The Cellular Structure of the Mammalian Nervous System

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The Cellular Structure of the Mammalian Nervous System

A re-examination, and some consequences for neurobiology

by Harold Hillman

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the Director of the Unity Laboratory of Applied Neurobiology,
University of Surrey



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There is no remembrance of former things; neither shall there be any remembrance of things that are to come with those that shall come after.

Ecclesiastes (200 B.C.)

But it still moves.

Galileo Galilei (1633)

I admit that the judgement can be biased in many ways, and to an almost incredible degree, so that while exempt from external control, it may be so dependent on another man's words, that it may fitly be said to be ruled by him; but although this influence is carried to great lengths, it has never gone so far as to invalidate the statement, that every man's understanding is his own.

B. Spinoza (1760)

I propose to describe briefly what I have seen during fifty years of work, and what an investigator can verify for himself.

S. Ramon y Cajal (1954)

DEDICATION

This monograph is dedicated to:

- the pursuit of scientific truth
- my wife Elizabeth
- Alexander, Annie Rachel, Benedict and Sophia, whose eyes look into the future.

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Foreword

It would seem an appropriate time to re-examine the cellular structure of the mammalian nervous system for the following reasons. Firstly, there is considerable confusion in the literature about the appearance of the different kinds of neuroglia by light and by electron microscopy, and this is complemented by widespread disagreements among distinguished neuropathologists about the international classification of tumours of the central nervous system. Secondly, there is an increasing volume of experiments on the physiology and biochemistry of tissue cultures of neurons and different kinds of neuroglia, whose validity depends upon the accurate identification of both the parent tissue and also of the cells subsequently growing in culture. The biochemical classification in recent years has often tended to become independent of the cellular identification, which makes the use of the neuroglial cell names doubtful and the significance of the biochemical properties of the cells difficult to relate to the physiological properties in vitro or in vivo (Table 1).

Table 1 Studies dependent upon the characterization of cell types in the nervous system by light microscopy

Classification and prognosis of tumours and infections in biopsies and histological sections
Identification of cells in electron micrographs
Identification of cells in tissue culture
Histochemical localizations in sections
Immunocytological localizations
Identification of cells in developing nervous system
Localization of physiological and biochemical properties in whole animals
Localization of transmitters and receptors
Characterization of cell separation by bulk separation methods
Identification of sites of action of drugs
Identification of biochemical properties of subcellular fractions from different cell types
Identification of microdissected cells
Identification of sites of action of bacterial and viral infections

Thirdly, histologists have not been sufficiently concerned with the distortions of the shapes, volumes or appearances of the cellular elements, resulting from the procedures they employ (Hillman, 1982b), nor how much the properties of the stained sections reflect the properties of the living tissue. Fourthly, most stains intended to be specific require a considerable art and skill to obtain the appearance which is agreed to be characteristic of the particular kind of cell. However, more seriously, most of the classical stains used for the central nervous system were developed before the concept of control experiments became as important as it is now agreed to be. Most stains which were said to be 'specific' were judged to be so by well-known neuropathologists, who did not generally test rigorously whether or not their particular stain showed up, for example, neurons as well as a particular kind of neuroglial cell. Fifthly, electron microscopists have attached much importance to whether they were cutting relatively thick (100 nm) or relatively thin (10 nm) sections, but virtually no attention has been given to the multitude of different geometrical shapes which must result from cutting a neuron and its processes in a number of different orientations (please see page 51). Sixthly, I believe that research in the causation and mechanism of neurological diseases such as multiple sclerosis, cerebral tumours and cerebral thrombosis, and psychological illnesses, such as manic-depression and schizophrenia, has been remarkably unsuccessful. I have previously suggested that this is partly due to the widespread use of biochemical procedures which themselves may change the tissues excessively and therefore obscure differences between normal and pathological tissue, or may destroy the unstable factors causing disease which they are intending to identify (Hillman, 1972). We have further suggested that the lack of success is also partly due to the considerable effort and resources devoted to the examination of structures apparent after electron and light microscopical preparation, which could not exist in living cells (Hillman and Sartory, 1980a).

Acknowledgements

I have great pleasure in acknowledging the careful and meticulous contribution to this work of Mrs Dalveen Taylor, in respect of assistance with the experiments and the literature. Mr James Kirby of St Peter and St Paul's Hospital, London, carried out considerable histological work using many staining procedures, and Miss Julie Howarth of the Biochemistry Department, Surrey University, also helped with histology. Two kind electron microscopists, who prefer to remain anonymous, nevertheless helped with some preparations. Mrs Nita Foale, Mr Kevin Shaughnessy and the whole Audio-Visual Aids Unit, directed by Mr Russell Towns, aided me very much with the photography and illustrations. The Librarians of Surrey University, particularly Mr Denis Hewlett and Mr Glyn Davis, and the many Librarians of the British Medical Association, the University of London, University College London and the Science Museum Library, could not have been more helpful with the bibliography, especially of older source material. Miss Elizabeth Bruce and Mrs. Linda James typed out the various drafts and corrections of the manuscript, which was an awesome task. Mr. David Jarman helped with the manuscript. Dr. Mary Hearns and Mr. Robert Calderwood helped with the literature. To all these colleagues, I express my profound gratitude.

I will be grateful to any reader who wishes to discuss, or take issue with, the views expressed, - about which I would be willing to take part in any dialogue, private or public.

Philosophy

The philosophy behind this monograph may be summarized: firstly, we are seeking to elucidate properties of living cells by direct examination or extrapolation. A corollary of this is that information from living or unfixed tissue is to be regarded as more valuable than that obtained from tissues subjected to treatments such as killing, fixation, freezing, dehydration, staining, homogenization, centrifugation, sonication, addition of inhibitors, or the application of powerful non-physiological chemicals, when the data derived from these two kinds of experiments are mutually incompatible. For example, information about the structure of unfixed neurons derived from phase-contrast microscopy is considered more valuable than that found by freeze etching (Hillman, 1976). Secondly, Occam's Razor is a useful instrument. Thirdly, Popper's insistence on the necessity to seek to falsify one's own hypothesis is regarded as a valuable scientific modus operandi. Fourthly, geometry has to be respected, irrespective of the size of the objects being examined. Fifthly, one should not adopt an agnostic position about an important theory in one's own discipline, which one is propounding; either one concludes that sufficient evidence has been proffered to accept the validity of a particular theory, or one regards the evidence is being insufficient to be persuasive - in which case one should reject the particular viewpoint. One should not espouse a theory in a subject central to one's discipline, and then hide behind one's own ignorance of the evidence upon which it is critically based. Sixthly, findings, or a theory based upon them, cannot be accepted as true if major assumptions inherent in the use of the technique have been shown experimentally to be wrong, or contradict the laws of optics, physics or thermodynamics - unless the effects of the assumptions being wrong have been calculated (or shown) to make no significant difference to the results or interpretation of the experiments. Seventhly, an experiment is only as good as the control observations made on the effect of the technique itself on the system under study. Obviously, no amount of repetition of experiments in which major assumptions have been ignored or the control observations have not been made can make them valid or justify deriving conclusions from them.

I do not consider that these philosophical attitudes are in any way controversial, but would be interested to hear from anyone who does. Certain reviewers have said or implied that they do not agree with some of the elements of the philosophy summarized here, but they have not stated precisely or at all which are the particular ones to

which they take objection, or why they do (Lucy, 1973; Robards, 1981; Gregson, 1981).

The writing of the monograph arose from the re-examination of the structure of the living cell (Hillman and Sartory, 1980a), and the application of the same philosophy and geometrical considerations to the examination of the mammalian nervous system. However, it coincided with an attempt to answer a question which has been facing me daily for nearly 20 years. During this period I have been using the method of Hyden (1959) to dissect out by hand neurons from the cranial nerve nuclei of rabbits, rats, guinea pigs and mice. In this technique one cuts transverse sections about 1 mm thick of the fresh medulla, and stains it slightly with weak methylene blue in 0.25 mol/l sucrose solution. The sections are laid flat on a wax block, and under a dissecting microscope the neurons appear marine blue on a background of intensely white tissue. One is struck by the relatively small area that the somas of these neurons - defined as the cells which stain blue - occupy as a proportion of the total area of the tissue. At the same time, one frequently asks oneself 'What is the cellular nature of the tissue between the neurons?' One may surmise that this question was in Virchow's mind (1846), when he gave the name neuroglia to this tissue (Figures 1 and 6).

The present monograph examines the normal mammalian nervous system, although its implications for neuropathology will be considered later (please see page 205). Much of the literature on neuroglia has been derived from studies on sections from brains which have infections or tumours. In my opinion the demonstration that cell types described as neuroglia in healthy tissue are identical with those given the same names in abnormal tissue or in tissue cultures has not been done rigorously, especially in view of the knowledge that the appearance and biochemistry of cells change as a result of infections, tumours or growth in tissue cultures.

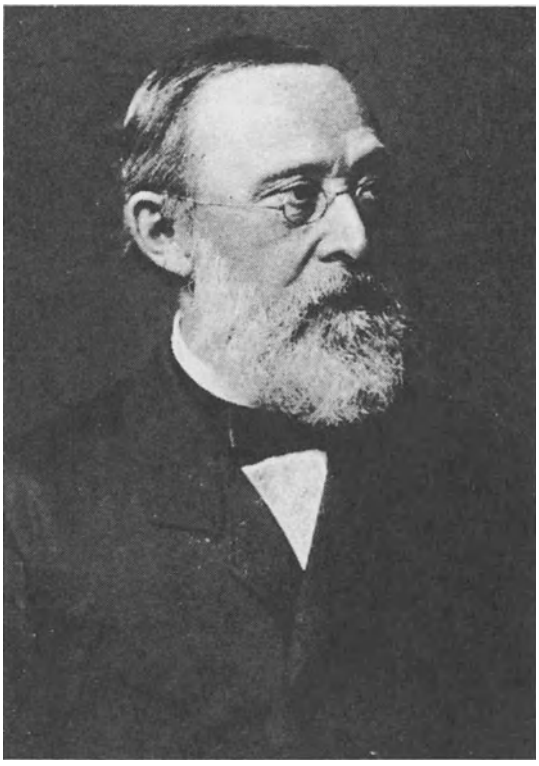


FIGURE 1 Dr Rudolf Virchow, who described the neuroglia as a nerve glue in the 1840s

1
Landmarks in the Cell Biology of the Nervous System

In this chapter the landmarks in the development of current views on the cellular structure of the mammalian nervous system will be reviewed. It is not intended to be a comprehensive disquisition, but a critical review of the thinking about the important advances and their limitations and validity. However, I have indulged in the luxury of employing the more questioning attitudes that have evolved since the epoch of the pioneers, and have quoted findings since then which are relevant. In all cases I have attempted to identify the originators of each technique, finding or theory. Some of the citations refer to findings in other tissues, but they were subsequently regarded as applicable to the nervous system. Agreement on the initiators or the first publications is not always universal.

There is a large literature on the history of the histology of the nervous system. The reader is referred to the authors listed below. However, I would like to emphasize that the critique of these findings is largely my own, and should not be taken as that of the authors cited, even when they have given permission to use illustrations from their works. The following references have been found to be invaluable: Barker, 1899; Robertson, 1899; Stieda, 1899; Singer, 1921; Garrison, 1924; Tiegs, 1927, 1929; Bourne, 1942; Baker, 1948-1953; Fulton, 1949; Brazier, 1951; Bauer, 1953; Ramon y Cajal, 1954; Glees, 1955; Baker, 1958; Hyden, 1961; Zaunick, 1961; Hamberger, 1963; Bradbury, 1967; van der Loos, 1967; Bunge, 1968; Clarke and O'Malley, 1968; Romeis, 1968; Peters, Palay and Webster, 1976; von Kirsche, 1977; Rottenberg and Hochberg, 1977; Niessing, Scharrer, Scharrer and Oksche, 1980; Ruska, 1980. Most of these publications were intended to be historical reviews; some of them have served this function incidentally, although they were not written with that intention in mind.

DESCRIPTION OF THE CELL

The description of cells in cork and the juices in them (Hooke, 1667) is often taken as the first description of cell walls and cytoplasm. The understanding that the intracellular phase was a liquid was reinforced by the more firmly based observations of Leeuwenhoek (1719) and Fontana (1784) that the axoplasm appeared fluid after it had been cut transversely (Figure 2).

The generalization of the cell theory to plants and animals formulated by Schleiden and Schwann (1838, 1847) was recognized as applicable to nervous tissue but Schwann (his

page 229) was mistaken about the structure of neurons. He drew them located within the axons, and he was evidently more interested in, and knowledgeable about, peripheral nerves and ganglia than about cerebral neurons.

FIXATION AND CUTTING SECTIONS

The microtome was invented for sectioning wood by Cummings before 1770 (see Hill, 1770), but, of course, wood is a hard tissue, and distorts less than soft tissue when cut. Sectioning of nervous tissue was not very satisfactory until Reil (1809) fixed samples of brain by immersing them in alcohol for several days. He observed that this procedure diminished the volume of the tissue by a quarter, and that small pieces had to be used to permit penetration. Hannover (1840) fixed brain and spinal cord with chromic acid, and noted that their gross appearances were not changed by the reagent. (The cellular structure of the nervous system had not yet been examined.) The microtome was much improved and made suitable for soft animal tissues by Von Gudden (1875).

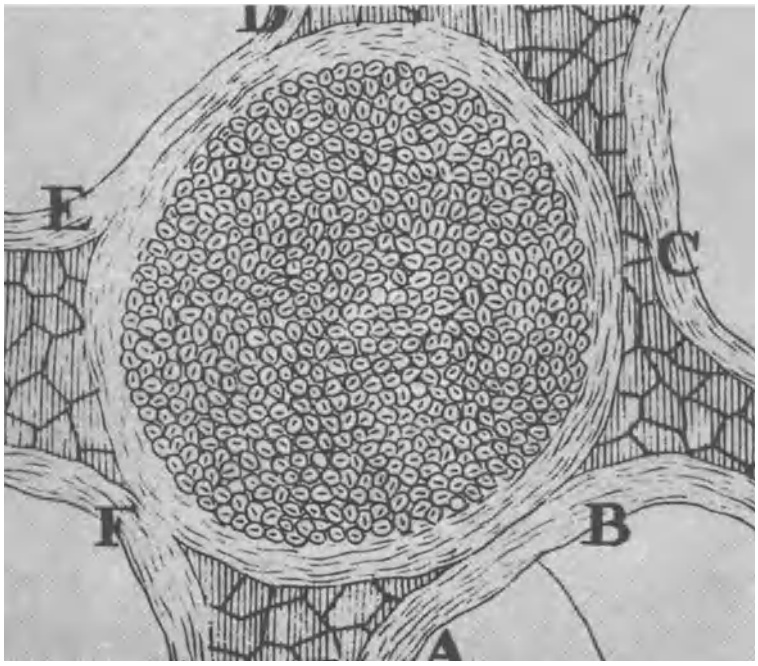


FIGURE 2 A cross-section of a peripheral nerve of cow or sheep (from Leeuwenhoek, 1719). This is probably the first illustration of a myelinated nerve fibre

ACHROMATIC OBJECTIVES

The great era of light microscopy which lasted more than 100 years - and for whose return we must fervently strive - began with the development of the achromatic objective by Lister (1830). By combining lenses of two kinds of glass he managed to diminish spherical and chromatic aberrations to a degree which made the microscope a useful and accurate device.

EARLY FINDINGS IN NEUROHISTOLOGY

Ehrenberg (1833) and Valentin (1836) described neurons, nuclei and nucleoli (Figure

3), and Remak (1838) saw fibres originating from nerve cell bodies. At about the same time, Purkinje (1838) (Figure 4) and later Deiters (1865) were cutting pieces of cerebrum and cerebellum and dissecting out neurons and nerve fibres by hand (Figure 5). This was an important advance in that whole cells could be isolated and drawn. Incidentally, it should be noted that Purkinje did not report knobs on the cell surface, although Deiters did. Parallel with this development, other histologists were cutting sections and attempting to elucidate the three-dimensional appearance of cells by examining them.

The difficulty of such reconstructions was appreciated by Stilling and Wallach (1842), who conceived the idea of cutting serial sections of the spinal cord. With the benefit of hindsight, one must say that those nowadays who extrapolate simply from the two-dimensional histological sections to the three dimensions of life, often fail to appreciate fully the consequences of the shrinkage and distortion of the cells which has already occurred during the preparation of histological slides (Baker, 1958).

NEUROGLIA DESCRIBED

Virchow (1854, 1856) made what must be regarded as the most significant histological observation about the tissue adjacent to the ventricles. It 'becomes continuous with the interstitial matter, the real cement, which binds the nervous elements together, and that in all its properties, it constitutes a tissue different from the other forms of connective tissue; [this] has induced me to give it a new name, that of the neuroglia [nerve cement]' (Figure 6).

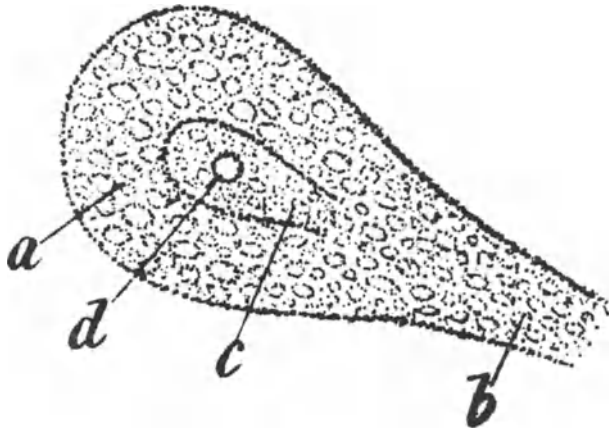


FIGURE 3 Drawing from Valentin (1836) of a cerebellar neuron. His nomenclature was a, parenchyma; b, tail-like appendage; c, nucleus; and d, nucleolus. The latter arrow may indicate that he observed a nucleolar membrane. It should be noted that he did not observe a difference in the appearance between the nucleoplasm and the cytoplasm.



FIGURE 4 J.E. Purkinje, who was probably the first person to dissect the nervous system by hand. This is reproduced from Jan Purkyne. Purkyne-Symposion. Nova Acta Leopoldina. Johann Ambrosius Barth Verlag: Leipzig. Page 5. 1961

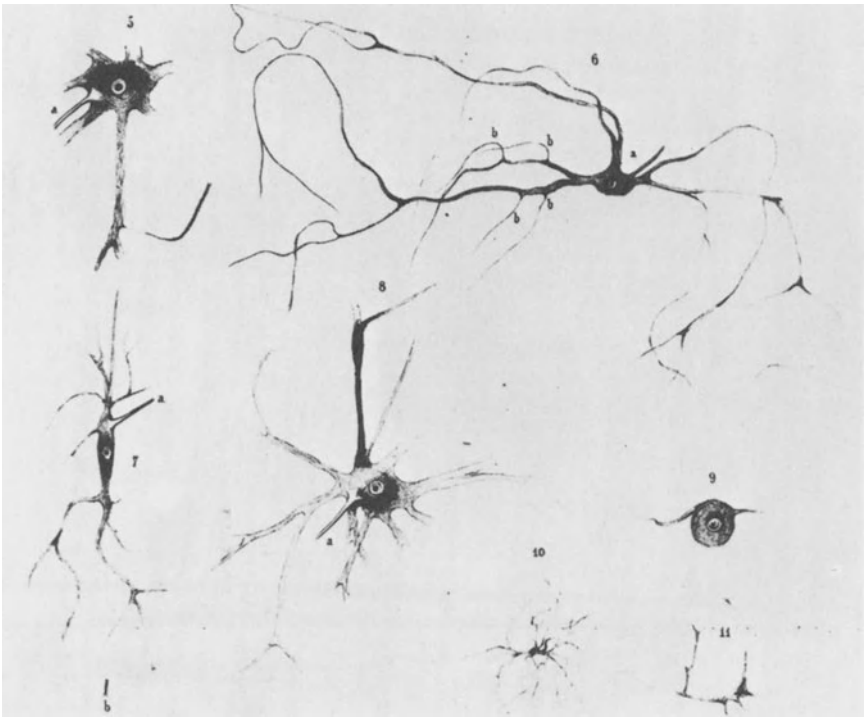


FIGURE 5 Deiters' (1865) drawings of isolated nerve cells. He has not drawn granules (synapses) on them. Please compare with Figure 44

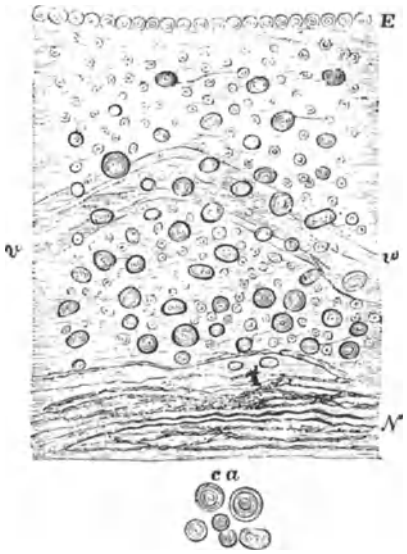


FIGURE 6 Drawing by Virchow (1871) of brain adjacent to the ependyma, E. The fibres are indicated as N, and the tissue between them and the amylaceous corpuscles (ca) is the neuroglia. This illustration is reproduced from Virchow, R. (1971). Cellular Pathology (Dover Publications, New York), page 313, by kind permission of the publishers

His illustration (Figure 94 of Virchow, 1863, republished in 1971, page 313) is reproduced here (Figure 6). Virchow saw a number of concentric bodies, which he thought were starch granules, as well as a number of nuclei. The 'corpora amylacea' may have been neurons observed under suboptimal optical conditions. The nuclei were probably identical with what were subsequently called 'Deiters' cells'; they were also seen by Nissl (1899), and they may also be the cells which del Rio Hortega (1919a) called microglia, and I will call 'naked nuclei' in this monograph (please see page 189).

STAINING PROCEDURES

Gerlach (1858) introduced carmine for staining tissues, and Golgi (1879a,b, 1886) devised his classical bichromate and silver staining procedure which was subsequently modified by Cox (1891). Improvements of the microscope (Clay and Court, 1932; Bradbury, 1967) and the development of the aniline dye industry in the late nineteenth century and early twentieth century, especially in German-speaking central Europe, led to an enormous multiplication of staining procedures (Bolles-Lee, 1885; Sharpey-Schafer, 1885; Bohm and Davidoff, 1895; Mann, 1902; Carleton, 1926; McClung, 1928). Most procedures for neurons, neuroglia and fibres involved depositing on them salts of heavy metals such as silver, gold and osmium, although some of them coloured other elements of the tissues at the same time (Golgi, 1886; Bielschowsky, 1904; Ramon y Cajal, 1913a; Glees, 1946).

CONTROL EXPERIMENTS

The concept of control observations was first used by Darwin (1875) in a study of the bladders of the plant species Utricularia, but it did not gain popular currency among experimenters at the time. Hankin (1890) used unimmunized control mice in a study of the effects of tetanus. I would venture to suggest that the empirical nature of the

relationships between clinical medicine, gross pathology and histopathology, conditioned a philosophy in which control experiments were not thought necessary. Histochemistry has been much less complacent in this respect (Barka and Anderson, 1963; Adams, 1965; Pearse, 1972, 1980), but, unfortunately, to date control experiments have been carried out far too infrequently, in experimental histochemistry (Hillman, 1972, page 52). The considerable improvement of experimental design resulting from Fisher's pioneering work in applied statistics (1935), has until recently failed to make sufficient impact on histochemistry, histology or electron microscopy. This probably can be attributed to the considerable number of observations and analyses which would have to be made in small pieces of tissue to achieve statistically significant results.

BRAIN AS A SYNCYTIUM

In 1854 Virchow wrote a paper whose title in English was 'On a substance presenting the chemical reaction of cellulose found in the brain and spinal cord of man'. Golgi (1875), using his newly described method in 'thick' sections of brain, saw a nerve net. Weigert (1895) showed an apparently random arrangement of interstitial fibres in the neuroglia. There were three senses in which the brain was described as a syncytium. Gerlach (1872) regarded the neurons to be a continuous reticulum (please see below), and this would be a syncytium. Many other authors regarded the syncytium to be present in neuroglial 'nerve-glue' in the sense of Virchow (Virchow, 1846, 1871; Hardesty, 1904; Held, 1909; Holzer, 1923). A third view, whose last major exponent was Bauer (1953), regarded the syncytium as being continuous through the neuron and the neuroglial material (Figure 7 is reproduced from Bauer, op. cit.) The whole idea that there might be a syncytium was vehemently and widely opposed by the majority of neurohistologists (Taft and Ludlum, 1929; Penfield, 1932; Glees, 1955; most authors in Windle, 1958). Both Virchow (1846) and Hess (1953) examined the chemistry of the 'ground-substance'.

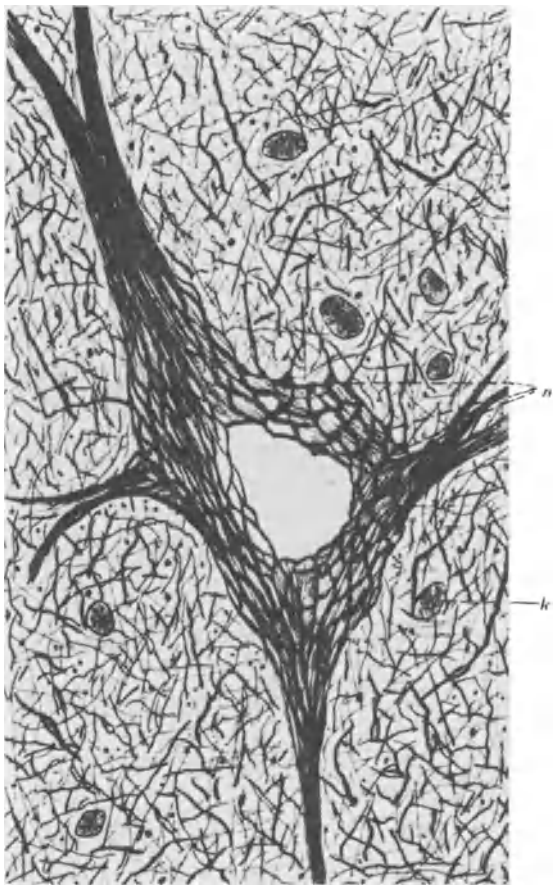


FIGURE 7 Bauer's (1953) view of the syncytium of the nervous system. He believed that the fibrils in the cytoplasm were continuous with those outside the cell bodies. This concept of the syncytium is very different from the present one (please see page 11 and Figure 78). This figure is reproduced from Bauer, K.F. (1953) Organisation des Nervengewebes und Neurencythiumtheorie, Urban and Schwarzenberg, Munchen, by kind permission of the publishers

THE RETICULAR VERSUS NEURONAL DOCTRINE

On the basis of observations using his gold chloride staining method Gerlach (1872) put forward the reticular theory, which united the whole central nervous system by fibres joining all its elements together. Golgi (1883, 1959) also accepted this view, and did not conceive the central role generally believed nowadays to be played by the neuron and all its processes. The reticularist view was questioned by observations of His (1887, 1888) and Forel (1887), and the neuronal doctrine was formulated by Waldeyer-Hartz (1891), although his contribution to the concept was relatively little. They believed that each neuron with its axon and dendrites was autonomous and had no direct connection with any other. Ramon y Cajal in 1933 (translated in 1954) summed up his views in a strongly worded book, entitled 'Neuron Theory or Reticular Theory', which is generally regarded as the definitive summary of the current view. On pages 8-9, he wrote:

> We have made careful investigations of the course and connections of nerve fibres in the cerebral and cerebellar convolutions of man, monkey, dog, etc., and we have never seen an anastomosis between the ramifications of two different protoplasmic expansions, nor have we observed them between filaments emanating

> from the same expansions of Deiters. The fibres intermingle in a most complex manner, producing a thick and intricate plexus, but never a net. It could be said that each element is an absolutely autonomous physiological canton.

Ramon y Cajal indicated that this statement was a summary of his views gathered from observations over a period of 50 years (Ramon y Cajal, 1954). This view was frequently used as an argument for the existence of synapses - that is - neurons are in contact, but not in continuity. I believe that Ramon y Cajal meant what he said, literally, that there are very few connections in the mammalian central nervous system which are visible by the light microscope.

AXONS AND DENDRITES

Deiters (1865) differentiated between the single axon and the several much finer dendrites (Figure 5), a view which is generally accepted nowadays. Golgi (1883) classified neurons into a Type I with a long axon, and a Type II with a short axon. He does not seem to have examined serial sections, nor does he seem to have appreciated that it is difficult to know with certainty the length of an axon from a single section.

Peters, Palay and Webster (1976, page 10) gave thirteen different criteria for distinguishing between axons and dendrites. For example, the axon extends either from the cell body or dendrite, while the dendrite extends from the cell body; the axon is unique in most cells but there are some examples of multiple origin, while the dendrite is usually multiple; the axon may be myelinated or unmyelinated, but the dendrite is rarely myelinated, and - if so - thinly; the axon usually gives rise to branches of the same diameter as the parent stem, but the dendrites usually divide into branches smaller than the parent stem. The only criterion which Peters, Palay and Webster indicated as absolute was that the axons extend outside the central nervous system, which the dendrites do not.

MITOCHONDRIA

Mitochondria were originally seen by La Vallette St George (1867) but Altmann's staining method using picric acid, and his clear illustrations (1890) led to them being called 'Altmann's granules' for a long period subsequently. They were probably seen in neurons for the first time by Held (1897) and Alzheimer (1904 and 1910). The cristae were first seen by electron microscopy by Palade (1953) and Sjostrand (1953a). However, no comment was made at the time on the fact that the cristae appear in the mitochondria of neurons and most other tissues far more frequently in the same plane of section than geometry would permit; see, for example, the latter authors and others (Rhodin, 1975; Peters, Palay and Webster, 1976, page 89; Fawcett, 1981, page 456). Perhaps the failure to notice the inadequacy of the geometry of the cristae arose from the beauty of the mental image of the oxidative enzymes situated in rows along the cristae.

GOLGI BODY

In the early 1890s Golgi described a 'reticular' apparatus in neurons of the barn owl

(Figure 8) and then in other cells (Golgi, 1898, 1899). Other authors later described the Golgi 'body', 'apparatus', or 'complex' in neurons as a shell of granules around the nucleus (Holmgren, 1900), or as a single paranuclear mass (Bourne, 1942). There was much discussion about whether the Golgi apparatus was an artifact or not. Walker and Allen (1927) and Walker (1828) made deposits of inorganic materials, which looked like Golgi apparatuses under the microscope, when they had been similarly prepared. Baker (1942) showed a silver grain under the electron microscope which looked disarmingly like a Golgi body. A full symposium of the Royal Microscopic Society in 1955, whose papers were published in 1956, debated its reality. However, its existence came to be accepted generally when the electron microscopists saw a lamellar structure throughout all tissues, which they identified as the Golgi body (Palay, 1958b; Novikoff and Goldfischer, 1961; Beams and Kessel, 1968). Unfortunately, no-one had compared the dimensions or appearances of, on the one hand, the rete diffusa, the shell and the paranuclear mass, with, on the other hand, the shape of a piece of an onion (please see Hillman and Sartory, 1980b).

Before lysosomes were described, research workers generally identified any particles in the cytoplasm, which were evidently not mitochondria, as Golgi bodies, or one of the more than a hundred other names used to describe the structure (Kirkman and Severinghaus, 1938). The relationship of the Golgi body to the endoplasmic reticulum or any other element of the 'cytoskeleton' remains to be clarified.

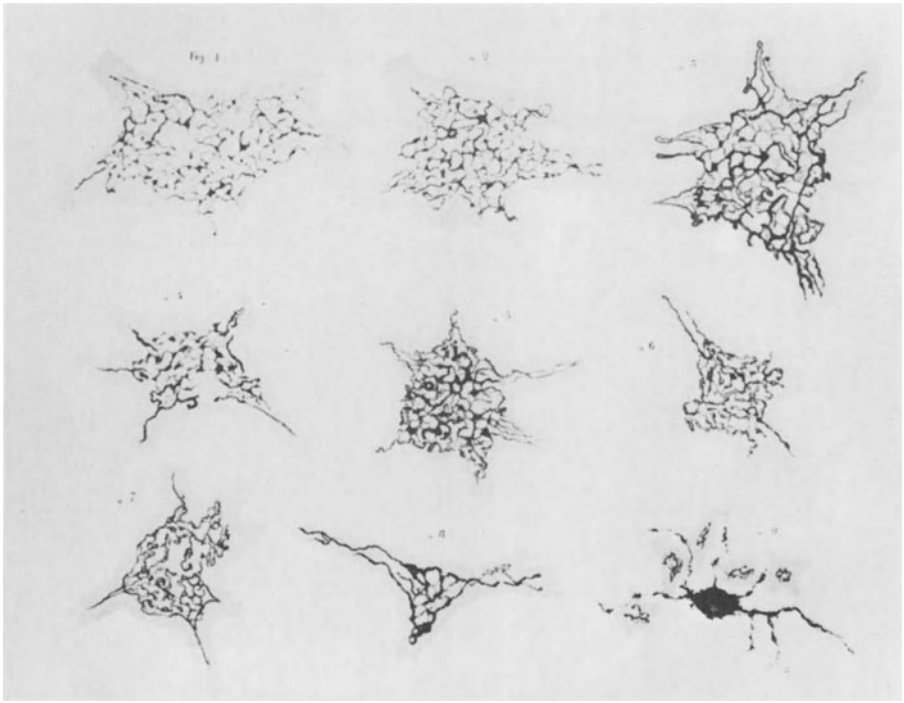


FIGURE 8 Golgi's view of the Golgi apparatus (1883) in a spinal neuron

NEUROMUSCULAR JUNCTION

The neuromuscular junction was first seen by Kuhne (1862). Since the interest in transmission, it has been classed with the synapse as a junctional tissue. This has enabled much which has been written about the structure and physiology of the neuromuscular junction to be taken as descriptions of the properties of synapses.

SYNAPSES

'End-feet' were seen in human neurons by Held (1897), and in rabbit spinal cord by Auerbach (1898). Illustrations from their papers show granules easily visible by light microscopy but some distance from the neuron somas (Figure 9). At about the same time, Sherrington's study of reflexes led him to postulate connections, which he called 'synapses' (Sherrington, 1897). It was not long before the events and properties which the physiologists described were ascribed to the 'end-feet' or 'end-knobs'.

When the electron microscopists started looking at the mammalian central nervous system, they soon saw 'pre-' and 'post-' synaptic thickenings (Palay, 1956a, 1958b). Synaptic 'vesicles' were seen in the early 1950s, first in retina (Sjostrand, 1953), and subsequently in brain and spinal cord (Palade, 1954; Palay, 1954, 1956b). They appear as circular or slightly oval bodies 20-60 nm in diameter, and are remarkably uniform in their dimensions in a particular tissue (references in Table 26). They were named in frog and earthworm by de Robertis and Bennett (1955), who noted that they predominated on one side of the synapse. Indeed, it is usually said that they occur on the pre-synaptic side, although, one could say that the pre-synaptic side is identified as the one with most vesicles.

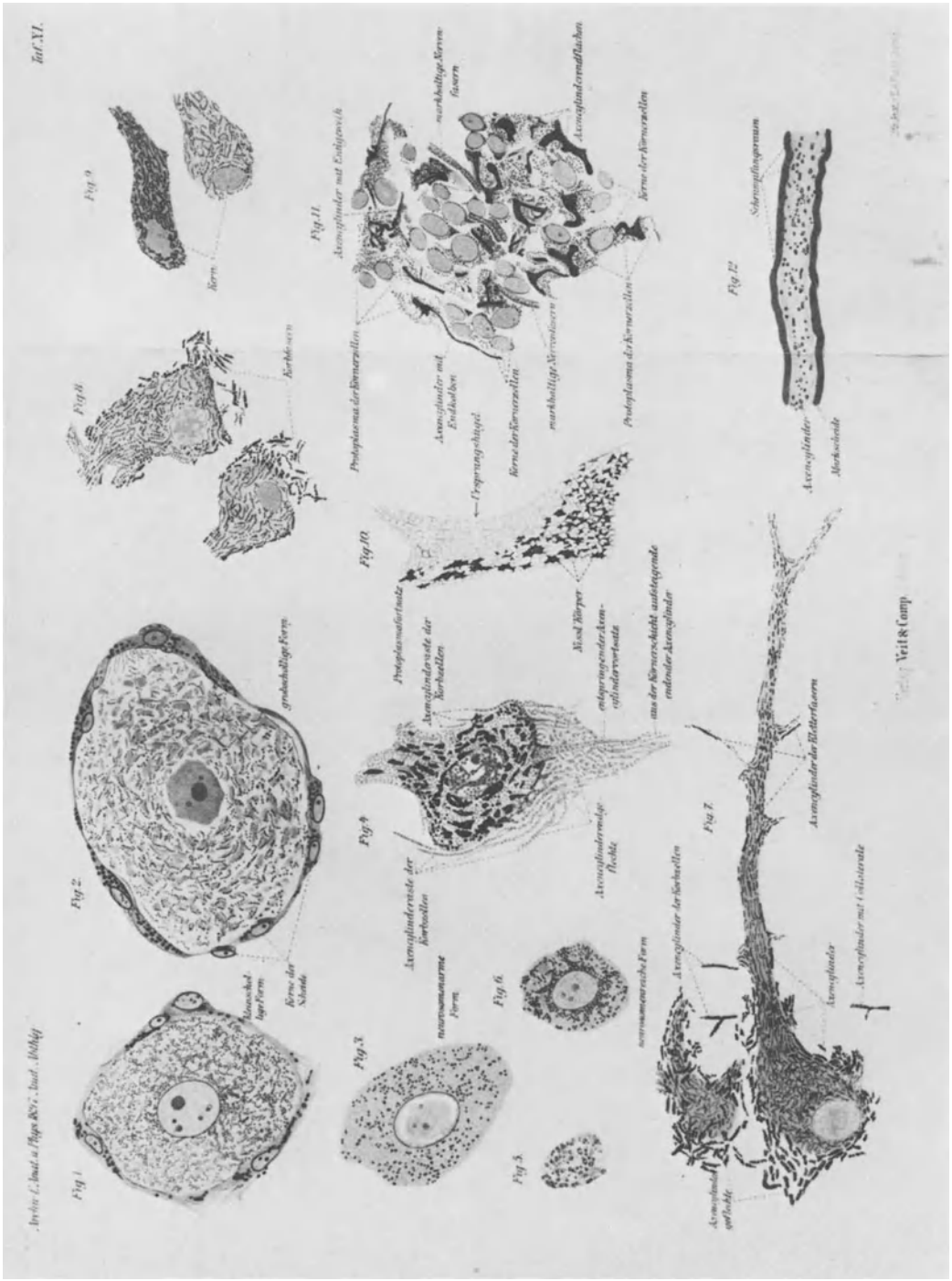


FIGURE 9 Drawings of neurons from rabbit trapezoid nucleus showing silver stained end-feet, which are considered one of the first demonstrations by light microscopy of synapses. Note particularly figures 1, 2, 4, 7, 8. These illustrations are from Held (1897, his plate XI)

Nowadays, electron microscopists use three different criteria for identification of a synapse; firstly, it must be in the central nervous system or ganglia; secondly, it must have a pre- and post-synaptic thickening, and, thirdly, synaptic 'vesicles' must be present, mainly on the 'pre-synaptic' side. Ramon y Cajal (1954, page 11) listed 11 different kinds of synapses, his classification being based entirely on the propinquity of fine fibres from different parts of the neurons. The more recent list from the electron microscopists comprises axo-dendritic, axo-somatic, axo-axonal, dendro-dendritic, somato-dendritic, dendro-somatic, somato-axonic, dendro-axonic, and glomerular, synapses (Peters, Palay and Webster, 1976, pages 159-174); (this does not include neuromuscular junctions). It means that each element of the total neuron can synapse with every other one. The latter authors made the point (page 159) that the 'existence of dendro-axonic and somato-axonic synapses must bring about radical changes in our thoughts about the function of connections between neurons'. Using the above criteria Henrikson and Vaughn (1974) found axo-glial synapses between the 13th and 14th days after gestation in the mouse spinal cord. Similar synapses have been found in the cerebral cortex of turtle (Ebner and Colonnier, 1975).

TRANSMISSION

The idea of a chemical or electrical excitation was first put forward by Du Bois Reymond (1877), but Elliott (1904) was the first person to suggest in a Communication to the Physiological Society that adrenalin might be able to transmit excitation. Incidentally Davson and Eggleton (1968) in a footnote remark that this concept is usually wrongly quoted as having been published by Elliott in a longer paper in the same year as the Proceedings; they noted that 'Apparently he was dissuaded from publishing this suggestion in his large paper, because it was too revolutionary'. From this early idea, and some seminal experiments of Fatt and Katz (1952) on frog nerve-muscle preparations, del Castillo and Katz (1956) proposed the vesicle hypothesis, for neuro-muscular transmission (for summary please see Katz, 1969). It is interesting to note that in the latter publication 13 years after the original concept, he wrote 'I should like to introduce a working hypothesis'.

This hypothesis has gradually been accepted as being applicable to central nervous system synapses, and also as being substantial supportive evidence for interpreting electron microscopic findings about synapses and synaptic vesicles. The criteria for transmitters have been enumerated frequently (see, for example, Hillarp, Fuxe and Dahlstrom, 1966; Werman, 1966; Barchas, Akil, Elliott, Holman and Watson, 1978). The latter authors give a long list, to which Osborne (1981) has added a few to make a total of 50 (reproduced here as Table 27). The vesicle and transmitter hypothesis is dealt with on pages 226-230.

NEURONS AND NEUROGLIA

When Deiters (1865) looked at the brain, he saw neurons and 'cell equivalents'; the latter had little cytoplasm and were subsequently called Deiters' cells. Henle (1871), Boll (1874) and Weigert (1895) all observed the same skein of fibres attached to the neuroglia. However, Golgi (1873), using his own stains, was not certain that the neurons and neuroglia were different cells. In 1894 he showed that the astrocytes

were connected to blood vessels and that their incidence increased with age. He also thought of the intercellular substance as a syncytium, in which he noted the fibres. Stricker and Unger (1879) made the point that there were various transition forms between neurons and neuroglia, perhaps implying, somewhat obliquely, that one cannot distinguish between the neurons and the neuroglia unequivocally (please see Tables 2-5).

Deiters (1865) described the microscopic appearance of neurons in detail (reproduced here as Figure 5). Cohnheim (1872) also saw the Deiters' cells without membrane or cytoplasm, and these free neuroglia were considered for many years to be important vehicles for gliosis. However, Golgi (1885), Weigert (1895) and Ramon y Cajal (1813a,b) said that these free nuclei did, indeed, have processes, which earlier staining procedures had not shown up. This then became generally accepted, as it is still today. However, it does leave open a number of important questions. How does one know that the membranes and cytoplasm are present around the naked nuclei if one cannot see them? How does one know that Deiters was staining the same kind of cells as Golgi, Weigert and Ramon y Cajal? What relationship do the nuclei seen by Virchow (Figure 6) or by Deiters, or in any cursory examination of any healthy part of the central nervous system (Figures 36 and 60) bear to the neuroglia which occur in acute or chronic gliosis in infection and injury? Are they the same cells from which glioblastomas and gliomas arise? What relationship, if any, is there between Deiters' nuclei and the amoeboid glia of Alzheimer (1904 and 1910)?

Although neuroglia had been seen and their classification had been debated earlier, it was to Ramon y Cajal (1892) that we largely owe the current classification of neuroglia - star-shaped cells with processes (astrocytes), adendritic cells (oligodendroglia) and those with elongate nuclei (microglia). Further detailed descriptions, especially of the oligodendroglia and microglia, were given by del Rio-Hortega (1919a,b, 1920). The latter author, like Alzheimer before him (1904 and 1910) and Dodgson (1948) after him, suggested that tissue around neurons could move and was elastic.

NISSL BODY

In the late nineteenth century the Nissl body was described by Key and Retzius (1876) and by Flemming (1882), but it was shown up much more clearly by Nissl, using his own staining system (Nissl, 1894). It then came to be named after him. Hyden (1961), in reviewing the Nissl body, concluded that it was identical with the 'endoplasmic reticulum' (his inverted commas). He then goes on to make the point that

> 'one must bear in mind that these discrete particles in membranes are observed in fixed, alcohol-treated, and embedded material. The pertinent question, therefore, is whether they really exist and have their counterpart in the living cell. In view of existing studies on the nerve cells there is reason to regard with caution such small structures at high resolution' (Hyden, 1961).

GRANULES

By light microscopy one may see granules. If they appear in the nucleoli they are called nucleololi or nucleolonema (Estable and Sotelo, 1951). In the nucleoplasm granules would

be called nucleoli, specks of dirt or artifacts; in the cytoplasm they would be called Golgi bodies or centromeres by older workers and lysosomes or ribosomes by modern electron microscopists; in the synapses they would be called synaptic vesicles, dense core vesicles, endocytotic or exocytotic vesicles. They all look like granules of different sizes (see Figure 10).

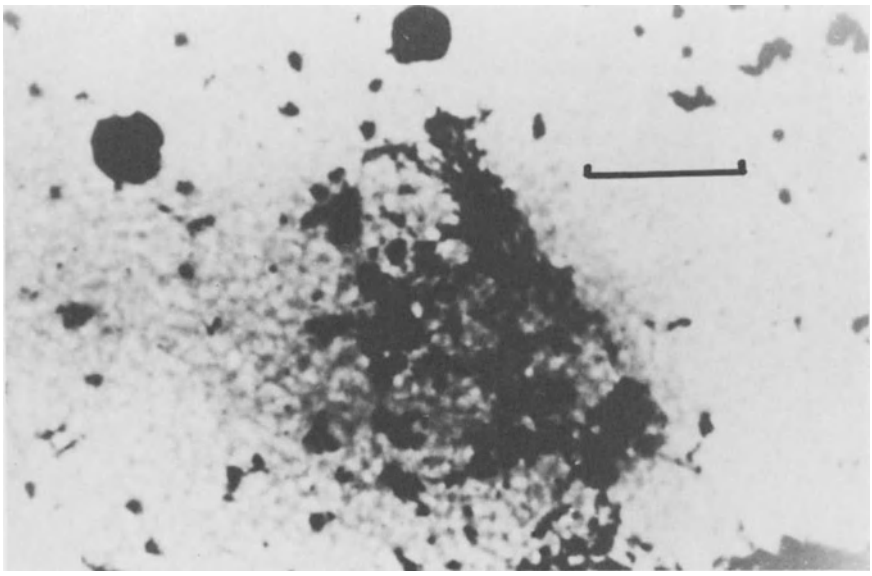


FIGURE 10 Drops of egg albumin were smeared and stained with haematoxylin and eosin. Please note the non-uniformity of the deposit, which could appear as showing fibres, granules, reticula and cytoskeletons. The bar is 20 μm.

MYELINATED FIBRES

In 1717 Leeuwenhoek (see 1719) saw myelinated fibres (Figure 2), but they were more clearly described by Fontana (1781). Remak (1836), and subsequently Koelliker, Dalton and Todd, obviously saw the node (Stirling, 1881), but it fell to Ranvier (1871) to give the definitive description, and consequently his name to the node of Ranvier. Incisures in the peripheral nerves were seen by Schmidt (1874) and Lantermann (1877), whose names were also given to them. Although they can be seen in unfixed peripheral nerves by light microscopy, their existence was not regarded as 'confirmed', until the electron microscopists also saw them in peripheral nerve and spinal cord (Blakemore, 1969; Hildebrand, 1971 and sources quoted there).

The history and appearance of the myelin sheath as revealed before electron microscopy was reviewed by Young (1945). The myelin sheath was examined by low-angle diffraction (Schmitt, Bear and Clark, 1935; Schmidt, 1936, Schmitt and Bear, 1939),

and its lamellar structure was described by electron microscopy in the whole nerve (Fernandez-Moran, 1950b; Rozsa, Morgan, Szent-Gyorgyi and Wyckoff, 1950; Sjostrand, 1953a); the same lamellar structure was also seen in homogenates (Fernandez-Moran, 1950a; Sjostrand, 1950). The beautiful concentric appearance is the pride of the literature (for example, Rhodin, 1975; Landon, 1976; Peters, Palay and Webster, 1976, chapter VI, pages 181-230).

THE SCHWANN CELL

This was described by Schwann (1838), and his illustration is reproduced here in Figure 11. From studies of chick embryo, Geren (1954) described how the Schwann cell wraps around the axon to form the myelinated sheath. The central end of the sheath has been called the internal mesaxon, and the peripheral 'entry' point of the axon, the external mesaxon (see, for example, Robertson, 1959, 1969, 1980; Fawcett, 1981, and Figure 71 from the latter book).

NUCLEOLI AND NUCLEOLAR MEMBRANE

Valentin (1836) drew a nucleolus which looked as if it had a membrane around it and was empty (reproduced as Figure 3). He described the nucleus as containing in its centre a 'circular corpuscle', which was both clear and solid (this seems somewhat contradictory). Purkinje (1838) and Deiters (1865) also showed clear nucleoli. 'Nucleolus-associated chromatin' was seen in neurons by Hyden and Hamberger (1945) and Hertl (1957). The nucleolonema was first seen by Estable and Sotelo (1951). In unfixed rabbit neurons it has been shown to be in continual movement, which was stopped by the addition of phenol (Sartory, Fasham and Hillman, 1971). However, the nucleolonema is not seen as well by electron microscopy as it is by phase-contrast (compare Figures 31-33 and 16, 18 and 57), so it is rarely described in modern textbooks. A nucleolar membrane was described in all unfixed neurons of the cranial nerve nuclei and anterior horns of the spinal cord of rabbit, rat and guinea pig (Hussain, Hillman and Sartory, 1974).



FIGURE 11 The myelinated axon as drawn by Schwann (1838). Please note the position of the nucleus

THE NUCLEOLOLUS

Mauthner (1860) first saw a small body in the nucleolus, which he called the nucleololus. Some authors since then have used the term for one or more minute granules, while others have used it for the appearance of a vacuole (Shantha, Manocha, Bourne and

Ariens-Kappers, 1969, and authors quoted there). It is difficult to conceive that the appearances of a granule and a vacuole could represent the same object, unless the former were viewed out of focus. It is doubtful whether serious microscopists could make the latter error. The nucleololus has been seen by both light and electron microscopy (Dutta, Siegesmund and Fox, 1963), but it has not been sighted in the textbooks recently.

