driving force, there is no net Na or K flux.

The most direct evidence (*2*) comes from experiments on giant axons of the squid (up to 1 mm in diameter). These axons can be cannulated, and about 95 percent of the cell plasm replaced by solutions of known composition. Normal action potentials are generated when the internal solution has a composition similar to that of the axoplasm. The fluxes of Na and K ions vary with the composition of the internal and external solutions and with transmembrane voltage (voltage difference between inside and outside solutions) in the direction predicted from the electrochemical gradients for these ions. A perfused axon can generate 300,000 action potentials. It is difficult to attribute these results to the residual cell plasm, and one is forced to a membrane theory of action potentials powered by Na and K gradients across the membrane. This and much other evidence firmly establish that excitable cells have Na and K ion activities which are respectively lower and higher than these activities in the solution bathing the cells, that is, Na and K ions are *not* in thermodynamic equilibrium across the cell surface. The energy for generating action potentials comes from the concentration differences of Na and K ions and these differences are maintained throughout life. Thus the necessity for (but not the existence of) ion pumps in excitable cells is established.

Those who contend that the properties of structured water are the complete or major explanation of Na and K ion distributions between cells and bathing media must not only "prove" that ion pumps are energetically impossible in normal excitable cells but must also provide an alternative equilibrium explanation of how excitable cells can produce net flows of Na and of K across their surfaces in the absence of electrochemical gradients of these ions. The latter is difficult, since the second law of thermodynamics apparently has to be violated.

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References

1. A. L. Hodgkin and A. F. Huxley, *J. Physiol. (London)* **117**, 500 (1952); *Proc. R. Soc. London Ser. B* **18**, 1 (1958); *Science* **145**, 1148 (1964); A. F. Huxley, *ibid.*, p. 1154.
2. P. F. Baker, A. L. Hodgkin, T. I. Shaw, *Nature (London)* **190**, 885 (1961), *J. Physiol. (London)* **164**, 330 (1962); *ibid.*, p. 355; W. K. Chandler and H. Meves, *ibid.* **180**, 788 (1965).

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contact the neuroblasts temporarily wrap themselves around the fiber before undergoing differentiation and the migratory movements needed for the development of the mature *Daphnia* optic system. Levinthal thinks that the contact between the lead fiber and the neuroblasts is somehow involved in signaling the neuroblast to differentiate.

He says that these observations would not have been possible without three-dimensional reconstructions of the developing neurons at different stages of development. For example, the investigators would not have been able to distinguish in electron micrographs the stage in which the neuroblast wraps around the nerve fiber from ordinary nerve fibers with myelin sheaths. These sheaths are also composed of cells wrapped around nerve fibers.

Sydney Brenner and his colleagues at the MRC Laboratory of Molecular Biology in Cambridge, England, are now using computers to study the anatomy of the nervous system of a simple roundworm. The investigators have produced genetic mutants of the worms that have unusual behavioral patterns. They hope to determine how the neuronal anatomy of the mutants differs from that of normal animals and to unravel how genes control neuronal structure and function.

The techniques of computer reconstruction of neuronal structure are not limited to cells from simple invertebrate animals, however. Several investigators are using them to study the nervous systems of a wide variety of vertebrates including the frog, cat, rat, and even the human. One goal is to determine how environment affects the development of cells in various areas of the brain. For example, the visual responses of cats raised in a striped environment differ from those of normal cats. How these behavioral changes are related to neuronal structure is a major question that computer techniques may help answer.

Once a body of information about the structure of nerve cells and their interconnections has been acquired, whether from computer or other studies, it can be fed into an appropriately programmed computer in order to build models of whole areas of the brain. Llinas and A. Pellionisz of Semmelweis University Medical School in Budapest, Hungary, are using this approach to study the function of the frog cerebellum.

A great deal is known about the structure and activities of the different types of neurons that make up this portion of

the frog brain, but much less is known about how their interactions affect the properties of the whole cerebellum. With a reconstruction of the anatomy and interconnections of the cerebellar neurons stored in the computer memory, it is possible to simulate the stimulation of a particular group of neurons and determine the response pattern of the neurons with which they connect. The results of the computer experiments can then be compared with the results of physiological experiments to determine how accurate the model is. The model can also serve as a guide for designing the physiological experiments.

In addition to helping neurobiologists gather quantitative information about the three-dimensional structure of nerve cells, computers are assisting in the tracing of nerve fibers and their connections in the brain and spinal cord. One way to trace fibers involves injecting radioactively labeled amino acids into the vicinity of the nerve cell bodies. After being taken up by the cell bodies, the amino acids are incorporated into proteins which are transported along the axons.

Location and quantitation of the label in serial slices of brain and spinal cord can be done by autoradiography. The sliced tissue is placed on slides and coated with a photographic emulsion. Wherever there is radioactivity, the emulsion is exposed and silver grains are formed. Locating and counting the grains on a slide is extremely tedious when done visually, but now David Whitlock of the University of Colorado Medical Center and Cowan and Wann have developed completely automatic computer systems for counting them. This process can be completely automatic because recognizing silver grains is a much simpler task than recognizing the more complex structure of neurons.

Both groups of investigators use television cameras to convert the gray tones of the autoradiographic image into a signal in which the grains appear totally black on a white background or conversely. The computer then counts and records the number of grains along with the spatial coordinates of the grain locations. Scanning the fields and focusing are completely automatic, although the operator can monitor the process. Whitlock says, however, that the system is accurate enough that counting can be done overnight without monitoring. This is important because scanning large fields may require several hours. The systems are adaptable to any kind of autoradiograph, not just those from nerve tissue.

—JEAN L. MARX