CYTOARCHITECTONICS AND MYELOARCHITECTONICS

Cytoarchitectonics is a term used for the types and arrangements of cells in the central nervous system, while myeloarchitectonics refers to the fibres within it. It is generally assumed, implicitly rather than explicitly, that the cells are neurons, and the fibres arise from the neurons. It has also been generally assumed that cells which stain with 'neuroglial' procedures are not excitable, and, therefore, their processes do not conduct action potentials.

Meynert (1867, 1868), Bevan-Lewis and Clarke (1878) and Hammarberg (1895) made systematic histological studies of normal and diseased brains. However, the demonstrations by Fritsch and Hitzig (1870), and Ferrier (1876) that stimulation of particular regions of the parietal cortex could produce movement of limbs, generated an interest in the relationship between the histology of the brain and its physiology. In the early twentieth century the cellular and fibrous arrangements of the brain were recognized as having a pattern. Brodmann (1903, 1909), the Vogts (1903, 1919) and Campbell (1905) published extensive studies of the cellular pattern of the cerebral cortex, including descriptions of the five to eight laminae. The identification, relative thickness, and physiological significance of the layers, were the subject of much discussion. Further anatomical studies were pursued by Ramon y Cajal (1909-1911), von Economo and Koskinas (1925) Von Economo, (1929) and many others; they led to the current view that the cortices of the parietal, temporal and occipital regions have an external lamina, consisting of: I, plexiform; II, small pyramid; III, medium-sized pyramid; IVa, star pyramid; and IVb, star cell, layers; and an internal lamina, consisting of: V, large deep pyramids, and VI, spindle, layers. The four main types of cell seen are: those with long descending axons; those with short axons ramifying near the cell body; cells with ascending axons ramifying in cortical layers; and cells with horizontal axons. Brodmann (1903) carried out the monumental task of mapping 52 areas of the brain, while Campbell (1905) looked at 20 areas.

Both in respect of the cortical layers and of the cell types, authors have no difficulty in indicating 'typical' examples in most histological preparations, but there are many cells - perhaps the majority - which are difficult to identify unequivocally and objectively. Some of this difficulty probably arises from the fact that the thickness of a single section cuts only a fraction of the diameter of a cell soma, and an even smaller fraction of the length of a process.

WHITE MATTER

According to Clarke and O'Malley (1968, page 586), De Vieussens (1684) was the first to examine the white matter; he described it as 'composed of innumerable connected fibres divided up into many bundles so close to one another that there is no perceptible

space between them, [and] compose a continuous body, that is, the staff.' Von Gudden (1874) devised the technique of cutting the optic nerve or other cranial nerve to observe the degeneration of the fibres and thus map the tracts, using serial sections. Flechsig (1876, 1881) studied myelination in brain and spinal cord as another method of examining the courses of the tracts. The development of the Weigert (1882) and Marchi and Algeri stains (1885-1886) for degenerating fibres constituted another important histological technique for mapping tracts (Schroder, 1914; Minkowski, 1923; Von Economo and Koskinas, 1925). In discussing 'anatomical' techniques, one should mention the Labanic work of Lassek (1948) in classifying a million fibres and counting those of the different diameters in the human pyramidal tract (please see also Lorente de No, 1934).

A number of workers have counted the number of muscle fibres which are innervated by a single nerve fibre and presumably an anterior horn cell. The ventral roots were cut to allow the sensory fibres to degenerate, so that the remaining fibres were all motor. The following ratios of nerve to muscle fibres were found: soleus, 1:120; extensor longus digitorum 1:165 (Clarke, 1931); sartorius 1:70-117 (Van Harreveld, 1947). Of course, each muscle fibre contains a considerable number of sarcomeres in series, some of them several millimetres away from the end-plates. This multiplicity of sarcomeres served by a single nerve fibre has considerable implications for the anatomy and physiology of the neuromuscular junction (please see pages 156-157,209,226-230).

PHYSIOLOGICAL EXPERIMENTS ON TRACTS

Following Fritsch and Hitzig (1870) many authors stimulated cortical areas or extirpated them in a variety of species, including monkey and man (Hitzig, 1874; Ferrier, 1876; Beevor and Horsley, 1890a,b; Bidwell and Sherrington, 1893; Sherrington, 1906; Leyton and Sherrington, 1917). Dusser de Barenne (1910, 1916) applied strychnine to the cortex and recorded the spread of excitability, having reasoned that any region away from the area stimulated, which reacted, must have fibrous connections with it.

The idea that localized parts of the brain had particular 'functions' probably originated from the physiognomists who were active well into the beginning of the twentieth century. It was confirmed, of course, by the experiments on mapping of tracts, electrical and strychnine stimulation, animal ablation experiments, and the surgical excision of epileptic foci by Penfield and his collaborators (Penfield and Erickson, 1941). Yet the very important experiments of Goltz (1881), the arch opponent of localization, should not be overlooked. He showed that decorticated dogs could survive months with minimal motor disturbance (Goltz, 1892). Pathologists know that babies who have had unilateral hemispherectomies subsequently grow up with a remarkable degree of cerebral function. Sometimes, also, patients who have had large volumes of their brains replaced by metastases from tumours in their lungs or stomachs have unexpectedly good neurological and psychological function. This is attributed to 'plasticity', but this really is only another expression for the same phenomenon. Furthermore, the concept of 'centres', demonstrated by experiments showing that particular parts of the brain may be stimulated or extirpated to increase or decrease their activity, is somewhat naive. Such experiments only show that the apparent centre is part of the circuit involved in the motor or sensory response, not that it is

necessarily the 'centre', nor even that a 'centre' exists for that activity.

MICROSCOPICAL TECHNIQUES

The nineteenth century was an epoch of unprecedented developments in the following aspects of microscopy relevant to the study of cerebral histology: tissue fixation; mounting of slides; illumination systems; microtomy; neural stains; photomicrography; micromanipulation; measurement of dimensions of microscopical objects; illustration; manufacture of model systems illustrating the properties of living cells (colpoids); microscope design; surgical pathology; vital staining; polarizing and dark-field microscopy; control experiments; microspectrography; study of artifacts. In the early twentieth century the following further techniques became practical and common: intracellular injection; tissue culture; warm stages; staining of degenerating fibres; autoradiography; biopsy; cinemicrography; tissue transplantation; microsurgery; histochemistry; time lapse photography; immunocytology; development of microchemical techniques. Several new kinds of microscopy were developed; inverted; X-ray; reflection; phase contrast; anopteral; interference; ultraviolet; cryomicroscopy; automatic photography; electron microscopy; television; acoustic (Hillman and Richards, 1983).

TISSUE CULTURE

Harrison (1907, 1910) was the first person to culture nervous tissue in vitro. He showed that fibrils from frog embryo would grow on clotted lymph. Lewis and Lewis (1915) and Lewis (1923, 1931) observed mitochondria, granules and pinocytosis, and Lewis and McCoy (1922) examined the survival in culture of tissues from rats, guinea pigs, rabbit and man. Weiss and Wang (1936) described fibres in the cytoplasm of chick embryo ganglion cells. Hogue (1950) grew human foetal brain cells from dead embryos, and was able to identify the different kinds of cells in culture.

Phase-contrast microscopy was described by Zernike in 1934. Pomerat and Costero (1956) performed epoch-making observations on intracellular movements using time-lapse photography and phase-contrast microscopy on cat cerebellar cultures. It has also been shown that neurons in culture can fire (Crain, 1956; Hild and Tasaki, 1962). Until 1964, Hild believed that neurons lost their synapses in tissue culture, presumably because he could not see them by light microscopy. However, when he and his colleague (Callas and Hild, 1964) used the electron microscope, they saw membrane thickenings which they regarded as synapses.

Hild, Chang and Tasaki (1958) found electrical responses in cultured cat cells, which they thought were astrocytes, and which were quite different from the rapid action potentials of the cells they identified as neurons (Hild and Tasaki, 1962). Myelination of axons in culture was shown (Peterson and Murray, 1956; Hild, 1957). An explant from the brain stem was demonstrated to join up with one from the spinal cord (Crain, Peterson and Bornstein, 1968), and the same school showed that nerves can activate muscles in culture (Peterson and Crain, 1970).

Intracellular movements seen in tissue culture seem to be incompatible with an 'endoskeleton'. A suggestion has been made that there are 'roads' through the 'endoskeleton', which permit such traffic. Such traffic lanes have been sought by optimistic voyagers (Andres, 1961; Bunge, Bunge, Peterson and Murray, 1967).

What should one conclude if intrepid explorers cannot find a way out? (Please see page 198-200).

CELLULAR AND INTRACELLULAR MOVEMENTS

Metchnikoff (1893) described phagocytosis and Nissl (1899) saw microglia which seemed so similar to cells seen around infections of the brain that he supposed that they were motile. Del Rio-Hortega (1919b) was also of the opinion that microglia could move. The migration of neuroglia in developing embryos was concluded to be occurring after examination of brains of embryos at various stages (Kershman, 1938, 1939). Axonal flow in both directions of proteins, amino acids, nucleotides and transmitter-related enzymes, and its arrest by obstruction of the axons, was described by Weiss and Hiscoe (1948), and has since been studied extensively (Weiss, 1961; Lubinska, 1964; Grafstein and Forman, 1980). Leonhardt (1951) injected particles into the brain, and found them subpially subsequently. Considerable work showing intracerebral movements of granules and cells has been done by Klatzo and his colleagues (Klatzo, Piraux and Laskowski, 1958; Klatzo and Miquel, 1960). Oehmichen has more recently published a review of his own experiments on cellular movements in the nervous system (Oehmichen, 1978).

THE CELL MEMBRANE

Cells have been recognized since the time of Hooke by the walls which surround them, and the chemistry of this wall has fascinated physiologists and biochemists for more than 90 years. Overton (1895, 1902) first suggested that the cell surface was lipid. Gorter and Grendel (1925) extracted the lipids from red blood cells of several species and calculated that there would be enough to make a bimolecular layer around each cell. Fricke (1925), Fricke and Curtis (1835), and Cole (1937), measured the capacity and resistance of several eggs, and Schmitt (1936, 1939) and Schmitt and Bear (1939) examined red cells by birefringence, and concluded that membranes were 1-10 nm thick. Davson and Danielli (1943) reviewed a great deal of evidence of: selective permeability of membranes to ions; oil-water partition coefficients of anaesthetics; high capacitances and resistances of membranes; birefringence studies; chemistry of the membranes, their surface tension and elastic properties, etc.; they then proposed their hypothesis that the cell membrane consisted of two protein molecules sandwiching a lipid layer (Figure 12).

A few comments about this seminal hypothesis should be made. Among the measurements of membrane thickness, the electrical measurements gave maximum values, while the birefringence measurements gave minimal values; all of these had a large range and most of the measurements gave larger values than the simple Davson-Danielli hypothesis would suggest. Most values were derived from membranes of plant cells, erythrocytes and marine eggs, but their results have generally been regarded as applicable to <u>all</u> membranes. Many of the measurements adduced were made on hydrated tissues (Davson and Danielli, 1936; Danielli, 1942). However, measurements from electron micrographs and low angle diffraction - particularly of myelin sheaths - usually made on completely <u>dehydrated</u> tissues, were used to support the Davson-Danielli model, without taking any shrinkage into account.

Robertson (1959) described the 'unit membrane', also known as the 'trilaminar'

appearance. It was originally thought of as the characteristic appearance of the cell membrane, but, subsequently, it was generalized to include the nuclear membrane, the endoplasmic reticulum, the myelin sheath, and occasionally the lamellae of the Golgi apparatus.

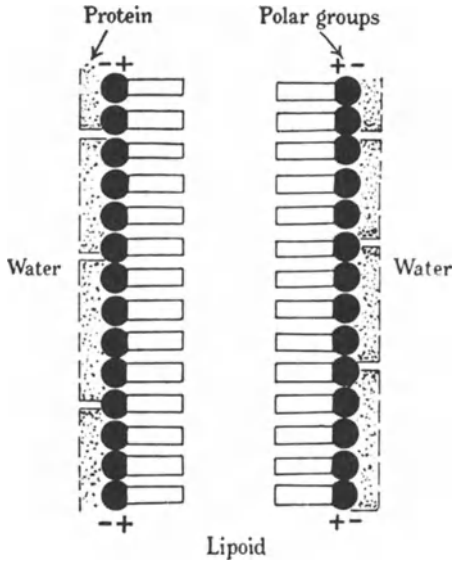


FIGURE 12 Diagram of the 'lipoid double-layer' as proposed by Davson and Danielli (1936). This figure is reproduced from Davson, H. and Danielli, J.F. (1936). The Permeability of Natural Membranes (Cambridge University Press, Cambridge), by kind permission of the authors and publisher

The fluid mosaic hypothesis of membrane structure was first put forward by Singer and Nicholson (1972) and is now widely accepted as if it were a finding (Semenza and Carafoli, 1977; Repke, 1980; Kates and Kuksis, 1980). It originated from some thermodynamic considerations, and studies on the distribution of antigens applied to red cell membranes and detected by freeze etching (Figure 13).

ELECTRON MICROSCOPY

The first electron microscope was made by Knoll and Ruska (1932), and the instrument was used to examine biological specimens not long after (Driest and Muller, 1935). During the three decades after the Second World War, neural tissues were the subject of intensive examination. The following structures were detected by the authors indicated in parentheses: endoplasmic reticulum (Porter, Claude and Fullam, 1945); cristae (Sjostrand, 1953a); ribosomes (Palay and Palade, 1955); 'unit membrane' (Robertson, 1959); lysosomes (de Duve, 1963); neurofilaments (Palay, 1964); microtubules (Porter, 1966); microtrabeculae (Wolosewick and Porter, 1976). The existence of the Golgi body, hitherto seen by light microscopy, was regarded as being 'confirmed', when a body which could be seen in the cytoplasm by electron microscopy was given the same name (Beams, van Breeman, Newfang and Evans, 1952). A 'cytoskeleton' which is claimed to consist of the following elements, whose mean diameters (nm) are given, has been reported: microtrabeculae, 3-6 (Wolosewick and Porter, 1979); actin filaments, 4-6 (Fine and Bray, 1971; Puszkin and Berl, 1972); glial filaments, 7-8 (Wuerker, 1970); neurofilaments, 10 (Palay, 1964; Wuerker and Kirkpatrick, 1972);

endoplasmic reticulum, 10 (Porter, Claude and Fullam, 1945); microtubules, 20-26 (Elfvin, 1961); Golgi body, 500-1000 (Hillman and Sartory, 1980a, references indicated on page 83). For reviews, see Wuerker and Kirkpatrick (1972), Shelanski (1973), Lazarides (1980), International Congress (1981).

The original techniques of detection of fluorescence (Williams and Chase, 1967-1977; Nairn, 1969; Shelanski, 1973; Weir, 1973; Sternberger, 1974) have been adapted for use with antibodies, for immunocytochemistry (Franke, Schmid, Osborn and Weber, 1978; Cold Spring Harbor Symposium, 1982). The antibodies are said to be specific, but are sometimes cross-reactive. The various elements of the 'endoskeleton' are viewed by light microscopy, usually in tissue cultures. The cells may have to be 'permeabilized', in addition to being fixed, frozen or stained. The dimensions of the elements of the 'endoskeleton' are measured by electron microscopy although during the preparation much of the lipid is lost.

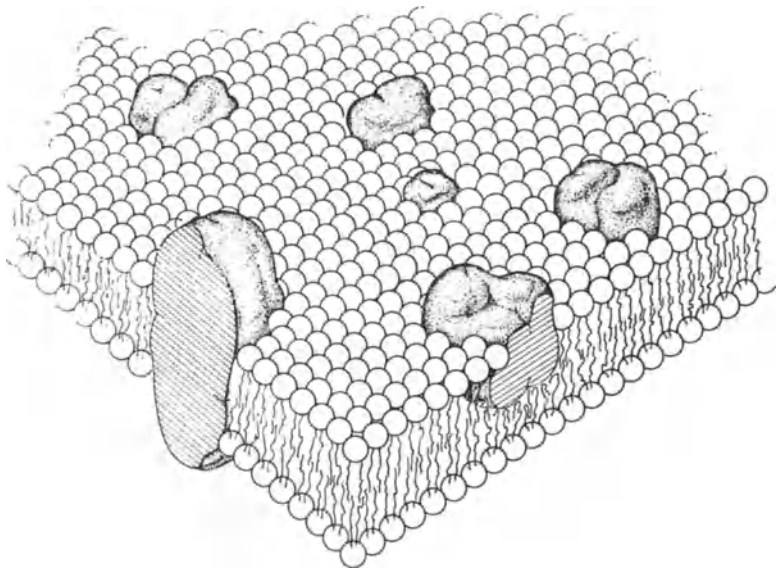


FIGURE 13 Diagram of the 'fluid mosaic' model of the cell membrane, according to Singer and Nicholson. The solid bodies with stippled surfaces 'represent the globular integral proteins, which at long range are randomly distributed in the plane of the membrane'. This diagram is reproduced from Singer, S.J. and Nicholson, G. (1972). The fluid mosaic model of the structure of cell membranes. Science, 175, 720-731, by kind permission of the authors and the American Association for the Advancement of Science

The electron microscope has also been used to detect the nuclear pores or pore apparatuses (Callan and Tomlin, 1950; Hartmann, 1953; Palay and Palade, 1955; Fernandez-Moran, 1957; Franke, 1974).

There have been many important developments in the techniques of electron microscopy. Among these are freeze etching (Meryman and Kafig, 1955; Steere, 1957; Moor, 1969), high-voltage electron microscopy (Dupouy, 1972) and others (for reviews, see Williams and Chase, 1967-1977; Meek, 1976; Stolinski and Breathnach, 1975; Hayat, 1970-1978; Weakley, 1981). All the latter techniques involve dehydration, but Parsons (1974, 1978) has attempted to examine tissues in wet chambers, to obviate the shrinkage. Unfortunately the pressure in his electron microscope is well below the vapour pressure of the water at that temperature so it is difficult to see how the specimens remain fully hydrated.

2
Methods of Studying Neural Structure

The following techniques are used to study the cellular structure of the nervous system:

(a) microdissection of unfixed tissues;
(b) light miscroscopic histology;
(c) observation of tissue cultures;
(d) electron microscopy;
(e) subcellular fractionation.

MICRODISSECTION

Microdissection of the mammalian central nervous system was originally pioneered by Purkinje in the 1840s (Van der Loos, 1967). It was widely used subsequently for many non-neural systems (see, for example, Gray, 1931; Chambers and Chambers, 1961; McClung-Jones, 1967). However, its modern renaissance is due to Hyden (1959, 1961, 1967) (Figure 14). The neurons are dissected out by hand under direct vision from sections of brain (Figures 15 and 16). The value of this method is that neurons can be separated with comparatively little disruption, leaving the soma intact, although, of course, the dendrites and axons are severed. The tension on the processes and placing the cells in unphysiological environments undoubtedly has some effect on them. Even this comparatively simple procedure implies fourteen separate assumptions (Hillman, 1983), but most of these are relevant to biochemical measurements made in them, and probably not to their structure. It is likely that placing them in unphysiological media might alter the osmotic conditions within the cells and therefore the total and relative dimensions of their subcellular organelles. It seems unlikely to alter the shapes of any part significantly. Nevertheless, after isolation these neurons respire linearly for several hours and have resting membrane potentials whose values can be altered reversibly (Epstein and O'Connor, 1965; Hertz, 1966; Hillman and Hyden, 1965). It is not known whether they can generate action potentials, since - to the author's knowledge - no systematic effort has yet been made so far to elicit them. It would be a very worthwhile project upon which to embark, since demonstration of the excitability of these cells would help to justify the very extensive biological studies already carried out on these cells, as well as the technique of isolation.



FIGURE 14 Professor Holger Hyden of Goteborg, modern pioneer of separation by hand of mammalian neurons. This photograph was taken in 1981. He is dissecting out the neurons with stainless steel wires

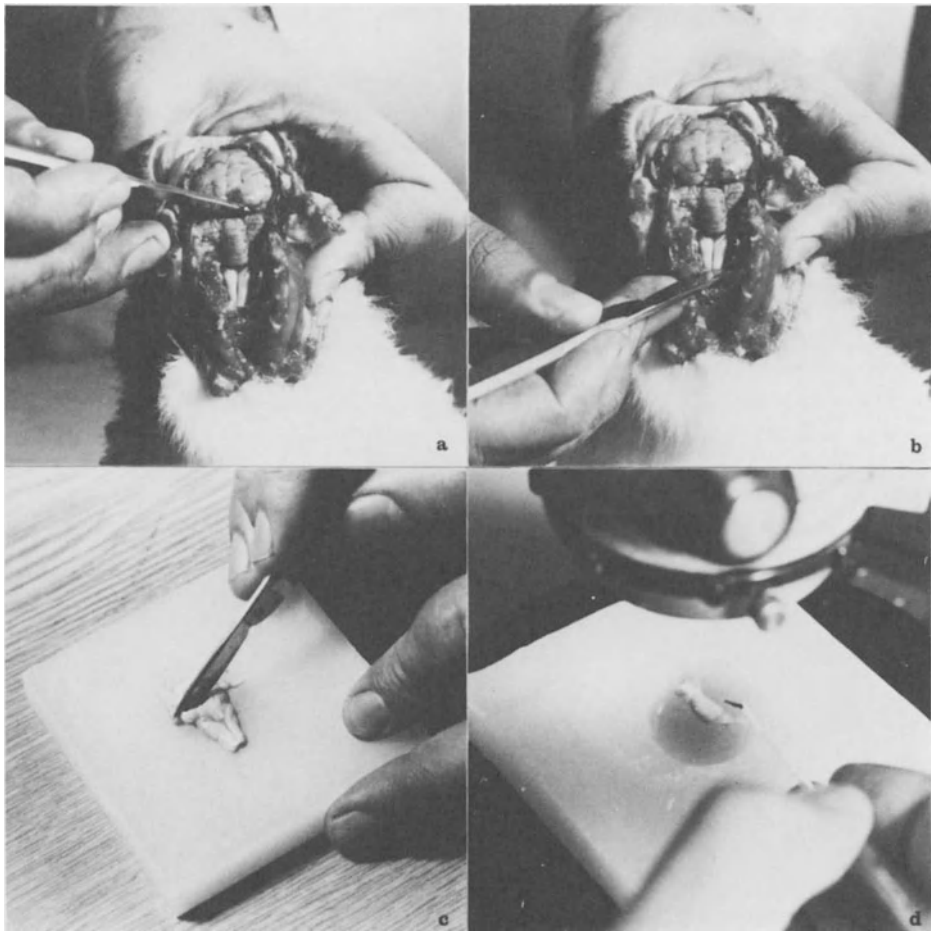


FIGURE 15 Dissection by hand of neurons of the cranial nerve nuclei, by the method of Hyden (1959). Rabbits have large neurons but any mammalian species can be used. The same technique can be used to separate spinal motor neurons: a, the brain is exposed and the first cut is made between the cerebrum and the cerebellum; b, the second cut is made below the medulla; c, several transverse cuts are made through the medulla with a scalpel; d, the slivers are placed on a wax block, stained slightly with methylene blue in 0.25 mol/l sucrose solution which is then washed off with more sucrose, and the stainless steel wire is used to lift out neurons of the cranial nuclei under direct vision using a dissecting microscope. The cells are then placed in a drop of sucrose solution in a cavity slide. These photographs were taken in collaboration with Dr T.S. Hussain

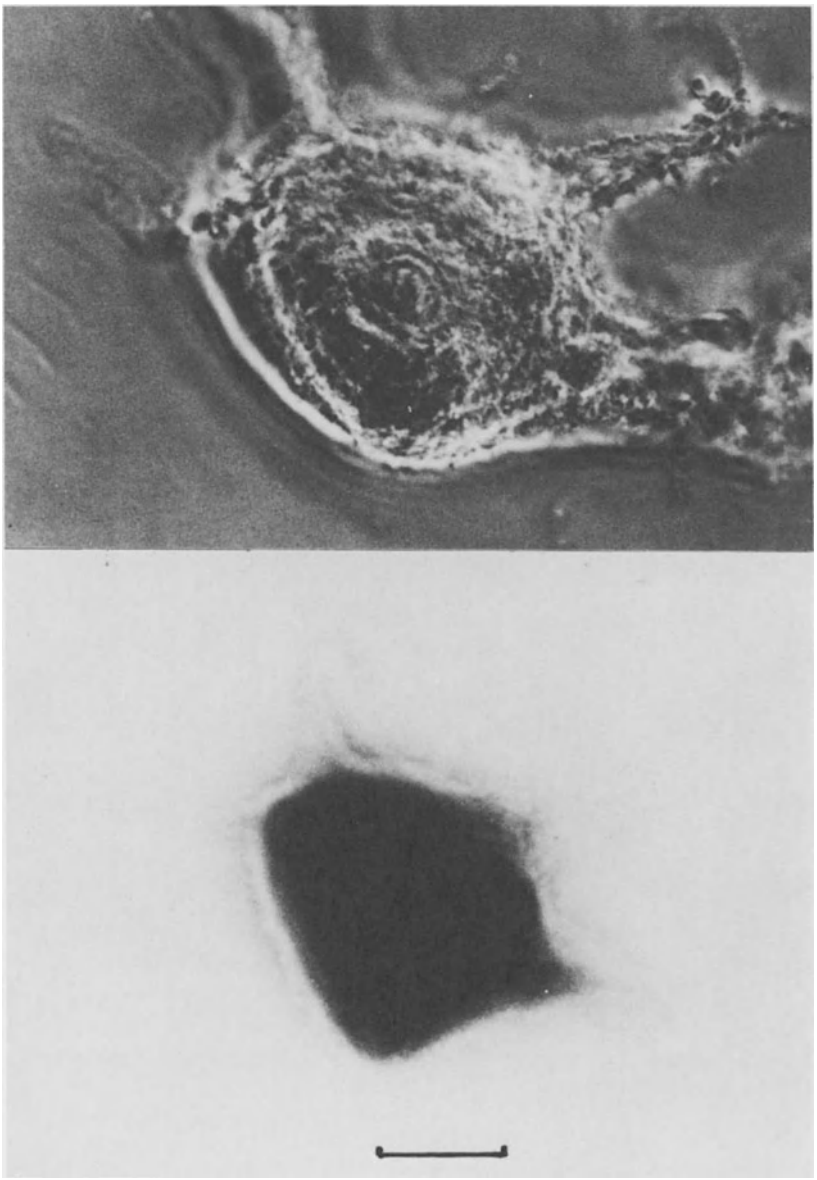


FIGURE 16 Isolated rabbit neuron, viewed by phase-contrast microscopy. Upper unfixed before staining with osmium tetroxide, lower after embedding in Araldite (it was not placed in the electron microscope or sectioned). The bar is 20 μm

The value of the experiments on isolated neuron cell bodies depends upon the truth of the assumption that the 0.25 mol/l sucrose or 0.15 mol/l saline in which they are dissected (Hyden, 1959; Hussain, Hillman and Sartory, 1974) does not significantly distort their shapes from those in vivo. There is no way of testing this. One can only plead that this technique of examining cells probably subjects them to less reagents than any other technique in vitro.

The relatively large dimensions of the cell bodies of mammalian cranial nerve nuclei and anterior horn cells has so far permitted a considerable accumulation of data about their structure, biology and pharmacology, which is not matched by findings about their processes or surrounding neuroglia.

In addition to neurons from the cranial nerve nuclei in the medulla, neurons can

also be isolated in large numbers from dorsal and sympathetic ganglia (Figures 17 and 18). Trypsin is often used for softening their capsules (Varon, Raiborn, Seto and Pomerat, 1963) but this powerful proteolytic enzyme may be avoided by the simple expedient of tearing open the capsules using dissecting needles (Hillman, 1966). Mammalian axons can also be teased apart with needles (Figure 19).

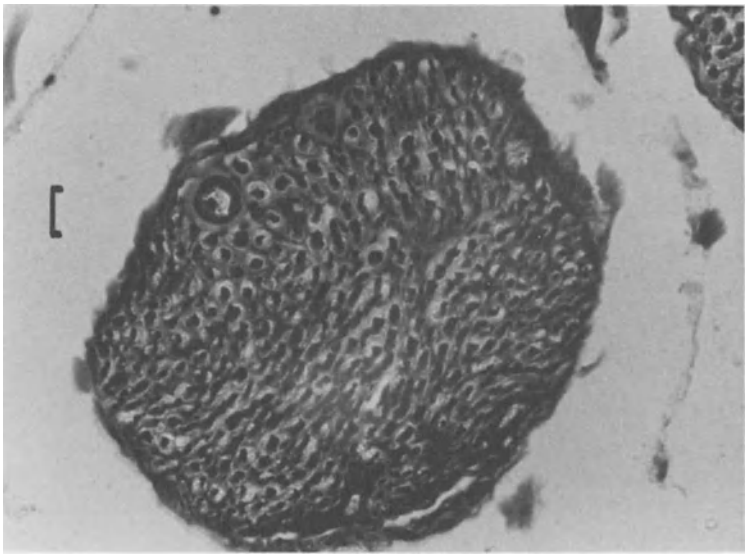


FIGURE 17 Human dorsal root ganglion, sectioned near the equator, stained with Palmgren's stain. The bar is 500 μm long

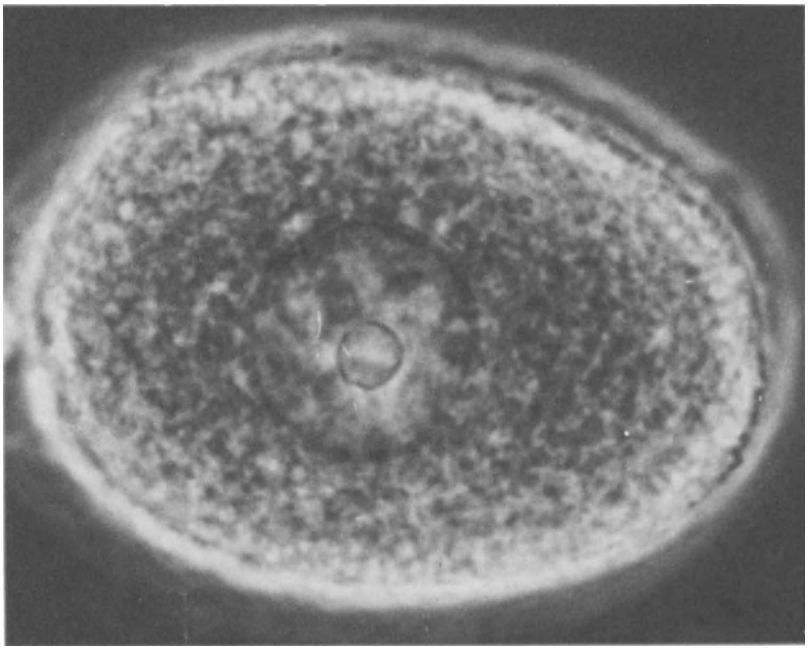


FIGURE 18 An unfixed rabbit dorsal root ganglion cell in normal saline viewed by phase-contrast microscopy. Please note the nucleolar membrane. The maximum diameter of the cell body is about 60 μm

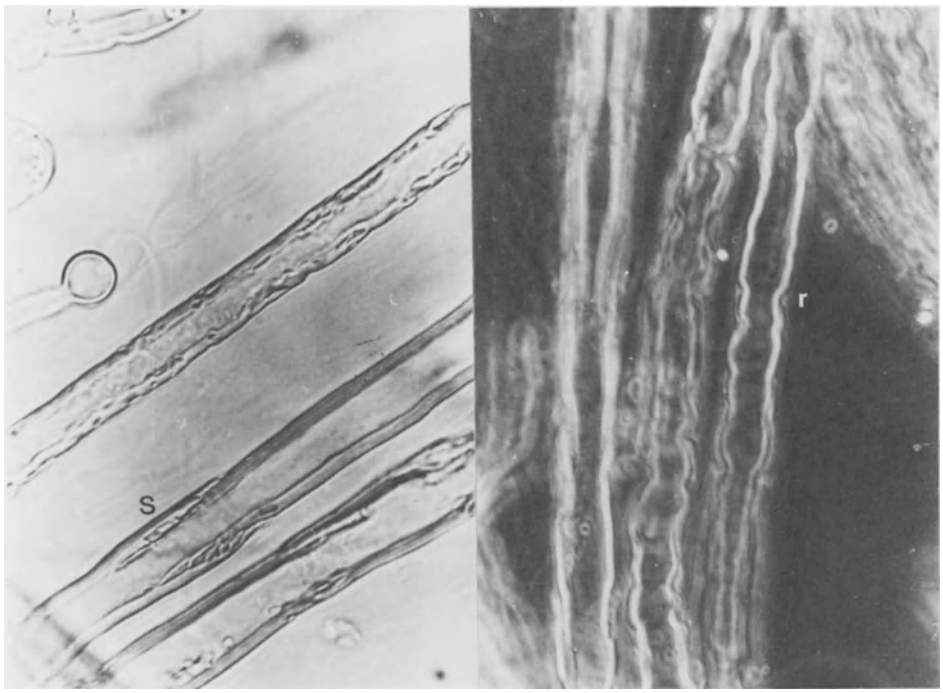


FIGURE 19 Myelinated axons from sciatic nerves, teased out, unfixed, placed in 0.25 mol/l sucrose (left) from a rat by bright-field illumination, showing Schmidt-Lanterman clefts, s; (right) from a rabbit by dark ground, showing nodes of Ranvier, r. The axons are 10-15 μm in diameter

Unfixed neurons, axons and neuroglia can be examined under the best optical conditions using phase-contrast, anopteral, dark-ground, polarizing and interference, as well as bright-field microscopy (Hussain, Hillman and Sartory, 1974). It should be stressed that the simplicity of the preparation, and the paucity of the reagents to which the cells have been subjected before they are observed, make these techniques the most valuable for examining the closest reality which can be achieved to the structure of the living cell in situ.

It is of considerable interest that hand dissection of the brain isolates neurons and 'neuroglial clumps' (Hyden, 1959; Hyden and Pigon, 1960). Hyden regarded these clumps as containing oligodendroglia. Hamberger (1963) found in 'vascular' glia a few astrocytes (Figure 20) because some of their cells were connected to blood vessels, which has been regarded as a criterion for astrocytes historically (Ramon y Cajal, 1913).

Mammalian peripheral nerve fibres have the advantage that they can conduct action potentials after isolation which are not significantly different from those in vivo (Erlanger and Gasser, 1937).

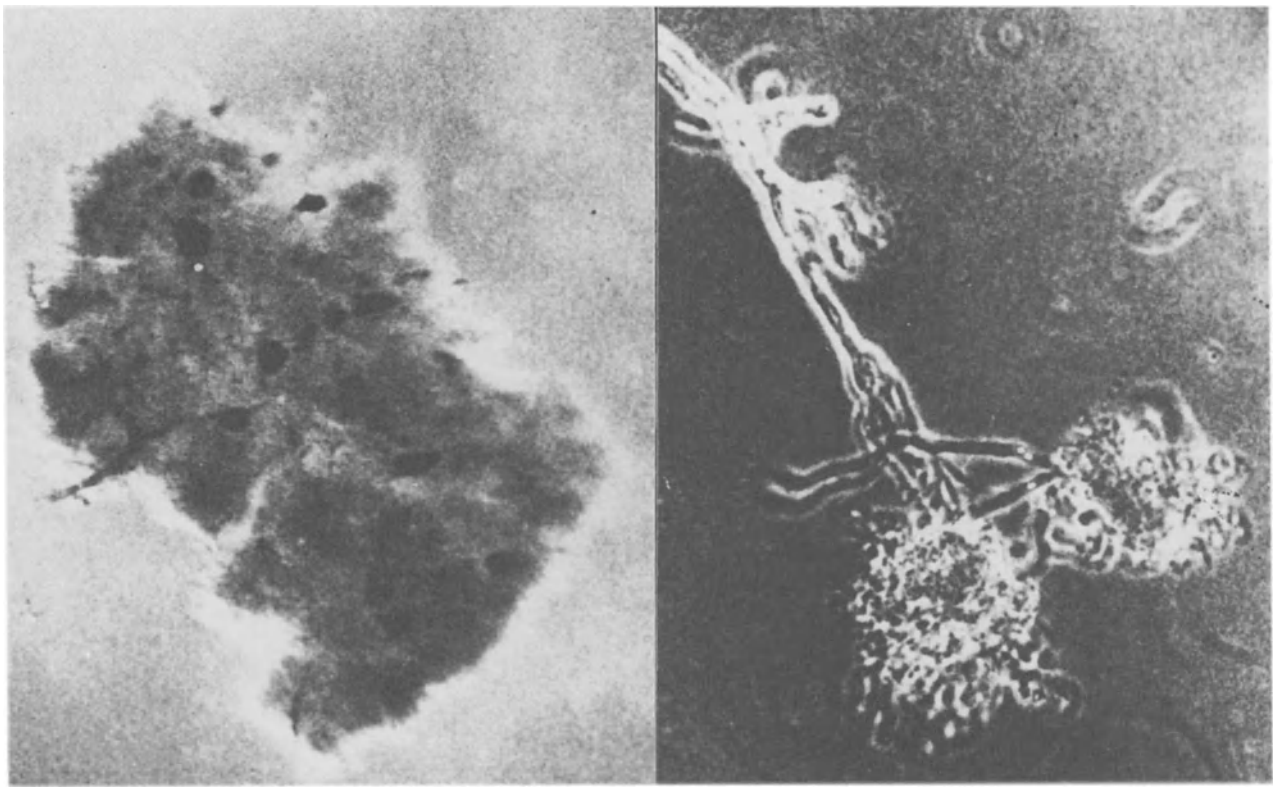


FIGURE 20 Flake of tissue (left) and capillary glia (right) from the lateral vestibular nucleus of rabbit, stained with methylene blue. The dark nuclei visible in the upper figure are regarded by Professor Hamberger as being oligodendroglial, as are most of the cells visible in the capillary glia. This figure is reproduced by kind permission of the author from Hamberger, A. (1963) Differences between isolated neuronal and vascular glia with respect to respiratory activity. Acta Physiol. Scand. 58, Suppl. 203, pages 1-58

HISTOLOGICAL TECHNIQUES

Histology developed as a branch of pathology for the correlation of the appearances of diseased tissues with clinical findings from patients. The correlations are empirical and do not imply that the tissue has the same appearance after staining as it did before. Tissue for histology has come from a dead animal; it has been fixed, dehydrated, embedded in a non-aqueous medium, cut a few μm thick, rehydrated, stained, probably counter stained, dehydrated again, cleared and embedded in a non-aqueous mountant. The effects of these procedures on the size, shapes, textures and colour of the cells and their organelles, are not only everyday observations for histologists, but have been carefully studied over a number of years (Tellyesniczky, 1898; Stowell, 1941; Ross, 1953; Baker, 1958; Gersh, 1959; Hillman and Deutsch, 1978).

In a single section one cannot usually know the original dimensions of a cell, the presence, position, dimensions of any of its organelles or any of their shapes (page 51). When one has made a number of serial sections, one can measure the dimensions as seen after preparation for histology. Unfortunately, even reconstructions from serial sections do not show the dimensions of the original cells in vivo. The cells and all their organelles have shrunk during dehydration, not all in the same proportion (Hertwig, 1931; Lodin, Mares, Karasek and Skrivanova, 1967; Hillman, Hussain and Sartory, 1976). The simplest explanation for the unequal shrinkage of parts of cells is that they each have different contents of water and are chemically very different. The experiments quoted above showed different shrinkage of nuclei and cytoplasm only. There is little information about the shrinkage of membranes during dehydration, except for the low angle diffraction studies of Finean (1957, 1960). He interpreted the diminution of the repeating period by 2-3 nm as being due to the loss of water, although it has also been proposed that it was due to loss of neutral mucopolysaccharide (Robertson, 1959; Wolman and Hestrin-Lerner, 1960). The myelin sheath has generally been regarded as a model for mammalian membranes of all kinds (Robertson, 1959; Adams and Davison, 1965; Fernandez-Moran, 1967).

The main changes occurring after histological preparation are as follows:

1. Shrinkage of the cytoplasm causes the neuron which was rather spherical in the unfixed state to become more concave between its main dendrites.
2. The cytoplasm of the neuron which is normally a fairly translucent liquid containing a few mitochondria precipitates, and crystalline deposits and granules appear within it.
3. Brownian movement, mitochondrial movement, rotation of the neuronal nuclei, and axonal flow, stop, probably due to denaturation of cytoplasmic, axonal and nucleoplasmic proteins.
4. The nucleoplasm, which was a translucent uniform fluid in both neurons and neuroglial nuclei, precipitates and becomes non-uniform.
5. The nucleolar contents in the neurons (the neuroglial nuclei do not appear to contain nucleoli) precipitate, making the nucleolonema appear amorphous and blurring the distinction between the nucleolonema and the pars amorpha. The precipitation also obscures the nucleolar membrane seen in neurons (Hussain, Hillman and Sartory, 1974). The inability to see the nucleolonema or nucleolar membrane in stained histological sections or in electron micrographs probably explains why their existence has been almost completely ignored. This implies that information derived from the latter preparations is more true than that found by examining unfixed untreated neuronal cell bodies, when a finding is made in the latter preparation, but not found in histological sections (Hillman, 1976).
6. The mitochondria, which appear clearly in the cytoplasm of unfixed cells in tissue culture, and both inside and outside neurons in isolated neurons (Hillman, Deutsch, Allen and Sartory, 1977; Hillman and Deutsch, 1978b), are difficult to see in either of these locations in sections of tissue. The simplest explanation is that they float out of the cytoplasm from the face of the very thin slivers of tissue into some of the reagents between the stages of sectioning and of final mounting. Another possibility is that they disintegrate due to 'osmotic shock' of hypotonic solutions,

or due to chemical attack by other agents. Such misfortunes may not befall the mitochondria in a whole uncut neuron or neuroglial clump.

7. Apparently empty holes or vacuoles may appear, which did not exist in life.
8. Membranes may become closer or more separated.

Thus, on the one hand one may see loss during preparation of some free mitochondria or granules which were present in the living tissue. On the other hand one may see appearing new 'structures', which are dehydrated cytoplasm, nucleoplasm, mitochondrioplasm or extracellular fluid, or are reaction products of any of the reagents used with them, or are composed simply of the reagents themselves. One must continuously try to peer into these glazed windows of our knowledge to perceive the blurred shadows of the shy reality which is the living tissue.

With all these reservations in mind, examinations of histological sections can give valid information about:

1. The presence of pathological states in tissue.
2. The number of a particular kind of cell or subcellular component in a particular section.
3. The relative positions of the components.
4. Their shape after shrinkage - an object which is still spherical after shrinkage has probably preserved its original shape. This is not necessarily true for electron microscopy, in which the metal deposit is exposed to electron bombardment and irradiation under high vacuum, and enough energy is liberated to make particles of nanometre dimensions or less become spherical.
5. The presence or location of a particular feature of the preparation, either if it always occurs in a particular situation, or if it has been shown statistically to occur at a more significant incidence in a particular part of the cell than it is found elsewhere.

Staining with haematoxylin and eosin is probably the most popular procedure used throughout histology and pathology. It is as well to remind ourselves that this technique has 14 steps and employs 11 different reagents. Neurons were isolated by hand dissection, but were not embedded and sectioned; they were fixed, stained with haematoxylin, counter stained with haematoxylin, counter stained with eosin, and the whole cell bodies were embedded in DPX medium. These steps resulted in a shrinkage of the cytoplasm to a mean of 25%, and the nuclei to 15%, of their original unfixed areas, respectively (Hillman, Hussain and Sartory, 1976; please also see Figure 16).

Obviously, different staining systems and different reagents change tissues to different extents, and it may be assumed that the fewer the reagents which are used the more accurately the histological section reflects the structure of the living tissue. In order to make comparison of the appearances of different kinds of cells easier and to diminish the number of reagents, a different approach has been tried. Six different tissues were embedded in paraffin wax and sectioned but not stained. They were examined under phase-contrast microscopy, and their appearances compared with those of sections stained with haematoxylin and eosin and viewed by bright-field illumination (Hillman and Sjuve, 1980).

Of course, light microscopy in general has its repertoire of artifacts, such as chromatic aberration, spherical aberration, Arey's discs, glare, coverslip thickness corrections, tube length corrections, errors due to the refractive index of the media, etc. These are well recognized, and can usually be avoided by well-trained microscopists (Hillman, 1982b).

A much more serious source of error are the precipitates, shrinkage artifacts, tears and cracks which result from the dehydration. Among these are the Golgi apparatus (Hillman and Sartory, 1980a, pages 63-66) and the cytoskeleton (please see ibid, pages 43-60)., as well as many apparent structures seen by electron microscopy (please see Table 17). It is appropriate to stress here two points; firstly, any solute must precipitate during dehydration if it does not diffuse away in, or is not dissolved in, the dehydrating agents; secondly, the constancy of any appearance is no evidence whatsoever, that it is not artifactual, pace many referees of journals.

It is extremely difficult to avoid some degree of subjective judgement in experimental histology. The following steps of the procedure contain subjective elements even in the hands of the most expert research workers: choice of species; choice of anaesthetic; method of killing the animal; selection of the sample; selection of the staining procedure to be used; decision on the precise duration of each step in the procedure; choice of type of microscopy, including the illumination and filters used; decision of magnification to be used to examine the tissue; decision on duration of the observations; decision on the number of observations to satisfy the histologist; choice of area to be photographed; choice of figures for submission for publication; reproduction of figures submitted for publication in the final printed form; decision on the nature of the features to be labelled; decision on which parts are to be labelled. In addition, there is some degree of subjectivity on the identification of the seven different major artifacts of light microscopy (White, 1966).

In considering the staining of neurons, astrocytes, oligodendrocytes and microglia, one has to bear in mind that the specificity of a particular stain for one of these elements rested mainly on the authority of the great histologists, such as Golgi, Cajal, Penfield, Del Rio-Hortega, Mallory and Glees. Most of them carried out their major research before the concept of control experiments had achieved the currency that it has today. These giants established the identifications of particular cells in an empirical fashion, and these have generally been accepted by neurologists and neuropathologists as working practices. Yet most authors nowadays, while diagnosing tumours both by the histological identification and by the neurological history of the patient, are well aware of the uncertainty of their identification of the cell types. In textbooks, each author shows an illustration of what he believes undoubtedly to be a clear and unequivocal example of a particular cell type. At the same time, there would be many cells in the brain which could not be identified certainly as being of a particular type, or about which two good histopathologists would disagree in good faith, without any objective way of deciding its identity. Thus one can draw up a table of authors who say quite specifically that one cannot distinguish clearly between any two kinds of cells by light microscopy (Table 2).

This understanding poses a considerable threat to the neurohistologists, who thought that they had been saved by the electron microscopists. Unfortunately, the

electron microscopists cannot be too much help, because they agree on the difficulty of distinguishing beyond any reasonable peradventure on the differences, particularly between the different kinds of neuroglia (Table 3). Also, they are usually using too high a magnification to examine many cells in one field.

Many authors cannot distinguish between astrocytes, oligodendrocytes and, sometimes, microglia, and admit this implicitly by using the term neuroglia, without stating to which variety they believe they are referring (Table 4).

The electron microscopists, the tissue culturists, the biochemists and the immunologists cannot help the light microscopists with the identification for a more general reason - that is - they themselves fundamentally depend upon the light microscopists to whom they refer their own preparations, or who have initially named the tissue of origin from which they took their pieces of tissue to examine or grow in culture. The different kinds of neuroglia were characterized by light microscopy more than two decades before the electron miscroscope was introduced to biology (Glees, 1955).

TABLE 2 Light microscopic studies in which the authors point to their inability to distinguish neurons and the different kinds of neuroglia

Cannot distinguish between neurons and astrocytes	Cannot distinguish between neurons and oligodendrocytes	Cannot distinguish between neurons and microglia
Deiters(1865)	Deiters(1865)	Deiters(1865)
Gerlach(1871-72)	Gerlach(1871-72)	Gerlach(1871-72)
Boll(1874)	Boll(1874)	Boll(1874)
Golgi(1883)	Golgi(1883)	Golgi(1883)
Spatz(1918)	Spatz(1918)	Brownson(1956)
Glees(1955)	Glees(1955)	Ramon-Moliner(1958)
Lewis(1976)	Ramon-Moliner (1958)	Brownson, Suter and Diller(1963)
Kennedy, Lisak and Raff(1980)	Kennedy, Lisak and Raff(1980)	

TABLE 3 Electron microscopic studies in which the authors point to their inability to distinguish between different kinds of neuroglia, or use the generic term 'neuroglia' without indicating a particular type

Cannot distinguish between astrocytes and oligodendrocytes	Cannot distinguish between astrocytes and microglia	Cannot distinguish between oligodendrocytes and microglia	'Neuroglia' used without differentiation into types
Luse(1956)	Farquhar and Hartmann(1957)	Farquhar and Hartmann(1957)	Wendell-Smith and Blunt(1965)
Farquhar and Hartmann(1957)	Palay(1958)	Kruger and Maxwell(1966)	Mori and Leblond(1969)
Maynard, Schultz and Pease(1957)	Schultz(1964)	Glees and Meller(1968)	Glees(1973)
Dempsey and Luse(1958)	Glees and Meller(1968)	Schultz(1964)	Schnapp and Mugnaini(1975)
Palay(1958a)	Orkand(1977)	Cammermeyer(1970)	Kuffler and Nicholls(1976)
Gerschenfeld, Wald, Zadunaisky and De Robertis(1959)	Privat(1978)	Matthews and Kruger(1973)	
	Roots(1978)	Lewis(1976)	
Gray(1959)		Peters, Palay and Webster (1976)	
Hartmann(1953, 1962)		Orkand(1977)	
Schultz(1964)		Privat(1978)	
Davson(1967)		Roots(1978)	
Cammermeyer(1970)			
Orkand(1977)			
Privat(1978)			
Roots(1978)			

TABLE 4 Light microscopic studies in which the authors point to their inability to distinguish between different kinds of neuroglia, or use the generic term 'neuroglia' without indicating a particular type

Cannot distinguish between astrocytes and oligodendrocytes	Cannot distinguish between astrocytes and microglia	Cannot distinguish between oligodendrocytes and microglia	'Neuroglia' used without differentiation into types
Deiters(1865)	Deiters(1865)	Deiters(1865)	Stricker and Unger(1879)
Jastrowitz(1870-71)	Jastrowitz(1870-71)	Jastrowitz(1870-71)	Held(1909)
Gerlach(1871-72)	Gerlach(1871-72)	Gerlach(1871-72)	Hess(1953)
Boll(1874)	Boll(1874)	Boll(1874)	Joseph(1954)
Golgi(1883)	Golgi(1883)	Golgi(1883)	Sholl(1956)
Penfield(1924)	Metz and Spatz(1924)	Del Rio-Hortega(1920)	Bairati(1958)
Rydberg(1932)	Urechia and Elekes(1925)	Metz and Spatz(1924)	Nurnberger(1958)
Bairati(1948;1958)	Bergman(1926)	Penfield(1924)	Lumsden(1953)
Ramon-Moliner(1958)	Creutzfeldt and Metz(1926)	Urechia and Elekes(1925)	Hyden(1961)
Koenig, Bunge and Bunge(1962)	Jakob(1927)	Bergman(1926)	Hamberger(1963)
Sulzmann(1962)	Pruijs(1927)	Creutzfeldt and Metz(1926)	Mori and Leblond(1969)
Schultz(1964)	Besta(1929)	Metz(1926)	Shantha, Manocha, Bourne, and Ariens Kappers(1969)
Truex and Carpenter(1969)	Cammermeyer(1963)	Jakob(1927)	Glees(1973)
Cammermeyer(1970)	Schultz(1964)	Pruijs(1927)	Watson(1974)
Orkand(1977)	Orkand(1977)	King(1937)	Kuffler and Nicholls(1976)
Raff, Fields, Hakamori, Mirsky, Pruss and Winter(1979)		Bauer(1953)	Fischbach and Nelson(1977)
		Clemente and Holst(1954)	Currie and Kelly(1981)
		Polak(1956)	
		Field(1957)	
		Ramon-Moliner(1958)	
		Cammermeyer(1963)	
		Estable-Puig, De Estable, Tobias and Haymaker(1964)	
		Schultz(1964)	
		Hamberger, Hansson, and Sellstrom(1975)	
		Lewis(1976)	
		Orkand(1977)	

The different elements of the 'cytoskeleton' are identified by immunofluorescent antibodies which are said to be more or less specific, and often also cross-reactive (Moll, Franke, Schiller, Geiger and Krepler, 1981). The elements are viewed under ultraviolet microscopy, but measured by electron microscopy. They are often identified in tissue cultures, but, to my knowledge they have never been seen in living cells. The cells may have to be 'permeabilized', that is fixed, frozen or stained for electron microscopy and dehydrated. The biochemistry of each of the elements is worked out after extraction of the tissue with powerful reagents, and then comparing the diameters from electron microscopy, the fluorescence from light microscopy and the biochemical properties of the extracts.

The accuracy of the identification of each element depends upon:

1. the nature of the fixative (including freezing) and any detergent used;
2. the degree to which fixation can prevent diffusion, whether or not 'permeabilization' has been used;
3. the specificity of the antigen, or any first or second antibody;
4. the penetration into the cell of the antibody from incubating medium;
5. the degree of the change of dimensions of each cellular constituent during preparation for histology, histochemistry or electron microscopy;
6. the effect of any of the reagents on the intensity of the antigen-antibody reaction;
7. non-specific uptake of antibody by elements in the tissue;
8. the effect of any of the reagents on the intensity of the fluorescence;
9. when immunofluorescence is used on tissue cultures, the intensity of the fluorescence may not be the same as it would be in the parent tissue from which the culture was taken.

Thus the light microscopist's view remains crucial and central, although apparently by no means certain. It thus behoves us to re-examine the whole fabric of current belief about these cells.

TISSUE CULTURE

Until now, techniques have not yet been developed for seeing living cell bodies in intact adult mammals. The nearest one can approach to knowledge about the living cell in vivo is to study preparations which have been minimally interfered with. These include cells in tissue culture, peripheral axons which fire, microdissected unfixed somas, and neural explants in the anterior chamber of the eye. One can also look at large non-mammalian cells either without removing them or in circumstances in which they can be visualized, or in conditions in which they fire after isolation: among such preparations are leech neurons and neuroglia, squid and cuttlefish axons, lobster stretch receptors, goldfish Mauthner cells and puffer supramedullary neurons. It is probably a reasonable assumption that the basic anatomy and the physiology of neurons from non-mammalian species are similar to those from mammals, although their biochemistry may be different.

Cells in tissue culture are normally judged to be living by observing intracellular movements with the non-invasive camera. The time-lapse films of Pomerat and his co-workers and Hansson and Sourander (1964) using retina, have given us new insights

into living cellular anatomy. There has also been an enormous development in the past decade of biochemical studies on the cells in culture (Fedoroff and Hertz, 1977; Schoffeniels, Franck, Tower, and Hertz, 1978; Pevzner, 1979; Raff, Fields, Hakamori, Mirsky, Pruss and Winter, 1979). While interesting in themselves, the biochemical properties of the different kinds of cells, particularly the neuroglia, depend crucially on their identification in the whole tissue and their later certain recognition in culture.

Tissue cultures give one a unique method of observing living cells, and the extrapolation is generally accepted that the findings from its use are true for the whole animal in vivo. This is an inescapable assumption. Some years ago we tried to observe living cells on the surface of the rat brain using vertical illumination microscopy, but unfortunately the tissue turned out to be too transparent. Nevertheless, such endeavours are still worthwhile pursuing in the future.

When cells or tissues are first put in culture there is a latent period during which they tend to be spherical and exhibit minimal intracellular movements. Subsequently, they begin to put out processes, which later develop into dendrites. The whole cells then gradually become flatter and may form a monolayer. Cells, which Pomerat and Costero (1856) identified as astrocytes, showed undulating movements. The elegant web-like dendrites are very difficult to differentiate from glial processes, unless one has decided on whether they take origin from a neuron or a neuroglial cell. It is of particular interest also, that the latter authors head one of their descriptions of the culture as 'Cells having some of the characteristic features of neurons'.

From the anatomical viewpoint a few generalizations may be made about the different sort of cells in culture. Firstly, in early cultures most cells, including those from non-neural tissues, look very similar. Tissues for culture must normally be taken from embryos, from very young animals or from tumours. The fact that the living cells can be examined in culture permits us to use them to describe general properties of living cells, which can be seen to be lost when the cells are fixed or killed. Among these properties, some cells show intracytoplasmic movements, clear nuclei, cell pulsation, electrical excitability, conduction, physiological synaptic activity, neuromuscular activity, myelination and intranucleolar movements. It therefore seems reasonable to entertain the assumption that the structures engaging in any of these phenomena are present in culture, and probably represent the minimal machinery which can exhibit them. Thirdly, many authors of papers on tissue cultures cannot distinguish the particular cells (Table 5) or do not describe the anatomy of the cells which they claim to identify (Table 6). A sample of the relatively few descriptions of the cells is given, and they can be seen to be vague and overlapping, both as seen by light and by electron microscopy. The commonest modes of identification are those derived from knowledge of the tissues or tumours from which they originated, or from identification by the use of biochemical 'markers' (Table 7). The greatest danger lies in the latter approach, since the correlations between the biochemical markers and the cells which they are believed to show up are not as good as they should be. When pressed, the research workers often adduce other evidence from classical light microscopy.

Fourthly, as mentioned above, the appearance of cells in culture changes. They 'mature', they de-differentiate - especially if cultured as a cell line - they degenerate, they may autolyse, and sometimes they disappear, presumed to have exploded. Of

TABLE 5 Light microscopic studies of tissue culture in which the authors point to their inability to distinguish neurons and the different kinds of neuroglia

Cannot distinguish between astrocytes and oligodendrocytes	Cannot distinguish between astrocytes and neurons	Cannot distinguish between oligodendrocytes and neurons	'Neuroglia' used without differentiation into type
Lumsden(1958)	Lumsden(1958)	Lumsden(1958)	Geiger(1957)
Murray(1965)	Fedoroff(1978)	Fedoroff(1978)	Lumsden(1958)
Lindsay, Barber, Sherwood Zimmer and Raisman(1982)	Lindsay, Barber, Sherwood Zimmer and Raisman(1982)	Lindsay, Barber, Sherwood Zimmer and Raisman(1982)	Bornstein and Appel(1961)
			Hild and Tasaki(1962)
			Varon, Raiborn, Seto and Pomerat(1963)
			Murray(1965)
			Vernadakis and Culver(1979)

TABLE 6 Authors using illustrations of light microscopic studies of tissue cultures who have not indicated anatomical criteria that they use for identification of particular cell types

Tissue	Authors
Human, monkey and rabbit cortex	Geiger(1957)
Kitten corpus callosum	Murray(1958)
Rat cerebellum	Bornstein and Appel(1961)
Rat and cat cerebellum	Hild and Tasaki(1962)
Rabbit cerebral hemispheres and cerebellum	Varon, Raiborn, Seto and Pomerat(1963)
Kitten thalamus and cerebellum	Murray(1965)
Rat cerebral hemispheres	Dittman, Sensenbrenner, Hertz and Mandel(1975)
Rat cerebral hemispheres	Moonen, Cam, Sensenbrenner and Mandel(1975)
Mouse dorsal root ganglion	Shahar, Grunfeld, Spiegelstein and Monzain(1975)
Mouse and rat spinal cord	Crain(1976)
Mouse cerebellum	Seil and Leiman(1977)
Mice brain hemispheres	Fedoroff(1978)
Rat optic nerve	Raff, Mirsky, Fields, Lisak, Dorfman, Silberberg, Gregson, Leibowitz and Kennedy(1978)
Rat brain	Lindsay(1979)
Rat corpus callosum, optic nerve, cerebral cortex, cerebellum	Raff, Fields, Hakamori, Mirsky, Pruss and Winter(1979)
Rat and mouse brain hemispheres	Hannson, Sellstrom, Persson and Ronnback(1980)
Rat brain	Abney, Bartlett and Raff(1981)
Rat cerebellum	Bartlett, Noble, Pruss, Raff, Rattray and Williams(1981)
Rat cerebellum	Cohen and Selvendran(1981)
Rat cerebellum	Currie and Kelly(1981)
Rat brain	Lindsay, Barber, Sherwood, Zimmer and Raisman(1982)

TABLE 7 Ratios of the activities in neurons and neuroglia of 51 different enzymes from the literature

Enzyme	Glia	Ratio of activity of neurons to Astrocytes	Ratio of activity of neurons to Oligodendroglia
Group I. Oxidoreductases			
Lactate dehydrogenase	1.8		
Malate dehydrogenase	2.6		
Malate dehydrogenase	0.2		
Isocitrate dehydrogenase	0.7		
6-Phosphogluconate dehydrogenase	0.6		
Glucose-6-phosphate dehydrogenase	0.4		
α-Glycerophosphate dehydrogenase	0.5		
Succinate dehydrogenase	0.8		
Succinate dehydrogenase	0.2		
Glutamate dehydrogenase	1.6		
Glutamate dehydrogenase	2.9		
Monoamine oxidase	1.8		
Monoamine oxidase	1.3		
Monoamine oxidase	1.4		
Monoamine oxidase	2.2		
Monoamine oxidase	2.8		
Monoamine oxidase	2.3		
Monoamine oxidase	1.1		
Monoamine oxidase	2.7		
Cytochrome oxidase	0.4		
Cytochrome oxidase	0.8		
Cytochrome oxidase	0.9		
Cytochrome oxidase	0.4		
Succinate: cytochrome c reductase	0.2		
Succinate oxidase	0.5		
Succinate oxidase	1.9		
Succinate oxidase	0.4		
Succinate oxidase	1.4		
Succinate oxidase	2.8		
Group 2. Transferases			
Choline acetyl transferase	1.1		
Galactosyl transferase		1.2	0.6
Sialyl transferase	2.4		
Aspartate amino transferase	2.0		
Aspartate amino transferase	1.0		
Hexokinase	3.2		
DNA polymerase	1.7		
DNA polymerase	0.2		
Acetylneuraminate cytidyltransferase	1.3		
Cerebroside sulphotransferase	0.1		
Group 3. Hydrolases			
Phospholipase A_2	5.5		
Butyryl cholinesterase	1.1		
Butyryl cholinesterase	2.5		
Butyryl cholinesterase	3.6		
Acetylcholinesterase	1.7		
Acetylcholinesterase	3.1		
Acetylcholinesterase	2.2		
Acetylcholinesterase	1.0		
Phospholipase A	8.1		
Alkaline phosphatase	6.0		
Acid phosphatase	1.4		
DNase:			
acid	6.1		
alkaline	4.3		

Enzyme	Glia	Ratio of activity of neurons to Astrocytes	Oligodendroglia
Group 3. Hydrolases (cont)			
2',3'-cyclic nucleotide 3'-phosphohydrolase		1.0	0.1
2',3'-cyclic nucleotide 3'-phosphohydrolase		0.2	0.2
2',3'-cyclic nucleotide 3'-phosphohydrolase	0.1		
Aryl sulphatase	0.9		
β -glucosidase	1.1		
β -glucosidase	6.8		
β -galactosidase	0.9		
β -galactosidase	0.9		
β -galactosidase	9.9		
β -mannosidase	0.8		
β -mannosidase	1.3		
β -glucosaminidase	0.9		
β -glucosaminidase	1.2		
β -glucosaminidase	9.2		
β -glucosaminidase		0.3	0.1
β -glucuronidase	1.5		
β -glucuronidase	3.4		
Glucocerebrosidase	1.4		
Glucocerebrosidase	1.1		
Galactocerebrosidase		1.1	0.9
N-acetyl-β-galactosaminidase	1.0		
N-acetyl-β-galactosaminidase	1.0		
N-acetyl-β-galactosaminidase	0.6		
Arabinosidase	3.4		
Cathepsin	6.8		
ATP ases			
Mg^+activated	5.7		
K^+,Na^+ activated	0.1		
K^+,Na^+ activated	0.4		
K^+,Na^+ activated	0.2		
Ouabain-insensitive	0.2		
Group 4. Liases			
Glutamate decarboxylase	1.6		
DOPA decarboxylase	0.9		
Carbonic anhydrase	0.1		
Carbonic anhydrase	0.1		
Carbonic anhydrase	0.2		
Guanylate cyclase	2.1		
Group 5. Isomerases			
Glucosephosphate isomerase	2.0		
Group 6. Ligases			
Glutamine synthetase	1.7		

It should be noted that no ratio exceeds 10, and that few authors distinguish the different types of neuroglia. Pevzner concludes that 'in our opinion, the existence of a strictly neuronal or glial enzyme marker is inherently unlikely'. This table is reproduced from Pevzner, L.Z. (1979) Functional Biochemistry of the Neuroglia (Consultants Bureau, New York and London), pages 51-63, by kind permission of the author and publishers

course, de-differentiation represents the greatest difficulty for the microscopist trying to identify cells. Identification of neurons is facilitated by their producing classical action potentials when they are stimulated. Cells which produce slower transients of lower voltage are presumed to be neuroglia (Hild and Tasaki, 1962; Crain, 1976) but another possibility is that they are neurons whose spike-generating machinery has been changed by their isolation or the culture media.

Fifthly, probably the staining properties, the biochemical properties and the antigenicity of the original cells change during the isolation and growth in culture. There seems to be a totally inexplicable and dangerous lack of experiments, in which (a) the classical stains of the different kinds of neuroglia have been used to demonstrate the same kinds of cells in culture, (b) the biochemical properties in culture have been shown in tissue sections in regions where the characteristic cells occur in large numbers (it is argued here that in the tissues, as opposed to the cultures of 'pure' kinds of cells, particular kinds of cell occur all mixed up with others, which would make the biochemistry difficult to disentangle); (c) with a few exceptions, most of the 'specific' markers of cells in culture have not been shown to behave similarly in sections, and never in vivo; (d) nearly all immunological techniques used fixed and frequently dehydrated tissue cultures (Pickel, 1981 and sources quoted there). It is argued that the antibody could not enter the living cell. Yet a fixed, dehydrated cell is a dead cell, and antigen-antibody reactions should be expected to work better in living cells. There is a great paucity of experiments in tissue culture, in isolated cells or in whole tissue, showing such properties in live tissues. This is a particularly crucial question for those studying catecholamines, endorphins, enzyme markers, etc., in the nervous system in relation to the possible roles of these substances in mental diseases, or in animal models of those diseases.

ELECTRON MICROSCOPY

The electron microscope has a much higher resolution than the light microscope - in practice better than 0.3 nm (Cosslett, 1982). No biological tissue can survive the electron beam, the high vacuum and the radiation, so the tissue must be stained, that is, have deposited on it salts of heavy metals; this deposit is examined in the electron microscope. If one looks at an unfixed cell in a physiological medium by light microscopy one is looking only at a real tissue; when one looks at a stained histological preparation one is examining tissue minus substances removed during preparation plus added reagents - it thus represents tissue plus artifact. In the electron microscope one is only looking at metal or metal salt, that is, the image is of an artifact. Of course, that does not mean to say that the electron microscopic artifact has no value, but clearly it has less value than the fresh tissue as a source of information.

In light microscopic histology one may be looking at a colour image due to, say, eosin, dichromate or pictrate, staining the tissue, or a deposit of salt of silver or osmium. The former solutions impregnate certain parts of cells, while the latter react with and precipitate on the structures; the former are colour stains, the latter are solid deposits and have grain. All the 'stains' used in electron microscopy - commonly, salts of osmium, lead, tungsten - have relatively poor solubility;

they precipitate on reaction with the tissue. Although I have often been told that electron micrographs have been published, or are in the possession of research workers, showing clear detail in tissues without any heavy metal stain, used at any time during the procedure so far no-one has referred me to any such pictures, nor favoured me with samples of their own work. Often, electron microscopists have used osmic acid as a fixative, or 'post-osmicated' a tissue, and have implied that the osmic acid was thus not used as a stain. Does this presume that this expensive salt knows when it is meant to act as a fixative, and when it is meant to act as a stain?

Incidentally, osmic acid, which is generally believed nowadays to stain proteins, has been used in light microscopy as a stain for fats since the nineteenth century (Mann, 1902).

Obviously, a deposit will react with both sides of a membrane of single thickness. Thus, any heavy metal reacting with a real membrane will be seen as two lines if the magnification is high enough. In our view that is one reason for the 'unit' membrane of Robertson (1959). Every single 'membrane' seen by electron microscopy, except that seen around 'lysosomes', appears double. Of course, a 'lysosome' is a solid particle, so the heavy metal deposit has only one ring around it. (For further discussion, please see pages 132-133).

Attempts have been made to examine living or hydrated cells by electron microscopy, either by using very high voltages like 3 MeV (Dupouy, 1972; Glauert, 1979), or by placing the tissues in wet chambers (Parsons, 1974, 1978). One awaits with hope the discovery with these instruments of new findings or greater detail, which so far have eluded classical light microscopy or electron microscopy.

Electron microscopy shares with histology all the difficulties arising out of the thickness of section (please see pages 49-62) and of the preparation techniques (Boyde, Bailey, Jones and Tamarin, 1977; Hillman and Deutsch, 1978a). In addition the technique as applied to biology has further problems:

1. The results of observation on what must be minute samples of the specimen are rarely controlled statistically; Cragg (1972) is exceptional in this respect. Whereas in light microscopy most histologists would agree on the identity of a particular structure, there would not necessarily be such wide agreement by electron microscopists. They have many fewer samples to examine, and they miss the dimension of colour.
2. Many structures seen by electron microscopy, whose existence in life we deny, are not seen in serial sections to fit together, or we have failed to find published evidence of these in the literature (please see pages 126-127). We would be interested to be referred to any such publications or receive copies of electron micrographs of serial sections. The structures in question are listed in Table 17. I would like to reiterate our previous assertion that any alleged structure of which one cannot make a three-dimensional transparent model must be an artifact or an optical illusion. No exercise of intellectual acrobatics can avoid the hurdles of geometry.
3. Attempts have been made to use biochemical evidence to support the electron

microscopic findings, but of course, since current biochemical findings are interpreted by reference to the electron microscopic images, the biochemistry cannot be used as evidence in this respect. It would be a circular argument to do so.

4. The difficulty of calibrating the electron microscope has been regarded as both an instrumental problem and as a difficulty of choice of object for calibration (Reimer, 1965; Bahr and Zeitler, 1965; Baumeister, Fringeli, Hahn, Kopp and Seredynski, 1976). Little attention has been paid to dimensional changes in the test objects under the conditions of the electron bombardment, which are almost certainly very different from the dimensional changes of the metal deposits and epoxy resins under the same regime.
5. After these reservations have been fully accounted for in our measurements, calculations and conclusions, electron microscopy can be used to give similar information to that derived by using histological techniques (please see pages 34-35).

SUBCELLULAR FRACTIONATION

If one wishes to draw conclusions about the structure of living cells or their organelles from subcellular fractionation, one is assuming that homogenization, centrifugation, separation and preparation for electron microscopy, have no significant effects on the size, shape and staining properties of the organelles under study (Hillman, 1972).

Biochemists who work with the mitochondrial fraction use the same term for the body in a section of tissue as they do for the same apparent structure in the subcellular fraction of the same name. It is worth noting that in both cases the whole mitochondria appear in the full range of shapes which geometry would dictate. Unfortunately, the cristae do not appear so. It is remarkable how frequently in a 'good' preparation they appear to be normal to the plane of section. A biochemical preparation said to be mitochondrial is seen to be packed with what are undoubtedly mitochondria. However, the preparation supposed to be composed largely of endoplasmic reticulum is called the 'microsomal' fraction; this is because it is also believed to contain cell membranes, as well as other intracellular membranes. There is obviously an assumption implied here that intracellular and extracellular membranes are structurally and biochemically similar. Such an assumption has not been proved. It would obviously not survive acceptance of the view that the endoplasmic reticulum and the cytoskeleton are artifacts. Nonetheless, the use of the term 'microsomal' obscures this assumption, and also permits one to conclude that an enzyme, marker, transmitter or chemical activity found in the microsomal fraction may be intracellular or intramembranous. It also makes the results of such experiments compatible with the findings of other authors, who claim that any such substance is located in either of these regions.

3
Cellular Identification – General Comments

The identification of each particular cell type and of the intercellular tissue, and the description of its structure, depends upon the following characteristics:

1. Their appearances by light and electron microscopy, including their relationship to other cells (Tables 8 and 9).
2. The specific staining systems by which they are demonstrated (Table 10).
3. Their physiological properties.
4. The markers, such as enzymes, proteins and transmitters they contain, and the antibodies and toxins for which they show affinity.
5. Their appearance in tissue culture (Table 11).

APPEARANCE OF CELLS

It cannot be stressed too much that the identification by light microscopy of the neurons, the neuroglia and the ependymal cells, is crucial to the understanding of neurohistology, since this was the technique by which the cells were described originally, and all other identifications of cells are still ultimately derived from these original descriptions (please see Table 1). It must be emphasized that none of the other categories under 1 to 5, nor those listed in Table 1, represent independent categories for classification. It has sometimes been said that when, for example, the identification of a particular cell by electron microscopy has failed to be unequivocal, it can be confirmed independently by the presence of a particular marker shown by immunocytochemical techniques.

GEOMETRY OF CELLS

In examining the structure and appearance of cells, a number of questions arise. Can one describe the three-dimensional appearances of cells merely by looking at a few sections? The simple answer is that one can only do so either by making and studying serial sections systematically, or by using very thick sections, or by isolating the particular cells. The necessity for serial sections is demonstrated by the following simple model (Figure 21). Three eggs of the same size were taken as models of a neuron whose soma had a maximum diameter of 70 μm. Eleven sections equivalent to 6 μm thickness were cut in three different co-ordinates. If one considers the egg

yolk at the nucleus, and the whole egg as the cell, it becomes obvious that, without serial sections or very complex stereological calculations, one cannot describe: the dimensions of the cell, the nucleus or any intracellular body in any plane; the shape of the cell, the nucleus or any intracellular body; the absence of the nucleus or any intracellular body; the number of any small intracellular organelles; the position of the nucleus or any intracellular body; the absolute or relative intensity of staining of any membrane or part of the cell; the number of attachments of synapses to the cell bodies. If one were then to attach prolongations representing axons or dendrites to this model, it would then become clear that, without serial sections, one could also not tell: the number of such processes; their thicknesses; their lengths; their directions; their absolute or relative intensities of staining. Of course, one could not know if any parts of the cells were moving.

In addition to these geometrical difficulties, the total and relative changes in dimensions during histology make it problematic whether one could make real measurements - reflecting the state of the living tissue - of the total or relative dimensions of any membrane or intracellular body without knowing the changes that have occurred.

One can go some way to calculating dimensions:

1. if one assumes that the cells have similar dimensions in a plane at right angles to the section as they do in the plane of the section - (this will not be true for any asymmetrical body which is randomly orientated, or for any body which is not randomly orientated);
2. if one has in a single section a very large number of randomly orientated cells or intracellular bodies;
3. if one has many sections;
4. if one cuts sections whose thickness is much greater than the average maximum dimension of the cells or intracellular bodies;
5. if one examines serial sections, or isolated cells.

The problem of shrinkage can only be solved by measuring the degree of shrinkage of the body in which one is interested as the staining proceeds, and then correcting for it.

Thick sections have considerable advantages from the geometrical viewpoint, but optically they are not so satisfactory. The injection intracellularly of such markers as horseradish peroxidase to define the axon and dendritic trees of cells represents an important advance for cell localization, but unfortunately the tissues are normally subjected to histological procedures subsequently (Muller and McMahan, 1976; Kitai and Bishop, 1981). Isolating cells by hand dissection involves pulling off some of their processes, which makes it difficult to count them unless one can see all the broken ends.

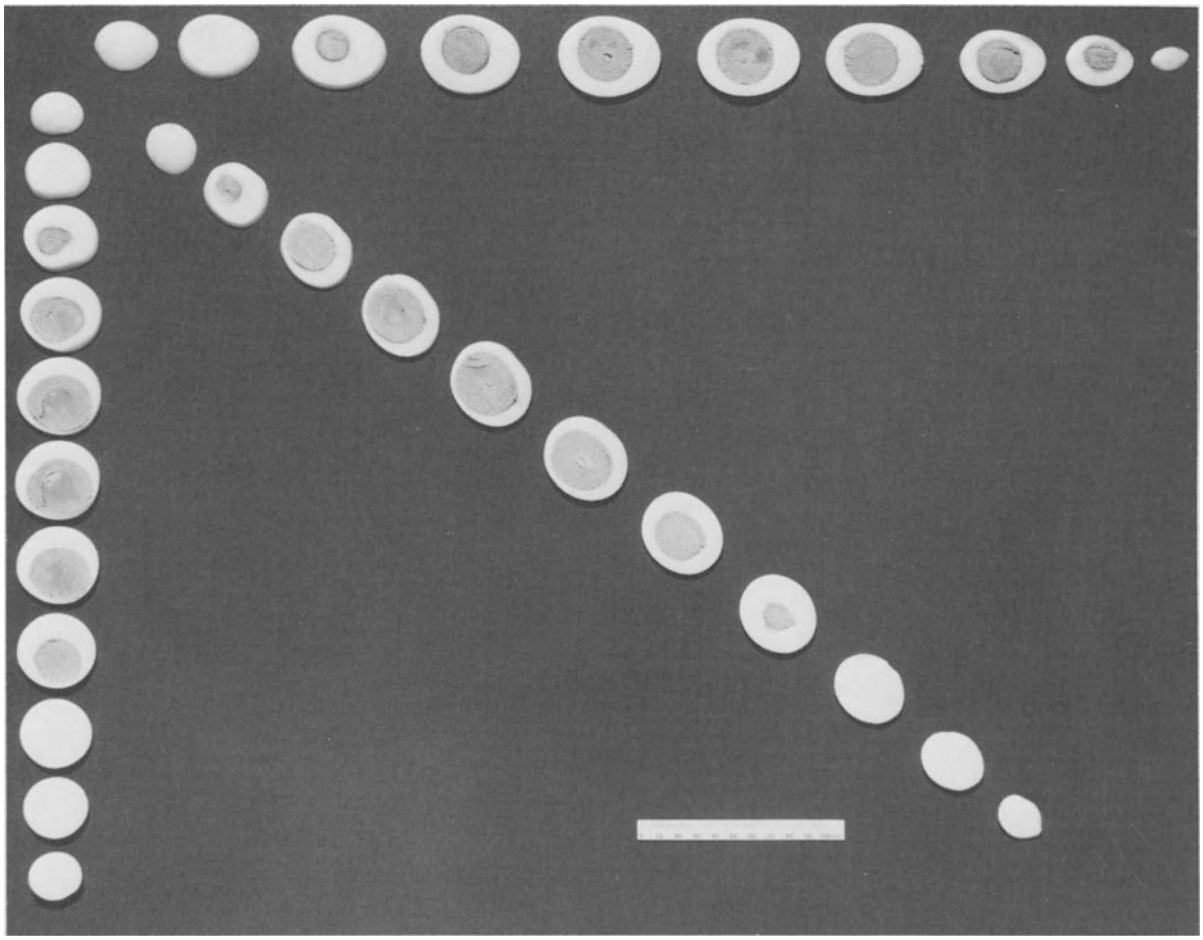


FIGURE 21 The different appearances, dimensions and positions of the nuclei which could be obtained from sections of a cell cut vertically, horizontally and diagonally. Three eggs as models of neurons of 70 µm maximum diameter were sectioned at a thickness equivalent to 6 µm corresponding to that of a histological section

Classical histology can yield information about the numbers of cells in a particular plane, their relative positions in a tissue, and their general appearances. A few examples may be given; they can be used to find out: whether cells have divided recently; whether they are adjacent; if an epithelium is columnar cell or squamous; if cells are secretory; if they are symmetrical; if they originate from particular tissue, such as brain, muscle, liver or kidney; if they are granular; if they are pleiomorphic; if they are phagocytic; if they are lysing; whether there is much connective tissue around them; their approximate shapes if they are relatively symmetrical (see also pages 34-35).

These geometrical and histological reservations make one very careful in interpreting much data in the literature reporting structures or dimensions of cells or their

Table 8 The appearance of allegedly different cellular elements in the central nervous system as described by the authors. Lack of information under any heading indicates that it was not supplied in that publication

Form	Size	Processes	Nucleolus
APPEARANCES OF NEURONS BY LIGHT MICROSCOPY			
Irregular		Varying, number of dendrites which repeatedly bifurcate: single large axon from cell body, second system of small axons from dendrites	Present
Polygonal Fusiform: markedly elongated, slender ovoid cells with smooth contours Triangular cells of greater bulk than the fusiform Unipolar fusiform cells. Elongated spindle shaped, more or less uniform		Four to six, slender, irregular highly branched, originating from angles of cell body Thick rectilinear protoplasmic expansion takes origin from the two poles of the cell body Three divergent, infrequently branching expansions	
Spherical, flask-shaped, multi-polar, pyramidal and spindle	4-435 μm diameter	Many branching dendrites, single axon	Present
		One axon and several dendrites unipolar, bipolar or multi-polar	Large and spherical
More or less conical perikaryon or spherical		Fine, smooth axon, fine dendritic processes	
Varies from almost spherical to slightly flattened prolate spheroids	Largest cell, highly variable in size	Vary from a few to thousands	One or more present
	100 μm width and 100-200 μm long	Extend for up to 300 μm and branch several times	
Vast range of shapes	Golgi type I: long axons and large cell bodies Golgi type II short axons and usually small cell bodies	Vary in number from one to a score and each dendrite may branch scantily or into a fantastic bush: also has one axon which may branch	Generally conspicuou
Fusiform, stellate, pyramidal, polyhedral spherical, conical etc.		Unipolar, bipolar or multi-polar; one axon and one or more dendrites	Usually more than one nucleolus present,with a nucleolus rich in RNA

Nucleus	Cytoplasm	Author/tissue
Present	Granular	Deiters (1865) ox - spinal motoneurones
Present		Ramon Y Cajal (1892) brain cells
Present	Fibrillary, Nissl granules present (tigroid masses)	Barker (1899) mammalian
Large and spherical	Contains mitochondria, Golgi apparatus, Nissl granules and neurofibrils, pigment granules	Ranson and Clark (1948) vertebrates
		Sholl (1956) cat - visual cortex
Nuclear membrane appears as a thin linear structure	Nissl substance present; mitochondria	Hyden (1961) rabbit Deiters' neurons
	Presence of lipochrome pigment (yellow granules) grouped at one or both ends of cell body. Cytoplasm was a firm gel	Roots and Johnston (1964) ox - red nuclei and lateral vestibular nuclei
Commonly large, and stains lightly, the chromatin network being composed of fine diffused fibrils	Mitochondria present as tiny, rod-like bodies Nissl bodies appear in cell and dendrites but not the axon, and vary in shape, size arrangement and number	Elliott, (1969)
Single nucleus usually centrally located, nucleoplasm is gelatinous. Cajal's accessory body sometimes present; intranuclear rodlet sometimes seen	Cytoplasm is of a soft gelatinous nature, in which mitochondria, Golgi apparatus, ergastoplasm and vacuoles are suspended; Nissl substance present and neurofibrils, and lysosomes	Shantha, Manocha Bourne and Ariens - Kappers (1969) brain cells

Table 8 continued

Form	Size	Processes	Nucleolus
Polygonal, pyramidal spherical or flask-shaped			Prominent
Complex, angular or polygonal with a slight concavity of cytoplasmic surfaces between the sites of the emergence of the processes	Large	Several short radiating dendrites and single long axon; axon may branch; multipolar normally	Single, conspicuous
Varied	Varied, 5-80 μm diameter	One axon, one or more dendritic processes	Large central nucleolus present-spherical
Unipolar, bipolar or multipolar		One axon and a variable number of dendrites	
Multiangular		A number of tapering, twisting and ramifying dendrites	Large, conspicuous and spherical
APPEARANCES OF ASTROCYTES BY LIGHT MICROSCOPY			
Star-shaped		Processes radiate in all directions, possess one or more perivascular expansions which are applied to the surface of small vessels	
		In fibrous astrocytes fewer straighter processes than in protoplasmic astrocytes. Both types attach to small blood vessels by one or more perivascular feet	None
		Fewer in number, longer and thinner-fibrous astrocytes one or more end-feet per cell	Present
		Attached to blood vessels by processes terminating in perivascular feet	Absent
Stellate or star-shaped	Protoplasmic astrocytes are generally smaller than fibrous ones (2-35 μm long)	Processes radiate in all directions, numerous, some scarcely branch, some branch a lot	Often absent
		Varying number radiating in all directions; have end-feet to blood vessels; sometimes envelops nerve cells with its processes	

ıcleus	Cytoplasm	Author/tissue
ingle nucleus is well visible, ırge and round	Cytoplasm can be abundant or small in amount, Nissl substance obvious; neurofibrils, mitochondria and Golgi apparatus are present	Ralis, Beasley and Ralis (1973) human - central nervous system
ırge, pale, spherical or lightly ovoid, usually centrally laced in perikaryon; very fine niformly dispersed chromatin ırticles	Cytoplasm - perikaryon, contains neurofibrils chromophilic substance or Nissl bodies, Golgi apparatus, mitochondria, a centrosome and various inclusions	Bloom and Fawcett (1975) man, rat - central nervous system
esicular nucleus, contains ıcleoplasm and chromatin ıcleolar satellite present	Nissl granules present, mitochondria and neurofibrils	Greenfield (1976) mammalian - brain and spinal cord
ononucleate		Landon and Hall (1976) vertebrate - central and peripheral nervous system
ırge, round vesicular, located n centre of cell body	Nissl bodies present in different sizes and shapes (absent in axons but present in perikarya and dendrites); Golgi body present in mitochondria	Peters, Palay and Webster (1976) rat - cortex
	Granular cytoplasm which contains gliosomes	Ramon Y Cajal (1913) human - cerebrum
ırgest glial nuclei; irregul-rly oval; small amount of cattered chromatin	Granular or finely reticular; centrosome present; Golgi apparatus visible only in young mammals pigment granules present and gliosomes	Penfield (1932) mammalian - cerebral cells
ırgest glial nuclei, usually gg shaped	Less perinuclear cytoplasm - fibrous astrocyte; gliosomes, mitochondria, centrosomes fuchsinophile granules, pigment granules all present	Glees (1955)
ame size as small nerve cells rregularly oval, stain lightly, ontains granules of chromatin		Ranson and Clark (1959)
val or egg-shaped. Consider-bly larger than those of ligodendroglia. Chromatin is ispersed in the karyoplasm	Somewhat granular cytoplasm contains vacuoles sometimes scanty; centrosome, Golgi body, gliosomes, glial fibres, pigment granules all present	Hosokawa (1963)
ound or oval, occasionally longated; larger than that of ligodendroglia or microglia; ıt usually only 1 nucleus, but ccasionally two or more of ifferent sizes		Mannen (1963) mammalian

Table 8 continued

Form	Size	Processes	Nucleolus
		Long wavy processes contain fibres (fibrous astrocytes); numerous, branched feathery; (protoplasmic astrocytes)	
			Conspicuous
		Multiple - called neuroglial fibres; perivascular feet attached to walls of small blood vessels and capillaries	
		Numerous, rather thick processes many being attached to the blood vessels and the pia mater by expanded pedicles; or long relatively thin smooth processes	
		Numerous, branching processes, sometimes attached by vascular end-feet to capillaries	
		Long and branched, occasionally capillary end-feet seen	
APPEARANCES OF OLIGODENDROCYTES BY LIGHT MICROSCOPY			
	Smaller than astrocytes	Smaller, more delicate expansions than astrocytes	
	Smaller than astrocytes	Few, slender, relatively free from branches	
Round, oval, triangular spindle-shaped, pear-shaped etc	Much small than astrocytes 3-3.5 μm	Few thin fibrous processes furnished with delicate, knot-like swellings	
		Few extensions	
		Few, short processes, or none at all	
Round to pear-shaped		Few, extremely delicate	

ıcleus	Cytoplasm	Author/tissue
ırgest glial nuclei; oval with ine, evenly distributed ıromatin		Gray (1964)
ırgest (5-10 µm diameter) and ılest of glial nuclei; round/ ral/egg-shaped. Fine chromatin etwork		Elliott (1969)
naller than neurones; larger ıan oligodendrocytes and less ense	Pale, little or no detail	Curtis, Jacobson and Marcus (1972)
ral; bundles of fibres inside ell body		Ráliš, Beasley and Ráliš (1973) human - central nervous system
ıirly large, light & indented ıclear envelope has even ıromatin lining		Ham (1974) rabbit brain
ırger than oligodendroglia ıd microglia	Neuroglial fibres present	Bloom and Fawcett (1975) man, rat, central nervous system
ırge	Granular, may contain fibres	Culling (1975)
ntral, round or ovoid	Cytoplasm, surrounding the nucleus extended as processes of varying length	Hamberger, Hansson and Sellström (1975) rabbit - cortex
ıaller than astrocytes, and ›under, and contain more ıromatin; dark	Small in amount and granular; appears roughly oblong; no end-feet or fibres; has centrosome, Golgi apparatus, fuchsinophile granules,and gliosomes	Penfield (1932) mammalian - brain and spinal cord
ıaller than astrocytes, stain ıch darker		Ranson and Clark (1959) vertebrates
arly oval, more chromatin ıan in astrocytes	Scanty granular cytoplasm surrounds the nucleus; centrosome, Golgi body, and gliosomes present	Hosokawa (1963)
›under and smaller than that astrocytes	Perinuclear mass, is small; form interfasicular glia	Mannen (1963) mammalian
ıaller than astrocytes, ›unded; dense 'blobs' of ıromatin, especially ›ncentrated at nuclear ıvelope		Gray (1964)
centric, round and smaller ıan those of astrocytes	Are situated next to blood vessels or at a short distance from them, which they contact with a thickening	Cammermeyer (1966) rabbit - cerebral cortex

Table 8 continued

Form	Size	Processes	Nucleolus
Compact, well-outlined bodies with a distinct cell membrane		Few, scantily branching	
Often pear-shaped	Small	Few, delicate processes	
	Smaller than astrocytes	Small number of slender, poorly branching processes	
	Smaller than astrocytes	Few, slender processes with few branches	
	Small	No vascular feet, processes fine and rarely branch	
Oval	Small	Small number	

APPEARANCES OF MICROGLIA BY LIGHT MICROSCOPY

Cytoplasm passes out into expansions or give a bipolar or tripolar appearance; the expansions have numerous branches and terminal spines			
		Vary in number, length and thickness, and number of secondary and tertiary branches	
Majority are multipolar but some are bipolar	Very small	Two, three or more spiny branching processes	

ıcleus	Cytoplasm	Author/tissue
ɜnerally smaller than astro- ɣtes, (4-5 µm diameter); ɜnerally elongated, and oval, r rounded, pearshaped or ɲdented; dense clumps of ıromatin	Gliosomes and centrosomes may be demonstrated by special preparation	Elliott (1969)
ɲaller than astrocytes, ɔund and eccentric	Scanty with no fibrils	Truex and Carpenter (1969)
ɲall and darkly staining	Thin rim of cytoplasm around nucleus	Curtis, Jacobson and Marcus (1972)
		Ráliš, Beesley and Ráliš (1973) human - central nervous system
ɔstly medium sized; spherical r oval with density inter- ɜdiate to astrocytes and ıcroglia; small number have ɜry light, large nuclei, ɜually with a large central ıcleolus; a fair number have small dark spherical nucleus		Ham (1974) rabbit - brain
ɲaller than astrocytes	No true neuroglial fibres are related to them	Bloom and Fawcett (1975) man, rat - central nervous system
	Stain intensely - no fibres present	Culling (1975)
	Rounded, darkly stained	Lewis (1976)

ɲall and often irregular or lɔngated; contains rather ɜavy chromatin elements		Penfield (1924) cat, rabbit - brain
ɲallest and darkest in the ɜrvous framework; ɔlymorphic; numerous granules ɲd a large granule represent- ŋg the nucleolus	Scanty in cell body, more abundant in prolongations; spongy protoplasm; no Golgi apparatus; specific granules and mitochondriaalso absent; some inclusions. Argentophile granules and, in some cases, lipoid granulations	del Rio Hortega (1932) rabbit, kitten - brain
ıcleus surrounded by ɜanty cytoplasm; smallest, ɔst darkly staining nuclei		Ranson and Clark (1959)

Table 8 continued

Form	Size	Processes	Nucleolus
Rod-like to irregularly shaped; monopolar, bipolar, multipolar	Average cell length including processes 52-62 μm, sometimes applied to capillary walls as perivascular satellites; when near to a nerve cell, it is a perineuronal satellite		Present
		Several with characteristic angular branching	
Stellate, elongated or bulb-like		One, two or more, long tortuous thick processes with delicate secondary branches, which contact numerous neighbouring neurons	
Inconspicuous in normal issue			
	Small cells	Long; of variable thickness angular bends and few branches	
Pleomorphic, elongated to triangular perikaryon		Extremely tortuous, prominent cytoplasmic processes varying in number and size	
	Small	Many	
	Small		
Processes and body covered with considerable number of tiny pointed twigs or spines		Few, rather short and twisted	
Oval	Small	A thick process arises from each end of cell which branches freely	

ucleus	Cytoplasm	Author/tissue
rregular, somewhat twisted orm; round, oval, rod-like riangular etc. Rich in hromatin	Argyrophilic granules and sometimes lipoid granulation present; centrosome present, also Golgi body; gliosomes are absent and glial fibres	Hosokawa (1963)
mallest; tend to be longated		Gray (1964)
void, or, rarely, a rounded ucleus with coarse chromatin ranules	Situated juxtaposition to neurons and are rarely found along blood vessels	Cammermeyer (1966) rabbit - cerebral cortex
ame size as, or slightly maller than, in oligodendro-lia but more darkly staining	Have centrosomes, and various granular inclusions, but no gliosomes or mitochondria	Elliott (1969)
arrow, elongated, stains less ntensely than perikaryon, or rocesses		Mori and Leblond (1969) rats - corpus callosum
longated or round	Perikaryon frequently situated next to a neuronal perikaryon or vascular wall; cytoplasm finely vacuolated; presence of PAS red stained lysosomal or lipofuscin-like granules in older animals	Cammermeyer (1970) rabbit - brain
longated	In diseased conditions end up as 'glitter' cells when they are full of phagocytosed debris	Johnston and Roots (1972)
	Migrate rapidly by amoeboid movement; phagocytic	Rálič, Beasley and Rálič (1973) human - central nervous system
mall, elongated, with dense hromatin separated by light paces	Rarely distinguishable	Ham (1974) rabbit - brain
mall		Bloom and Fawcett (1975) man, rat - central nervous system
eeply staining		Culling (1975)

organelles. All these reservations apply to transmission electron microscopy as well as to light microscopy.

When one searches the classical literature for descriptions of the different kinds of cells, one finds that there are many descriptions by light microscopy of neurons but virtually none of astrocytes (Tables 8 and 9). Is this because astrocytes are rare in healthy tissue? Are they difficult to stain? Are their shapes so variable that they defy description? Are they so obvious in sections that they need no description? Is it possible that they do not exist?

When one finds that respected histologists say that one cannot distinguish between cell types (Tables 2 to 5), one must pose the question, Why then do they consider that they can recognize different kinds of cells? One cannot obviate this problem by quoting other criteria for the identification of the particular cells when one knows that these other criteria themselves are based on the histologists' initial classification of the cell type. The same difficulty exists with respect to the use of cell markers.

A very vivid indication of the difficulty of distinguishing cell types by light microscopy is seen in the illustration from the Frontispiece of Peters, Palay and Webster (1976), which they have kindly permitted us to reproduce (Figure 22). Brief perusal of their drawings leads to the realization that rotation of any of their 'typical' cells would give appearances which would be quite indistinguishable from each other. If one were to make three-dimensional models of their Golgi-stained neurons, astrocytes and oligodendrocytes, and if one were to cut sections of them of thickness equivalent to those used in histology, the sections would have a wide range of overlapping appearances, and so would be even less distinguishable from each other than whole cells. Presumably their drawings are intended to represent typical appearances, so one would be tempted to conclude that the cells' external appearances stained by the Golgi method cannot be differentiated by light microscopy.

Pathologists normally examine sections at relatively low-power magnification, and their diagnosis is based as much on the clinical appearance and natural history of the condition as on the appearance of individual cells at high-power magnification. The pattern of cells in the tumour helps diagnosis of its type, and the polymorphism of their shapes gives some indication of the prognosis, yet this polymorphism or pleiomorphism itself renders the identification of the predominant cell type more difficult. This difficulty is carried on to any identification of cells in culture from the tumour.

In many functional studies the location of the cerebral nuclei and the general myeloarchitectonics indicate the physiological role of the whole nucleus so that their morphology is not so important. For example, recording responses in the sensory cortex to stroking the footpads, or stimulating the motor cortex to produce epileptiform movements, has few implications for the morphology of the neurons involved in these physiological events.

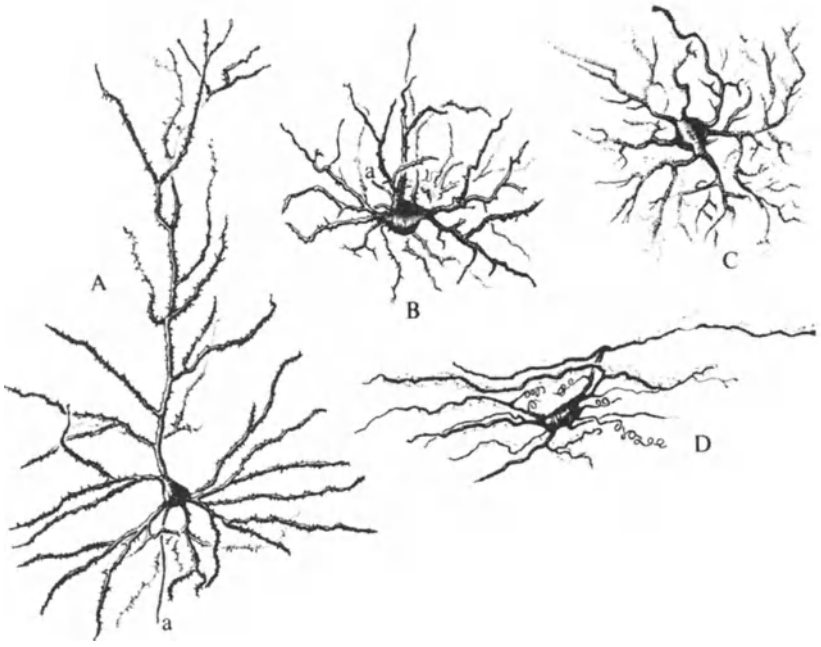


FIGURE 22 The original legend reads: 'Various aspects of nerve cells in light microscopic preparations: A: A small pyramidal cell from the visual cortex, Golgi method. The axon (a) descends from the cell body. B: A small neuron in the dentate nucleus of the cerebellum, Golgi method. The axon (a) is represented only by its initial segment. C: Protoplasmic (velate) astrocyte in the grey matter, Golgi method. D: Oligodendrocyte in the white matter, Golgi method.' Peters, A., Palay, A.L. and Webster, H. de F. (1976) The Fine Structure of the Nervous System. Frontispiece. (Philadelphia: W.B. Saunders Company). Please note that rotation of any of these cells, and cutting sections of them in various orientations, could produce pictures which would not distinguish any of them from each other. Please also see Tables 8, 9 and 10.

SPECIFICITY OF STAINS

Staining procedures can either be general ones, such as haematoxylin and eosin or cresyl violet, or they can be 'specific' stains for particular cell types or parts of cells. The identification of cells with both types of stains depends upon their shapes and colours. The colours may vary in intensity or distribution within the cell. Some salts, such as those of silver or gold, can stain a tissue grey or yellow, respectively, or deposit as black or brown granules, depending on the precise chemistry of the extremely complex mixtures of all the reagents and the tissue. The particular chemical milieu in which the reagent tissue mixture exceeds its solubility product is unknown and probably unknowable.

There have been many studies of the effects of reagents on particular compounds extracted from tissues (Mann, 1902; Baker, 1958; McClung-Jones, 1967; Symposium, 1965; Pearse, 1972, 1980). However, in view of the complexity of the chemistry of tissue and the alchemical concoctions of all the chemicals which are used in the procedure, it seems extremely unlikely that the simplified chemical studies are adequate. For example, the examination of the reaction of 10% formalin only with an enzyme preparation (Bruce-Casselman, 1959) is unlikely to represent all the chemical events

Table 9 The appearance of allegedly different cellular elements in the central nervous system as described by the authors. Lack of information under any heading indicates that it was not supplied in that publication

Form	Size	Processes	Nucleolus	Nucleus	Cytoplasm	Author/tissue
APPEARANCES OF NEURONS BY ELECTRON MICROSOPY						
		Axon, thin longitudinally orientated filaments, dendrites present	Nucleoli present eccentrically; vacuolated or solid and always lack a smooth contour; no limiting membrane	Great variety in apparent thickness and structural arrangement of nuclear membrane; indentations appear regularly; nucleoplasm has electron dense irregular clumps of fine granular material, less dense	Composed of minute scattered granules; mitochondria occur as spheres, short rods or elongated and possibly wavy filaments, vary in size from 300 to 3000 Å diameter	Hartmann (1953) rat - central nervous system
			Appears as a tangle or convoluted mass of dense and thick filaments made up of dense aggregations of fine granules; no membrane present	Has double nuclear membrane with perforations	Cytoplasm crowded with formed elements equivalent to Nissl bodies, vary considerably in size and profile, has endoplasmic reticulum orderly arranged within the Nissl bodies, and small granules in close contact with outer membranes of endoplasmic reticulum or scattered throughout matrix; have an orderly arranged agranular reticulum present, vesicles and a concentrically laminated, membranous structure has been found; also numerous filamentous, rod-like, or spherical mitochondria; neurofilaments - fine long threads 60-100 Å in diameter traverse the matrix, either all in one direction, or criss-cross in all directions, dense rounded bodies 350-660 μm in diameter are present	Palay and Palade (1955) rat - dorsal root ganglion, medulla oblongata and cerebellar cortex
			Large, conspicuous, of a dense granular nature	Poorly defined chromatin clumps; nuclear membrane appears double	Large numbers of granules present and very fine filaments; endoplasmic reticulum randomly orientated; Golgi apparatus present	Schultz, Maynard and Pease (1957) rat - cerebellar cortex and corpus callosum
		Many dendrites containing many canaliculi, mitochondria and membrane-bound cavities. Axon present		Chromatin granules occur in clusters; fine filaments present; nuclear envelope has pores	Granular endoplasmic reticulum, ribosomes, mitochondria, neuro-filaments, Golgi apparatus are present. Centrioles and other dense homogeneous bodies are sometimes present	Gray (1964)
				Nuclear envelope appeared intact and with paired membranes. large number of pores in envelope	Recognizable, granular endoplasmic reticulum with some ribosomes aggregated along the outer surface. Cytoplasm was packed with free ribosomes. Membranes of Golgi apparatus were preserved but dilated; mitochondria were well preserved and neurofilaments were recognizable	Roots and Johnston (1964) ox - red nuclei and lateral vestibular nuclei of brain
			Prominent	Lightly granular	Dense: Nissl bodies - granular endo-plasmic reticulum; Golgi apparatus a concentrically arranged group of cist-ernae; dense cytoplasmic granules; mitochondria generally smaller than in other cells; multivesicular bodies; dense granular inclusions; large hetero-geneous bodies: glycogen; lipofuscin pigment and basal bodies and cilia; neurofilaments	Ross (1964) rat - dorsal root ganglion

				Pale and often irregular	Dense, abundant ribonucleoprotein present; elaborate smooth surfaced membrane systems present; sparse disorderly arranged rough endoplasmic reticulum; oval and irregular electron dense bodies present	Nathaniel and Nathaniel (1966) rat – posterior horn of spinal cord
		Axon and dendrites; axons diameter 0.2-2.2 µm, contain vesicles 400-500 Å and 600-1400 Å diameter		(a) Irregular with folded and dilated nuclear envelope (b) More spherical	(a) Dark neuron-dense cytoplasm; numerous free ribosomes, dilation of cisternae of the vacuolar system; large Golgi complexes with dense cored verticles of 1000 Å in diameter close by; large dense bodies 3-4000 Å diameter also present (b) Pale neuron-less dense cytoplasm with fewer ribosomes; fewer cisternae in endoplasmic reticulum and Golgi complex less well developed	Etcheverry and de Iraldi (1968) rat – arcuate nucleus
Characteristic; round or irregular	Large, and varying		Large, round, dense, situated near centre of nucleous; nucleolonema present	Centrally located; round to oval; has nuclear envelope (double membrane 175-300 Å apart); nuclear pores present, also fibrils and particles	Contains numerous ribonucleoprotein particles – polysomes; granular endoplasmic reticulum present as orderly stacks or haphazard scattering of cisternae; Golgi apparatus scattered throughout cell body; multivesicular bodies present, round or oval; electron dense bodies present; round, oval or elongate mitochondria	Rapoport and Stempak (1968) rat – ventral horn cell
Multipolar usually	Varying – small to giant	Isodendritic	Off centre, nucleolus present	Usually large, spherical and eccentrically placed; contains fine filaments and associated granules	Nissl substance, small mitochondria, Golgi apparatus, neurofilaments, microtubules, lysosomes, pigment granules and vesicles of various sizes	Sotelo and Palay (1968)
				Large, eccentrically placed	Ribosomes, endoplasmic reticulum, Golgi complex, lysosomes present, numerous elongated random mitochondria	Bondareff and Hyden (1969) giant nerve cells of Deiters nucleus

APPEARANCES OF ASTROCYTES BY ELECTRON MICROSOPY

		Number variable		Ovoid, larger and less dense than other glial cells; 'vesicular' appearance	Abundant cytoplasm appears relatively 'empty'; few mitochondria, occasionally some Golgi material and a few small vesicles and particles; irregularly-shaped masses of finely granular material are sometimes present	Farquhar and Hartmann (1957) man and rat – cerebrum
Perivascular end-feet present	Large	Processes which form sheaths	Not present	Conspicuously, clumped chromatin masses, nucleoplasm rather watery	Abundant and wartery; occasional mitochondria, Golgi apparatus present, sparse and widely-spread endoplasmic reticulum	Schultz, Maynard and Pease (1957) rat – cerebral cortex and corpus callosum
		Processes present, practically filled with filaments (90 Å)		Oval, densely granular	Relatively thin layer; mitochondria or agranular reticulum present, and ergastoplasm with a high proportion of fine nucleoprotein granules; small dense, irregularly shaped clusters enclosed by single thin membrane	Palay (1958) rat – central nervous system

Table 9 continued

Form	Size	Processes	Nucleolus	Nucleus	Cytoplasm	Author/tissue
Vascular feet applied to blood capillaries			Prominent	Ovoid; dense accumulations of chromatin near the nuclear membrane	Low density 'watery'; mitochondria, Golgi material, endoplasmic reticulum and small number ribonucleoprotein particles present	de Robertis, Gerschenfeld and Wald (1960)
		Multiple, delicately branched refolding processes		Ovoid or reniform Large and light	Scant perinuclear cytoplasm Light and watery; gliofilaments sometimes present, form part of so-called neuropil	Luse (1962) Hosokawa (1963)
					Pale, watery; extensive bundles of fibrils in fibrous astrocytes; practically no granular endoplasmic reticulum; very few mitochondria	Gray (1964)
					Relatively clear filamentous cytoplasm with little endoplasmic reticulum	Mugnaini and Walberg (1964) cat and dog
		Contain osmiophilic bodies, glycogen granules and filaments			Granular endoplasmic reticulum is scarce but numerous free ribosomes present; number of mitochondria is relatively small; Golgi complex is well developed; osmiophilic bodies are frequently found; filaments present scattered or in bundles	Glees and Meller (1968) monkey
	Larger than oligodendrocytes	Many; which form glial sheets between fascicles of **axons**			Less electron-dense than oligodendrocytes; contain characteristic filaments and/or glycogen; microtubules are rare	Vaughn and Peters (1969) rat - optic nerves
		Pale processes, with glycogen present		Some condensed chromatin adjacent to nuclear material	Many filaments, few microtubules	Curtis, Jacobson and Marcus (1972)
				Nuclear membrane is double and seems to be continuous with endoplasmic reticulum; presence of pores	Cytoplasm packed with various inclusions mitochondria present as double walled capsules with ridged inner walls; normally have no centrosome, Nissl bodies formed of numerous flattened vesicles, tightly packed neurofibrils present, endoplasmic reticulum and Golgi apparatus	Elliott (1969)
		Unipolar, bipolar, or multipolar	Usually one or two present and consists of small dense granules and threadlike nucleolonemas	Prominent, enclosed in a nuclear envelope which is a double-membrane; contains chromatin granules	Filamentous and tubular structures present, granular endoplasmic reticulum and ribosomes associated with it; ribosomes also scattered freely and often grouped in rosettes. Also agranular endoplasmic reticulum present, mitochondria and a variety of other inclusions	Johnston and Roots (1972) ox - brain
Smooth perikarya 1. More irregular 2. outline, pear-shaped or multipolar	Large Large 17-35 µm in diameter	Few spines (3-4 per cell) More spines	Well developed; generally centrally placed in nucleus, associated with it can see a heterochromatic mass and a nuclear rodlet	Round and usually lobulated or dimpled at one pole; nucleoplasm is thin and flocculent and has small clumps of chromatin	Contains a myriad small, thin, mitochondrial profiles which appear singly or in clusters; small Nissl bodies; polysomes; Golgi apparatus; microtubules	Chan-Palay (1973) rat - lateral vestibular nucleus

Smooth or irregular	Small, 10-12 μm in diameter	3-4 more slender and tortuous than of large neurons	Prominent and central	Nucleus usually well lobulated, chromatin well dispersed	Cytoplasm is limited, little Nissl substance - scattered cisternae of endoplasmic reticulum and small collection of polysomes. A prominent Golgi apparatus rings the nucleus	Chan-Palay (1973) rat - lateral vestibular nucleus
Angular or polygonal		Multipolar, dendrites and the axon	Present	Nuclear envelope and pores	Aggregations of neurofilaments, granular endoplasmic reticulum and associated ribosomes. Golgi network, agranular endoplasmic reticulum, mitochondria, centrosome containing a pair of centrioles and other inclusions	Bloom and Fawcett (1975)
		Multipolar, contains microtubules	Large, spherical, conspicuous eccentric, extremely dense, made up of granules and fine filaments	Large, round, contains fine chromatin filaments; surrounded by the plasmalemma	Strands of chromatin, multivesicular bodies, lysosomes, Golgi apparatus present, and granular endoplasmic reticulum; occasional microtubules; mitochondrial profiles present; ribosomes and neurofilaments	Peters, Palay and Webster (1976) rat - cerebellar cortex
		The axon contains neurofilaments, sparser in dendrites; branching occurs in both	Normally single nucleolus present	Small deeply stained nucleus or lighter larger nucleus	Nissl substance, neurotubules and neurofilaments, membrane-bound ribosomes, rough endoplasmic reticulum, Golgi apparatus, mitochondria, microfilaments, microtubules	Jacobson (1978)
					Ribosomal endoplasmic reticulum, neurofilaments, and microtubules, Golgi complex, numerous free ribosomes, neurotubules, ovoid to round mitochondria	Han and Holmstedt (1981)
					Unusually lucent matrix; numerous fibrils - thinner than neurofilaments (diameter = 60-80 Å); lysosomes and glycogen granules present	Constantinides (1974)
		Several; long, irregular and branched; vascular end feet		Ovoid		Hamberger, Hansson, and Sellström (1975) rabbit - cortex
		Fibrous astrocytes are longer and branch less than protoplasmic astrocytes		Round/ovoid with smooth outline (protoplasmic astrocytes) or irregular and indented (fibrous astrocytes)	New density; small amount of rough endoplasmic reticulum, few enlarged mitochondria, and a Golgi complex, lysosomes and glycogen granules (20-40 nm) present	Blackwood and Corsellis (1976)
				Light rather large, often indented	Light, poor development of endoplasmic reticulum	Ham (1976)
				Irregular	Light, watery; some mitochondria, scanty endoplasmic reticulum	Lewis (1976)
Formation of 'end-feet' processes			Occasionally present	Bean-shaped or irregular	Fibrils present, and small amount of endoplasmic reticulum; ribosomes, Golgi apparatus, glycogen granules and elongated mitochondria present	Peters, Palay and Webster (1976) rat - optic nerve and cerebellar cortex
		May be attached to a capillary			Lucent cytoplasm, sparse cytoplasmic organelles, bundles of microfilaments 79-100 Å in diameter, may contain glycogen granules	Jacobson (1978)

Table 9 continued

Form	Size	Processes	Nucleolus	Nucleus	Cytoplasm	Author/tissue
Processes expand at their distal ends and abut onto capillaries -astrocyte feet		Varying number, some with few branches, some extensively branched			Numerous filaments, scattered or in discrete bundles; relatively few other organelles present so has 'watery' appearance, glycogen granules are characteristic inclusions; few microtubules	Roots (1978) vertebrate
Pericapillary end-feet		Numerous processes to less numerous	Medium-size to large	Various shapes, often oval; chromatin evenly dispersed, very few peripheral clumps	Relatively clear; mitochondria present sometimes with matrix filaments and angular cristae; granular endoplasmic reticulum present in cell body, some processes and end-feet; intermediate filaments numerous to scanty; microtubules present but scanty; heterogeneous dense bodies present (probably some specific); glyogen like granular material present in some processes and end-feet; cilium, Golgi apparatus, multivesicular bodies and centrioles present	Mugnaini, Osen, Dahl, Friedrich and Korte (1980)

APPEARANCES OF OLIGODENDROCYTES BY ELECTRON MICROSOPY

		Seldom seen		Round to oval; smaller and denser than astrocytes; chromatin often aggregated peripherally to form dark rim	Scanty; quite dense and contains an abundance of formed elements i.e. mitochondria, Golgi material, endoplasmic reticulum and small particles	Farquhar and Hartmann (1957) man and rat - cerebral tissues
	Small	Few		Dense	Scant rim, moderately dense; no **fibrils** moderate quantities of endoplasmic reticulum and mitochondria; dense RNA particles; sparse groups of Golgi apparatus	Schultz, Maynard and Pease (1957) rats - cerebral cortex and corpus callosum
Round	Small	Few		Nearly spherical	Crowded with dense nucleoprotein granules and small vacuoles	Palay (1958) rat - central nervous system
				Round, with double nuclear envelope; condensed chromatin constituted by fine filaments	Denser than that of astrocytes; Mitochondria present plus large quantity of ribonucleic-protein particles	de Robertis, Gerschenfeld and Wald (1960) rat - spinal cord
		Few broad processes which often branch dichotomously		Round/ovoid	Abundant; a little ergastoplasm; few mitochondria	Luse (1962)
				Nucleus smaller than that of astrocytes and more round and more electron dense	Less cytoplasm; and is denser than that of astrocytes	Hosokawa (1968)
				Chromatin in conspicuous 'blobs'	Narrow ring which contains granular endoplasmic reticulum, and many mitochondria	Gray (1964)

					granular endoplasmic reticulum	Walberg (1964) cats and dogs
			Present as a constant feature	Round and very seldom lobulated; unevenly filled	Ribosomes are densely packed, partially freely dispersed and partially attached to the cisterns of the endoplasmic reticulum; number of mitochondria varies; Golgi complex is well developed; considerable electron density	Glees and Meller (1968) monkey
		Few		More electron-dense than astrocytes; usually located near one pole of the cell; irregular clumps of chromatin beneath the nuclear membrane	More electron dense than astrocytes, Golgi apparatus present; short regular profiles of granular endoplasmic reticulum are scattered throughout and diffuse, electron dense material, numerous clusters of free ribosomes, and microtubules are present; no filaments	Vaughn and Peters (1968) rat - optic nerves
				Dense, nuclear chromatin, light patches adjacent to the numerous nuclear pores	Large quantities of free ribosomes/ ribosome rosettes; prominent microtubules; dark and light multivesicular bodies; granular inclusions; Golgi apparatus and mitochondria present; no fibrils or glycogen granules	Truex and Carpenter (1969)
				Located towards pole of cell; chromatin heavily clumped	Denser, but scantier than neurons; many microtubules - few neurofilaments; dense clumps of endoplasmic reticulum; clusters of polyribosomes	Curtis, Jacobson and Marcus (1972)
		Fewer than astrocytes		Eccentric; clumped chromatin	Abundant, denser than astrocyte cytoplasm; almost no fibrils - large number of microtubules; prominent Golgi vesicles; marked development of endoplasmic reticulum; many granules but no glycogen; numerous ribosomes	Constantinides (1974)
				Rounded/ovoid/irregular shape; dense	Dense; microtubules in processes; very few cytoplasmic fibrils of glycogen granules; Golgi apparatus, endoplasmic reticulum, mitochondria, and rosettes of free ribosomes present	Blackwood and Corsellis (1976)
			Well marked	Round, oval or irregular	Closely packed ribosomes, polyribosomes and endoplasmic reticulum	Lewis (1976)
		Few		Round, oval, or irregular; chromatin tends to clump, dense	Dense, well-developed Golgi apparatus; inconspicuous mitochondria; few fibrils and glycogen granules; abundant microtubules; short cisternae of endoplasmic reticulum	Peters, Palay and Webster (1976) rat - optic nerve and spinal cord
					Fewer cytoplasmic processes than astrocytes, have abundant endoplasmic reticulum and ribosomes, high density of cytoplasmic organelles, contain prominent Golgi apparatus and vesicles and many cytoplasmic microtubules	Jacobson (1978)
		Fewer processes than astrocytes			Large number of organelles especially microtubules, ribosomes and various dense bodies; glycogen is not found	Roots (1978) vertebrate

Table 9 continued

Form	Size	Processes	Nucleolus	Nucleus	Cytoplasm	Author/tissue
		Few to numerous, often thin	Small	Round or oval, chromatin in clumps; swollen nuclear envelope	Usually dense; mitochondria few and small; granular endoplasmic reticulum abundant in cell body but not in distal processes; short cisterns with wide transparent lumen; intermediate filaments usually not present; numerous microtubules both in cell bodies and processes; heterogenous dense bodies present (some specific); glycogen-like granular material not observed or cilium; Golgi apparatus, multi-vesicular bodies and centrioles present	Mugnaini, Osen, Dahl, Friedrich and Korte (1980); see also Mugnaini and Walberg (1964)

APPEARANCES OF MICROGLIA BY ELECTRON MICROSCOPY

		Several processes with many branches		Great density and variable shape, chromatin evenly distributed and is uniformly dense	Quite dense and contains abundant formed elements (i.e. mitochondria etc); amount is intermediate between that of astrocytes and that of oligodendrologia, frequently there is a large amount of cytoplasm near one pole of the cell	Farquhar and Hartmann (1957) man and rat - cerebrum
				Dense, angular and irregular	Extremely dense; conspicuous Golgi apparatus; moderate amount of endoplasmic reticulum; few, small mitochondria	Schultz, Maynard and Pease (1957) rat - cerebral cortex and corpus callosum
	Small	Very small with no mitochondria inside them		Elongated nuclei	Dense, heterogeneous, cytoplasmic masses present, containing fine granules, rounded larger masses and packets of thin, parallel, sheet-like material	Palay (1958) rat - central nervous system
Irregularly indented				Longish and irregularly shaped, finely granular and of high electron density	Dense, and dark inclusion bodies are often present	Hosokawa (1963)
				Dense, highly crenated	Densely packed granules, pale endoplasmic reticulum present	Gray (1964)
		Processes extend regularly	'Unique'	Smaller than any other cell; elongated; dense chromatin masses; light nucleoplasm	Thin rim around nucleus - of same density as interchromatic nucleoplasm; no fibres; infrequent Golgi, endoplasmic reticulum and ribosomes; mitochondria and lysosomes present;	Mori and Leblond (1969) rat - corpus callosum

				cytes, more than astrocytes; few or no tubules or filaments; long, stringy cisterns; fat droplets and laminar dense bodies present		Marcus (1972)	
				Sparse; no fibrils or microtubules; no glycogen; few microvilli, some dense bodies		Constantinides (1974)	
			Dense chromatin	Little rough endoplasmic, but fair amount of dense bodies		Ham (1974)	
			Small, shape relates to number of processes; extremely dense	Extremely dense; moderate amounts of 'angular reticulum', endoplasmic reticulum and mitochondria		Greenfield (1976)	
			Oval, or elongate with prominent chromatin clumps	Thin rim around nucleus, and forms broad processes; long narrow cisternae of endoplasmic reticulum; Golgi apparatus present and large numbers of inclusion bodies		Peters, Palay and Webster (1976) rat - cerebellar cortex and spinal cord	
			Small, containing dense chromatin clumps and light nucleoplasm	Scanty cytoplasm containing lipid inclusions, lysosomes and **abundant** vesicles but sparse endoplasmic reticulum, scattered ribosomes, and few or no microfilaments or microtubules		Jacobson (1978)	
				No abundance of filaments or microtubules, but other inclusions are numerous and much more common than in either oligodendrocytes or astrocytes		Roots (1978) vertebrate	
		Few, both thin and large, with thin side arms	Oval or indented, chromatin in large clumps, small to large nucleolus	Dense, or moderately dense; mitochondria few and small, with matrix and dense granules; granular endoplasmic reticulum has few cisterns with electron-dense content; some of these are long with narrow lumen; not usually any intermediate filaments present; microtubules present but scanty; heterogeneous dense bodies present, often numerous, some containing lipid droplets; no glycogen like granular material observed; cilium not observed; Golgi apparatus, multivesicular bodies and centrioles present		Mugnaini, Osen, Dahl, Friedrich and Korte (1980); see also Mugnaini and Walberg (1964)	

which occur to enzymes in tissue when it is fixed. Most modern electron microscopists believe that osmium salts stain proteins specifically, while light microscopists for a long time have considered them as a suitable stain for lipids (Romeis, 1968, pages 260-261).

Sometimes the authors of new staining procedures or modifiers of well-accepted procedures claim that they are 'specific' for one cell type (Table 10). It is extremely rare that they are quite so definite about the specificity. The following degrees of certainty are expressed about the specificity of a staining procedure for a particular cell type:

1. it is specific; it does not stain other cell types;
2. it is specific, but may stain other cell types, for example, if permitted to 'overstain', or 'by accident', or occasionally;
3. it is said to be specific but no information is given about whether or not it has been tested on other cell types;
4. it is not explicitly claimed to be specific but is listed as staining one cell type, or is 'recommended' for staining that cell type, and is not listed under staining procedures for other cell types;
5. it stains one type of cell with a range of colours which overlaps grossly the range of colours which it also stains another type of cell;
6. one has to assess subjectively by exercise of judgement, skill or experience at what moment, or with what concentration of reagents at a particular stage in the procedure, the particular cells in which one is interested are stained adequately. The literature is replete with examples of this practice. In discussing the demonstration of protoplasmic astrocytes in the cerebral cortex by the method of Cajal, Gray (1954, page 537) says: 'The successful application of this technique depends on the ability to judge the exact shade of purple which indicates a satisfactory termination of the staining process.' Carleton and Drury (1962, page 221) comment: 'The complexity of the intervention of factors as yet unknown make it often difficult to stain particular elements of the nervous system in the same way on different occasions.' These reservations, regrettably, are much more frequently expressed in the older literature. The more recent literature has tended to regard the specificity of cells as being defined more accurately by the electron microscopist than by the light microscopist, forgetting that the electron microscopist takes his cue from the light microscopist. (Please see also Appendixes 1 and 2).

In assessing the literature on specificity of staining, one frequently comes across the use of the term 'neuroglia', without a more precise indication of whether the author is referring to astrocytes, oligodendrocytes or microglia. This can only be interpreted as meaning either that the author believes that the particular staining procedure does not differentiate between the types of neuroglia, or that it stains all of them, or that he cannot distinguish between them. So far histologists have not indulged in the luxury of wondering whether, indeed, they are different types of cells.

The study of different kinds of neuroglial cells - if indeed they do exist - is made much more difficult when one compares the specificity of particular stains as listed in authoritative standard textbooks. Frequently, different authors, using the more or less

explicit expressions listed above either assign rather different specificities to a particular staining procedure than do their equally distinguished colleagues, or describe them as being much less specific. Examples of this occur commonly in the literature, but are most easily appreciated by comparing the texts (Table 10). (Please see also Appendix 1.)

In addition to using the term 'neuroglia', other generic terms used include 'nervous system stain', 'neural stain', 'central nervous system stain', 'neural element stain', 'spinal cord stain', 'cellular element stain in the nervous system'. All these terms carry the implication that one cannot distinguish between the different cellular elements of the nervous system. Dare one also comment that most 'specific' stains also show up capillaries in the nervous system?

If one accepts that the staining procedures are not 'specific' for particular kinds of cells, one could still say that it shows them up, even if in a number of defined and undefined circumstances, it may also stain other cells, to which at the time one was not directing one's attention. Unfortunately, this does not help the histologist who is attempting to classify the cells in the nervous system, since it begs the important question of whether there are any criteria for distinguishing the different kinds of cells; that is, what is the evidence for believing that neurons, astrocytes, oligodendrocytes and microglia are different cells?

In order to claim that a particular stain shows up a specific cell type, one has to show also that, firstly, it does not show up any of the other cell types in that tissue, and secondly, that staining procedures which are believed to be specific for other cell types do not also stain the one for whom the specific staining is claimed. These requirements for specificity apply equally to any claim for the specificity of any marker for a particular cell type.

The specificity of the stains depends heavily on these 'control' experiments, but the great era for the development of stains preceded that in which the concept of control experiments, of testing of statistical significance, or even of questioning upon what evidence any authority had claimed specificity for his staining procedure, had arrived. Since that era, the belief in specificity has apparently continued to be accepted without question.

PHYSIOLOGICAL PROPERTIES

In general, neurophysiologists penetrate cells with intracellular electrodes, or record extracellularly. It is easier to encounter a large cell body than a small one, and it is less damaged by the electrode. Therefore, if a stable membrane potential can be recorded and the cell body can be excited, that cell is usually described by the neurophysiologists as a neuron. If a large resting potential is recorded, but surface, orthodromic, antidromic or intracellular stimulation fails to excite that cell, it is generally felt that it is likely to be a neuroglial cell. From such reasoning, and the fact that neuroglia in culture give very slow potential changes, which look quite different from those in neurons (Hild and Tasaki, 1962), it is generally believed either that neuroglial cells do not fire or that they are too small or too difficult to penetrate with intracellular electrodes. It might be advantageous for those who believe that astrocytes and oligodendrocytes are different cell types to try to give a definitive

Table 10 The cell types or parts of cells believed by different authors to be stained by particular procedures

Bielschowsky's silver	Cajal's gold sublimate	Cresyl violet	Golgi-Cox silver	Haematoxylin and eosin	Holzer	Marsland, Glees and Erikson	Palmgren	Patay
			Nerve cells and processes - dark brown Background - pale brown Other elements not reported	General Nuclei - blue Cytoplasm - pink Neuroglia fibres - pink				
Peripheral nerve fibres Axis cylinders - black Myelin - red-violet Axis cylinders Connective tissue - violet or blue-violet Other elements not reported	**Protoplasmic neuroglia** Neuroglia cells, Astrocytes } dark purple Third element - unstained Background - light purple Other elements not reported		**Axis cylinders and dentrites** Nerve cells and processes, Neuroglia, Connective tissue, Fibres } silver impregnation	General	**Neuroglia** Other elements not reported			
Cell bodies, axon and dendrites - black Neurofibrils Other elements not reported	**Protoplasmic and fibrous astrocytes** Microglia Oligodendroglia Neuroglia cells - purple red Nerve cells - pinkish Nerve fibres - nearly or quite unstained		**Nerve cells and processes, Neuroglia** } dense black	**General**				
Neurofibrils - brown Neuroglia - may be slight colouring Other elements not reported	**Astrocytes** - black Other elements not reported			**General** Nuclei - blue Cytoplasm - pink	Glial fibres - blue Other elements not reported			
	Astrocytes Other elements not reported				Glial fibres Other elements not reported			
			General					
Nerve cells and processes Other elements not reported	**Neuroglia** - dark purple Other elements not reported	**Nervous tissue** **Nissl granules**	Nerve cells and processes Other elements not reported	**Nerve cells and processes** Other elements not reported	**Neuroglia** Other elements not reported			General Nervous tis grey
Axons and dendrites - black **Neurofibrils** - black Neuroglia - may be lightly stained Other elements not reported	Astrocytes - opaque purplish-black Other elements not reported	**Nissl substance** - deep purple-blue Nuclei - blue Some cytoplasmic processes of neurones - blue Other elements not reported	**Neurons** - black **Neuroglia** - black	Nuclei - blue-blue/black Neuroglial fibres, Axons, Nerve endings } Not stained Cytoplasm - pink	**Fibrous glia** - deep blue Other elements not reported		**Nerve fibres** - black Other elements not reported	
Neurofibrils in cell body, axons and dendrites of neurons - black Reticulum fibres - black Other elements not reported	**Astrocytes and processes** - black Nerve cells - pale red Nerve fibres - not stained Other elements not reported	**Nissl substance** - purple Other elements not reported	**Nerve cells and processes** Other elements not reported					
Neurofibrils, axon and dendrites Neuroglia Nerve endings Other elements not reported	**Astrocytes** - black Neuron bodies, Microglia nuclei, Nerve endings } also stained Oligodendroglia, Axons and myelin sheath } not stained	**Nervous tissue** Nuclei - violet Plasma - blue Amyloid, mucin and mast cells granules - red	**Neurons** - black **Neuroglia** - black	**General** Fibroglial fibrils - not stained Nuclei - blue				
Nerve cells and processes, Neurofibrils, Neuroglial processes } black Other elements not reported	Astrocytes, Oligodendrocytes } black Background - light pinkish purple Other elements not reported			Nerve cells - pink Nuclei of nerve cells, Oligodendroglia, Astrocytes, Microglia } blue Other elements not reported				

ld's silver ate	Phosphotungstic acid haematoxylin	del Rio-Hortega's silver	Weigert-Pal	Weil and Davenport	Author	Comments of authors cited
			Myelinated nerves – blue Nerve cells and large axis cylinders – brown Neuroglia, Fibrin, Nuclei } blue		Mann (1902)	
			Medullated nerve fibres – blue-black Other elements not reported		Hartridge and Haynes (1930)	
lendroglia – black glia – light brown elements not orted	Nuclei – blue Intercellular substances – pink	**Protoplasmic and fibrous neuroglia** **Microglia** Other elements not reported	*Myelin sheaths – blue; background – colourless Other elements not reported *Glial fibres and nuclei – blue Cytoplasm – colourless Other elements not reported	**Oligodendroglia** – brown **Microglia** – brown Other elements not reported	Gatenby and Painter (1937)	Bielschowsky – "According to our experience good results are rarely obtained". Golgi – "and of the fact that the impregnation may be limited sometimes to certain elements, sometimes to others, care should be excercised in the interpretation of the results obtained. A further source of possible error is found in the formation of precipitates which may, up to a point, simulate dendrites and other structures".
		Microglia Other elements not reported	**Myelinated nerve fibres** – black Other elements not reported		Carleton and Short (1960)	
	Pathologic astrocytes Mitochondria Nuclei, Fibrin, Fibroglia, Microglia } blue Other elements not reported	Neurofibrils – black Other elements not reported	**Myelin sheaths** Other elements not reported		McManus and Mowry (1960)	
lendroglia and micro-ia elements not orted			Glial fibres		Hosokawa (1963)	
					Mannen (1963)	Golgi – "As this technique is not specific for nerve cells and glial cells, both can be seen".
lendroglia, glia } purplish-blue-grey ytes	**General**	**Microglia** – dark grey Oligodendroglia Other elements not reported	**Myelin sheaths** Nerve cells and processes All nervous tissue	**Microglia** – brown Other elements not reported	Gray (1964)	Penfield – to show astrocytes as well – "simply leave the sections too long in the silver stain" del Rio Hortego – "nor does it stain oligodendroglia except by an occasional accident." Author uses term "recommended for"
lendroglia } black glia ytes – lightly ained elements not ported	**Astrocytic glia** Nuclei } deep Fibrin } blue Myelin – light blue Other elements not reported	***Astrocytes** – brown-black ***Oligodendroglia** – black ***Microglia** – black Neurons not reported	**Myelin sheaths** – deep blue-black to blue Other elements not reported	Pathological glial – grey-black Other elements not reported	Drury and Wallington (1976)	
lendroglia, glia } dark grey to black elements not reported					Humason (1967)	Penfield's method – "long fixation tends to increase staining of microglia and astrocytes"
lendroglia, glia } brown ytes s not reported	**Fibroglial fibrils** Myoglial fibrils Neuroglial fibrils } blue Fibrin Other elements not reported	**Astrocytes** **Oligodendroglia** } grey **Microglia** Neurons not reported	**Myelinated nerve fibres** – dark blue Other elements not reported		McClung Jones (1967)	Golgi – "best staining procedure for classical neuroglia"
	Glial fibres – blue Other elements not reported	Microglia – black Other elements not reported	Myelinated nerve fibres – blue Other elements not reported		Cruickshank, Dodds and Gardner (1968)	

Table 10 continued

Bielschowsky's silver	Cajal's gold sublimate	Cresyl violet	Golgi-Cox silver	Haematoxylin and eosin	Holzer	Marsland, Glees and Erikson	Palmgren	Patay
					Astrocytes Other elements not reported			
Nerve cells and processes; neurofibrils Untoned: nerve fibres and neurofibrils - brown to black After gold toning - fibres - grey-black Background - pinkish grey to purplish Other elements not reported	**Protoplasmic and fibrous astrocytes** Astrocytes - black Background - unstained or light brownish purple Nerve cells - red Nerve fibres - not stained	**Nissl granules and nuclei of neuroglia cells** Nissl granules - purple to violet Nuclei of neuroglia and endothelial cells - slightly bluer than Nissl granules Other elements not not reported	**Nerve cells, axons and dendrons** Nerve cells and processes - black Neuroglia - astrocytes sometimes stained black Background - dull yellow	**Nuclei** - blue Other elements not reported				
Neurofibrils in cell body, axons and dendrites of neurons - black	**Astrocytes and processes** - deep ruby to dark purple Neurons and axons - deep ruby to dark purple	**Nissl substance** - purple **Glial cells** - lilac Background - colourless	**Nerve cells and fibres** - black Neuroglia - sometimes blue	**General** Nuclei - blue-black		Nerve fibres - dark brown-black Other elements not reported	**Nerve fibres** - brown-black Other elements not reported	
Neurofibrils Other elements not reported		Nissl bodies in neurons Other elements not reported	Nerve cells and processes Other elements not reported	Nuclei - dark purple or blue Cytoplasm - pink				
Neurons, neurofibrils axons and dendrites - black Other elements not reported	**Astrocytes** - red-black Other elements not reported			Nervous system		**Neurons, axons and dendrites** - black Other elements not reported		
	Astrocytes Other elements not reported			Nerve cells Reactive microglia Astrocytes Microglia Other elements not not reported				
Nerve fibres - black Nuclei - brown Other elements not reported				Nuclei - blue-black Cytoplasm - pink-red		**Axons** and dendrites - black Other tissues light grey to yellowish brown		
		Nerve cell bodies and dendrites - blue Nuclei of oligodendrocytes - blue Nissl bodies in nerve cells - blue Other elements not reported	Nerve cells and processes - brown Nuclei of oligodendrocytes - brown Thin nerve fibres - brown-black Other elements not reported	Nerve cells and processes - purple Nuclei of oligodendrocytes - blue Cytoplasm - pink Small nerve cells - purple				
Cell bodies, axon and dendrites - black Neurofibrils Other elements not reported	**Protoplasmic and fibrous astrocytes** Microglia not Oligodendroglia stained Neuroglia cells - purple-red Nerve cells - pinkish Nerve fibres - nearly or quite unstained		**Nerve cells and processes** - black Neuroglia cells and processes - black	Nuclei - blue Karyosomes - dark blue Plasmasomes - red Cytoplasm - shades of pink				
			Neurons Astrocytes red Glial cells brown					
Bielschowsky (1904) neurofibrils - black						Marsland, Glees and Erikson (1954) Rabbit neurons, anterior horn cells and fibres, boutons termineaux and free terminals - black precipitate Other elements not reported	Palmgren (1948) **Nerve fibres and endings** Cell bodies - red Nerve fibres - black "Hence within certain limits the selectivity of the nerve fibre staining is directly proportioned to the delay of the precipitation"	

Cell types in **bold** indicate that the authors stated explicitly that the stain is specific for that cell type. Other cell types not in bold have been indicated to be stained by the procedure, but not specifically. Absence of an entry under a particular heading means that the author did not mention that procedure, sometimes because it had not yet been described. 'Other elements not reported'means that the authors have not mentioned whether or not other cell types or organelles are stained by that procedure. 'General'means that all neural elements are stained by that procedure, although not necessarily the same colour or intensity. An asterix(*) means that the author mentions slight modifications of the procedure. Please see also Appendix 1.

eld's silver nate	Phosphotungstic acid haematoxylin	del Rio-Hortega's silver	Weigert-Pal	Weil and Davenport	Author	Comments of authors cited
	Astrocytes Other elements not reported				Uyeda, Eng and Bignami (1972)	
dendrocytes dark grey **glia** to black round - pale grey elements t reported	**Tissue elements in general** Neuroglia fibres Fibroglia fibres Myoglia blue Nuclei Fibrin	***Protoplasmic and fibrous astrocytes** Astrocytes - black Background - unstained or light brownish purple Nerve cells - red Nerve fibres - unstained Not reported ***Oligodendrocytes** Processes and cytoplasm - black Nuclei - practically unstained Backgound - grey Not reported ***Microglia** - black Other glial cells dark grey to black Background - pale	**Myelin sheath** - dark blue Other structures - unstained		Clark (1973)	Hortega's method for microglia is very similar to his method for oligodendroglia. "Although this method is probably the most selective for oligodendrocytes, other types of cells may be stained particularly microglia. To secure staining of the former only, the time of fixation should be kept within 2 days, since long fixation tends to cause staining of microglia, astrocytes and even nerve cells".
glia black **dendroglia** elements not ported	**Astrocytes** - blue Neurons - pink Neuroglia Nuclei, fibrin blue Glial fibres Microglia	***Microglia** - black **Oligodendroglia** - black ***Astrocytes** - black to dark grey Neurofibrils - black Neurons not reported	**Myelin sheaths** - dark blue-black Other elements not reported	**Microglia** - black **Oligodendroglia** - black Other elements not reported	Ralis, Beasley, and Ralis (1973)	"Cresyl violet can be used to show **general cytoarchitecture** of brain and spinal cord. Weil and Davenport's method may give some non-specific staining. Hortega's method for astrocytes may give some non-specific staining. Holzer-normal glia are unstained".
dendroglia - black glia - black glia elements not ported	**Nervous tissue** Nuclei, centrioles, fibroglia, myoglia, blue and neuroglia, fibres, fibrin		Myelin Other elements not reported		Bloom and Fawcett (1975)	
	Astrocytes and fibres Microglia Nerve cells Other elements not not reported	Nerve cells Neurofibrils Oligodendroglia Other elements not reported	Myelin sheaths - deep blue-black Not reported Other elements not reported	**Oligodendroglia** - black Microglia - black Neuroglia Other elements not reported	Culling (1975)	
			Myelinated nerve fibres Other elements not reported		Greenfield, Blackwood and Corsellis (1976)	
	Nuclei Fibroglia Myoglia Neuroglia fibres light blue Centrioles dark blue Mitochondria Myelin Fibrin Other elements not not reported				Smith and Bruton (1977)	"Silver impregnations for nerve fibres are capricious and frequently fail". Phosphotungstic acid haematoxylin - "The fact that a number of structures are demonstrated by one colour in no way detracts from its usefulness, as the colour is present in many shades and the structure identification is helped by its morphology". "The length of staining time will determine the elements stained and degree of intensity".
	Nerve cells - blue Nerve fibres - grey Myelin - pale Other elements not reported		White matter, i.e. nerve fibres in tracts, stains dark grey. Nerve cells in grey matter stain lighter. Other elements not reported		Leeson and Leeson (1979)	
lendroglia bluish- **glia** grey elements not orted	**Neuroglia** - blue Fibrin - blue Other elements not reported		**Myelin** - blue-black Other elements not reported	***Protoplasmic astrocytes** - violet ***Fibrous astrocytes** ***Oligodendrocytes** - grey ***Neuroglia** - deeply stained Astrocytes faintly Oligodendrocytes stained Glia fibres	Carleton, Drury and Wallington (1980)	Haematoxylin and eosin - "Specialized structures such as neuroglial fibres, dendrites etc. are not brought into evidence" Weigert-Pal - "A simple and rapid not specific method". Penfield - "Differentiation between the two is easy on morphological grounds"
				Neurons not reported	Millhouse (1981)	
eld (1930) **dendroglia** purple- glia grey elements not orted	Mallory (1900) **Neuroglia fibres** Nuclei, neuroglia fibres and fibrin - blue Axis cylinders and ganglion cells - pale pink Connective tissue - deep pink "The blue colour is a little sensitive to strong light, and on prolonged exposure will fade to pink" Other elements not reported	del Rio Hortega (1971) Neuroglia and connective tissue; Microglia and oligondendroglia - black Neurons not reported	Weigert (1885) Medullated fibres - dark blue to blue-black. Has a variety uses Other elements not reported	Weil and Davenport (1933) Oligodendroglia and microglia - brown Particularly good for "pathologic glial proliferations" Other elements not reported		

answer to the question, 'What are the electrical properties of neuroglial cells in the living intact adult mammalian central nervous system?' Obviously, this question is not answered by dubbing those cells as neuroglia, which one cannot see being penetrated by the electrode or subsequently marked histologically. Quite explicitly, the statement that an unexcitable cell is a neuroglial cell, followed by the deduction that neuroglial cells are not excitable, is a circular argument. It will be concluded later in this monograph that besides neurons, the only kinds of neuroglia are the naked nuclei free in the ubiquitous syncytium, and that what are generally called astrocytes and oligodendrocytes are neurons (page 189), so this problem does not arise for us.

Neuroglia have been widely characterized as cells 'supporting' or 'satellite' to neurons (Muller, 1930; Krypsin-Exner, 1952; Brownson, 1955, 1956; Hyden, 1962; Koenig, 1964; Friede, 1965; Peters, Palay and Webster, 1976, pages 231-266; Pevzner, 1979). Prima facie, it is difficult to know what is meant by this implicitly teleological term. It is highly likely that every sort of cell in any tissue interacts with its neighbouring extracellular fluids and cells. In this sense all tissue fluids and cells are interdependent, and are therefore mutually supporting. One cannot do the experiment of withdrawing particular cellular elements from any living tissue and examining the behaviour of the remaining tissue afterwards, as one does classically in ablation experiments of cerebral nuclei or endocrine organs.

In the middle 1960s at a meeting of the Brain Research Association in London, the distinguished neurobiologist, J.Z. Young, was talking about memory in the neurons of octopuses. He was asked by a questioner in what way his experiments could be interpreted as locating the memory to neurons rather than to the nuclei upon which he was experimenting. He agreed immediately that one could not attribute the properties to the neurons rather than to the central nervous nuclei as a whole. Nevertheless, it is widely believed that memory and many other probably cerebral functions are located in neurons, although there is no unequivocal evidence for this belief; in many senses this view is complementary to the view that neuroglia are 'supporting' cells.

The idea that the neuroglial cells support the neurons, or are 'satellites' of them, probably originated from the fact that some small neuroglia adjacent to neuron somata look like 'satellites' (Figure 23). Many biochemical experiments have been carried out on the cytochrome oxidase, succinic dehydrogenase, and ATPase in isolated neurons and their enzyme activities have been compared with those of 'neuroglial clumps' (Cummins and Hyden, 1962; Hamberger, 1963; Hyden, 1967). Their oxygen uptake was different, and the clumps responded to high potassium ions by increasing their oxygen uptake, while the neurons did not (Hertz, 1966, 1979). Of course, it is not at all surprising that, since all the cellular elements in the nervous system stain differently from the tissue between them, they should show different enzymic and other biochemical properties. It does not follow that if the same properties change in the opposite or in the same direction in neurons and neuroglia that one of them is nutritive, supporting, or satellite in relation to the others. Indeed, although much has been written about the neuron-glia relationship (Hertz, 1965; Hyden, 1967; Johnston and Roots, 1972; Schoffeniels, Franck, Tower and Hertz, 1978; Pevzner, 1979; Giacobini, Vernadakis and Shahar, 1980), it would be difficult, within the context of neurobiology, to define these terms expressing the concept that the neuroglia were somehow 'secondary' to neurons.

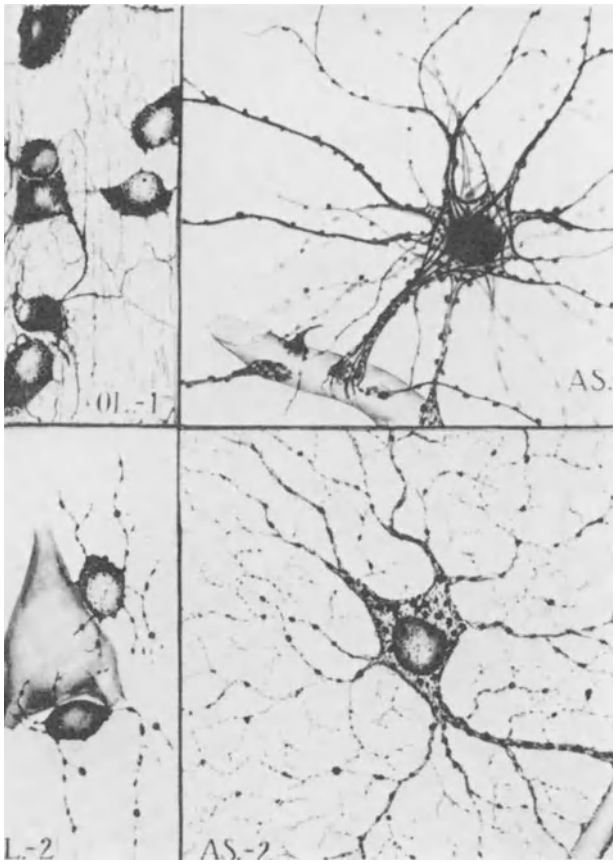


FIGURE 23 Penfield's (1928) drawings of OL-1 oligodendroglia, AS-1 fibrous astrocyte, OL-2 satellite oligodendroglia, AS-2, fibrous astrocyte. This figure is reproduced from Penfield, W. (1928) Neuroglia and microglia: the interstitial tissue of the central nervous system. In Special Cytology, edited by Cowdry, E.V. New York: Hoeber, page 1032.

Cells identified as neuroglia in tissue culture generally do not fire (Hild and Tasaki, 1962; Krnjevic and Schwartz, 1967; Nelson, 1973; Hosli, Andres and Hosli, 1980). Although it is generally believed that neuroglia do not fire, I think that insufficient determined experiments have yet been devoted to posing the questions with an affirmative expectation. The slow potential changes due to the application of aspartate and glutamate are similar in neurons and astrocytes (Hosli, Andres and Hosli, 1979). If one were to show clearly that astrocytes and oligodendrocytes, which had been precisely identified by independent and dependable criteria - under precisely the same conditions as neurons - did not fire, one could use this criterion to distinguish the neurons from the two types of neuroglia. It may be that the necessary exhaustive examinations will be carried out in the future, and electrical excitability will then become a useful criterion.

Microglia and reactive astrocytes are generally considered to be phagocytic. Microglia cannot often be identified with certainty in tissue culture, and it is not at all clear from the literature whether protoplasmic and fibrillary astrocytes are considered to be the same cells as reactive astrocytes, or transformed cells or different cells.

SPECIFIC CELL MARKERS (TABLE 10)

In claiming that a particular marker is 'specific' for a cell type, one would not be

satisfied if it were present in preparations of other cell types to any significant degree, since its presence at all indicates that those cells can also synthesize the marker. In some conditions they may synthesize more or less, but the ability to synthesize a naturally occurring marker must derive from the genotype. Therefore, even an apparent concentration in one cell type, in a particular preparation of ten times the concentration in another, does not merit the characterization of a substance as a specific marker, especially in view of the variability of marker concentrations between whole homogenates, synaptosomes, organ cultures, tissue cultures, cell clones, tumours, etc. of the same parent tissue origin. Pevzner (1979, pages 51-63) shows a table of 51 enzymes in 186 preparations, none of which show the enzymes too low for their activities to be measured (Table 7). Therefore, none of these enzymes can be considered specific to a cell type.

Whereas the proteins, transmitters and enzymes occur naturally in or around the cells, the toxins and the antibodies do not. These substances are usually detected using histochemical or immunocytochemical techniques. The use of such techniques - as has been stressed previously - carries the implicit assumption that none of the steps of the procedures used affects the optical properties of the cells substantially. This should have been demonstrated for each step in each procedure for every preparation in the range of conditions under which it is being studied. Markers are sometimes demonstrated in tissue after homogenization - a procedure whose users must also believe that it does not affect the apparent concentration, or distribution, of the marker.

Of the markers, the enzymes and proteins are located within the cells and the toxins are outside the cells. The transmitters may be present inside or outside the cells, and the antibodies usually gain access to the inside of the cells after they have been 'permeabilized'.

It would be fair to say that all the microscopic techniques for indicating markers are qualitative rather than quantitative. They depend upon the following variables: each of the reagents used in the procedures; the concentrations of these reagents; the time for which the tissue has been exposed to each of them; the degree of 'permeabilization' of the cells; the penetration of the substrate or toxin; self-absorption by the specimen; the specificity of the reaction used; the system of microscopy and illumination employed; interference of the reaction demonstrating the marker by substances present in the tissue other than the marker. The effects of most of these variables can be minimized by using suitable 'control' preparations, but the empirical nature of most of the procedures - in addition to one's ignorance about the full impact of these variables on the measurement system - will probably ensure that these techniques will remain largely qualitative for quite a long time to come. Perhaps we will have to await a new epoch, when research workers will have a better stomach for 'control' experiments than they do at present.

APPEARANCES IN TISSUE CULTURE (TABLE 11)

Observations on tissue culture have the advantages that one is observing living whole cells. The cells are not dehydrated, shrunk or sectioned, and virtually all the geometrical problems are obviated. One can compare the relative dimensions of the somas and nuclei and the lengths of the dendrites and glial fibres. In the literature

there are some clear descriptions of the different kinds of cells in culture, but I cannot find any statistical studies comparing the measurable dimensions of the latter parameters between the different kinds of cells in culture. In tissue cultures the shapes of cells depend upon the following factors: the location in the parent tissue; the age of the parent tissue; the age of the culture; the nature of the culture medium; the frequency of the changes of medium; the number of passages of the culture. The culture may originate from: a whole embryonic organ; a whole adult organ; dissociated embryonic cells; dissociated adult cells; a natural tumour; an experimentally induced tumour. Tumours may be slowly or rapidly growing.

The real difficulty of studies of cell structure in tissue culture is the similarity of appearance of most cells in culture at some time or other during development. I think that tissue culturalists with many years' experience might have great difficulty in distinguishing, for example, cultures of fibroblasts at some stages from cultures of neurons, astrocytes or oligodendrocytes or tumours from them, purely on their appearances alone. It is very likely that they would immediately request knowledge of their tissue origin and clinical history before pronouncing on the identity of a whole culture and the cells in it. Most cells in culture - other than endothelial cells - appear 'neuron'-shaped, and have long processes which grow out in all directions. The processes and the cell bodies sometimes have a thin spread of granules on them. They show intracellular movements.

THE CURRENT VIEW OF THE DIFFERENT KINDS OF CELLS AS DESCRIBED IN THE LITERATURE

With these generalizations and reservations in mind, we may now examine the authoritative literature for descriptions of the different kinds of cells by light and electron microscopy. The intention of this section is to try to find out by what criteria histologists, electron microscopists, histochemists, neuropathologists, immunocytochemists, physiologists and tissue culturalists, distinguish the different cell types, and how real these distinctions are.

In Tables 8-11, one often sees empty columns, in which comparative information is absent from a particular publication, presumably because that information was not investigated in that series of experiments. An occasional space probably has no significance, but I would venture to suggest that sometimes there may well be some good, albeit esoteric, reason for frequent absence of data under a particular heading. This would have been regarded as 'singular' by a celebrated psychobiologist studying an aphasic dog, and would certainly have generated interpretable clues (Holmes, 1905). Clearly, one must distinguish between absence of information and an unequivocal statement denying the possession of a property. The English term, 'fail to show' can either mean that a research worker has tried but failed so far to demonstrate a property he believes to be possessed by his system, or that he has not yet investigated that property by accident or intention. One is entitled to seek an explanation of why such crucial information has not been recorded.

4
Specific Identification

LIGHT MICROSCOPY OF HISTOLOGICAL SECTIONS (TABLE 8)

The literature indicates that neurons may be spherical, elongated, pear-shaped, spindle-shaped, pyramidal, fusiform, conical, unipolar, bipolar, multipolar, polygonal, stellate, irregular or varied in shape; their maximum diameters in stained sections appear to be between 4 μm and 435 μm. The astrocytes are rarely described, but one description is implied in their name, star-shaped; they are the largest glial cells with diameters of 20 μm to 35 μm. Oligodendrocytes are round or oval. They are generally described as 'small' but the only measurement of their diameters is 10 μm to 20 μm. Microglia in normal tissue are, stellate, elongate, spherical, oval, bulb-like, bipolar, tripolar, multipolar, amoeboid, pleiomorphic; they are small or very small, or cannot be seen, or do not exist.

The number of processes - given the name dendrites - arising from neurons is variously put at being from one to thousands, in addition to one thicker axon. They taper, twist, ramify or bifurcate for a few to hundreds of μm, except for the axons which may reach tens of centimetres in length. Astrocytes also have many processes, which are seen to branch, and end frequently on capillaries or blood vessels; the latter connections are often regarded as specific criteria for identifying astrocytes. Oligodendrocytes are generally described as having small, delicate, few processes, or occasionally none at all. Microglia have few processes which branch two or three times; the processes may be straight or tortuous.

By light microscopy the nuclei of neurons appear large, spherical or ovoid and diffuse chromatin is spread within them; they contain conspicuous nucleoli, which are usually centrally placed. The nuclei of astrocytes are 5 μm to 10 μm in diameter, smaller than those of neurons and larger than those of oligodendrocytes. Oligodendrocytic nuclei are often described as eccentric, and often stain darkly. The nuclei of microglia appear spherical, oval or elongate, and stain darkly; nucleoli are rarely reported in microglial nuclei, and most authors seem to regard them as absent.

Neuronal cytoplasm contains Nissl granules, which do not appear in the cytoplasm of other cells. Many authors see a Golgi apparatus, mitochondria and cytoplasmic fibrils; lipofuscin granules may be present, and centrosomes may sometimes be identified. The cytoplasm of astrocytes is not so frequently described. It is sometimes said to be paler and Nissl granules are not reported in it, but Golgi apparatuses,

mitochondria and centrosomes can be seen. Oligodendrocytes either have a thin layer of cytoplasm with no fibrils, or they are said to contain Golgi apparatuses, fibrils, lipofuscin and centrosomes. Microglia either have scanty or barely distinguishable cytoplasm. In some publications they contain dense cytoplasm with granules and mitochondria but no Golgi apparatuses or Nissl granules.

APPEARANCES BY ELECTRON MICROSCOPY (TABLE 9)

From the tables one can see that few publications describe the overall appearances of cells by electron microscopy, presumably for the following reasons: firstly, it is believed that the appearances have already been characterized by light microscopy; secondly, the section thicknesses are such that many serial sections would be necessary to describe the whole outline of a single cell; thirdly, one cannot describe the shapes accurately because they are distorted during preparation for electron microscopy; fourthly, the particular authors were not interested in the shapes of the cell bodies.

The neurons appear round, pear-shaped, multipolar, polygonal, angular, irregular or smooth (Table 9). Descriptions of the cell bodies of astrocytes are rare, but they deal mainly with the processes. The processes end on vascular capillaries; astrocytes have less processes than neurons do; they are branched and delicate. There are also few descriptions of oligodendrocytes; they are said to have few to numerous processes, often thin and branching. Microglia are small, may be indented, and have several processes.

The cytoplasm of neurons contains granules believed to be lipofuscin. The cytoplasm of neurons and all types of neuroglia contain Nissl bodies, endoplasmic reticulum, Golgi bodies, mitochondria with cristae, ribosomes, polyribosomes, granules and varying proportions of filaments, tubules and fibrils. The astrocytes have paler cytoplasm than the neurons, whole the oligodendrocytes have denser cytoplasm; the microglia have been described as having no cytoplasm, a little cytoplasm or dense cytoplasm (Table 9).

The nuclei of neurons appear 'large'. They may be spherical, oval, lobulated or indented. They may be centric or eccentric. The nuclear membranes appear to be trilaminar, and to be punctuated by nuclear pores or nuclear pore apparatuses. The nuclei contain granules or filaments of chromatin. The nuclei of astrocytes appear as having various shapes: irregular, ovoid, indented, or reniform. They may be centric or eccentric, and appear large or 'prominent'. They also have nuclear pores or nuclear pore apparatuses, and a trilaminar membrane. The nucleoplasm contains masses or granules believed to consist of chromatin.

The nuclei of oligodendrocytes are round or oval, and appear dense. They also have a trilaminar membrane with nuclear pores, and they contain clumps of chromatin. The nuclei of microglia are also oval, elongate, angular, variable or irregular; they are usually dense or light. They contain clumps of chromatin. There seems to be little information on whether or not they have nuclear pores.

The nucleoli of neurons are 'large', centric or eccentric, but have no limiting membranes, visible by electron microscopy. They have a dense granular or filamentous mass, the nucleolonema. Occasionally there is more than one nucleolus in a neuron. The nucleoli of astrocytes are described as absent, occasional, constant or prominent; their shapes have not been examined in detail.

In respect of nucleoli, there is unfortunately a lack of information about oligodendrocytes, as there is with microglia. One cannot tell from the literature whether the nucleoli cannot be seen, or cannot be recognized, or do not exist or have not been examined specifically and systematically, or have not been examined at all.

The references given in these tables were not selected for their diversity of views, but represent all the authorities available to the author. Under these circumstances, careful perusal of the original references, or Tables 8 and 9, or my summary of them, leads to the following inescapable conclusion. In the literature the descriptions of the different kinds of cells in the nervous system by light or electron microscopy either overlap so considerably, or are so evidently lacking in comparative data, that one cannot conclude with confidence that the different kinds of cells in the nervous system can be distinguished from each other by light or electron microscopy.

APPEARANCES IN TISSUE CULTURE (TABLE 11)

Prima facie, one would hope that descriptions of cells in tissue culture would be similar to those of cells identified as particular types in histological sections made from adult tissue. Neurons in culture may have cell bodies which are spherical, bipolar or multipolar. They may be relatively large, like Purkinje cells, or small, like dorsal root ganglion cells. They have fine, branching, filamentous processes; filaments are seen in the cell bodies of some of them (Table 11).

Astrocytes have stellate cell bodies with wide undulating processes at their origins, or with multiple branching. Some astrocytes are often described as 'fibroblastic' in appearance. The cell bodies may be contractile, and they may contain more than one nucleus.

Oligodendrocytes may be difficult to distinguish from astrocytes in culture. They appear globose and often have dense nuclei. They also have extensively branching processes, sometimes ending in membraneous expansions, but usually fewer in number than the astrocytes possess. The appearance of microglia in culture does not seem to have been described, although motile phagocytic cells are often seen, especially by time-lapse photography.

One may conclude from these descriptions in the literature that neurons, astrocytes and oligodendrocytes cannot be distinguished from each other in culture by their appearances alone, since they overlap so obviously. Identification usually requires knowledge of their tissue origin or pathology, their staining properties and their possession of markers believed to be characteristic for their cell type.

SPECIFIC STAINING

Many colour dyes used in histology, such as methylene blue, haematoxylin, eosin and cresyl violet, are nowadays considered non-specific. The metal stains were developed for biological purposes from their use in photographic techniques, and they were nearly all variations of reactions involving reduction of silver salts. Indeed, before the modern sophisticated mixtures were concocted for colour photography, there were already in commercial use complete systems of colour photography based on silver salts of different hues.

One can list the increasing intensities of colour with which cells in the nervous

Table 11 The appearance of allegedly different cellular elements in tissue cultures as seen and described by the authors by phase-contrast microscopy. Lack of information under any heading indicates that it was not supplied in that publication

Neurons	Astrocytes	Oligodendrocytes	Microglia	Author/tissue
Purkinje cells identified by large size and characteristic structure in living preparations, their affinity for silver salts, their content of neurofibrils and basophilic substance. Also present large numbers of small granule cells with delicate processes which could be confused with oligodendrocytes using phase optics and methylene blue. Granules have a clear nucleus and a prominent nucleolus. Well-developed non-membranous processes	Processes form inter-lacing networks, are dense and characterised by presence of undulating membranes; processes have a broad cone shaped base	Similar size cells to granule cells but the perinuclear zone often obscured; processes generally arise directly as a narrow process from the perinuclear cytoplasm; often appeared to glow under phase contrast microscopy, due to their globose form and their rich granular content which cast a halo around the perinuclear cytoplasm		Pomerat and Costero (1956) cat - cerebellum
Identified pyramidal cells, giant pyramidal cells, giant polymorphous neurons and bipolar spindle neurons Cell bodies very from 30 to 300 µm in length; some binucleate and polynucleated neurons; generally one nucleolus present, but 2 or 3 may appear and 1 or 2 nucleolar satellites may be present; neuro-fibrils present in cell bodies as well as in neuronal processes				Geiger (1957) human, rabbit and monkey - cerebral cortex
Cell bodies are small and spherical and show marked refractility processes are fine, short and anastomose richly; difficult to distinguish from astrocytes in some cases	Stellate or angulated cells with processes of very great length; processes are slender, arise fairly sharply from the perikaryon; they branch and anastomose freely, appear sticky, elastic, lateralized rather polymorphic nuclei, many are binucleated, some-times nuclei show amitotic features	Cannot distinguish between primitive oligodendrocytes and astrocytes in culture		Lumsden (1958) human - brain
	In the early stages of cultivation astrocytes from different parts of the brain form a three dimensional net-work of various densities; the cells are connected by their processes; character-istic shaking movement of the cell bodies; in a thin zone of outgrowth astrocytes present a typical network of stellate processes but at the periphery there is a gradual transformation so more similar to ependyma cells	Small cells with globose, vase-shaped or rounded perinuclear zones rich in highly refractile granules and droplets which tend to obscure the nucleus. A small number of processes arise abruptly (i.e. no cone-shaped base) from the perinuclear cytoplasm		Pomerat (1958) cat - brain and spinal cord
Neurons extended long, delicate processes that form inter-connecting bundles; heterogeneous in size and shape	Fibroblastic	Processes which are usually long extensively branched, dark and thorny; some have small, dark nuclei and dark cytoplasm, some are larger and lighter; processes often end in large membranous expansions	Macrophages - vac-uolated cytoplasm which frequently contain debris; occasionally extend short processes	Raff, Fields, Hakamori, Mirsky, Pruss and Winter (1979) rat - optic nerve, cerebellum, corpus callosum and cerebral cortex
10% of cells in dorsal root ganglia culture have typical neuronal morphology, with large refractile cell bodies and long, fine processes	GFAP+ (glial fibrillary acid protein) cells - some large with large vesicular nuclei, others smaller and darker with multiple branching processes	GC+ (galactocerebroside) cells are usually small, with dark nuclei and cytoplasm, 50% of these have multiple, sometimes branching processes, others have rudimentary processes or none at all		Kennedy, Lisak and Raff (1980) human fetuses - optic nerve, spinal cord, dorsal root ganglion
Small and bipolar, or large and or multipolar	Most are flat, fibroblastic - like cells with filament bundles extending into 1 or 2 long processes, some are smaller and darker (GFAP+)	Dark nuclei and cytoplasm; and usually have branched processes (CG+)		Abney, Bartlett and Raff (1981) rat - brain

system are stained: blue-violet coloured solutions precipitate as black granules; grey also precipitates as black granules; red-ruby-purple solutions precipitate as brown or black granules. Examination of Table 10 shows that virtually all 'specific' stains eventually show up on cell bodies or fibres as black or brown granules of silver salts or silver metal. One can scan the vertical columns of Table 10, and one cannot fail to see that there is no single staining procedure which all, or even most, authors would agree to be specific for a type of cell or fibre in the nervous system. Of course, individual authors do claim or believe in the specificity of particular procedures indicated in the tables. Part of the problem here is the fundamental one that it is difficult to judge the relative value of different observations by different authors using basically - but not exactly - similar procedures and reagents, when they have often studied cell types in different mammalian species, of different ages, in different nuclei. (Please see also Appendix 1).

Here again, we must be humbled by the conscience of doubt, as well as our unwillingness to adopt an agnostic position, into concluding that staining procedures do not permit us to identify unequivocally the four major types of cell in the nervous system or their processes.

MARKERS (TABLE 12)

'Markers' may be proteins, enzymes, amino acids, transmitters or antibodies. Obviously, the concept of a marker implies that it is thought to be relatively or completely specific for the cell type it is believed to mark. In the list of markers (Table 12) many of the authors have not stated whether or not they have examined the reactions of cell types other than those they believe to be shown up by the markers; in the table these are indicated by the term 'not reported', whereas others, marked 'not labelled', mean that the authors have shown that the markers were not present in the particular cell types. Unfortunately, even the clear demonstration of absence of a marker cannot be used as a certain criterion for the identification of a particular cell type, since frequently the absence of the reaction is itself the only criterion for the identification of the type of cell itself. Thus, it would be a circular argument to define the specificity of a marker for a particular type of cell by the belief that the particular cell is identified by the specificity of the marker.

Many markers, such as tetanus toxin, glial fibrillary acid protein, S-100 or glutamate synthetase, were initially regarded as specific markers for particular cell types until their presence in, or affinity for, other cell types was examined. However, when we take a bird's-eye view of a very large number of markers nestling in the literature and the specificities claimed by the different authors, we come to the inescapable if disappointing conclusion that there is no marker upon whose specificity all authors would agree. (Please see also Appendix 2).

Most of the markers have been tested in tissue cultures, and their specificity may change quantitatively or qualitatively during the life of the culture, and may also be different in the host tissue than in the culture.

One could possibly differentiate the cell types by the intensity with which they are marked, but the concept of specificity rather implies an 'all-or-none' phenomenon, especially in relation to genetic origin or immunological reactions.

CONCLUSIONS FROM THE LITERATURE

The evidence in the literature indicates massive and sincere differences of opinion among responsible neurobiologists about the identification of what are believed to be different types of cell, in relation to the following criteria: appearances in adult tissues by light and electron microscopy; light microscopy of cell types in tissue culture; staining procedures believed to be specific; markers generally regarded as specific. In addition, many authors have made the point that they themselves cannot distinguish between the cell types, especially of the neuroglia, or have used the term neuroglia, which implies obviously that they cannot distinguish between the different types of cells described by this term.

We will attempt to draw a conclusion about the reason for all of these difficulties (please see page 205).

Table 12 Enzymes, proteins, antibodies, transmitters, and a toxin, which are widely used as markers for different neural elements in tissue culture or immunochemistry. There are no markers for microglia

Marker	Neurones	Astrocytes	Oligodendrocytes	Author
Enzymes	Carbonic anhydrase	Carbonic anhydrase*	Carbonic anhydrase*	Giacobini (1961)
	Glutamate decarboxylase	Glutamate decarboxylase	Glutamate decarboxylase	Roberts (1962)
	Gamma aminobutyric acid transaminase	Gamma aminobutyric acid transaminase	Gamma aminobutyric acid transaminase	
	Beta-galactosidase	Beta-galactosidase*	Beta-galactosidase*	Hirsch (1972)
	Ceramide glucosyltransferase	Ceramide glucosytransferase	Ceramide glucosyltransferase	Radin, Brenkert, Arora, Sellinger and Flangas (1972)
	Beta-galactosidase	Beta-galactosidase	Beta-galactosidase	Raghavan, Rhoads and Kanfer (1972)
	Alpha-glycerophosphate dehydrogenase	Alpha-glycerophosphate dehydrogenase	Alpha-glycerophosphate dehydrogenase	Sellinger and Santiago (1972)
	Beta-galactosidase	Beta-galactosidase*	Beta-galactosidase*	Sinha and Rose (1972)
	Carbonic anhydrase	Carbonic anhydrase*	Carbonic anhydrase*	Murai (1973)
	Carbonic anhydrase	Carbonic anhydrase*	Carbonic anhydrase*	Nagata, Mikoshiba and Tsukada (1974)
	Carbonic anhydrase	Carbonic anhydrase*	Carbonic anhydrase*	Hamberger and Sellstrom (1975)
	Not reported	Not reported	2',3'-cyclic nucleotide 3'-phosphohydrolase	Benda (1978)
	Not reported	Not reported	Glycerol-phosphate-3-dehydrogenase	
	Not reported	Glutamate dehydrogenase	Not reported	Berl, Nicklas and Clarke (1978)
	L-glutamate decarboxylase	L-glutamate decarboxylase	L-glutamate decarboxylase	Schousboe (1978)
	Not reported	Glutamate synthetase	Glutamate synthetase	Stahl, Spence, Coates and Broderson (1978)
	Not reported	Glutamine synthetase	Glutamine synthetase	Tower (1978)
	Glycerol-3-P-dehydrogenase	Glycerol-3-P dehydrogenase	Glycerol-3-P dehydrogenase	Tower (1978)
	Carbonic anhydrase	Carbonic anhydrase	Carbonic anhydrase*	Varon (1978)
	Not reported	Glycerol-phosphate dehydrogenase	Glycerol-phosphate dehydrogenase	
	2',3'-cyclic nucleotide 3'-phosphohydrolase	2',3'-cyclic nucleotide 3'-phosphohydrolase	2',3'-cyclic nucleotide 3'-phosphohydrolase	
	Not reported	Glutamine synthetase	Glutamine synthetase	
	Not reported	Not reported	Galactosyltransferase	
	Not reported	Not reported	Cerebroside sulfotrans-	
	Not reported	3-hydroxy,3-methyl glutaryl co enzyme A reductase	3-hydroxy,3-methyl glutaryl co enzyme A reductase	
	Not reported	Glutamine synthetase	Glutamine synthetase	Norenberg and Martinez-Hernandez (1979)
	Pyruvate phosphokinase	Pyruvate phosphokinase	Pyruvate phosphokinase	Pevzner (1979)
	NAD pyrophosphorylase	NAD pyrophosphorylase	NAD pyrophosphorylase	
	Glutaminase	Glutaminase	Not reported	Schousboe, Nissen, Bock, Sapirstein, Juurlink and Hertz (1980)
	Not reported	Carbonic anhydrase	Carbonic anhydrase	
	Glutamate decarboxylase	Not labelled	Not reported	
	Not reported	Glutamine synthetase	Not reported	
	Not reported	Not reported	Carbonic anhydrase	Sensenbrenner, Labourdette Delaunoy, Pettman, Devilliers, Moonen and Bock (1980)
	Not reported	Glutamine synthetase	Not reported	Vernadakis, Parker and Norenberg (1980)
	Not reported	Not reported	2',3'-cyclic nucleotide-3'-phosphohydrolase	
	Not reported	Not reported	Glycerol phosphate dehydrogenase	
	Not reported	Not reported	Carbonic anhydrase isoenzyme II	Ghandour, Derer, Labourdette, Delaunoy and Langley (1981)
	Not reported	Not reported	2',3'-cyclic AMP phosphohydrolase	
	Not reported	Butyrylcholinesterase	Not reported	
	Not reported	Glutamine synthetase	Not reported	White, Dutton and Norenberg (1981)

Table 12 continued

Marker	Neurones	Astrocytes	Oligodendrocytes	Author
Enzymes	Not reported	Not reported	2',3'-cyclic nucleotide-3'-phosphohydrolase	Hansson (1982)
Proteins	S-100	S-100*	S-100*	Hyden and McEwen (1966)
	Not reported	Glial fibrillary acid	Not reported	Eng, Vanderhaeghan, Bignami and Gerstl (1971)
	14-3-2	14-3-2	14-3-2	Packman, Blomstrand and Hamberger (1971)
	Not reported	Glial fibrillary acidic	Not reported	Bignami, Eng, Dahl and Uyeda (1972)
	S-100	S-100*	S-100*	Moore (1972)
	14-3-2	14-3-2	14-3-2	
	S-100	S-100*	S-100*	Satake (1972)
	14-3-2	14-3-2	14-3-2	
	S-100	S-100*	S-100*	Uyeda, Eng and Bignami (1972)
	Glial fibrillary acidic	Glial fibrillary acidic*	Glial fibrillary acidic*	
	Not reported	S-100*	S-100*	Jacque, Jørgensen, Baumann and Bock (1981)
	14-3-2	Not reported	Not reported	
	Not reported	Glial fibrillary acidic	Not reported	
	S-100	S-100*	S-100*	Hamberger and Sellstrom (1975)
	S-100	S-100*	S-100*	Hertz (1977)
	14-3-2	Not reported	Not reported	
	Not reported	Glial fibrillary acidic	Not reported	Benda (1978)
	S-100	S-100	S-100	
	Not reported	Glial fibrillary acidic	Not reported	Fedoroff (1978)
	Not reported	Glial fibrillary acidic	Not reported	Gheuens, Lowenthal, Karcher and Noppe (1978)
	Not reported	Glial fibrillary acidic	Not reported	Hertz, Schousboe, Boechler, Mukerji and Fedoroff (1978)
	Not reported	S-100	S-100	Jacobson (1978)
	14-3-2	Not reported	Not reported	
	Not reported	Gial fibrillary acidic	Not reported	
	Not reported	S-100	Not reported	Jacque and Baumann (1978)
	14-3-2	Not reported	Not reported	
	Not reported	Glial fibrillary acidic	Not reported	
	Not reported	Not reported	Wolfgram W1 and W2	
	Not reported	Glial fibrillary acidic	Not labelled	Raff, Mirsky, Fields, Lisak, Dorfman, Silberberg, Gregson Leibowitz and Kennedy (1978)
	Not reported	Glial fibrillary acidic	Not reported	Schousboe (1978a)
	S-100	S-100	S-100	Varon (1978)
	Not reported	Glial fibrillary acidic	Not reported	
	Not reported	Glial fibrillary acidic	Not reported	
	Not labelled	Glial fibrillary acidic	Not labelled	Raff, Fields, Hakamori, Mirsky, Pruss and Winter (1979)
	Not reported	Glial fibrillary acidic	Not reported	Balazs, Regan, Meier, Woodhams, Wilkin, Patel and Gordon (198?)
	Not labelled	Glial fibrillary acidic	Not reported	Bignami, Kozak and Dahl (1980)
	Not labelled	Glial fibrillary acidic	Not labelled	Currie (1980)
	Not labelled	Glial fibrillary acidic	Not labelled	Kennedy, Lisak and Raff (1980)
	Not reported	Glial fibrillary acidic	Not reported	Schousboe, Nissen, Bock Sapirstein, Juurlink and Hertz (1980)
	Not reported	Glial fibrillary acidic	Glial fibrillary acidic	Schürch-Rathgeb and Monard (1980)
	Not reported	Glial fibrillary acidic	Not reported	Sensenbrenner, Labourdette, Delaunoy, Pettman, Devilliers, Moonen and Bock (1980)
	Not labelled	Glial fibrillary acidic	Not labelled	Abney, Bartlett and Raff (1981)

Table 12 continued

Marker	Neurones	Astrocytes	Oligodendrocytes	Author
Proteins	Not labelled	Glial fibrillary acidic	Not labelled	Bartlett, Noble, Pruss, Raff, Rattray and Williams (1981)
	Not reported	Glial fibrillary acidic	Glial fibrillary	Cohen and Selvendran (1981)
	Not reported	Glial fibrillary acidic	Not reported	Currie and Kelly (1981)
	Not reported	Glial fibrillary acidic	Not reported	Ghandour, Derèr, Labourdette, Delaunoy and Langley (1981)
	14-3-2	Not reported	Not reported	Jacque, Jørgensen, Baumann and Bock (1981)
	Not reported	Glial fibrillary acidic	Not reported	
	Not reported	Glial fibrillary acidic	Not reported	White, Dutton and Norenberg (1981)
	14-3-2	Not reported	Not reported	Hansson (1982)
	Not reported	Glial fibrillary acidic	Not reported	
	Not reported	Alpha-albumin	Not reported	
	Not reported	Not reported	Myelin basic protein	
	Not reported	S-100	S-100*	
	Not reported	Glial fibrillary acidic	Not reported	Meier, Regan, Balazs and Wilkin (1982)
Anti-bodies	Dl, D2 and D3 antigens	Not reported	Not reported	Jacque, Jørgensen and Bock (1979)
	Not reported	NSl antigen	NSl antigen	Benda (1978)
	Not reported	Gl and G2 antigens	Gl and G2 antigens	
	Nl and N2 antigens	Not labelled	Not labelled	
	Dl, D2 and D3 antigens	Not reported	Not reported	Jacque and Baumann (1978)
	Not reported	NSl antigen*	NSl antigen*	Varon (1978)
	Not reported	Anti corpus callosum sera*	Anti corpus callosum sera*	
	Not reported	Gl and G2 antigens*	Gl and G2 antigens*	
	Not reported	Not reported	Anti oligodendrocyte serum*	
	Neural filament antisera	Not labelled	Not labelled	Bignami, Kozak and Dahl (1980)
	Not labelled	Neural-antigen 2 (Ran 2)	Not labelled	Abney, Bartlett and Raff (1981)
	Not labelled	Neural-antigen 2 (Ran 2)	Not labelled	Bartlett, Noble, Pruss, Raff, Rattray and Williams (1981)
	Dl, D2 and D3 antigens	Not reported	Not reported	Jacque, Jørgensen, Baumann and Bock (1981)
	D5 antigen	D5 antigen	D5 antigen	
	Dl, D2 and D3 antigens	Not reported	Not reported	Meier, Regan, Balazs and Wilkin (1982)
Trans-mitters	Gamma aminobutyric acid	Gamma aminobutyric acid	Gamma aminobutyric acid	Roberts (1962)
	Dopamine	Not reported	Not reported	Hillarp, Fuxe and Dahlstrom (1966)
	Noradrenaline	Not reported	Not reported	
	5-Hydroxytryptamine	Not reported	Not reported	
	Gamma aminobutyric acid	Not reported	Not reported	Obata (1972)
	Gamma aminobutyric acid	Not labelled	Not labelled	Schousboe, Svenneby and Hertz (1977)
	Gamma aminobutyric acid	Gamma aminobutyric acid	Gamma aminobutyric acid	Kelly and Dick (1978)
	Gamma aminobutyric acid	Gamma aminobutyric acid*	Gamma aminobutyric acid*	Schousboe (1978a)
	Not reported	Acetylcholine*	Acetylcholine	Varon (1978)
	Not reported	Dopamine*	Dopamine*	
	Not reported	Adenosine*	Adenosine*	
	Not reported	Nor-epinephrine*	Nor-epinephrine*	
	Gamma aminobutyric acid	Gamma aminobutyric acid	Not reported	Currie and Kelly (1981)
	Gamma aminobutyric acid glycine	Gamma aminobutyric acid glycine	Gamma aminobutyric acid glycine	Wilkin, Csillag, Balazs, Kingsbury, Wilson and Johnson (1981)
Toxin	Tetanus	Not labelled	Not labelled	Dimpfel, Neale and Haberman (1975)
	Tetanus	Tetanus	Tetanus	Mirsky, Wendon, Black, Stolkin and Bray (1978)

Table 12 continued

Marker	Neurones	Astrocytes	Oligodendrocytes	Author
Toxin	Tetanus	Not reported	Not reported	Raff, Mirsky, Fields, Lisak, Dorfman, Silberberg, Gregson, Leibowitz and Kennedy (1978)
	Tetanus	Not labelled	Not labelled	Raff, Fields, Hakamori, Mirsky, Pruss and Winter (1979)
	Tetanus	Not reported	Not reported	Balazs, Regan, Meier Woodhams, Wilkin, Patel and Gordon (1980)
	Tetanus	Not labelled	Not labelled	Currie (1980)
	Tetanus	Not labelled	Not labelled	Kennedy, Lisak and Raff (1980)
	Tetanus	Not labelled	Not labelled	Abney, Bartlett and Raff (1981)
	Tetanus	Not labelled	Not labelled	Bartlett, Noble, Pruss, Raff, Rattray and Williams (1981)
	Tetanus	Not reported	Not reported	Cohen and Selvendran (1981)
	Tetanus	Not reported	Not reported	Currie and Kelly (1981)
	Tetanus	Not reported	Not reported	Meier, Regan, Balazs and Wilkin (1982)
Lipid	Not reported	Not reported	Galactocerebroside	Jacque and Baumann (1978)
	Not reported	Not labelled	Galactocerebroside	Raff, Mirsky, Fields, Lisak, Dorfman, Silberberg, Gregson, Leibowitz and Kennedy (1978)
	Not labelled	Not labelled	Galactocerebroside	Lisak, Abramsky, Dorfman, George, Manning, Pleasure, Saida and Silberberg (1979)
	Not labelled	Not labelled	Galactocerebroside	Currie (1980)
	Not labelled	Not labelled	Galactocerebroside	Kennedy, Lisak and Raff (1980)
	Not labelled	Not labelled	Galactocerebroside	Abney, Bartlett and Raff (1981)
	Not labelled	Not labelled	Galactocerebroside	Bartlett, Noble, Pruss, Williams (1981)

This table was compiled from the references indicated, and is not claimed to be comprehensive. 'Not reported' means that the authors have not stated whether or not they examined whether the markers were present in cells other than those indicated. 'Not labelled' means that the authors have shown that the particular marker was **not** present in the cells indicated. Extremely few authors state by what criteria they identify the particular cell types. An asterisk (*) means that the authors use the generic term 'neuroglia' without indicating a particular cell type.

5
Experimental Studies

HISTOLOGY

Hyden (1959) dissected out single neurons and the pieces of tissue which are adjacent to them (Figure 29) (Hyden, 1959; Hyden and Pigon, 1960; Hyden 1961). They usually picked out clumps of approximately the same volume as the Deiters' neurons they were studying, in order to facilitate comparison of the biochemical properties of the neurons and neuroglia. In view of the paucity of histological studies, we took out pieces of tissue adjacent to neurons, and stained them with the following routine procedures: haematoxylin and eosin; Weil and Davenport (1933); Mallory's (1900) phosphotungstic acid haematoxylin; Patay's (1934) triple stain; chromosome red; Marsland, Glees and Erikson's (1954) stain; Gallyas's (1963) stain (Hillman, Deutsch, Allen and Sartory, 1977). The glial clumps were sandwiched between pieces of liver. With the exception of some areas stained according to Marsland, Glees and Erikson, all the other staining procedures showed: the presence of a small number of star-shaped neurons; the presence of a much larger number of what appeared to be 'naked' nuclei; the tissue between these structures appeared largely unstained by any of the procedures; membranes were not detected by these procedures between the neurons and the naked nuclei, although Weil and Davenport's; Mallory's; Marsland, Glees and Erikson's; and Gallyas's stains are believed to show up neuroglia (Table 13). However, the most surprising, clear and repeatable observation was that with every one of these procedures the membranes between the liver cells could be detected quite clearly (Hillman, Deutsch, Allen and Sartory, 1977). We then concluded that there were no cell membranes in the glial clumps between the neurons - which, of course, have their own membranes - and the 'naked' nuclei. This meant that most of the tissue in neuroglial clumps was either part of a syncytium, or was extracellular. The 'naked' nuclei either represented the nuclei of such a syncytium, or they were cells with little or no cytoplasm. Indeed, the spherical nuclei which are sometimes described as reactive astrocytes, sometimes as oligodendrocytes and sometimes as microglia (Table 8) are usually characterized as having little or no cytoplasm, or having cytoplasm which does not stain with the particular procedures used.

TABLE 13 The staining procedures used in present experiments and the structures or compounds they are believed to show. These staining procedures can be found in Gray (1958), Gallyas (1963), Romeis (1968) and Ralis, Beasley and Ralis (1973)

Staining procedures	Used to show	Colour
Cajal	Astrocytes	Dark ruby to dark purple
Cresyl violet	Nissl substance Glial cells	Purple Lilac
Gallyas	Nuclei and neuroglial processes	Black or grey-black
Golgi-Cox	Nerve cells and fibres Neuroglia	Black Sometimes stained blue
Haematoxylin and eosin	All cells	Nuclei - blue black Other components - pink
Holzer (1923)	Glial fibres	Blue
Marsland, Glees and Erikson	Nerve fibres	Dark brown to black
Osmium tetroxide	Proteins and lipids	Black
Palmgren (1948)	Nerve fibres	Dark brown to black
Patay	Nuclei	Red
Weigert-Pal	Myelin sheaths	Dark blue to black
Weil and Davenport	Microglia Oligodendroglia	Black Black
Phosphotungstic acid haematoxylin	Astrocytes	Blue

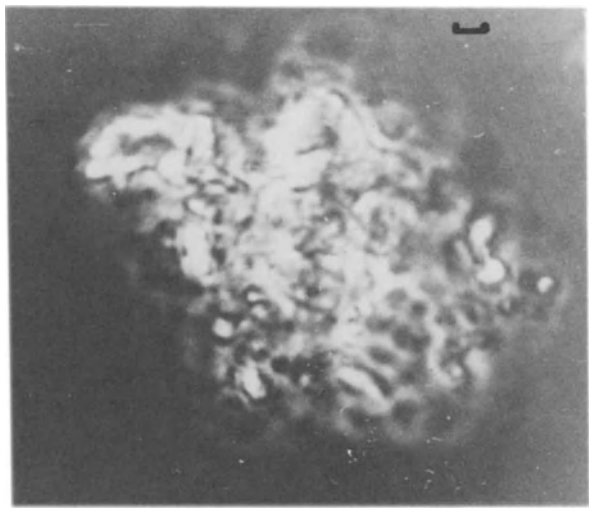


FIGURE 24 The mitochondria can be well seen in the frontal cortex of rat. The tissue was fixed and embedded in Carbowax (but not stained) and viewed by phase-contrast microscopy (Hillman and Sjuve, 1980). The bar is 5 μm

It was relatively easy to distinguish between the 'naked' nucleus as being surrounded by a syncytium or a curious kind of cell with little cytoplasm. We dissected out neurons and neuroglial clumps, and viewed them both by phase-contrast microscopy, and also after staining them with Janus Green-B neutral red. In all cases, the neuroglial clumps - like the neurons - were absolutely replete with mitochondria (Figure 24) in those parts of the brain (Hillman and Deutsch, 1978b). Therefore, the neuroglial material must be intracellular. This finding, together with the absence of membranes between the neurons and naked nuclei, led to the inescapable conclusion that the neuroglial clumps were, indeed, part of a syncytium.

Subsequently we did a further series of experiments, each on at least 10 pieces of the same tissues under each of the same conditions from 12 rabbits and 12 rats. The following additional experiments were carried out: firstly, whenever central nervous tissue was stained, pieces of kidney and liver from the same animals were taken out and stained simultaneously and with the same procedures; secondly, instead of examining tissue only from the medulla, we then studied pieces from the frontal lobes; the parietal lobes; the occipital lobes; the cerebellum; the thoracic spinal cord and the lumbar spinal cord; thirdly, instead of picking out neuroglial clumps under microscopic vision we placed the stainless steel wire at random points just below the surface of transverse 1 mm sections of the latter parts of the nervous tissue, and picked out pieces of tissue of 500-1000 μm in diameter. Thus, we were examining representative parts of the whole central nervous system, rather than tissue defined by its proximity to neurons or capillaries.

Once again, all these tissues showed the occasional neuron and the frequent naked nuclei in the ubiquitous syncytium (Figures 25-27). With all the staining systems - of which haematoxylin and eosin, cresyl violet and Weil and Davenport are shown - and Mallory's phosphotungstic acid haematoxylin also tested in this series, the membranes

around the kidney and liver cells could clearly be seen. In addition to staining the central nervous system with the latter stains it has also been examined after staining with all the nine other procedures in Table 13. In all cases no membranes could be seen between the neurons and the naked nuclei.

EXAMINATION OF UNSTAINED TISSUES

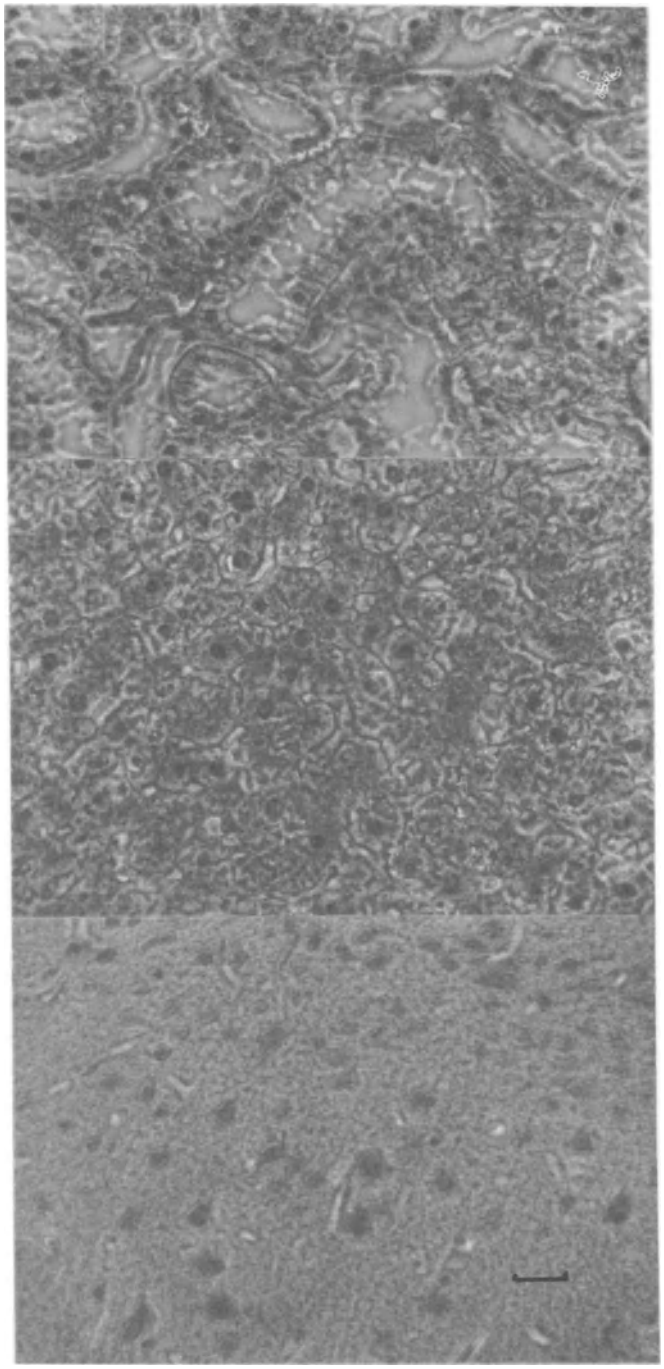
Different staining procedures distort the elements of a tissue to different extents. It may not be appreciated that the haematoxylin and eosin, the most popular procedure in use, involves 11 reagents in 14 steps. So we devised a simplified procedure, which ensures that all the tissues to be compared have been subjected to the same reagents and manipulations. This technique, involving five reagents in 10 steps, consisted of embedding tissues in paraffin wax, cutting sections 6 μm thick, mounting them in DPX, and examining them under phase-contrast microscopy (Hillman and Sjuve, 1980). The procedure shows up membranes clearly, as the embedding paraffin wax exaggerates phase differences to the detriment of colour differences, with which histologists and pathologists are much more familiar.

Once again, one saw only two elements, the apparently larger cells with processes, and the naked nuclei. No continuous membranes could be seen between the neurons and the naked nuclei (Figure 28). In addition to embedding the same seven tissues in paraffin wax, we also stained samples of each of them with 2% osmium tetroxide and embedded them in Epon. 'Thick' sections (2 μm) were cut for examination by phase-contrast microscopy, and 'thin' sections (80 nm) for electron microscopy. Once again both of these techniques revealed the same general features (Figure 29): (1) the 'large' approximately spherical cell with cytoplasm and processes, which are generally agreed to be neurons, and whose relative sparsity is known to every jobbing electron microscopist; (2) the spherical nuclei.

When 'naked nuclei' appear by light or electron microscopy to be attached by a 'foot' to a capillary, they are designated astrocytes. The problem with this identification is that, if one cannot see astrocytic 'feet' in a field, unless one examines serial sections, one cannot know whether the 'feet' cannot be seen because they are not in the relatively shallow plane of the section, or because they are not present. Of course, sections for electron microscopy are one-twentieth to one-fifteen-hundredth of the thickness of those used for light microscopy, so that the appearances of any processes would be much rarer on electron micrographs.

CELLS WITH APPARENTLY THIN FILMS OF CYTOPLASM

The cells apparently with little or no cytoplasm were examined carefully by bright-field illumination using approximately x960 magnification in preparations of rat and rabbit frontal, parietal, occipital, cerebellar, medullary sections, and thoracic and lumbar spinal cord sections, stained by all the staining procedures indicated (Table 13). It rapidly became obvious that the nuclei which appeared to be surrounded by thin rims of cytoplasm were simply out of focus. In any single section one can see a number of nuclei, and since the focal plane at this magnification is only about 1 μm deep, most nuclei are not clearly in focus at any particular setting of the fine adjustment.



FIGURES 25-27 Sections of rat kidney, liver and frontal cortex were stained simultaneously either with haematoxylin and eosin (Figure 25), or with cresyl violet (Figure 26), or with Weil and Davenport (Figure 27), and viewed by phase-contrast microscopy. These three staining procedures (shown) as well as phosphotungstic acid (not shown), all gave the clear appearance of a membrane around the renal tubule and hepatic cells, but none at all between the neurons and naked nuclei in the cortical cells (lower micrographs). It was thus concluded that no such membrane occurs in the latter situation. The bars in figures 25-27 are 50 μm

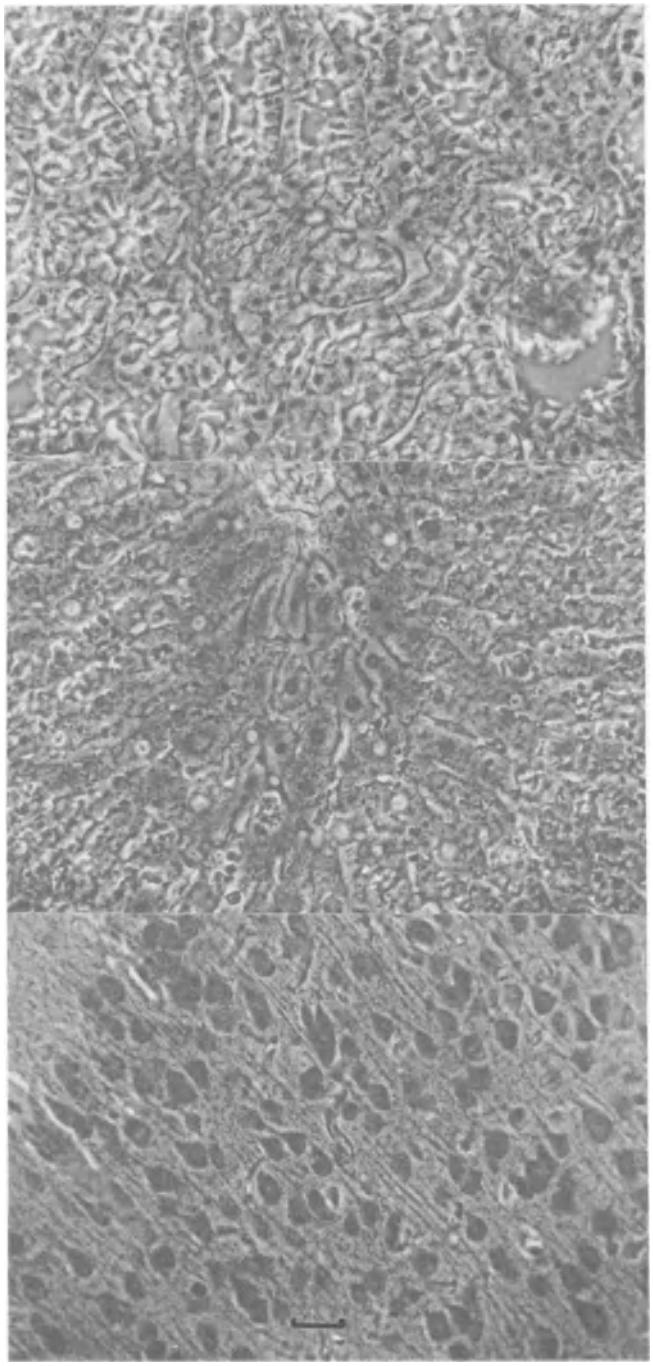


FIGURE 26

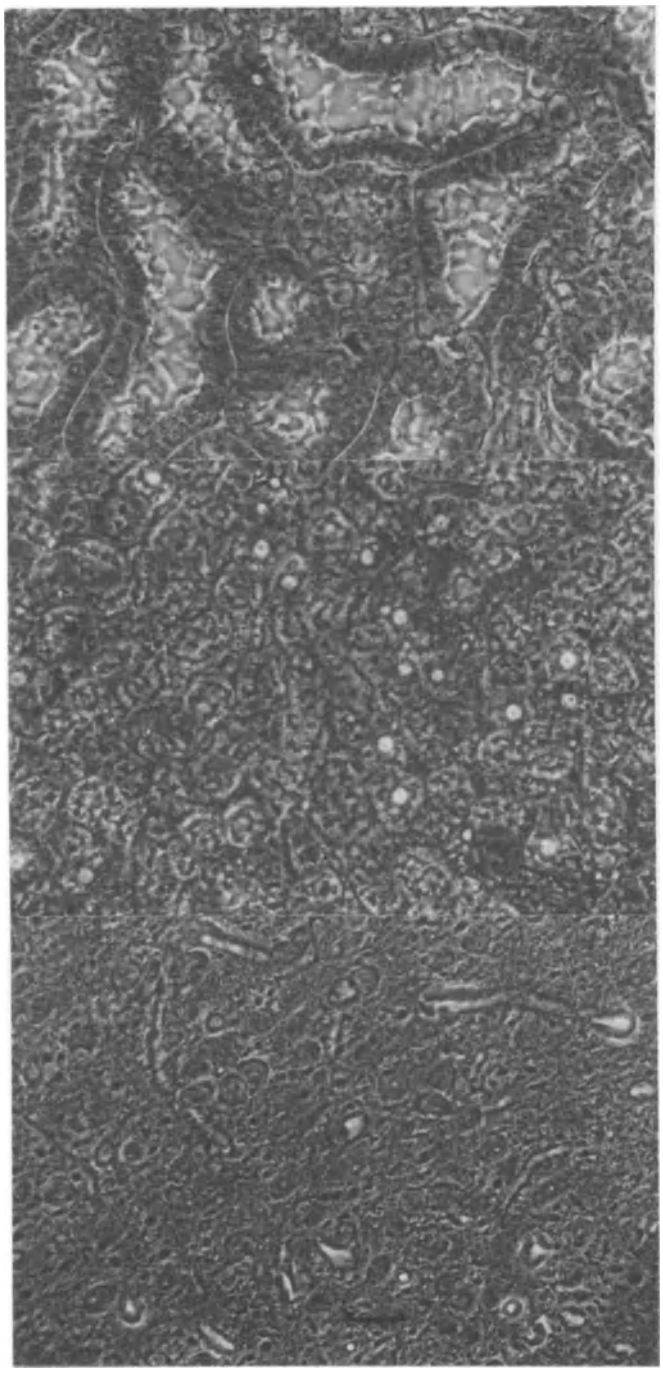


FIGURE 27

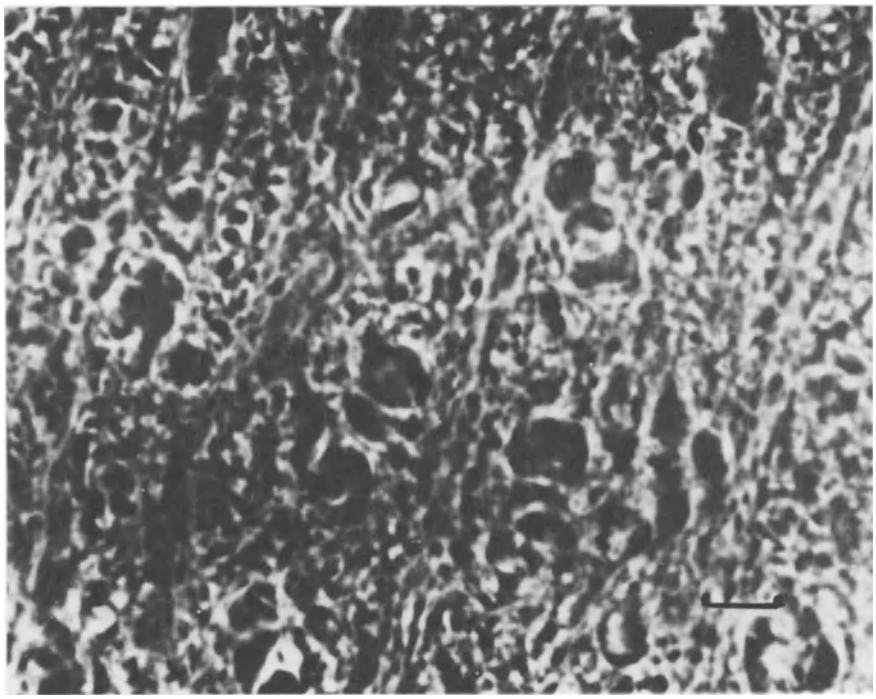


FIGURE 28 Frontal lobe embedded in paraffin wax, not stained, but viewed by phase-contrast microscopy. The bar is 50 μm

The cells out of focus all seem to be encircled by a fine rim of cytoplasm. If one refocuses on one of the latter nuclei, others go out of focus, and they then appear to be surrounded by this much-described thin layer of cytoplasm.

The widespread differences of opinions among distinguished histologists about the specificity of particular staining procedures led us to pose the question, How sure are we that different procedures do indeed show up different cells? We examined this in the following way.

SERIAL SECTIONS

Serial sections were made of the five different brain areas and the two regions of the spinal cord, and each section in a series was stained with a different procedure in the order: haematoxylin and eosin; Patay's triple stain; Weil and Davenport; Mallory's phosphotungstic acid haematoxylin; Gallyas's; Marsland, Glees and Erikson. In these time-consuming experiments we were greatly aided by Mr James Kirby of the St Peter and St Paul's Hospital, London, to whom we wish to record our considerable indebtedness.

In some experiments we picked out an identifiable group of cells which appeared to be typical neurons, and followed them for two or three sections through the sequence of staining procedures. Obviously, since the sections were cut 6 μm thick, it was not easy to identify particular cell bodies, which appeared to have maximum diameters of

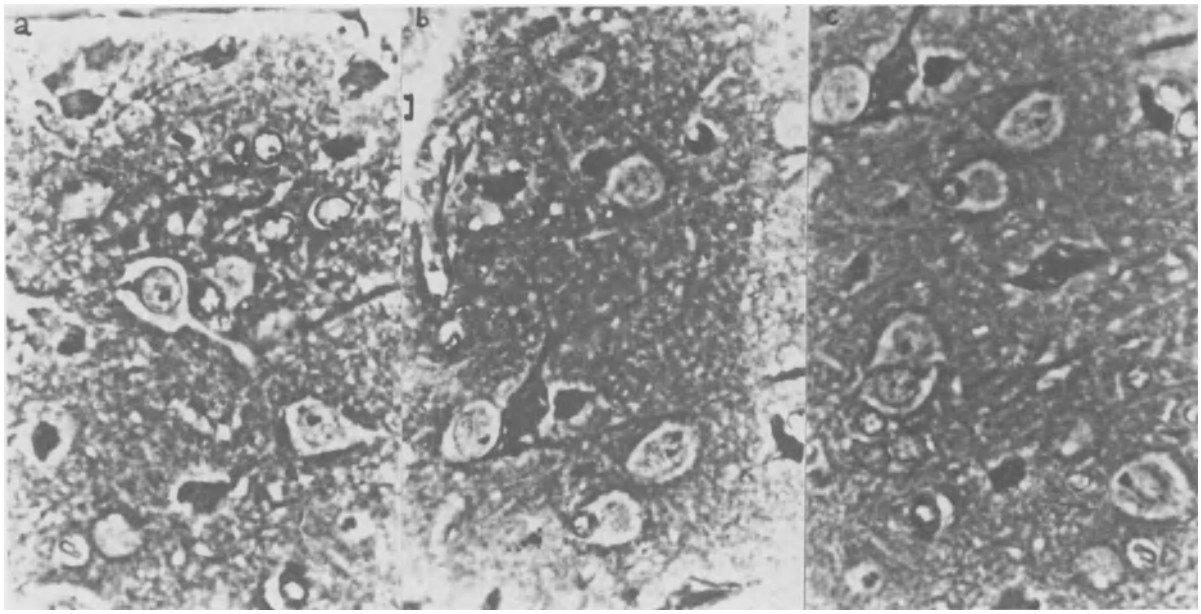


FIGURE 29 Sections of a (left) frontal, b (middle) parietal and c (right) occipital cortex of rat were made. They were fixed in glutaraldehyde, stained with osmium, embedded in Araldite, cut 2 μm thick and viewed by phase-contrast microscopy. Only two kinds of cells can be seen: the darkly staining neurons with processes, and the lightly staining naked nuclei usually surrounded by a shrinkage artifact. The bar is 50 μm

40-60 μm, through more than three to six sections. However, we could follow the same cells - when their positions formed a pattern - relatively easily in photographs of the same areas, and it became abundantly clear that the different procedures generally stained the same populations and not different ones (Figure 30). This could be demonstrated more precisely by counting what proportion of the cells with cytoplasm (other than single nuclei), which could be seen in one section could be identified in any subsequent one of a series. Examination of six serial sections of each of the same parts of the six areas under study showed that about 50% of the same cells could be followed from one section to the next (Table 14). Of course, if a staining procedure were specific for one cell type, only a small proportion of the cells in one serial section should show up in the next one in the series. More remarkably, approximately the same proportion of cells stained by one procedure appeared to be stained by a quite different procedure in the adjacent serial sections. In addition to the remarkable degree of overlap of elements stained, there was another very clear finding in every single section. That was that, with all stains, the bulk of the tissue was only lightly stained by any procedure. This observation was made not only in respect of the six stains mentioned above, but also for all the stains listed in Table 13.

TABLE 14 Serial sections were cut from rat frontal cortex 6 μm thick and stained with different stains. The same cells were identified by their positions from one section to the next serial section; the percentage of those seen in the second serial section originating from the first section is given in the third column of figures. The mean percentage for all stains and sections was 55%

	Number of cells stained	Number of cells identified in the first section seen in the second section	Percentage
1st series			
Gallyas: Weil and Davenport	50	24	48
Weil and Davenport: Patay	42	16	38
Patay: Haematoxylin and eosin	35	17	49
2nd series			
Gallyas: Weil and Davenport	84	40	48
Weil and Davenport: Patay	49	28	57
Patay: Haematoxylin and eosin	41	26	63
3rd series			
Gallyas: Weil and Davenport	55	19	34
Weil and Davenport: Patay	33	21	64
Patay: Haematoxylin and eosin	40	22	55
4th series			
Haematoxylin and eosin: Patay	71	30	43
Patay: Weil and Davenport	65	28	43
Weil and Davenport: Phosphotungstic acid haematoxylin	43	28	65
Phosphotungstic acid haematoxylin: Gallyas	44	19	43
5th series			
Haematoxylin and eosin: Patay	46	21	46
Patay: Weil and Davenport	39	19	49
Weil and Davenport: Phosphotungstic acid haematoxylin	37	18	49
Phosphotungstic acid haematoxylin: Gallyas	39	26	67

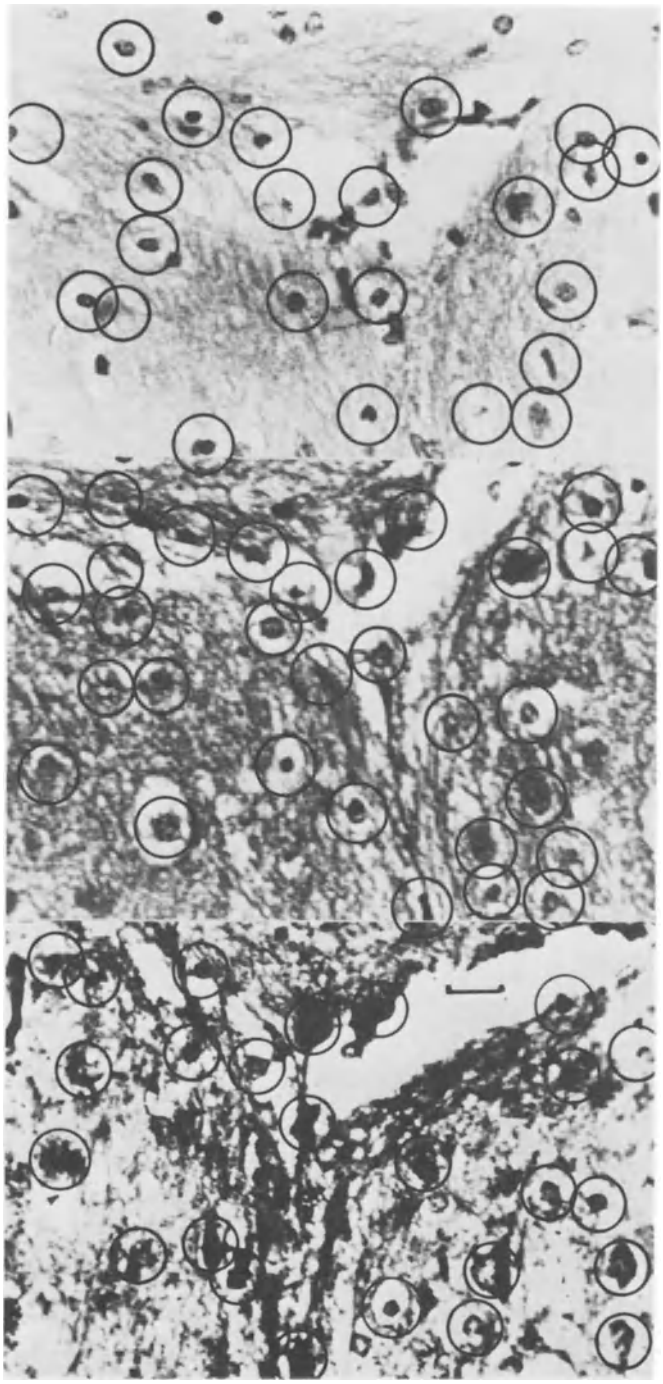


FIGURE 30 For legend please see page 104

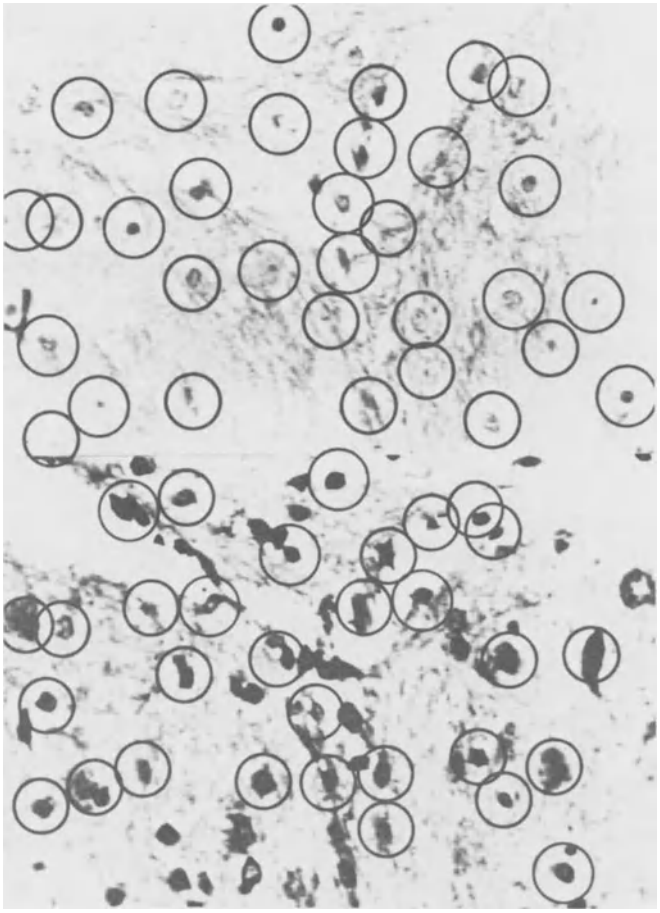


FIGURE 30 Serial sections of the septal region of rat were cut and stained with (page 3, upper) haematoxylin and eosin; (middle) Patay's stain; (lower) Weil and Davenport; (page 104 upper), phosphotungstic acid haematoxylin (Mallory) and (lower) Gallyas's stain. The cells in each section which appear to be identifiable in the next section are marked by circles on the subsequent pictures. Careful examination reveals that a substantial proportion of the cells stained by one procedure are also stained by the others, that is, the stains (several of which are neuroglial, please see Table 10), are not specific. The apparent shapes, dimensions and positions of the cells obviously change because one is cutting them at different places, because the different staining procedures and the microtomes distort and displace the cells to different extents. Therefore, the identity of cells in successive serial sections is based on their relative position rather than their shapes. The bar is 50 μm

QUANTIMETRY

We quantitated the proportion of the sections of each tissue which were stained by procedures which some authors believed to be specific. If each procedure were specific for neurons, astrocytes, oligodendrocytes and microglia, then the addition of the areas should together add up to approximately 100%. If they were not specific they might add up to more than 100%, and any significant degree of non-specificity would exaggerate the total proportion of the volume of the tissue stained by all the procedures.

We employed the stains classically believed to show: neural cells and their processes (Marsland, Glees and Erikson, 1954); astrocytes, phosphotungstic acid haematoxylin (Mallory, 1900); oligodendrocytes, glia and microglia (Weil and Davenport, 1933). We also used known non-specific procedures, such as haematoxylin and eosin, Patay's

triple stain and Gallyas's stain (please see Table 13).

The procedure was as follows: the brains of 24 rats were fixed with 10% formalin and embedded in paraffin wax. Over 400 sections were cut across the whole brain and stained with the above procedures. Of these sections, four fields across each of the frontal, parietal, and occipital lobes and of the medulla were taken at random; each of these fields had been stained with one of the six procedures listed in the preceding paragraphs. The fields were examined with bright-field illumination giving a magnification on the screen of x450.

In each slide, twenty different fields using x16 objective were scanned under a Quantimet 720 (Imanco Ltd), which has 500 000 Pix cells between the guard frames, and it scans 10 times per second. The microscope was focused and the intensity betweenn 0 and 63 (black to white) was selected for each field to show with maximum contrast the structures which the particular procedure is believed to stain. This adjustment of setting for each field - although time-consuming - corrected for differences of intensity of staining between different areas of a particular section, and also between the intensity and colour seen with different staining procedures. The number of Pix cells scanned and the number of elements counted appeared continuously at the edge of the screen. Each of these two numbers was read five times for a particular field and the mean values for each of the five readings were recorded. The areas stained were expressed as the number of Pix cells divided by 500 000 multiplied by 100; the mean and standard deviations were calculated for a particular staining procedure for all the sections of the particular area under study. Typical measurements are given in Appendix 3. The whole procedure was repeated using different slides but the same procedures, and no significant differences were found between the two sets of measurements. Therefore, the readings of the two sets were combined, and the values given represent eight slides, each counted in 20 different areas, for each of six different staining procedures, taken at random from over 400 slides originating from 24 different rat brains. In addition, slides of thoracic spinal cord, stained with haematoxylin and eosin only, were examined in a similar way.

The results are given in Tables 15 and 16. They show that

1. No single staining procedure, 'specific' or 'non-specific', shows up more than about 10% of the area of any section, or, put another way, most of the central nervous system is not stained by any of the procedures used.
2. If one adds up the areas stained by Marsland, Glees and Erikson (neural cells and processes); Mallory's phosphotungstic acid haematoxylin (astrocytes) and Weil and Davenport (oligodendrocytes and microglia), the total areas stained would only be between 14% and 20% of the total area of the tissue - that is, staining of all the elements together which are believed to be specific does not result in colouration of most of the tissue in the four different areas.
3. Even if one adds up the areas stained by the three 'specific' and the three 'non-specific' stains - whose overlap would much exaggerate the total areas stained - it still does not show up more than 33-41% of the total tissue.
4. Only 2.6-4.2% of the spinal cord was stained, which compares with means of 5.5-7.6% of the brain areas.

TABLE 15 Proportion, % (m ± s.d.) of areas of coronal sections of rat brain (and spinal cord) stained by the procedures indicated (n = 40 each).
The stained sections were scanned by a Quantimet particle counter, using a Leitz microscope, bright-field and a total magnification on the screen of x450. Please see also text (page 105) and legend to Appendix 3. Please note that the first three procedures are widely believed to be specific for the elements indicated in parentheses.

	Frontal	Parietal	Occipital	Medulla
Marsland, Glees and Erikson (neural cells and processes)	2.7 ± 1.0	5.2 ± 2.0	7.7 ± 3.3	5.1 ± 1.9
Mallory's phosphotungstic acid haematoxylin (astrocytes)	6.2 ± 2.0	3.6 ± 1.5	8.1 ± 2.1	6.6 ± 2.1
Weil-Davenport (oligodendroglia and microglia)	5.7 ± 1.8	5.2 ± 2.0	4.7 ± 1.9	4.7 ± 1.9
Haematoxylin and eosin (all cells)	5.5 ± 2.0	7.6 ± 2.1	5.9 ± 1.9	6.9 ± 2.6
Patay's triple stain (nuclei)	7.3 ± 2.0	5.1 ± 2.1	5.7 ± 2.6	7.2 ± 2.1
Gallyas (nuclei and neuroglial processes)	6.6 ± 1.7	6.6 ± 2.2	9.0 ± 2.3	9.0 ± 4.6
(Haematoxylin and eosin - spinal cord = 3.4 ± 1.3)				

TABLE 16 Mean percentages of stained areas as a proportion of total areas of 20 fields, each of two different brains

	Frontal	Parietal	Occipital	Fourth ventricle
Marsland, Glees and Erikson (neural cells and neural processes)	2.7	5.2	7.7	5.1
Mallory's phosphotungstic acid haematoxylin (astrocytes)	6.2	3.6	8.1	6.6
Weil-Davenport (oligodendroglia and microglia)	5.7	5.2	4.7	4.7
'Total mean area' assuming specificity of each stain	14.6	14.0	20.5	16.4

Please see also legend to TABLE 15

5. We then examined over 100 sections of brain, and thoracic and lumbar spinal cord of human beings, cats, rats, rabbits and guinea pigs, stained by all the procedures indicated in Table 13, using bright-field illumination and a magnification of x400 (but not the Quantimet).

In every section one sees that only a small proportion of the tissue is stained by any procedure believed to be 'non-specific', or 'specific' for particular elements. Only a small proportion of areas, such as the medulla or the anterior horn, which one is conditioned to believe contain a high concentration of nuclei or neurons, take up a significant quantity of stain. One's interest in particular nuclei conditions one to the expectation that most of that area is stained, whereas, in fact, only a small proportion of the area has been coloured. This phenomenon is analogous to that called 'closure' by psychologists.

ELECTRON MICROSCOPICAL STUDIES

We next looked at electron micrographs. They also show only two kinds of cells, those with processes and the 'naked' nuclei (Figures 31-33). In those with processes the cell membrane can be detected all the way around a substantial volume of cytoplasm. On the other hand the 'naked' nuclei exhibit clear membranes round the nuclei themselves, but evident and striking absence of membranes round an area which would be considered as their cytoplasm. However, all the space between the 'naked' nuclei and the cells with processes is replete with mitochondria.

Thus in unstained sections, sections stained by a variety of histological procedures, and in electron micrographs, the agreement of the findings was excellent. We had cells with processes and 'naked' nuclei, in a ubiquitous volume containing mitochondria.

After considering, in detail, the gulf of uncertainty about the 'specificity' of appearances, staining and markers of different kinds of cells which have been summarized (pages 63-91, Tables 8-11 and Figures 34, 35 and 36), we then sought out our more trusty instrument, 'Occam's Razor'. We concluded that structurally we could classify all cells in the central nervous system into two types - those with processes, and naked nuclei. Those with processes are probably all neurons, and the naked nuclei are the mobile nuclei of the syncytium. These conclusions will be examined in more detail below (please see pages 119-123).

However, we then tested the question directly by looking again at our own 500 or so slides stained by the procedures in Table 13. We could classify all cells seen into one of the three appearances, viz. cells with processes, naked nuclei, and occasionally larger, rounder, paler-staining cell bodies (Figure 36), which we believe to be neurons which have not shrunk or been stained as much as others.
In re-examining our histological, including serial, sections, we always bore in mind the many appearances which sections through a polymorphous object could give (Figure 21).

DORSAL ROOT GANGLIA (FIGURE 37)

These were teased out using mounted stainless steel needles, and their capsules torn open. Many oval cells with remarkably few processes came out (Figure 18). Staining with haematoxylin and eosin showed the same types of cells, presumably neurons

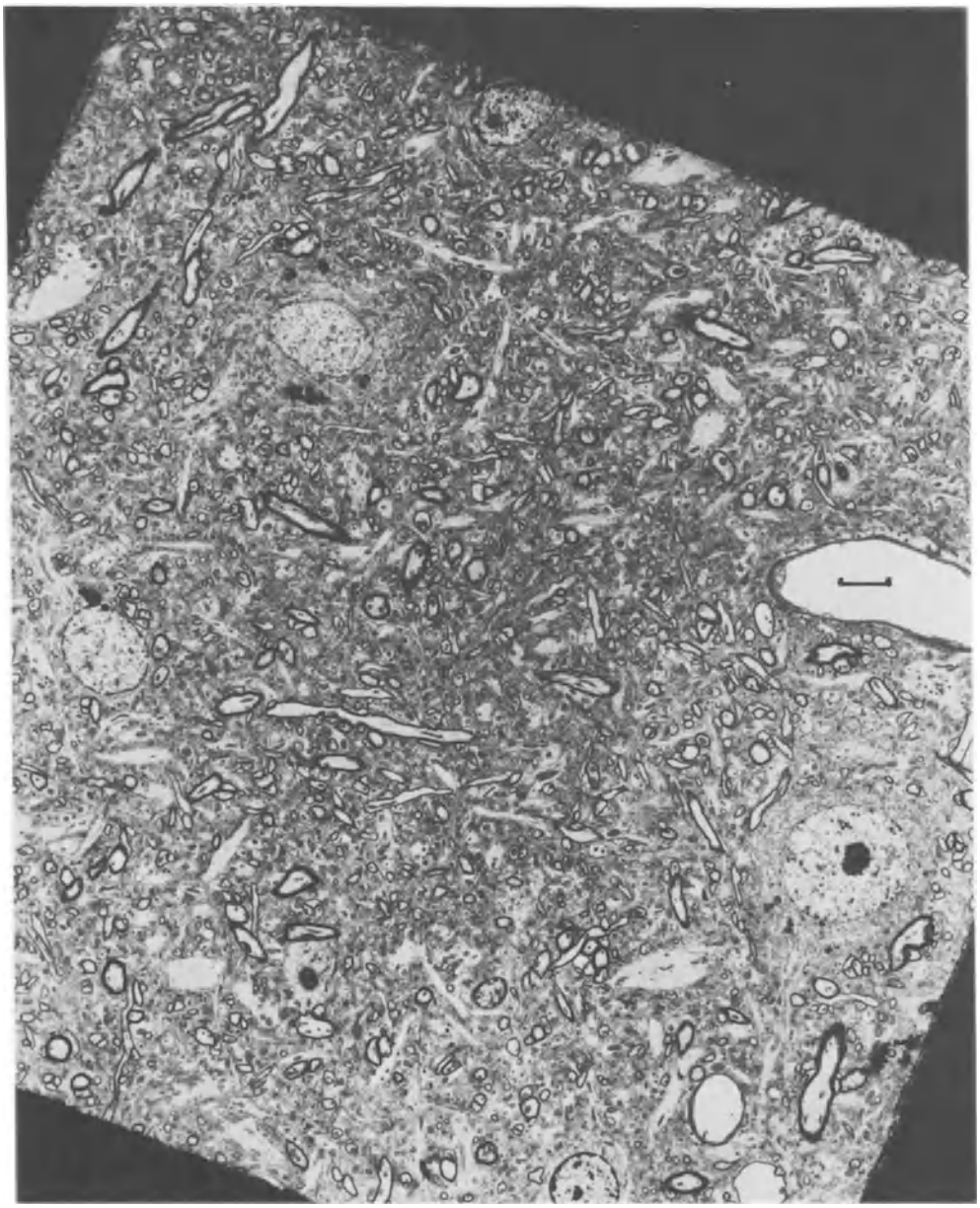


FIGURE 31 An electron micrograph of the parietal cortex of rat showing the relative sparsity of cells, surrounded by other material. Please compare with Figures 32 and 33. The bar is 5 μm

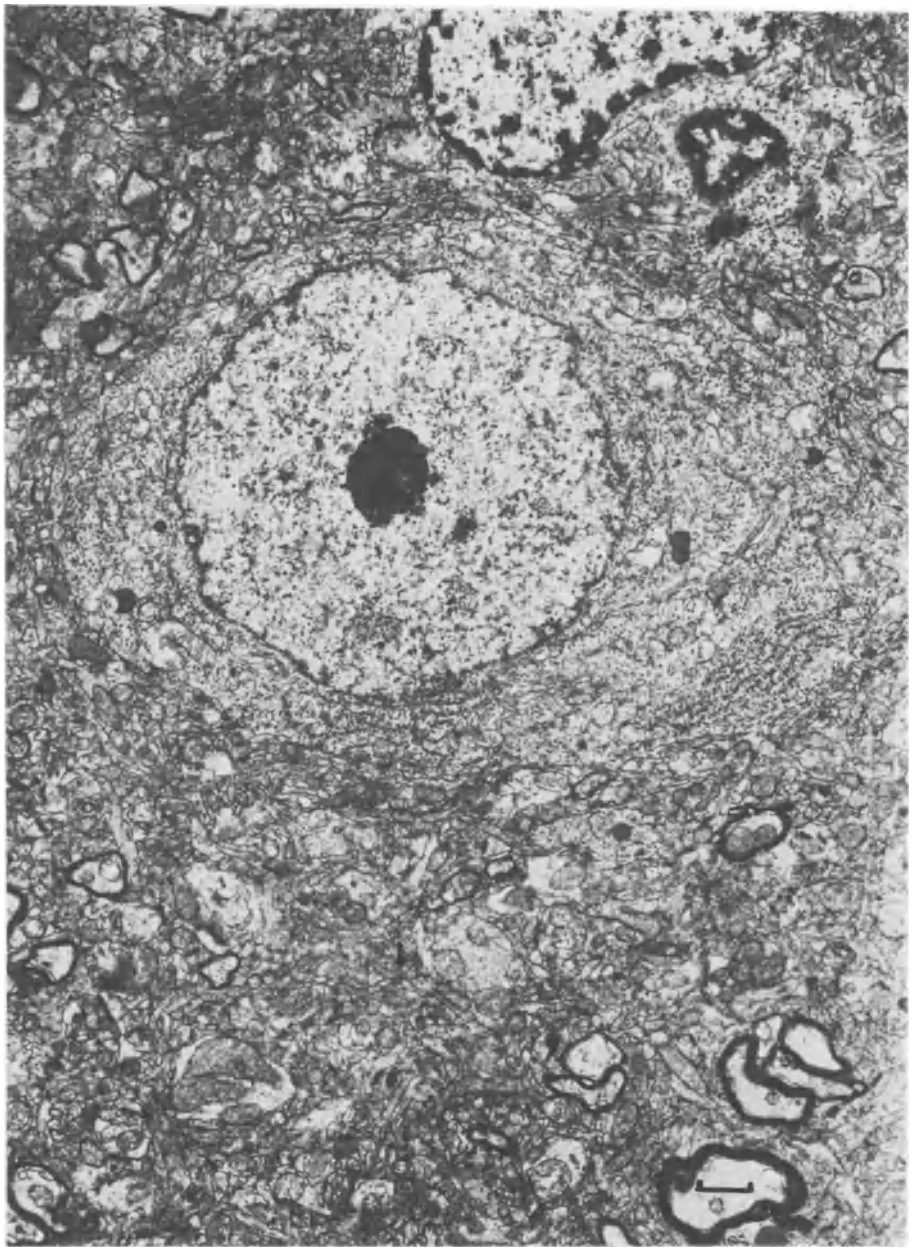


FIGURE 32 Electron micrograph of a neuron in the parietal cortex of rat. The nucleolonema can be seen in the nucleolus. The cell membrane can be detected sometimes as a clear line and sometimes by the change in texture of the cytoplasm on both sides of it. The bar is 5 μm

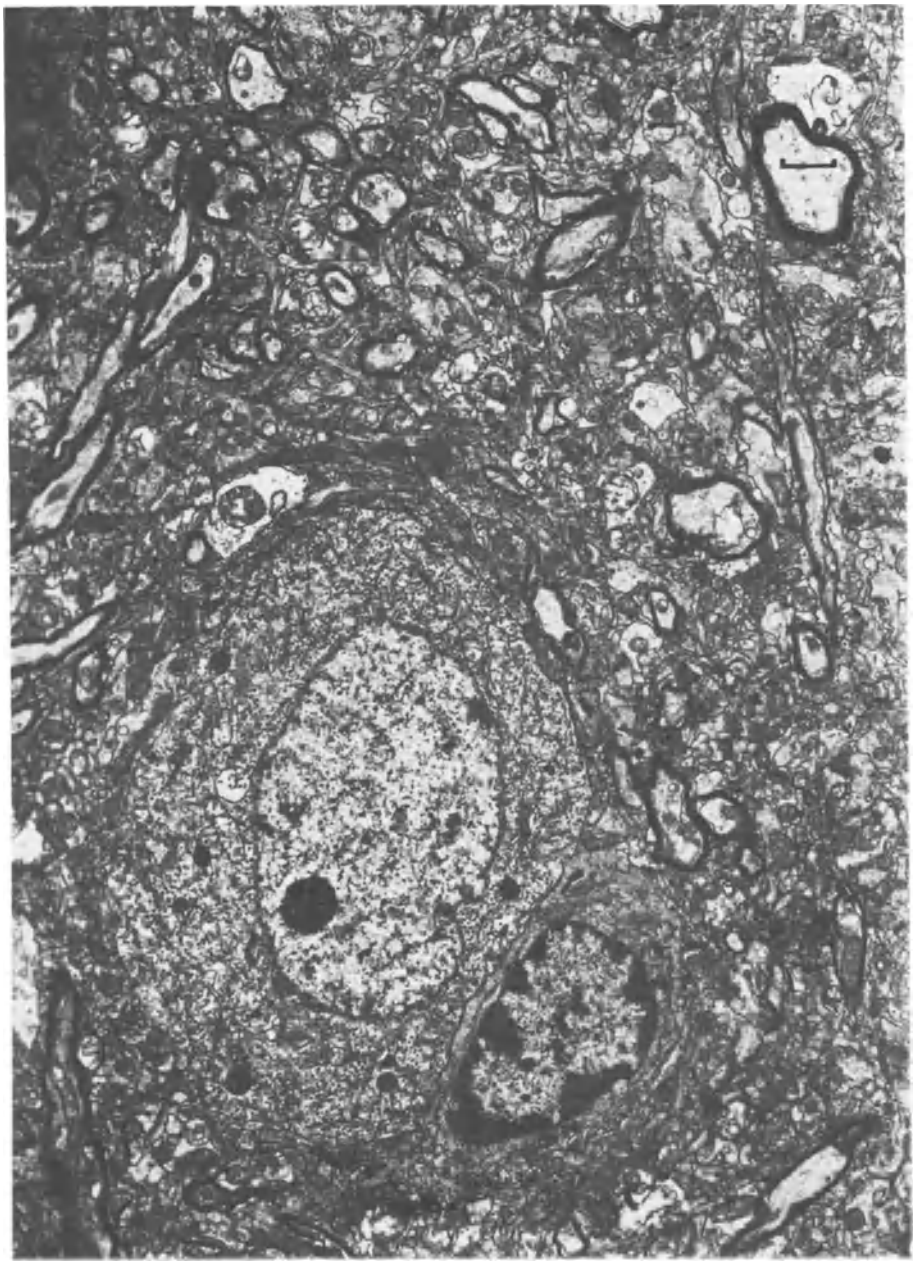


FIGURE 33 Electron micrograph of a neuron and 'satellite' cell in the parietal cortex of rat. The bar is 2 μm

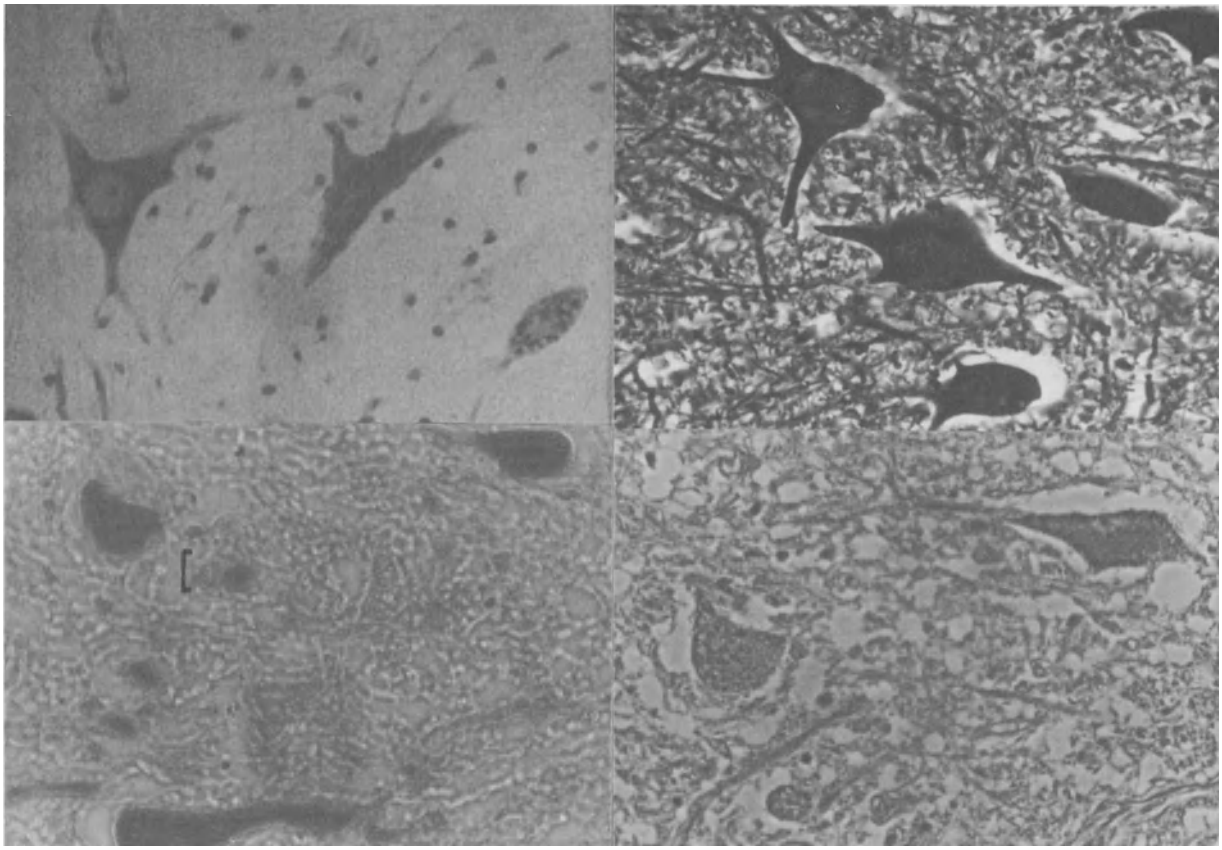


FIGURE 34 Human spinal cord showing ventral motoneurons stained with, (upper left) cresyl violet, (upper right) Palmgren's stain, (lower left) haematoxylin and eosin, and (lower right) phosphotungstic acid haematoxylin. The neurons show up with all these four procedures. The bar is 50 μm

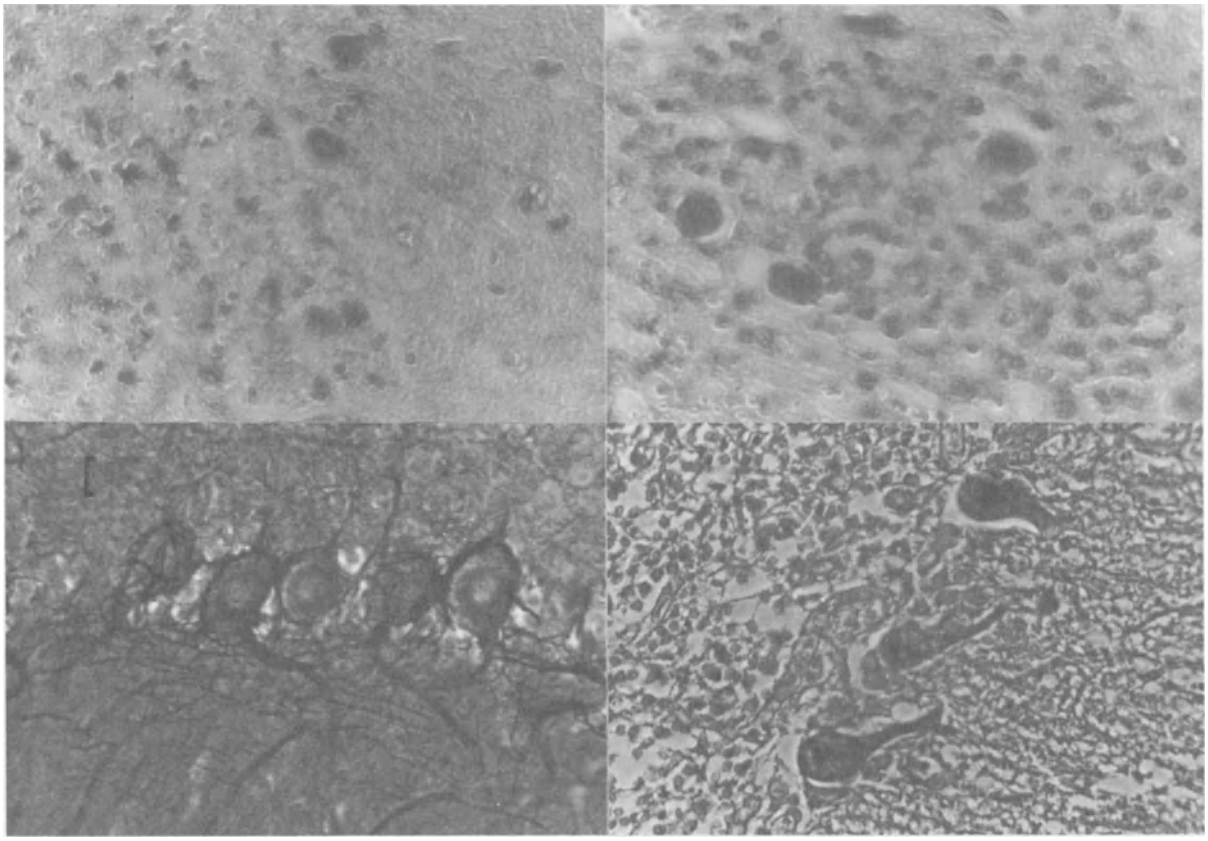


FIGURE 35 Purkinje cells, of human cerebellum stained with (upper left) cresyl violet, (upper right) haematoxylin and eosin, (lower left) cat cerebellum with Palmgren's stain, (lower right) human cerebellum stained with phosphotungstic acid haematoxylin. Please note the dendrites of the basket cells arborizing around the Purkinje cells with the Palmgren stain. These are the only dendrites in the mammalian nervous system which appear close enough to cell bodies to permit synapses to be present, but synaptic knobs or expansions cannot be seen even under high-power magnification with oil-immersion objectives. This figure should be compared with views of the cerebral cortex and retina (Figures 50-52), where there are no such intimate relationships. The bar is 50 μm

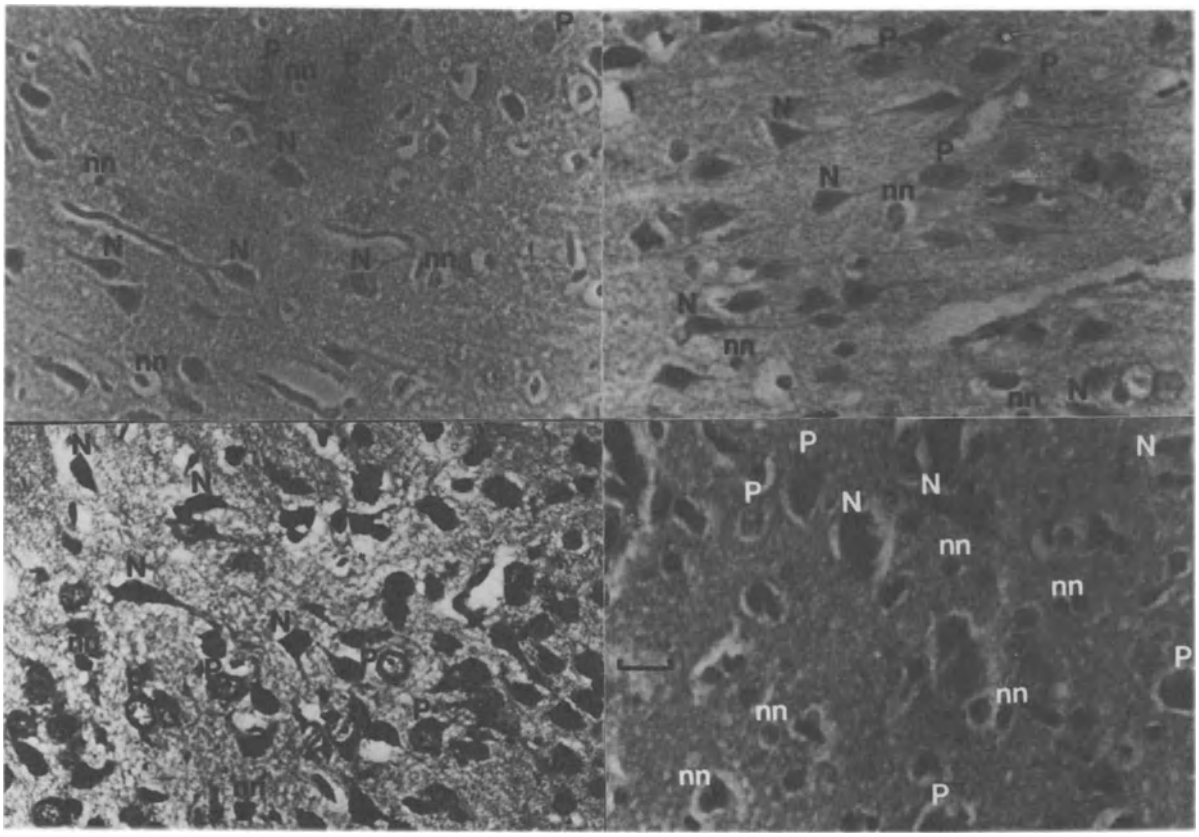


FIGURE 36 The frontal cortex of rat was stained with (upper left) haematoxylin and eosin, (upper right) phosphotungstic acid haematoxylin, (lower left) Marsland, Glees and Erikson, (lower right) Patay's stain. In all cases, only neurons N, naked nuclei nn and paler, more spherical cells P, could be seen. All cells in all histological sections can be classified as belonging to these three groups, or ependymal cells. The bar is 50 μm

(Figure 37). There are a number of small circles between these neurons, which are so small that it is difficult to say whether one is looking at granules or transverse sections of fibres. The neurons stain more or less intensely, which may reflect inhomogeneity of the population or uneven uptake of stain, but there is no clear evidence from light microscopy of a second population, which could be considered as neuroglial cells.

WHITE MATTER

In the white matter we must distinguish between two kinds of fibres. Those in the cerebral commissures or in the cerebellum, for example, are very fine; they have diameters of less than 1 μm, so that their lumens cannot be seen by light microscopy (Figures 38 and 39). The axons of the fibre tracts in the white matter of the spinal cord have greater dimensions - 2·5 μm in stained sections (Figure 40) - much less than those of myelinated peripheral nerves (Figure 41). Nodes of Ranvier and Schmidt-Lantermann clefts are not found in white matter.

Many further studies of the histology of the white matter of the rat, rabbit and human cerebellum and spinal cord were carried out using the routine staining procedures indicated in the legends of Figures 38, 39 and 40. It was noted that there appeared to be very few fibres in the white matter.

AXONS

Eight rats and twelve rabbits were anaesthetized with ether, and their carotid arteries cut. The brachial, femoral and sciatic nerves were excised using fine iris scissors, and the nerves were teased with mounted needles and examined by phase-contrast microscopy. Other nerves were embedded in paraffin wax and transverse sections 6 μm cut and examined under phase-contrast microscopy.

The traditional view of the unfixed preparation was seen - an axon surrounded by a myelin sheath, which was interrupted by nodes of Ranvier. Both the axoplasm and the cytoplasm of the sheath appear clear and translucent (Figure 19). There was no evidence in the cytoplasm of the Schwann cell of any solid structure or lamellae visible within the resolution of the microscope. On transverse section the stained myelin sheaths appeared as uniformly black circles (Figure 41).

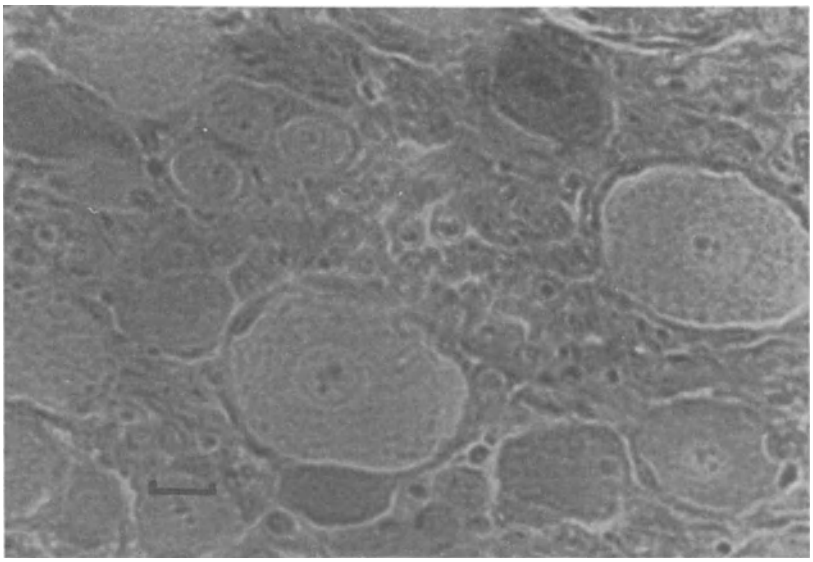


FIGURE 37 Transverse section of human dorsal root ganglion stained with haematoxylin and eosin. Note the large neurons, some of them staining much darker than others. Granules are seen between the neurons. The bar is 20 μm

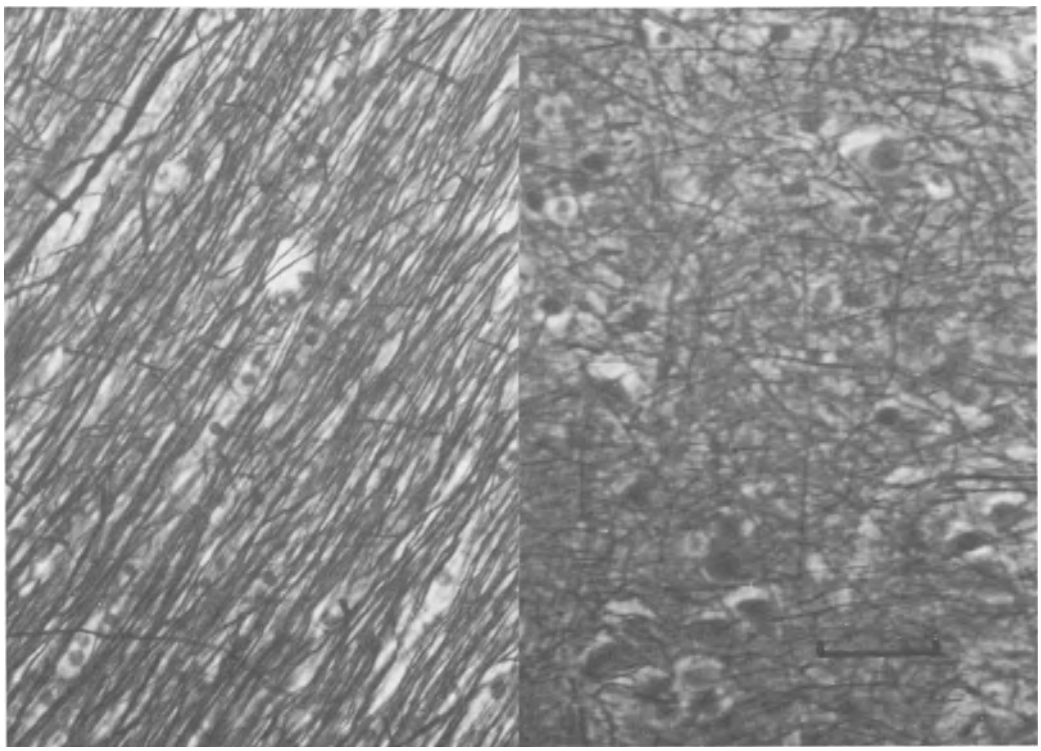


FIGURE 38 Anterior white commissure of human thoracic spinal cord stained with Palmgren's stain, showing a commissure of fibres (left) and syncytial fibres (right). Please see also legend to Figure 39. The bar is 50 μm

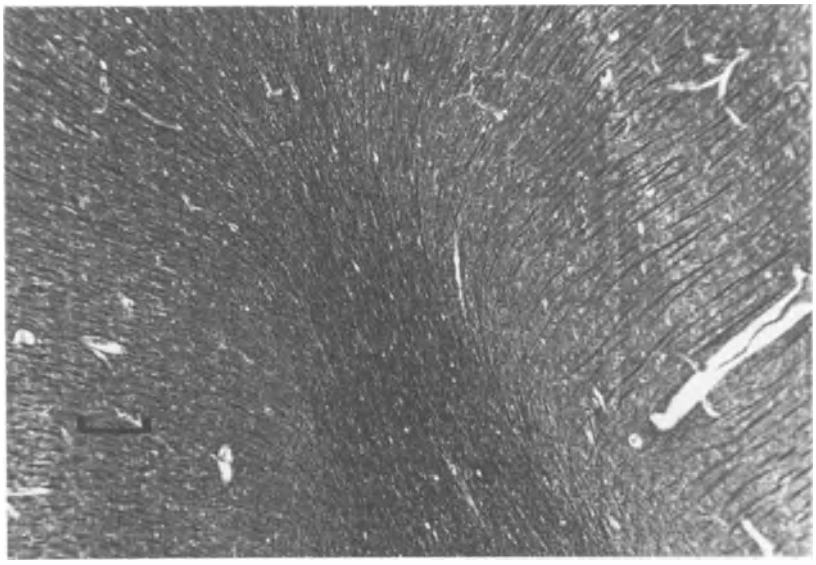


FIGURE 39 White matter of the human cerebellum stained with Palmgren's stain. Please note how the fibres in the plane of the field in the lower half swing up towards a transverse orientation at the upper half of the field. The bar is 50 µm

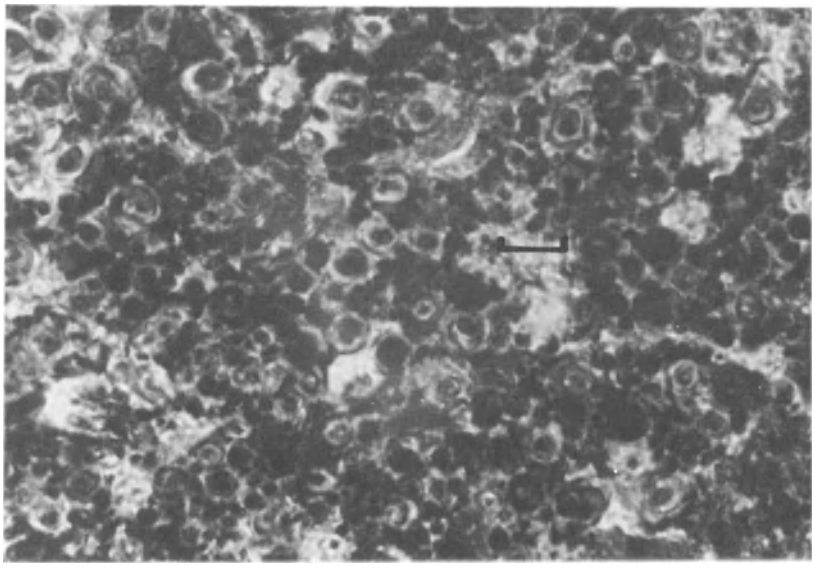


FIGURE 40 Transverse section of white matter fibres of human thoracic spinal cord, stained with Weigert-Pal's stain. The bar is 20 µm

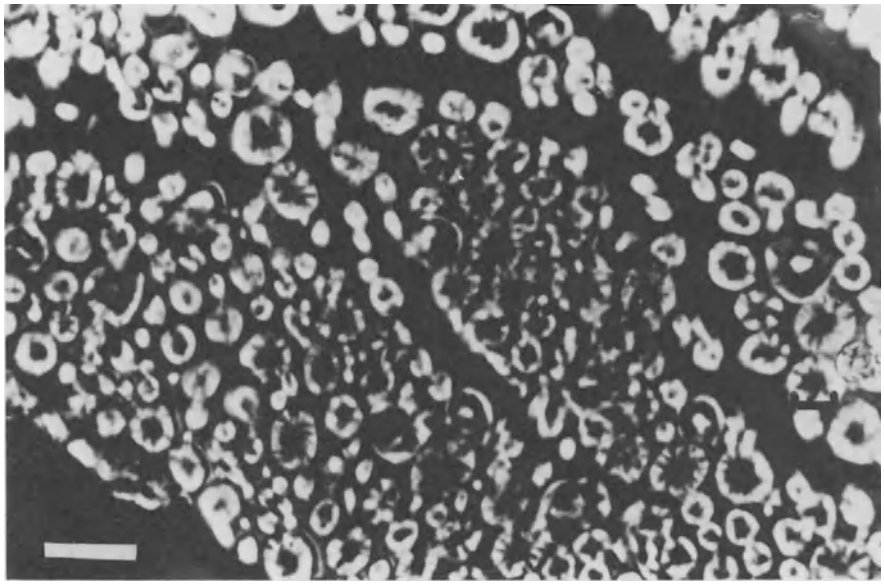


FIGURE 41 Transverse section of part of the sciatic nerve of a rat, embedded in paraffin, not stained, but viewed by dark-field illumination. The bar is 20 μm. Please note the myelin sheaths, and compare with Figures 19 and 40

6
Critique of these Experiments

The demonstration by well-known neurological staining procedures of membranes around kidney and liver cells, but not between nuclei and cells with processes (which we believe to be neurons), could be explained by supposing that the membranes of neuroglia are not visible by light microscopy, but this view is not tenable, since the neuroglia were originally characterized by light microscopy. Alternatively, someone might like to assert that some procedure other than those listed in Table 13 might show up these membranes.

Another possibility is that all cells with processes, which we are now calling neurons, are not just neurons, but also include astrocytes, oligodendrocytes and possibly microglia - in accordance with the current view. We believe that we have brought sufficient evidence to undermine fundamentally the view that the neuroglial cell types can be differentiated with certainty by any - and therefore all - the criteria normally employed; that permits us to proffer the simplifying suggestion that all cells with processes are neurons. This is a working hypothesis, and its implications for neurobiology will be discussed later (pages 205-241).

What then of the large volume of biochemical findings quoted in Table 12 from the groups of Raff in Britain, Hertz in Canada, Eng and Varon in the USA, Hamberger in Sweden, Pevzner in the USSR, Schousboe in Denmark, and many others? The accumulation in one table of these careful experiments carried out in prestigious laboratories all over the world points quite clearly to a Tower of Babel populated by biochemists.

Nonetheless, despite all these reservations, we are bound to accept that significant differences in quantities of markers have been found. Do these mean that the cell types are different? Not necessarily. The cell types have been separated by different procedures involving varying degrees and techniques of homogenization, filtration, and culture in different media. It is therefore not at all surprising that biochemical analysis of the cultures after their hazardous and wayward voyages should find them to be biochemically different. Only if they had all been separated by the same procedures and then illustrated profound biochemical or immunological differences, could one suppose that they were different cell types.

The overall uncertainty about the identity of cells based on the lack of specificity of the markers does not mean, either, that there are no biochemical differences between

living neurons and neuroglia in situ, or that both the neurons and the neuroglia are homogeneous in their biochemical properties. We would like to stress the distinction between, on the one hand, a dissatisfaction about the adequacy of the evidence identifying the cell types currently characterized by the alleged markers, and on the other, the probability that the population of cells in the living central nervous system is chemically heterogeneous. We regard the latter possibility as being completely open.

We have shown directly that most of the same cells, such as ventral horn motor neurons and Purkinje cells of cerebellum, stain with most of the staining procedures. When we looked at the multiplicity of views about what each procedure does show up (Table 10), we were induced to go back to the originators of classical methods, and ask what they said about the specificity of their own methods. The answer is as follows: the authors either (1) used the generic term 'neural' tissue; (2) did not assert that their procedures were specific; or (3) did not mention control tests on cells other than those in which they were interested. The simple fact is that none of the original authors claimed that their staining procedures were specific. Later authors assumed them to be so.

We have asserted that no continuous membranes could be seen between whole neurons and naked nuclei, either by light or by electron microscopy. We have considered how membranes are detected (Appendix 4). It is widely held that membranes cannot be seen unless either they are normal to the section (Horne and Harris, 1981), or they are not normal to the section (Ham, 1974). The latter two explanations are incompatible. Elsewhere we have shown how the expected incidence of the membrane being seen clearly normal to the section can be calculated, and that the actual incidence with which it is seen thus is much higher (Hillman and Sartory, 1980, page 40). Yet these calculations become unnecessary when one sees that the nuclear, mitochondrial and cell membranes can be detected along most of their courses by electron microscopy; therefore any cell membrane between the neurons and naked nuclei should also be seen as frequently. Liver and kidney cells were surrounded by such membranes. A categorical statement that we have never been able to detect such a membrane in the central nervous system in stained preparations by bright-field illumination, in unstained preparations by phase contrast microscopy, or by electron microscopy, should be taken as an invitation to neurobiologists to produce any evidence to the contrary - if indeed, such evidence exists. In particular we would ask them to show us these membranes in any species or by any histological or electron microscopic technique other than those which we have used.

It might reasonably be asked why we used the neural stains, such as Weil and Davenport's, Mallory's phosphotungstic acid haematoxylin and Marsland, Glees and Erikson's, when we have shown them to be non-specific. When we first used them we shared the widespread belief that they were, indeed, specific for particular elements. We then tested them, and found them to stain the same elements - the cells with processes and the naked nuclei - and sometimes the cell processes. We then searched the literature, and found out what a diversity of opinion there was about which element, and how specifically, it was stained (Table 10). Bearing in mind that the non-specificity would result in the addition of the stained areas being an underestimate of the elements

present, the staining could still properly be used to examine the proportion of the area occupied by cellular elements. Light microscopic histology is still the only way of quantifying the cellular elements in a significant volume of tissue.

An important potential error in attempting to assert the relevance of these measurements to the tissue in the intact living animal could arise if the cells with processes and the naked nuclei were to shrink significantly differently than the syncytium surrounding them, while they were being prepared for histology. If the former elements shrunk less than the syncytium, this would result in the measured proportion they occupied being exaggerated by quantimetry and if they shrunk more, it would result in the proportion being understated. Measurements of shrinkage in cerebral slices - representing the whole tissue - and in isolated neurons, showed that the differences in shrinkage were not very large (Hillman and Deutsch, 1978, and sources quoted there).

En passant, one many wonder why many neural staining procedures seem to show up cell bodies, but not - according to the traditional view - most of the cell's processes. The general exception to this proposition is that of the silver salts. We do not believe this to be the case; that is, we do not believe that fixation, staining or dehydration can cause any elements which are present not to show up at all, even though, obviously, some procedures do increase the visibility or contrast of some elements more than others. For example, the failure to see tracts of white matter by electron microscopy is often explained as being due to poor fixation. Such an explanation - which is obviously quite feasible - should not be accepted unless it had been demonstrated; this could be done quite easily.

One may ask why the mitochondria do not appear in a normal haematoxylin and eosin preparation of brain examined under high-power magnification. The simplest explanation is that, since they are moving in a liquid cytoplasm in life (Hillman and Sartory, 1980, page 57), they diffuse out during the rehydration of the sections before staining. Certainly they are seen in abundance in unfixed pieces of central nervous tissue (Figure 42). Another possible explanation for the disappearance of some organelles or fibres is that the vast changes in water content, osmotic pressure, permeability, fragility, pH and pressure occurring during preparation, could cause them to lyse, and then become unrecognizable.

The possibility that in the pieces of brain we were seeing bacteria rather than mitochondria can be discounted, since the preparations were always examined in freshly made up solutions, and the cerebral tissue always came from animals which had been newly killed. There would not be time for the multiplication of bacteria in such an abundance as we have seen.

The neuroglial 'clumps' of Hyden or 'flakes' of Hamberger were defined by their proximity to neurons, and their identification as neuroglial cells depended upon the belief that the neuroglial material was composed of cells. Hertz (1965, 1966) originally used this approach, as did the whole school of Hyden (please see Hyden, 1967), including ourselves (Hillman, Hussain and Sartory, 1973; Hillman, Deutsch, Allen and Sartory, 1977). After the latter experiments, when we realized the ubiquity of the neuroglial material, we then realized that the terms neuroglial 'clumps' and 'flakes' both meant material adjacent to the neurons; that is, all the neural substance other than the neurons and nuclei. We therefore, decided to examine this in a more general

way, and take out small pieces of brain and spinal cord at random, to find out if the neuroglial clumps and flakes were, indeed, typical central nervous tissue. This turned out to be the case (page 94).

The detection of large numbers of mitochondria between the neurons and the naked nuclei, as well as within the neurons themselves, indicated that the tissues were intracelluar. We detected the mitochondria because we examined the tissue both unstained by phase-contrast microscopy and after staining with Janus green (Hillman and Deutsch, 1978b). Of course, mitochondria are located intracellularly.

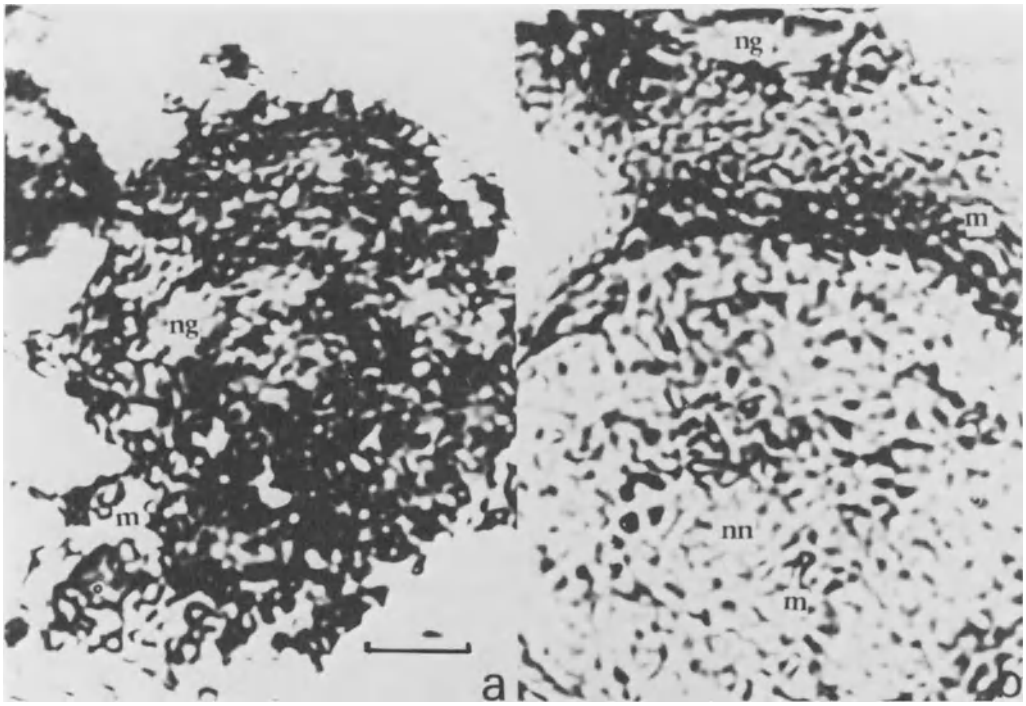


FIGURE 42 Mitochondria seen in (left) neuroglial clump, (right) neuron below with neuroglial clump above by phase contrast microscopy; nn is neuron, ng is neuroglia, m is mitochondrion. The bar is 10 μm

The hypothesis that the third kind of cells - the swollen relatively unstained cells - were neurons which had not shrunk due to dehydration, or had not been as intensively stained as other neurons, fits in with the frequent observation that in an identified population there is a wide variation in the degree of staining of individual cells. This is seen, for example, in the ventral horn cells in the spinal cord and in the Purkinje cells in the cerebellum (Figures 34 and 35).

The wide variation of the appearance of sections of an egg would suggest that sections of neurons could also show a rich range of appearances. A clear space around stained neurons has been recognized as an artifact for many years, and its incidence is widely believed to be diminished by perfusion fixation for light and electron microscopy. The effects of shear during section are not so widely recognized, but they can be appreciated rapidly by comparing the rather elegant appearance of the unfixed unsectioned neuron (Figures 16, upper, and 18) with the more distorted histological appearances of most neurons stained but not sectioned (Figures 16, lower, and 44). I have also noticed it in some unpublished observations. In the early 1970s I attempted to cut sections for electron microscopy of single neurons embedded in epoxy resins. The shrunken torn fragment could hardly be recognized as originating from the whole neuron.

The problems of detecting the presence of, and measuring the thickness of, cell membranes by light and electron microscopy are discussed in Appendix 4.

The fact that different staining systems produce apparently different appearances of cells, even of the same cells, is not at all surprising. One is using an array of fixatives, embedding media, stains, dehydrating agents, differentiating agents, clearing agents and mounting media. These vary in chemistry - aldehydes, heavy metal salts, organic solvents, oils, waxes and epoxy resins. The tissues are subjected to these reagents each with the following conditions varied: thickness of section, length of exposure to the reagent, concentration of the reagent, temperature at which it is applied, sharpness of knife, water solubility of reagent, stability of reagent, subjective assessment and skill of the histologist, the kind of microscopy used and the skill of the microscopist. The likelihood that variation in any of these parameters will affect the appearance and therefore the identification of the cells or their organelles is strongly implied by the necessity to reproduce the conditions of a particular staining procedure prescribed by its author in order to achieve the same appearances. Therefore, the reproducibility of the appearance of a particular structure using a very precisely defined series of conditions cannot be regarded as evidence either that, firstly, that is the original shape of the structure, or secondly, that different shapes resulting from different staining procedures arise from different structures (see also Appendix 5).

If one conceives that each cell in a population may stain and shrink to a different extent during the same histological procedures, one could suppose that a population would show a range of 'plumpness' to 'spideriness', and slight to intense staining. This could depend upon the degree of stress of the animal during killing, the metabolic state of the cells in the tissue at the time of fixation, the depth within the tissue specimen, the size of the specimen, the duration of application of each of the reagents, the distance between the microtome and the cell body in the section, and the proximity

of the cell to the surface to which the reagents are added or to the microscope slide. All these variables could also affect the staining of whole cells and each of their individual organelles.

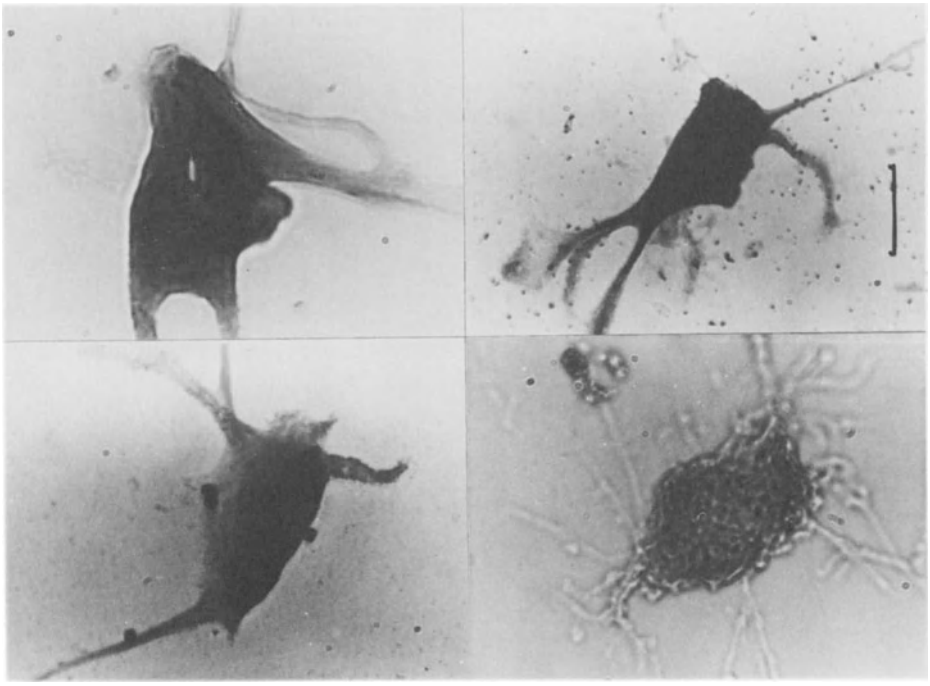


FIGURE 43 Rabbit neurons isolated by hand dissection, stained with, (upper left) Marsland, Glees and Erikson, (upper right) Gallyas's stain, (lower left) Mallory's phosphotungstic acid haematoxylin, (lower right) Weil and Davenport. The bar is 20 μm

In view of the protean appearance of sections of cells, it might be appropriate to ask the following question. How can one be sure that the appearance of 'naked' nuclei in sections is not also due to neurons - that is, that there are not two populations of cells, but only one? Is it not possible that the circles are transverse sections of neurons near the bases of processes. I believe that this is unlikely, because the naked nuclei stain the same colours as the nuclei in the neurons in the same sections by virtually all staining procedures (Figures 25, 26 and 27). Also, one would expect to see an occasional thorn sticking out of the circular structure, if it was indeed a section near the origin of a process of a neuron. I do not know of a report of any such appearance having been seen on the nuclei of classical neuroglial cells.

The naked nuclei seem to be significantly different from the nuclei of the neurons, in that the former do not appear ever to have nucleoli.

When the sections were stained and scanned with the Quantimet, 40 areas were examined in each particular coronal section. This examination, therefore, was a measure of the areas in the whole section and tissue, and not only in the cerebral cortex, or the cranial or spinal cord nuclei. While our specific studies were done on

rats and rabbits, we have also examined tissues from the other species mentioned, and stained by the other procedures listed (Table 13). We have never seen any stain which colours equally most of the area or volume of any nervous tissue. We would request any neurohistologist who has found any procedure which is claimed to be specific for any cell type in any species to scan it with a particle counter, or to send it to us to examine ourselves. Naturally, we would be particularly interested to see histological sections or light micrographs of any tissue in which most of the tissue has been stained. Nevertheless, we have shown that the usual assumption that the 'specific' neurohistological stains would together colour the totality of the tissue, and that, therefore, the neurons and neuroglial cells together make up most of the nervous system, is simply not tenable, at least for the rat and rabbit central nervous system.

7
The Fine Structure of the Nervous System

THE NEURON

The structure of the generalized cell has previously been reviewed (Hillman and Sartory, 1980a), and I would refer the reader to this publication for detailed evidence that the following structures could not exist in living cells: Nissl body, endoplasmic reticulum, ribosomes, lysosomes, Golgi apparatuses, cristae of mitochondria, nuclear pores, neurofibrils, neurofilaments, neurotubules, microtrabeculae, intermediate filaments and actomyosin filaments (other than in muscle) (Table 17). The evidence brought there may be summarized briefly. Living cells show intracellular movements which are visible under relatively low-power light microscopy (x200-1000) (Figure 44). Cytoplasmic movements are incompatible with the existence of a fine cytoskeleton, which requires 10-100 times greater magnification to see, and which is supposed to permeate the cytoplasm and to be attached to the cell membrane and to the nuclear membrane. Intracellular viscosity has been measured in a large number of living cells and has generally been lower than that of glycerol. Observations on many living cells indicate that the cytoplasm is a liquid. In addition to these biological grounds for doubting the existence of many of these apparent structures, most of them appear in the plane of the picture far too frequently than simple geometry would dictate; they are two-dimensional and could not exist in three dimensions. No three-dimensional transparent scale models in which intracellular movement might appear possible have ever been made.

On geometrical grounds it has also been shown that the trilaminar appearance of the cell membrane, the nuclear membrane, the mitochondrial membrane, and the neuromuscular junction, is an artifact, although the membranes themselves are not. The synapses will be dealt with below (please see pages 135-156).

In response to our assertion that the 'cytoskeleton' is an artifact of preparation for electron microscopy, it has been alleged that it can be seen by light microscopy. The filaments are fibrils and are believed to have diameters between 10 nm and 15 nm (Lazarides, 1980; Shelanski, Liem, Letterier and Keith, 1981). Until recently it has generally been accepted that the maximum resolution of the light microscope using a monochromatic source under optimal conditions is 200-250 nm. Therefore one could only see structures 10-15 nm in diameter by light microscopy, if they occurred in bundles whose total diameter would be sufficient to be resolved. However, when one looks at

the elements of the cytoskeleton by electron microscopy, they do not appear to be associated in close bundles (Table 18). It is obviously inconceivable that a structure which appears by light microscopy to have a minimum diameter of 200 nm would shrink to only 10 nm, bearing in mind that the preparations are observed after dehydration both in light and electron microscopy.

TABLE 17 Artifacts in nervous tissues and suggestions about their most likely origin during preparation

Artifactual structures	How they were caused
Trilaminar appearance of membranes	Groove caused by evaporation and explosion of tissue under thin metal deposit (please see Figure 45)
Golgi apparatus	Precipitate of cytoplasm with heavy metal
Endoplasmic reticulum Lysosomes Cytoskeleton neurofibrils neurofilaments actin filaments neurotubules microtrabeculae	Dehydration and precipitation of cytoplasm with heavy metal
Myelin lamellae	Dehydration and precipitation of contents of myelin sheaths
Anatomical synapses (light microscopy) (electron microscopy)	Metal deposits on regions of cell membranes
Structures in synaptic cleft	Dehydration and precipitation
Exo- and endocytotic vesicles	Granules adjacent to membranes around which metal has deposited
Nuclear pores	Bubbles in nucleoplasm or cracks in metal deposit on nuclear membrane

Recently, Allen and his colleagues have introduced a video-enhanced contrast system, with which they claim to see by light microscopy structures as small as 5 nm in diameter moving along nerve axons (Allen, Travis, Allen and Ilmaz, 1981; Allen, Metuzals, Tasaki, Brady and Gilbert, 1982; Brady, Lasek and Allen, 1982; Allen and Allen, 1982).

If one cuts sections of any fibrous tissue at random, the majority of fibres are very unlikely to be found well orientated to the microtome either longitudinally or transversely. Why are the fibres, filaments, fibrils and trabeculae - which are believed to be orientated randomly in tissues other than muscle - so shy of exhibiting their oblique profiles? One would not say that they are never seen obliquely, but they do appear in nerve cells far more frequently in longitudinal than in transverse section, and they appear obliquely hardly at all. Moreover, attempts to adduce biochemical or

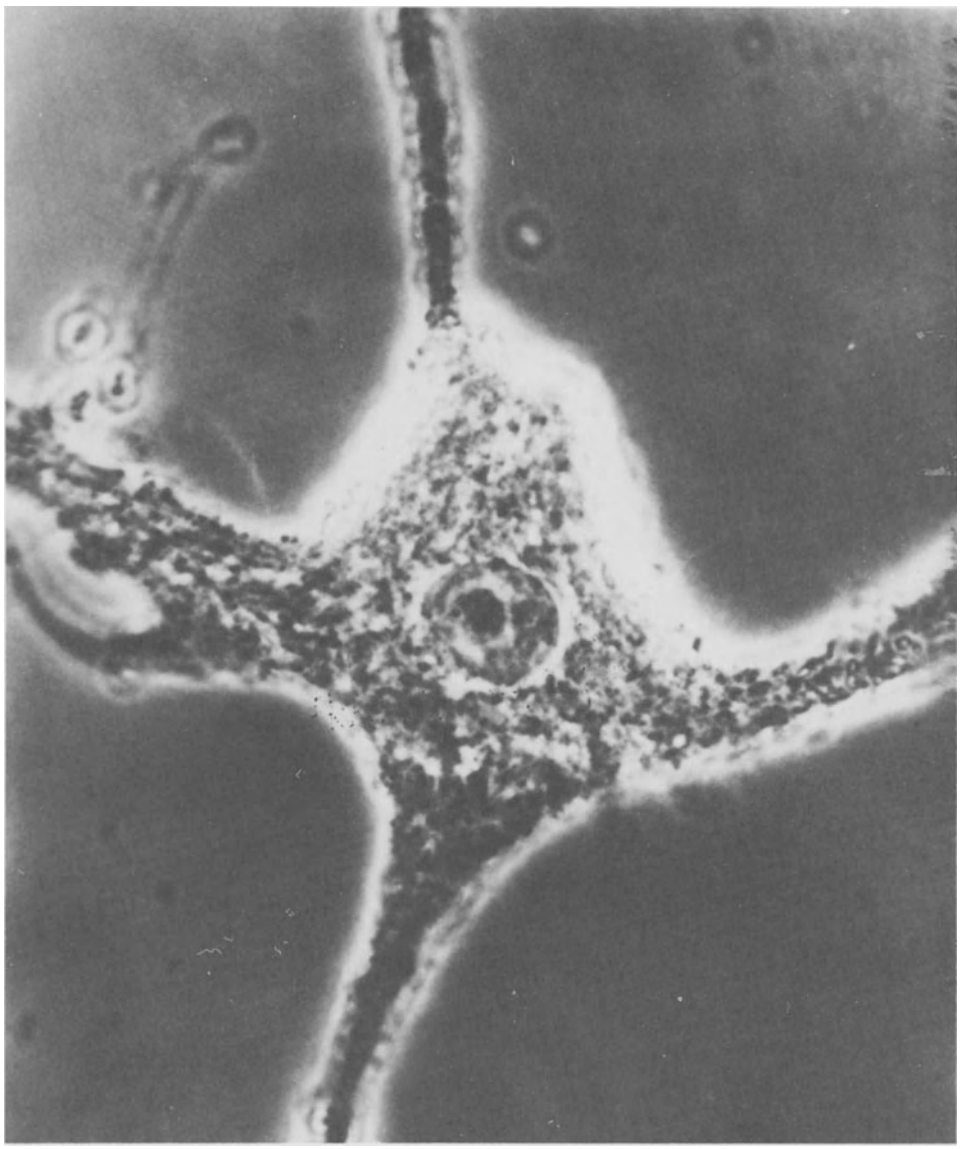


FIGURE 44a Intracellular movements in a rabbit Deiters' neuron. Changes in the appearance of the nucleolus can be seen clearly. The neuron was isolated by the technique of Hyden (1959) and placed in the '199' medium of Morgan, Morton and Parker (1950) in a parallel-walled chamber (Sartory, Fasham and Hillman, 1971). It was examined under phase-contrast microscopy, a, 28 hours after the animal was killed, b, 10 minutes later, c, 20 minutes later. The bar is 5 μm

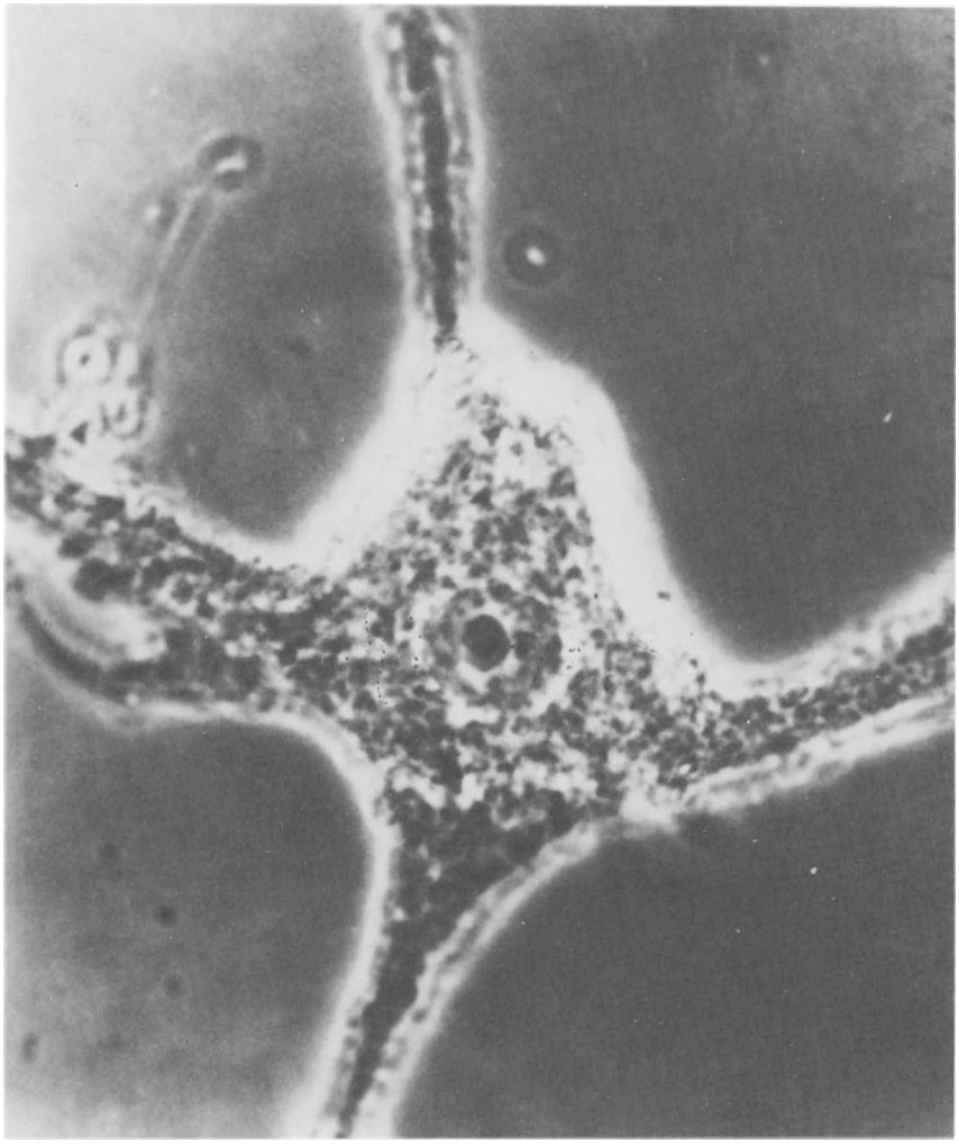


FIGURE 44b

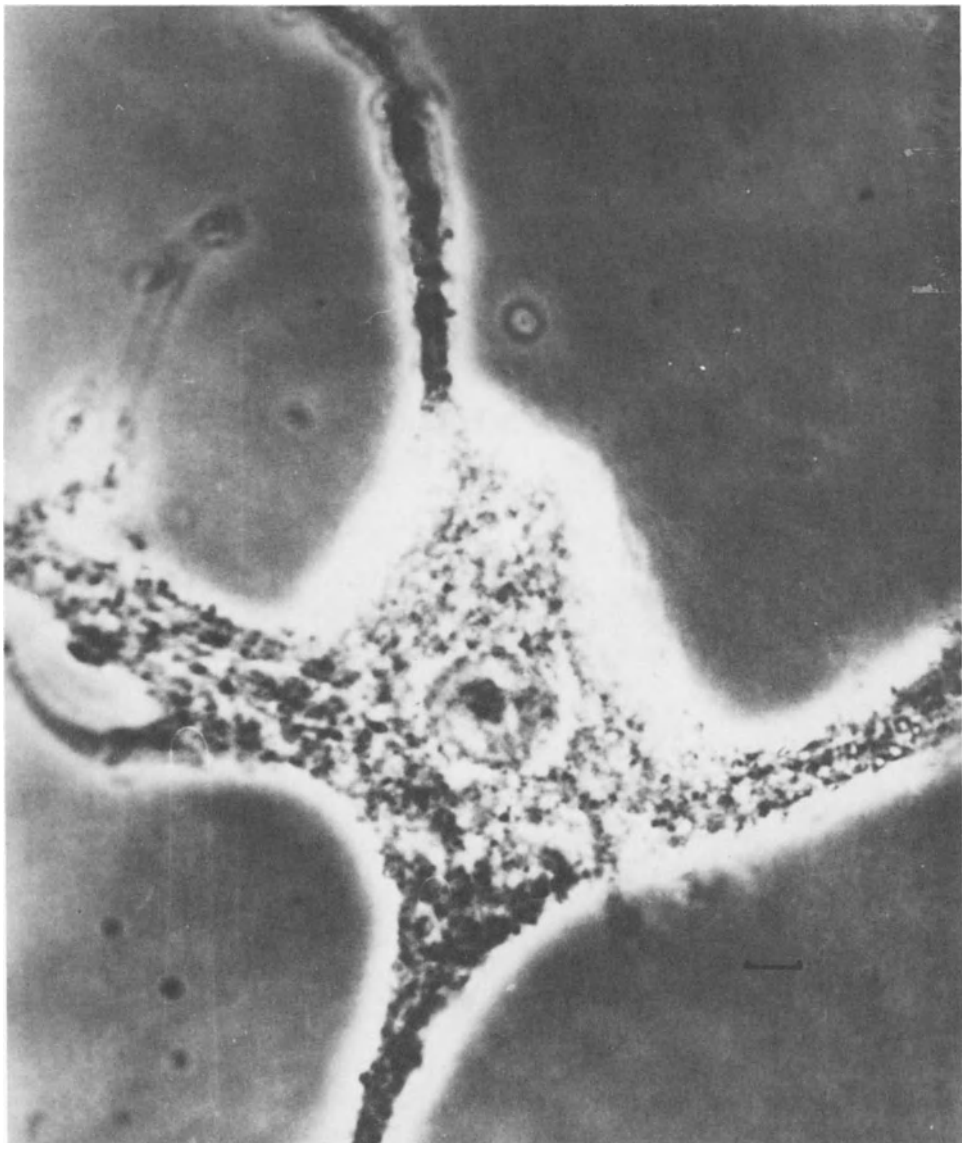


FIGURE 44c

TABLE 18 Publications of mammalian structures showing intracellular trabeculae, filaments and tubules in the nervous system.

Structures (nm)	Animal/tissue	Authors
Neurofilaments	Mammalian cerebral cortex	Rhodin(1963)
Neurofilaments (9-10) Microtubules (23-26)	Rat optic nerve	Peters and Vaughn(1967)
Gliofibrils Microtubules	Rabbit neocortex	King(1968)
Neurofilaments Microtubules (22)	Rat cerebral cortex	Peters, Proskauer and Kaiserman-Abramof(1968)
Neurofilaments (10) Microtubules (27)	Rat anterior horn cell	Wuerker and Palay(1969)
Neurofilaments Microtubules	Cat somatic sensory cortex	Jones and Powell(1970)
Filaments (9) Microtubules (25)	Rabbit spinal cord	Raine and Wisniewski(1970)
Neurofilaments Microtubules (22-27)	Rat brain, parietal lobe	Tani and Ametani(1970)
Microfilaments (4-6) Neurofilaments (9-10) Microtubules (24-28) Neurotubules (24-28)	Rat dorsal root ganglion	Yamada, Spooner and Wessels(1971)
Neurofilaments (6-10)	Rabbit nodose ganglion	Johnston and Roots(1972)
Neurofilaments Microtubules	Rat cerebellar cortex	Palay and Chan-Palay(1972)
Neurofilaments (10) Microtubules (25)	Rat cerebellar cortex	Wuerker and Kirkpatrick(1972)
Neurofilaments Microtubules	Monkey visual cortex	Palay, Billings-Gagliardi and Chan-Palay(1974)
Microtrabeculae (3-6) Microfilaments (6-10) Microtubules	Human fibroblast	Wolosewick and Porter(1979)
Neurofilaments (7) Microtubules (24) Neurotubules (24)	Human unmyelinated nerves	Han and Holmstedt(1981)
Microtrabeculae (3-12)	Rat cerebral cortex	Cohen, Wolosewick, Becker and Pappas(1984)

In all of these publications the structures in the illustrations shown appear single on the electron micrographs and not in bundles. Their diameters, as noted by the authors, are also indicated. It should be noted that as single strands their diameters are at least one order smaller than could be seen by the light microscope. These publications were not selected because the structures were not in bundles, and I have not been able to find any electron micrographs in the literature on mammals showing strands sufficiently close together that a bundle of them would be seen with the resolution of the light microscope

biophysical evidence to explain the geometrical paradox cannot be successful, since they are epistemologically of quite different dimensions.

If it is accepted that the 'trilaminar' appearance of membranes is artifactual - and I stress that the detailed evidence has been published elsewhere (Hillman and Sartory, 1980) - one is edged along to the conclusion that the cell membrane, the nuclear membrane and the mitochondrial membranes are all of single thickness. The difficulties both of detection and of measurement of membrane thickness are discussed in Appendices 3 and 4.

The 'trilaminar' appearance could be due to the two layers of metal deposited on either side of the water and lipid extracted membranes. Alternatively, it could be the groove resulting from the explosion of what was left of the cell membrane through the layer of heavy metal salt above it. This would occur when the highly energetic electron beam, passing through the atmosphere at a very low pressure in the electron microscope, hits the membrane beneath the heavy metal salt and vaporizes it. The resultant explosion would blow out a groove (Figure 45). When the thickness of the trilaminar membrane is measured in a dehydrated specimen, it must be underestimated.

The well-known Davson-Danielli hypothesis about the structure of the membrane dominated the textbooks between the 1930s and the early 1970s. It proposed that the membrane consisted of a lipid molecule sandwiched by two protein molecules (Figure 12). A critical study of the evidence for the hypothesis would be a valuable addition to the literature, but it is outside the main theme of this monograph. However, there are a number of anomalies about belief in this particular model, which will be dealt with here. The Robertson 'unit' membrane is considered by some to be the structural expression of the Davson-Danielli model, and by others to be independent here in terms of the former idea.

A 'microsomal' fraction believed to contain the cell membrane and the endoplasmic reticulum, has been separated by subcellular fractionation, and the analysis of its composition is generally believed to yield data about all the 'membranes' of the 'cytoskeleton' or 'endoskeleton'. Thus, the belief has gradually gained credence that all the 'membranes' are chemically similar, and - as a corollary - that unless a structure has been shown to have the particular characteristic chemical constitution, it is probably not a membrane.

Both the Davson and Danielli membrane and the 'unit' membrane are agreed to be 6-12 nm thick throughout the literature. With transmission electron microscopy the membrane appears quite smooth, although the use of freezing techniques frequently shows it to have little knobs on it. Such knobs are believed to be proteins, enzymes or receptors of a large and increasing variety (please see Table 29).

The fluid mosaic hypothesis of membrane structure (Singer and Nicholson, 1972) is now widely accepted, as if it were a finding (Semenza and Carafoli, 1977; Repke, 1980; Kates and Kuksis, 1980). It originated from some thermodynamic considerations and studies on the distribution of antigens applied to red cell membranes and detected by freeze etching. 'A prediction of the fluid mosaic model is that the two-dimensional long-range distribution of any integral protein in the plane of the membrane is essentially random' (Figure 13) (Singer and Nicholson, 1972). The following questions immediately arise: What does 'model' mean in this context? Is this what they believe the

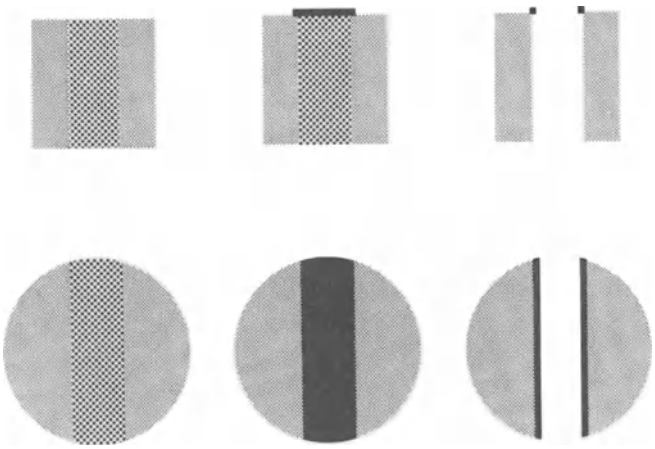


FIGURE 45 Suggestion about the mechanism by which a membrane appears as two lines by electron microscopy. Upper row side views, lower row plan views, of a membrane (coarse mottling), embedded in a medium (fine mottling). Left shows an embedded membrane; middle shows the deposit of heavy metal (black) on the surface of the membrane. The electron bombardment, heat and high vacuum causes the tissue to vaporize and explode through the metal deposit, leaving the two metal tracks at the edge of the previous location of the membrane, right

structure to be? What does a 'two-dimensional orientated viscous fluid' mean? Does it mean a thin layer? How can a fluid be two-dimensional? Do their experiments tell us any more than that the cell membrane contains water, and there is water on both sides of it? If one took a coverslip with water on it and placed a drop of protein on one corner, would it not diffuse across its surface? What would that indicate about the properties of the glass? If one took two adherent wet pieces of paper and injected antibody between them, would that not also spread 'two-dimensionally' as well as through the paper? How can one study a 'fluid mosaic' using freeze etching which freezes the tissue to below its eutectic point, until it becomes solid?

I think one should state unequivocally that the evidence upon which the theory is based can be accounted for by stating that living membranes contain water, and that diffusion occurs in aqueous media.

The idea that cell membranes might be fluid or liquid, implies the so-far unanswered question, 'How do the cells have sufficient tensile strength to contain the cytoplasm?' This is especially pertinent for myelinated nerves, muscle cells and epidermal cells, which may be exposed to considerable pressures. How could one pick out a neuron by hand dissection? How could Cummins and Hyden (1962) cut open neurons and pick up their membranes?

The real difficulty about both the Davson and Danielli and the Singer-Nicholson hypotheses is that neither of them can be demonstrated clearly to be true or untrue. Once again, we have evidence of relatively simple hypotheses, in proof of which cornucopias of complex experiments have been concocted, but which are not amenable to direct proof or falsification. The facility with which monolayers of lipids can be made does not and cannot prove that this is what happens in cell membranes, although many membranologists seem to believe it does.

The presence of water in a solid membrane should be no more surprising than water in an orange skin, which does not mean that the skin is fluid.

It would seem that much of the difficulty about any discussion about the solidity or fluidity of the cell membranes arises from two different uses of the word fluid. For example, glass has the mechanical properties of a solid, but physicochemically it is a fluid. If Singer or Nicholson were to draw a plan of a house, they would no doubt draw the windows as solid structures, even although they certainly know that glass is a fluid molecularly. The positions should be clarified. We believe that mechanically all the real cell membranes are solid structures, probably containing free water. Our view is mainly based on the ability with which whole neurons can be separated, and the possibility of cutting them open (please see page 231). We have no accurate knowledge of the precise chemistry of the membranes (Hillman, 1972). We are not at all clear whether Singer and Nicholson make the distinction between the mechanical and physicochemical meanings of the word 'fluid', and, if so, whether they are claiming that the cell membranes are fluid or liquid both mechanically and physicochemically. However, our main contention must be stated categorically, all the cell membranes in life are mechanically solid. Definitions of fluid, liquid or solid can be found in standard textbooks of mechanics or physical chemistry.

In relation to the membranes of the 'cytoskeleton' it is pertinent to ask why, in electron micrographs, the endoplasmic reticulum appears in sections as parallel lines, but their supposed subcellular offspring appear as rings. The electron microscopists and the biochemists both vehemently maintain that the two structures are identical. They assert that the reticulum 'rounds off' during separation. Yet it is simpler to suggest that the parallel lines and the circles represent different structures. In this sceptical age it is extraordinary that the identity of two such different shapes should be accepted as representing the same object on the basis of a verbal explanation, in the absence of demonstration of how the one shape could change into the other. If it did, it would imply the following train of events: pieces of two parallel lines representing a net or two layers of flattened vesicles would join together at two ends of a length of reticulum simultaneously; such two parallel strands or plates would expand to form a complete circle but would not burst; the ends beyond the join would disappear; the rings representing the thin sections would orientate themselves almost completely in the plane of the electron micrograph.

It should be noted that: (a) the microsomes are not seen joined at both ends on a sort of knot; (b) no intermediate shapes between the parallel lines and the almost perfect circles are seen; (c) segments like an arc of a circle, or a piece of orange skin, would be expected if the fraction contained pieces of reticulum not in the plane of the section; (d) unstuck pieces are not seen equatorially on microsomal circles. These vagaries of geometry are so striking that they shake one's faith in the identity of the endoplasmic reticulum and the microsomes, an identity lacking evidence.

Synapses and synaptosomal fractions are beset by similar structural difficulties. The electron microscopist's synapse is supposed to be a knob connected by a dendrite. When the knobs are separated in the sub-cellular fractions of nervous tissue (Pappas and Purpura, 1972; Cottrell and Usherwood, 1977, many papers) it is generally believed

that the resultant hole in the synapse closes up, so that the synaptosome looks like a perfect circle.

The evidence adduced here that the Davson-Danielli, the Robertson, and the Singer-Nicholson hypotheses, have serious and unacceptable inconsistencies (pages 251-254) should not be taken as denying that cells, nuclei or mitochondria have membranes. If we must be rigorous in our thinking, we can only conclude the following points about the cell membranes:

1. The latter membranes exist in life, although other 'cyto-membranes' do not (Table 17), (please see also Appendix 4 on detection of membranes).
2. The membranes are insoluble, and probably contain substantial amounts of proteins and lipids. The measurements made on subcellular fractions must underestimate the contents in the cells of all soluble compounds including proteins and fatty acids. These must be lost differentially from different fractions, since all procedures involve the additions of a large volume of water and other chemically active reagents. The precise quantity of materials lost from each location in the cell depends on: the chemistry of all the reagents; the time for which they are applied; the physical procedures used; the rate and total quantity of energy dissipated during the procedures; the affinity of each natural component of the tissue for each other and for all the reagents used.
3. Structurally the membranes are each one layer thick. At a molecular level they may be chemically heterogeneous, but it is likely that their dimensions dictate some sort of orientation to them. Semi-permeability, active transport and excitability are all phenomena which can be more easily visualised if the molecules in the membranes are orientated, but one must still be cautious about interpreting the orientation of molecules from studies of dehydrated tissues.
4. The thicknesses of the membranes in life are unknown, but are certainly more than the 6-8 nm seen in dehydrated specimens by the electron microscopist. Since they appear to be of uniform thickness at high magnification in electron micrographs, it is likely that this reflects a uniformity of thickness in the original living material upon which the heavy metal was deposited.
5. The nuclear membranes are not punctured by pores (for evidence, please see Hillman and Sartory, 1980b, pages 67-75).
6. In addition to shrinkage of the width of the membranes resulting from dehydration, it must also shrink in the longer axis. Therefore, in life the membranes around the cell, the nuclei and the mitochondria must contain greater volumes than are seen by histology or electron microscopy. They must be more convex in life than in histological preparations.

THE SYNAPSE

The emergence of the synapse and the neuromuscular junction first as physiological, and then as anatomical, entities has been discussed briefly (pages 15-17). The time has now come to examine the current evidence for their appearances. There are several references in the literature to the suggestion that synapses are difficult to find in the brain (Glees, 1944; Wyckoff and Young, 1954, 1956; Boycott, Gray and Guillery, 1960).

When one looks at the mammalian literature, most of the synapses seen by light microscopy are found in the spinal cord or in the cerebellum, or are descriptions of neuromuscular junctions, or are diagrams. We show here a fine drawing from Held (1897) (Figure 9) who described 'end-fusse', and a more recent light micrography of a silver preparation from Wyckoff and Young (1956) as Figure 46. Small silver granules can be seen on the surface of the neuron soma. Careful examination of the latter figure reveals two important features which are characteristic of all light micrographs: firstly, that granules are seen both on the somas, and also in the background between the somas; secondly, relatively few of the granules have pre-terminal fibrils which can be seen to be connected to other somas. In Table 19 we list 18 publications showing synapses in the illustrations of which granules of approximately the same sizes appear clearly outside the neuron somas. Few of the authors comment on this, although occasionally it has been explained by the suggestion that those particular granules are on the surfaces of somas which are out of focus. The short connections of the synaptic knobs are recognized in the diagram which De Robertis (1959) has put in a review of the subject, and has kindly permitted us to reproduce as Figure 47.

In respect of the granules or knobs being located away from the somas or on somas which are out of focus, one has, of course, no way - other than their appearances - of identifying these knobs as synapses in these particular preparations. Perhaps, in the future, an author who claims that the granules or synapses are on surfaces of somas out of focus will add an adjacent photomicrograph showing the surface of the somas in focus, with the granules neatly situated on them.

As regards the second feature - the shortness of the connections to these knobs - one has a further difficulty. The fact that one can see fibrils arising from the knobs in some instances (Figures 46-48) means that their diameters are wide enough to be within the resolution of the microscope used. As one can see the dendrites arising from the cell bodies on the same illustrations, one must ask why one cannot see the fibrils from the alleged synapses wandering for a good distance across the microscopic field. When one examines cerebral commissures or cerebellar white matter, stained, for example, with Marsland, Glees and Erikson's or Palmgren's stain (Figures 38 and 39), one sees many dendrites, and fine fibres all of which are sufficiently thick to be seen within the depth of 4-10 μm sections; this is evidence that one has sufficient magnification to see fibres connected to the synaptic knobs, if they were present.

The expected appearances of these connections on plan view and on side view have been indicated on a diagram (Figure 49). The latter are simply not seen. With the exception of the fibres from the basket cells surrounding the cerebellar Purkinje cells (Figure 35), I have been unable to find anywhere in the mammalian literature any light micrographs showing even fine fibres apparently investing somas; however, even on Purkinje cell bodies, knobs are not generally seen, and the encirclement of fibres does not necessarily mean that these fibres contact granules on the soma surface.

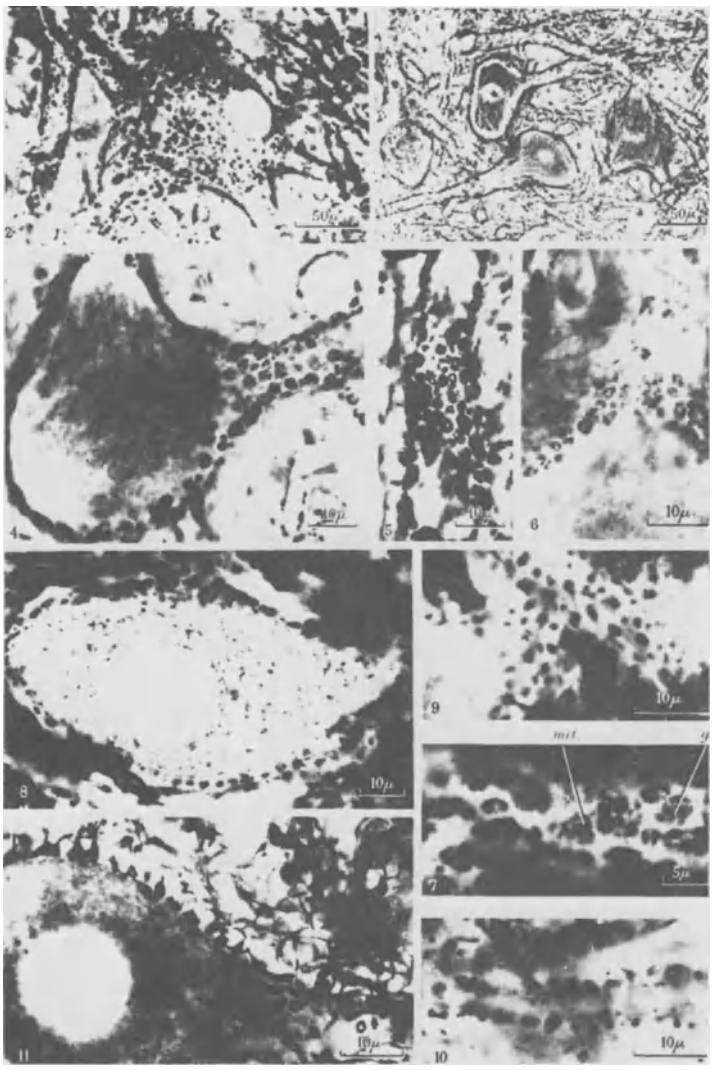


FIGURE 46 'End-feet' (synapses) on the neurons of the ventral horn of the thoracolumbar region of the cat. The 'end-feet' were stained with silver stains. Please note the following features: the diameters of the end-feet; the rarity, except on a few endfeet in 11, of preterminal fibres attached; the frequent presence of silver-stained granules and fibres, in regions not obviously on the neuron surface. This figure is reproduced from Wyckoff, R.W. and Young, J.Z. (1956) The motoneurone surface. Proc. Roy. Soc. B. Lond., 144, 440-450, by kind permission of Professor Young and the Royal Society

TABLE 19 Illustrations by light microscopy showing some knobs of similar size to synapses outside the cell body

Tissue	Author
Mammalian spinal cord	Phalen and Davenport (1937)
Cat ventral ganglion of acoustic nerve	Lorente de No (1938)
Cat ventral horn cell	Barr (1939)
Cat cervical sympathetic ganglion	Gibson (1940)
Cat spinal cord	Barnard (1940)
Human cerebral cortex	Meyer and Meyer (1945)
Mammalian nervous system	Weber (1955)
Cat visual cortex	Sholl (1956)
Cat spinal cord	Wyckoff and Young (1956)
Rat superior cervical ganglion	Couteaux (1958)
Kitten spinal cord	Madge and Scheibel (1958)
Dog spinal cord	Gelfan (1964)
Cat spinal cord	Sprague and Hongchien (1964)
Kitten spinal cord	Szentagothai (1964)
Cat reticular formation	Brodal (1969)
Cat medulla oblongata	Windle (1969)
Rat reticular formation Cat ventral horn cells	Curtis, Jacobson and Marcus (1972)
Rat anterior horn cell	Leeson and Leeson (1981)

The terms used are those of the authors

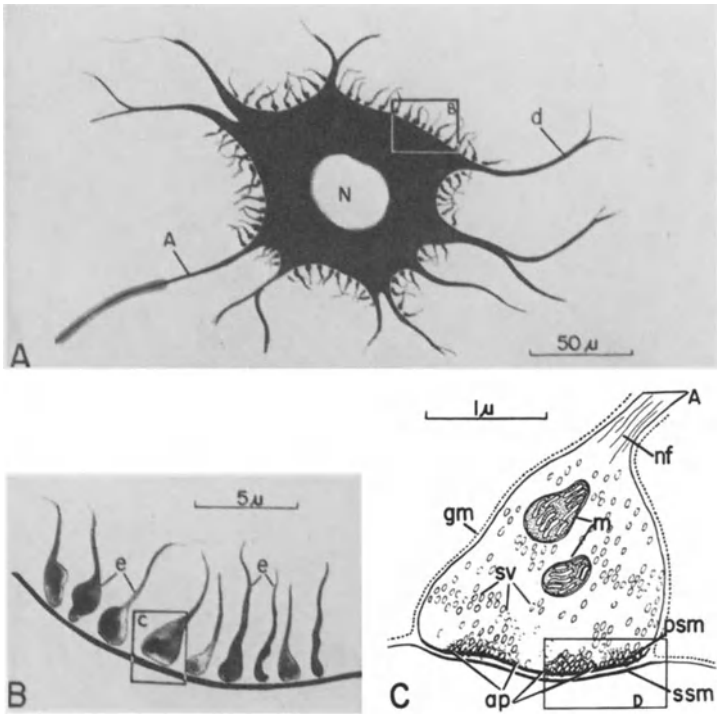


FIGURE 47 A diagram of the current view of the attachment of synapses to the neuron soma, from de Robertis (1959). Please note that the axon and dendrites are shown travelling a long way away from the soma in A, but neither in A nor B do the synaptic connections reach beyond a small distance from the soma. This diagram is reproduced by kind permission of the author and publishers from 8, 65 (Academic Press). This diagram should be compared with Figures 49 and 53, showing the expected view of synapses

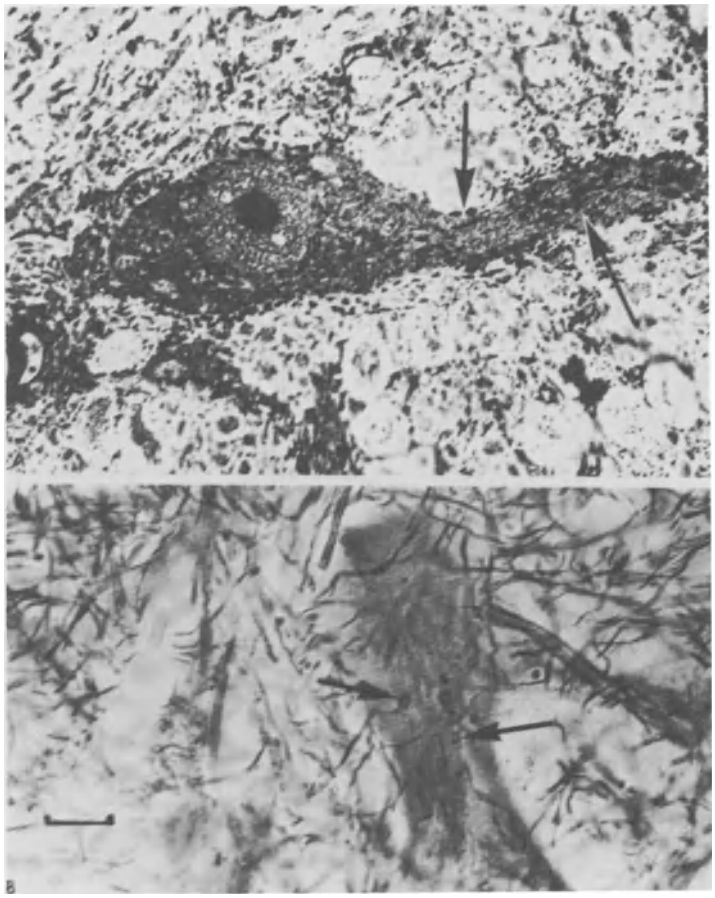


FIGURE 48 Synapses on neurons of A, rat reticular formation, B, ventral horn of cat, stained with silver stain. In the reticular section many stained granules are not obviously associated with neurons. In the ventral horn neuron many fibres can be seen, but they are rarely attached to the synapses. These illustrations are reproduced from Curtis, B.A., Jacobson, S. and Marcus, E.M. (1972) Introduction to Neurosciences, Philadelphia; Saunders, page 54, by kind permission of Dr Jacobson and the W.B. Saunders Company. The bar is 10 μm

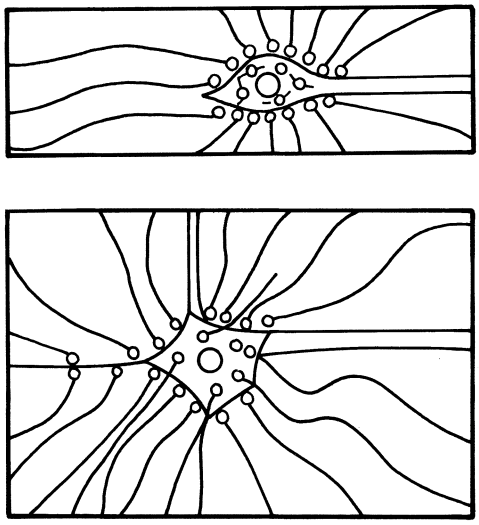


FIGURE 49 Expected views of synapses seen by light microscopy on the soma of a neuron. Please note the synaptic connections ('telodendroglia') spreading to the edge of the field. This should be compared with the previous Figures 46-48

In a Golgi preparation of brain, 150 μm thick, one can focus on the dendritic tree at various planes in the tissue; the dendrites form an aesthetic arborization (Figure 50). As one follows any particular dendrite under the microscope, especially using a x90 or x100 oil-immersion objective, one can see that there are no connections between the dendrites and axons from a particular soma to any other soma, just as the branches of trees in a closely planted grove do not make connections with each other. However, they appear to do so on a normal two-dimensional photograph (Figure 51).

In cats, Wassle (1982) demonstrated the dendritic trees of ganglion cells in whole mounts of retina after retrograde labelling with horseradish peroxidase, and has kindly provided us with Figure 52. Although occasional dendrites from one soma overlap one another, and fluorescent granules are seen on many dendrites, it is difficult to suggest that there are any connections between the cells or their processes. Boycott, Gray and Guillery (1960) explained the failure to see synapses frequently in the neo-cortex by their not being stained (see also Gray, 1966).

En passant, it should be noted in the illustration from Wyckoff and Young (1956) (Figure 46) that the synaptic knobs appear empty on light micrographs, while the electron microscopists fill them with a treasury of synaptic vesicles.

The histologists searched for the synapse, while the physiologists continued to describe the properties of reflexes, of excitation and inhibition, in the belief that they were all located in the anatomical structures which were still rather elusive. They were both ready to accept that the location by the electron microscopists of the synapses not only between neurons, somas and fibrils, but also between virtually every part of the neuron including the axon and many neuroglia (please see page 17). confirmed the older views about where the physiological events occurred. They did not appreciate the serious three-dimensional difficulties in interpreting the micrographs,



FIGURE 50 The original legend reads 'A section showing a number of the smaller pyramidal neurons in the outer part of the visual cortex of a cat stained by the Golgi-Cox method. Technique: mercuric chloride-dichromate. For details see Sholl (1953). Section 150 μm thick'. Apparent contacts between the processes can be seen to be extremely rare even in this photograph. This figure is reproduced from Sholl, D.A. (1956) The Organisation of the Cerebral Cortex. London: Methuen, page 14, by kind permission of the publishers

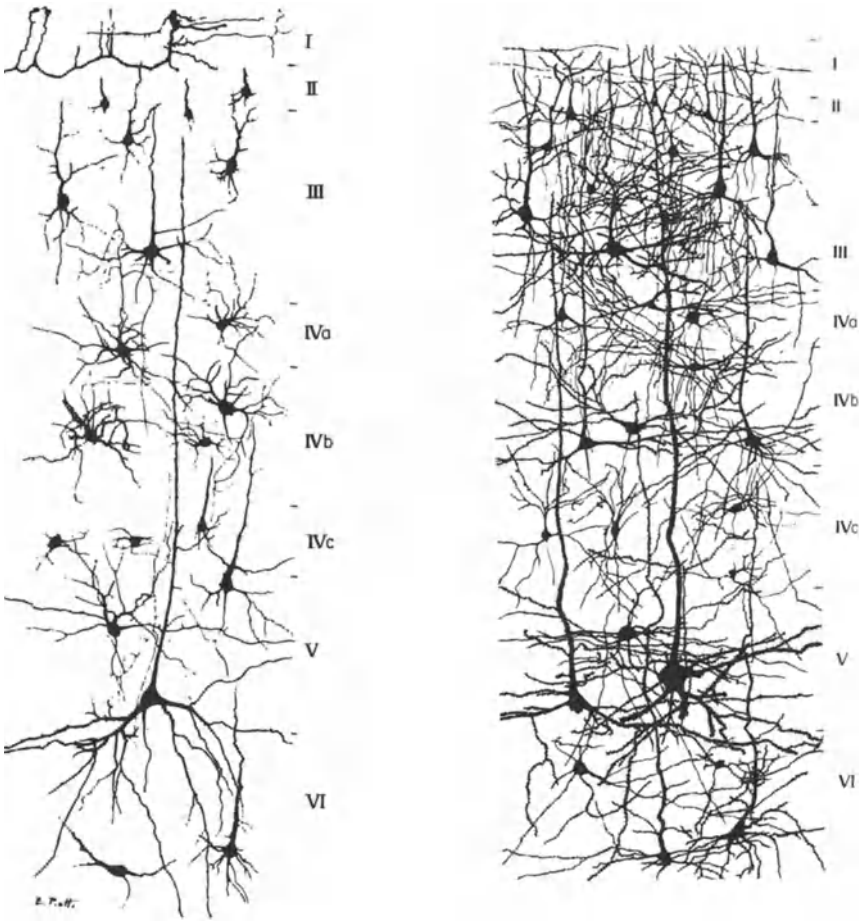


FIGURE 51 Dendritic arborizations in the human cerebral cortex: left, neonatal, right, after 24 months. These illustrations are reproduced from Conel, J.L. (1939, 1959) The Postnatal Development of the Human Cerebral Cortex, Vols. I, VI. Cambridge, Mass: Harvard University Press, by kind permission of the publishers and the President and Fellows of Harvard College. It is difficult to imagine that the bodies of neuroglial cells could fit in between these dense arborizations.

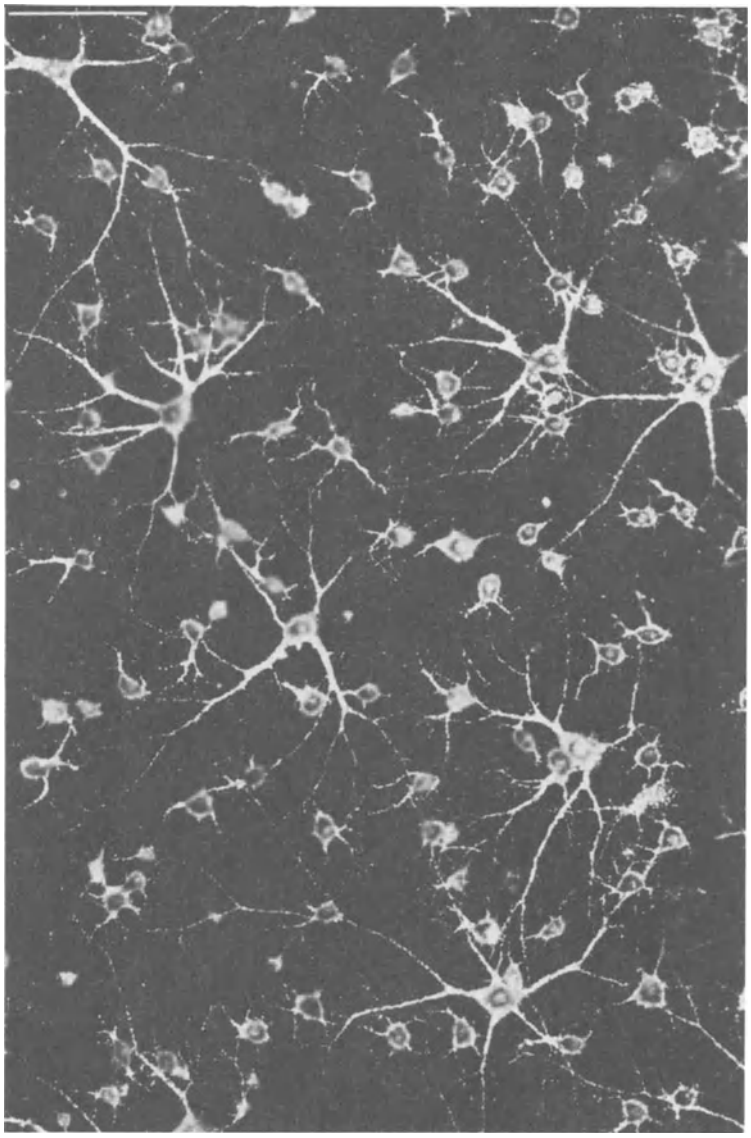


FIGURE 52 Micrograph from a whole mount of the cat retina after retrograde labelling of the ganglion cells with HRP injected into the ipsilateral lateral geniculate nucleus (scale 100 μm, eccentricity 7 mm, temporal retina). This figure is from Wassle, H. (1982) Morphological types and central projections of ganglion cells in the cat retina. In Progress in Retinal Research, 1, eds Osborne, N.N. and Chader, G.J. Oxford: Pergamon, page 143; it is reproduced by kind permission of the author and publishers. Please note that there is little evidence of contacts between dendrites

nor the probably irreconcilable discrepancies between the views of the light and electron microscopists of what are believed to be the same structures.

The geometrical problems are summarized in Figure 53; both kinds of microscopists have different problems. By light microscopy the vast predominance of views could be represented by a section through an egg lying on its side with its long axis parallel to the surface upon which it was lying. One does not see sections in all the other orientations in light micrographs. Obviously, if the synaptic knobs are distributed all

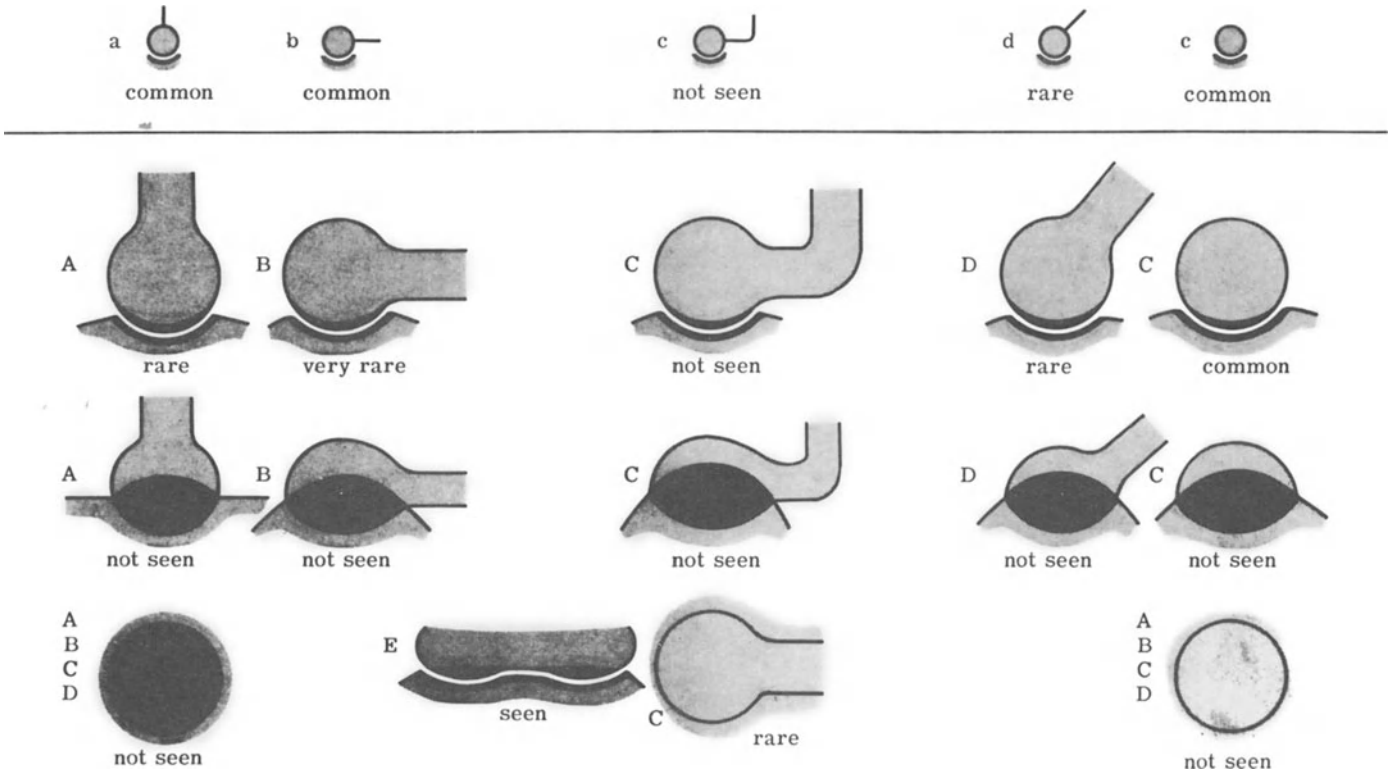


FIGURE 53 Drawings of possible appearances of synapses by light microscopy (top line, a,b,c,d) and electron microscopy (lower lines, A,B,C,D,E); the same letters should refer to the expected views of the same shaped synapse at different magnifications and orientations. The black lines and shading indicate pre- and post-synaptic thickenings; the grey shading indicates the cytoplasm of the synapses and the neurons. These diagrams are based on the assumption that the synapses are randomly distributed over the surface of the neurons, as shown, for example in the diagram of Barr, 1939. The appearances of the synapses by light microscopy should be completely compatible with their appearances by electron microscopy. Geometry dictates unequivocally that all the appearances of the electron microscopic images indicated by a particular capital letter should be seen, and that they should occur in approximately equal frequencies. Examination of the considerable literature on the structure of synapses fails to show a significant incidence of any of the images of the third line down (with oval pre- or post-synaptic membranes), nor of the arcuate sections of the synapses as seen at the bottom extreme left and extreme right. Light microscopic images corresponding to the 'double' synapse seen on electron microscopy (E) do not seem to have been reported either (please see references in Tables 21-26). It should also be noted that the common views by light microscopy a,b,c, do not correspond to the common view by electron microscopy, C, in the right-hand column

over the neuron surface, any section should go randomly through single synapses in a complete range of orientations and sizes. For example, the pear shape should not appear more frequently than the circular shape, which would be at right angles to the pear shape. We have tilted the model and drawn the resultant shapes which would be expected (Figure 53). In these diagrams the top line represents the views seen by light microscopy, with the exception of the c in the middle of the diagram.

One would expect to see the same structures on electron micrographs at higher magnifications than they are seen in light micrographs. These are represented by capital letters on Figure 53. Simple geometry again tells us that for an object which is randomly orientated it should be seen equally frequently in any two planes at right angles to each other. When one takes the top line of expected electron microscopic images A, B, C and D, one can then tilt them at 45° away from or towards the plane of the picture, and look at the resultant expected images depicted in the next line down using the same capital letters. One then obtains a series of images in our diagrams, which are simply not seen on electron micrographs. Put another way, one rarely if ever sees a synapse whose thickening obliterates the synaptic cleft and which bridges as an oval shape the pre- and post-synaptic region. If one then tilts the synapses a further 45° to examine their expected appearances at right angles to their long axes, one would expect to see the appearance of a black circle due to the synaptic thickenings not apparently connected to the membranes of either of the cells. Again, this appearance should occur as often as the right-hand top electron micrographic diagram C.

A further generalization may be made. One hardly ever sees the preterminal fibre of the synapse on electron microscopy. The top right-hand image C appears to be the commonest picture. Why is the connection of the synapse to the parent soma seen so rarely on electron microscopy, when - although too short for distant connections on light microscopy - it is at least seen sometimes (Figure 46). Fortunately, the neuromuscular junction represents a control tissue, in which the preterminal fibre is always seen by electron and by light microscopy. As far as the classification of synapses into Types I and II (Gray, 1959), represented in Figure 53 by E, it should be pointed out that that appearance does not seem to have any counterpart in light microscopy; perhaps the magnification is not high enough to show it. However, neither Gray himself nor those who accept his classification appear to have realized that a section through a Type II synapse E at right angles to, and in the long axis of, the one shown would give the typical appearance of a type I synapse; thus, there is no necessity to postulate the two types.

Hyden and his school (Hyden, 1961, 1967a) have published a large number of photographs of isolated unfixed neurons, similar to our micrographs (Figures 16 and 44). At the magnifications used one should be able to see synaptic knobs, if they exist, but they are difficult to recognize. This could be because (1) they have all been pulled off during the isolation by hand dissection; (2) the granular appearance of the neuron surface under phase-contrast microscopy is due to many small synapses; (3) the synapses in unfixed tissue have the same refractive index as the media in which the neurons are prepared (usually 0.25 mol/l sucrose); (4) synaptic knobs do not occur on neurons. Roots and Johnston (1965) found rather few synapses on isolated

neurons using electron microscopy and they also believed that the membranes of the neurons are largely stripped off during preparation. The latter explanation seems unlikely to us, since isolated neurons after isolation possess resting potentials normally believed to be associated with a membrane (Hillman and Hyden, 1965); these potential differences could be changed reversibly by altering the oxygen, potassium ions or sodium ions in the incubating media. Many of those who isolated unfixed neurons believe that the granular appearance is due to synapses.

We believe that the granular appearance is due to intracellular structures for the following reason. One can draw diagrams of the appearance of granules on the surface of the soma (that is, synapses), and compare the appearances with those of granules in the cytoplasm (that is, mitochondria) (Figure 54). When one examines cells by low-power light microscopy with a considerable depth of focus, the number of granules seen over the nuclei would be more if the granules were synapses than if they were mitochondria. Neurons which have synaptic physiology in tissue culture have been illustrated by several authors (Table 20). Invariably fewer granules appear over the nuclei than over the cytoplasm at these relatively low magnifications (Figure 55). (Of course, when one focuses on the nuclei with high-power light microscopy when the depth of focus is only 1-2 μm, one cannot distinguish whether granules are in the cytoplasm or on the surface of the cell.) The fewer granules over the nucleus than over the cytoplasm under low-power microscopy must be interpreted as meaning that the granules are intracellular - that is, mitochondria and not synapses.

One must readily admit the difficulty of divining the total specifications prescribed by the original designer of anatomical structures, but it is interesting to speculate on the reason for which the precise area through which the transmitters are believed to diffuse and attach - the pre- and post-synaptic regions - should have been so armorially thickened. It is strange that these should be chosen to be sites of the entry and exit of large insoluble molecules or granules - processes known as endocytosis and exocytosis.

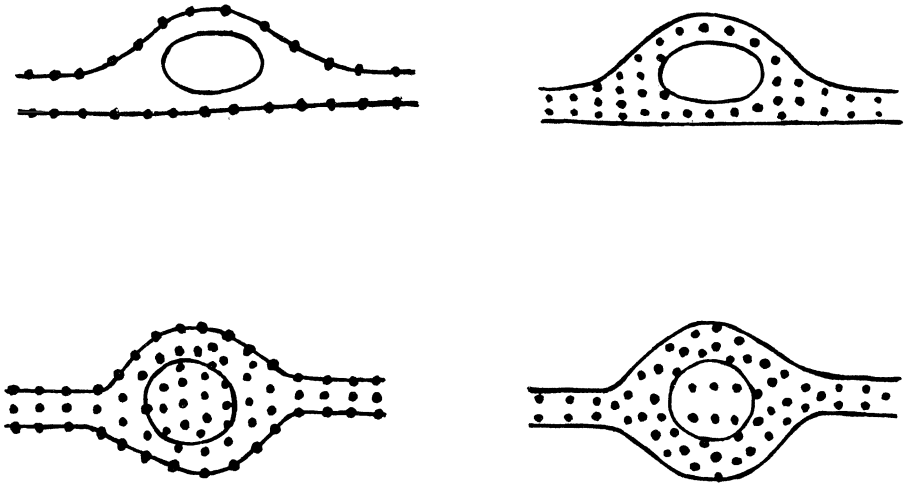


FIGURE 54 If one sees granules in a cell under the light microscope, one can determine if they are on the membrane of the cell (left-hand figures) or in the cytoplasm (right-hand figures); that is, whether they are synapses or mitochondria. The cell is viewed in transverse section (upper diagrams) and then from above (lower diagrams). If the granules are on the surface they will appear in only slightly less density over the nucleus than over the cytoplasm (lower left). If they are in the cytoplasm but not in the nucleus (upper right) they will appear from above in much lower density over the nucleus than over the cytoplasm (lower right). In cells examined under low-power magnification when the depth of focus is several micrometres, one can see, nevertheless, that fewer granules are visible over the nucleus than over the rest of the cytoplasm (Table 20), suggesting that the granules are intracytoplasmic, that is, they are mitochondria.

TABLE 20 Neurons in tissue culture in which the nucleus appears clearer than the cytoplasm, which indicates that the granular appearance is more likely to be due to mitochondria than to synapses

Human cerebral and cerebellar cortex	Costero and Pomerat (1951)
Human cortex	Geiger (1957)
Rat and cat cerebellum	Hild and Tasaki (1962) *
Rabbit cerebral hemispheres and cerebellum	Varon, Raiborn, Seto and Pomerat (1963)
Chick and rat dorsal ganglion	Pomerat, Hendleman, Raiborn and Massey (1967)
Mouse spinal cord and dorsal root ganglion	Nelson (1973) *
Mouse dorsal root ganglion	Shahar, Grunfeld, Spiegelstein and Monzain (1975)
Mouse spinal cord	Fischbach and Nelson (1977) *

* In these publications, neurons have been shown to fire. Please see Figure 54.

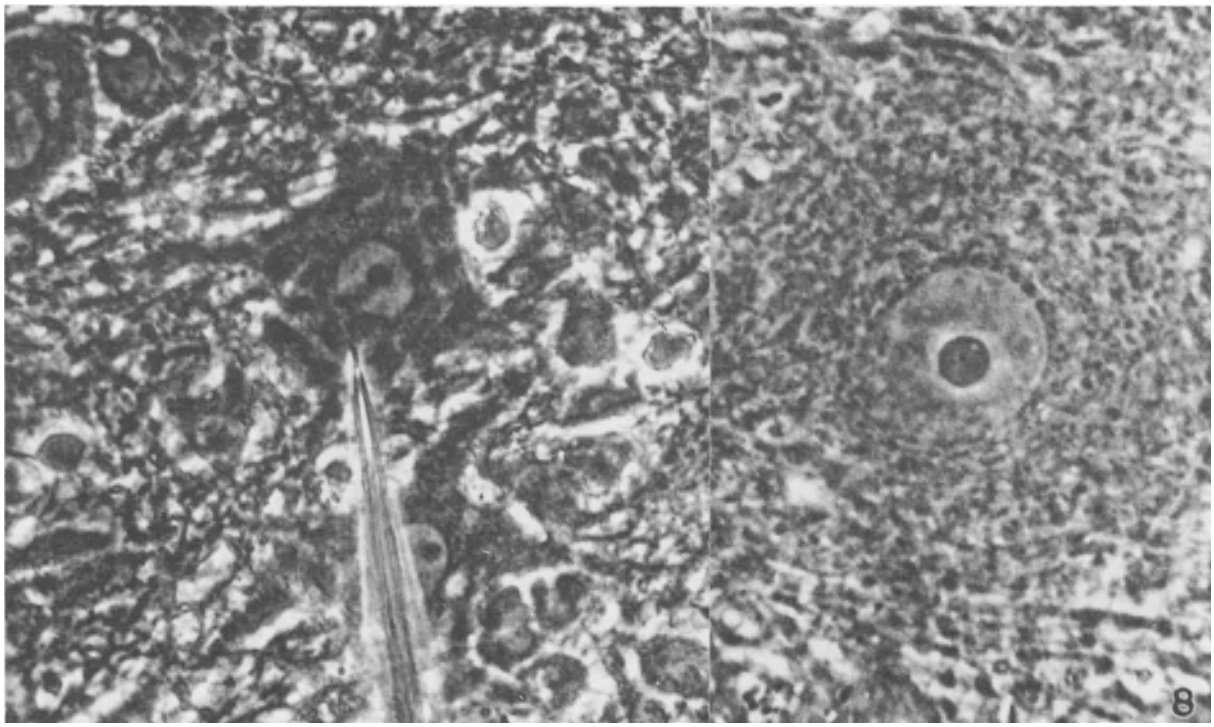


FIGURE 55 Purkinje cells of cat cerebellum in (left) a 25-day-old culture; (right) a 42-day-old culture. The cell on the left is being penetrated by an intracellular electrode, which records an action potential. This photograph is reproduced from Hild, W. and Tasaki, I. (1962) Morphological and physiological properties of neurons and glial properties of neurons and glial cells in tissue culture. J. Neurophys., 25, 277-304, by kind permission of the authors, the editors of the Journal of Neurophysiology, and the American Physiological Society. The cell on the right is reproduced from Pomerat, C.M. and Costero, I. (1956) Tissue culture of cat cerebellum. Am. J. Anat., 99, 211-247, by kind permission of the editors of the American Journal of Anatomy, and Alan Liss, Inc. Please note that in both the nucleoplasm appears less granular than does the cytoplasm

Practically every part of the neuron has been claimed to be seen on electron microscopy to synapse with other neurons, and, occasionally with neuroglial cells (please see references on pages 15-17). This raises a number of questions. Why do these synapses not appear as knobs on light microscopy? How widespread is their distribution? Does an electron microscopist examining the mammalian central nervous system see thickenings on the surface of cells which he had previously thought of as neuroglia, then proceed to reclassify the cells as neurons? Have systematic attempts been made to search for them and examine their incidence widely in relation to mammalian neuroglia? Are there any other certain and unequivocal criteria which a microscopist can use to identify a synapse?

It is generally held that the careful histological studies of Ramon y Cajal - summarized in 1954, please see page 13 in which he pointed out that neurons did not have contact with each other, support the existence of synaptic clefts. The apparent absence of continuity of neurons has been used as one element of evidence that synaptic transmission is chemical. What does one mean by 'contact'? In an electric plug,

a wire is screwed tightly on to a metal contact. Most authors see the synaptic cleft 20-30 nm wide (Table 21), which is probably much narrower than the gap in parts of a fully functional electrical contact. In a synapse, the pre- and post-synaptic sides would both be in a tissue fluid which is highly conductive.

Gray (1959) classified cortical synapses into two types, based on the width of the synaptic clefts, the depth of the post-synaptic thickenings, and whether the synapses appeared to be axodendritic or axosomatic. Subsequently, he found that the same classification could be extended to cerebellar synapses (Gray, 1961). Van der Loos (1965) doubted the distinctness of the two types, and Karlsson (1966) did not accept that the classification was applicable to other parts of the central nervous system.

TABLE 21 The width of the synaptic cleft in mammalian central nervous system measured by electron microscopy

Tissue	Width (nm)	Author
Rat abducens nucleus	20	Palay (1958b)
Rat abducens nucleus	20	Hyden (1961)
Rat dorsal cochlear nucleus	20	Eccles (1964, page 17)
Monkey lateral geniculate nucleus	20	Gray and Guillery (1966)
Cat subfornical organ	20	Akert, Pfenninger, Sandri and Moor (1972)
Rat cerebral cortex	30-40	Curtis, Jacobson and Marcus (1972, page 52)
Rat prepyriform cortex	20-30	Johnston and Roots (1972, page 35)
Rat cerebral cortex	20	Jones and Brearley (1972a)
Cat oculomotor nucleus	20	Pappas and Waxman (1972)
Rat ventral horn of spinal cord	20	Bloom and Fawcett (1975, page 37)
Neurons from various species	20-30	Peters, Palay and Webster (1976, page 135)
Neurons from various species	15-25	Brodal (1981, page 11)

The identification and therefore confirmation by electron microscopy of the existence of synapses, which had been previously seen and described by light microscopy, was generally regarded as having settled the problems of the elusiveness and shapes of the synapses. There are, however, further disquieting discrepancies. Comparison of the numbers of synapses per soma by light microscopy (Table 22) with the numbers by electron microscopy (Table 23), shows that - with a few exceptions - the former technique reveals incidences in the 10s and 100s, while the latter technique reveals 1000s to 10 000s. One many assume that when light and electron microscopists measure synaptic diameters, they publish values of average diameters, or their photographs show 'typical' synapses. One has to be a bit careful in comparing values for light micrographs, which are normally given by the authors (Table 24) with measurements from electron micrographs (Table 25). The electron microscopists have generally avoided giving values for the diameters of their synapses, so we have had to measure them off their figures. The precision of values given by authors of light micrographs is low because the synapses are too small to measure them accurately,

so they are usually estimates; in electron micrographs the precision is diminished by the accuracy with which measurements can be made from published micrographs. With these reservations firmly in mind one can compare the diameters of synapses measured by light microscopy (Table 24) with those measured by electron microscopy (Table 25). Although the range of the former is between 500 and 15 000 nm in diameter, and the latter is from 250 to 2500 nm, in general the overlap between the two sets of dimensions is so poor as to cast doubt as to whether the two sets of measurements were both of the same objects. The differences could not be accounted for by supposing that the shrinkage during silver staining for light microscopy would be less than that during osmic acid staining for electron microscopy, bearing in mind that shrinkage resulting from dehydration with ethanol is common to both techniques; also a linear dimension which is double another encloses a volume eight times that of the other.

We may summarize the conclusions about the synapses. Synapses seen by electron microscopy do not respect Euclidean geometry. They are seen in larger numbers, but in smaller dimensions, by electron than by light microscopy. Although some authors have shown the appearance by light microscopy of some areas where dendrites from different cells appear to overlap (Van der Loos, 1960, 1974; Van der Loos and Glaser, 1972). I have been unable to find in the literature micrographs showing a parent soma connected to a dendrite whose preterminal fibre is attached to a synapse. At a full meeting of the Physiological Society at University College, London, on March 28th, 1985, I invited colleagues to send me micrographs or references to literature showing this.

The synaptic thickenings of the electron microscopists may be due to the heavy metal stains attaching themselves to apparent rings believed to be parts of the membrane for which they have a greater affinity than other parts, or they may be due to random deposits of the heavy metal on such rings. There is no way of distinguishing between these two possibilities. Furthermore, even if the heavy metal does have a greater affinity for 'specific' sites on the cell membrane, that does not mean that these represent the sites of the physiological activities. Even if the measurements of the dimensions of the synapses were accurate to within one order of what they appear to be, the widths of the intracellular electrodes with the finest tips would be far too large to be of use in locating the site of a single synapse. Since one cannot specify the sites of physiological events of transmission, it follows that findings about physiological events do not necessarily support or deny any hypotheses about those events. (Please see also Hillman, 1985.)

The discrepancies between the light and microscopic views of synapses and their apparent absence in some sites does not necessarily mean that they do not exist, but they certainly raise some difficult questions. However, our discussion of the lack of cogent evidence for long connections between the synaptic endings and other dendrites presents a much more serious problem, especially for the physiologists. This is dealt with on page 197.

I would like to stress that I am not denying the existence of neuromuscular junctions or pre-terminal fibres to them. However, evidence from them may not be relevant to synapses, without demonstration of identity of the particular property in both tissues.

TABLE 22 Number of synapses per neuron soma reported by the authors or counted from their publications, as detected by light microscopy

Neuron	Synapses per cell	Stated (s) or counted (c)	Authors
Guinea-pig trapezoid nucleus	54	c	Barker (1899)
	48	c	
	46	c	
	36	c	
	26	c	
Mammalian spinal cord	0-10	s	Phalen and Davenport (1937)
Cat motor neurons	14	c	Lorente de No (1938)
	38	c	
	38	c	
	22	c	
	30	c	
Cat ventral horn cell	1250	s	Barr (1939)
Cat anterior horn cell	192	c	Barnard (1940)
	388	c	
	156	c	
	130	c	
	416	c	
	68	c	
	106	c	
	148	c	
	114	c	
	234	c	
	150	c	
	70	c	
Cat cervical sympathetic ganglion	Up to 13	s	Gibson (1940)
Monkey lateral geniculate body	1	s	Glees and Le Gros Clark (1941)
	1	c	
Human dorsal sensory and internuncial	97	s	Minckler (1942)
Human intermediate sensory and internuncial	152	s	
Human ventral sensory and internuncial	116	s	
Human nucleus dorsalis - thalamus	348	s	
Human intermediolateral - thalamus	122	s	
Human posteromedian - thalamus	362	s	
Human anteromedian - thalamus	675	s	
Human anterolateral - thalamus	1624	s	
Human posterolateral - thalamus	3990	s	
Human cerebral cortex	16	c	Meyer and Meyer (1945)
	24	c	
Cat ventral horn of spinal cord	2000	s	Wyckoff and Young (1956)
Rat superior cervical ganglion	12	c	Couteaux (1958)
	14	c	
Dog lumbosacral cord neuron	800	s	Gelfan (1964)
Dog motor neuron	6000-10 000	s	
Cat spinal cord	84	c	Sprague and Hongchien (1964)
Rat cerebral cortex	10 000	s	Johnston and Roots (1972)

The terms used in Tables 22-25 are those used by the Authors

TABLE 23 Number of synapses per neuron soma reported by the authors as detected by electron microscopy

Neuron	Synapses	Authors
Rat spinal cord	2000	De Robertis (1959)
Mammalian	Many hundreds	Eccles (1964)
Rabbit Deiters cell	10 000	Hyden (1964)
Rat lateral geniculate nucleus	133 83	Karlsson (1966)
Guinea pig cerebral cortex	800	Blackstad (1967)
Mouse visual cortex Mouse motor cortex Monkey visual cortex Monkey motor cortex	7000 13 000 5600 60 000	Cragg (1967)
Vertebrates	10 000	Johnston and Roots (1972)
Cat cerebral cortex	870	Kaisman-Abramof and Peters (1972)
Cat spinal cord	Several thousands	Conradi (1972)
Mammalian spinal motor neuron Mammalian cortical pyramidal cell Mammalian cerebellar Purkinje cell	500 30 000 Up to 80 000	Bullock, Orkand and Grinnell (1977)

TABLE 24 Diameters of synapses seen by light microscopy in the literature. The values were stated by the authors

Tissue	Width (nm)	Authors
Mammalian nervous system	500-7000	Ramon y Cajal (1909)
Monkey spinal cord	950 x 800-3330 x 2600	Phalen and Davenport (1937)
Cat spinal cord	500 x 100-4000 x 5000 (mean 1500 x 2000)	Barr (1939)
Cat spinal cord	7000 (mean 2000-3000)	Gibson (1940)
Cat ventral horn motor neuron	1000-5000	Bodian (1952)
Cat cerebral cortex	2500	Entin (1954)
Cat spinal cord	15000 x 8000	Szentagothai-Schimert and Albert (1955)
Rat spinal cord	1000-5000 (mean 2000)	Wyckoff and Young (1956)
Human cerebrum	800-2500	Smythies, Gibson and Purkis (1957)
Rat visual cortex	2000-4000	Boycott, Gray and Guillery (1960)
Cat spinal cord	1000-4000 few 15000	Gray and Guillery (1966)
Cat reticular formation	500-3000	Brodal (1969)
Rat cerebellum	1500-6000	Valdivia (1971)
Cat motor neurons	1000-3000	Curtis, Jacobson and Marcus (1972)
Cat granule and stellate cells in cerebellum	500	

TABLE 25 Diameters of synapses as measured from illustrations of electron micrographs in the literature. The accuracy of measurements is estimated to be about 50 nm from the printed micrographs

Tissue	Width (nm)	Authors
Rabbit ventral horn cell	1200	Wyckoff and Young (1956)
Cat upper lumbar spinal cord	600-1000	Gray (1959, 1961)
Rat hippocampus	500	Blackstad (1967)
Ox brain	500	Roots and Johnston (1964)
Rat prepyriform nucleus	800	Gray and Guillery (1966)
Rat medial mammillary nucleus	800	Heimer and Ekholm (1967)
Rat cerebellum	1500	Lumsden (1968)
Cat lateral vestibular nucleus	1300	Brodal (1981, page 10)
Cat subfornical organ Monkey spinal cord	500-800) 500)	Akert, Pfenniger, Sandri, and Moor (1972)
Rat cerebellar cortex	500-2000	Bloom (1972)
Rat prepyriform cortex	1300	Johnston and Roots (1972, page 38)
Rat cerebral cortex Cat oculomotor nucleus Cat sensorimotor cortex Cat hippocampus	1200) 1000-1500) 650-1000) 1200)	Pappas and Waxman (1972)
Rat median septal nucleus	500-2500	Raisman and Matthews (1972)
Rat olfactory glomerulus	600-1000	Reese and Shepherd (1972)
Monkey lateral geniculate body	1500	Glees and Hasan (1973)
Rat ventral cochlear nucleus	250-600)	Peters, Palay and Webster (1976, pages 118-180)
Rat cerebral cortex	250)	
Rat spinal cord	250-1000)	
Rat auditory cortex	500-750)	
Rat visual cortex	400-900)	
Monkey visual cortex	600	Somogyi and Cowey (1981)

The synapses are believed to contain 'vesicles' (Gray and Whittaker, 1962), which are 10-70 nm in diameter (Table 26). It is very likely that the vesicles are artifacts for the following reasons. Inspection of the references in Tables 23, 25 and 26 shows that in any individual preparation vesicles appear far too frequently of uniform size. If they were uniform in diameter in the living tissue, they should be seen in sections in a normal distribution of diameters. Such studies which have been done show certain diameters to predominate; very few small contours of vesicles are seen (please examine illustrations in Birks, Huxley and Katz, 1960; Pappas and Waxman, 1972; Gray, Gordon-Weeks and Burgoyne, 1982). Secondly, the number of vesicles seen depends upon the fixative used to stain them, and the concentration of magnesium ions in the preparation medium (please see page 229). Thirdly, much of the evidence which is believed to be compatible with the transmitter hypothesis, but in our opinion, does not necessarily bear upon it, has been reviewed in this monograph (pages 226-230), and has been shown to contain a number of important but unlikely assumptions. Fourthly, there are believed to be about 50 neurotransmitters (Table 27). One must ask if each

TABLE 26 Diameters of synaptic vesicles seen by electron microscopy in the literature. The values were stated by the authors

Tissue	Width (nm)	Authors
Guinea-pig forebrain	50	Gray and Whittaker (1960)
Rat abducens nucleus	20-65	Hyden (1961)
Rat cerebral cortex	20-70 (mean 40)	De Robertis (1963)
Mammalian central nervous system	30-60	Eccles (1964)
Rat cerebellar cortex	15-60	Palay (1964)
Rat prepyriform cortex	30-60	Gray and Guillery (1966)
Cat dorsal nucleus of lateral geniculate body	30-50	Peters (1968)
Rat ventral horn of spinal cord	20-65	Bloom and Fawcett (1969)
Cat reticular formation	30-60	Brodal (1969)
Rat cerebellum	40 50-60 x 25	Valdivia (1971)
Kitten subfornical organ	50	Akert, Pfenniger, Sandri and Moor (1972)
Rat cerebral cortex	10-20	Johnston and Roots (1972)
Cat oculomotor nucleus	40-60	Pappas and Waxman (1972)
Various mammalian neurons	40-50	Jones (1975)

TABLE 27 Substances which have been proposed to be involved in the communication between neurones (partly from Barchas, Akil, Elliott, Holman and Watson (1978) and partly from Osborne (1981)) Communication between neurons. Neurochem International, 3, 3-16.

Adrenalin
Dopamine
Noradrenalin
Tyramine
Octopamine
Phenylethylamine
Phenylethanolamine
Dimethoxyphenylethylamine(DMPEA)
Tetrahydroisoquinolines
Serotonin (5-hydroxytryptamine)
Melatonin
Tryptamine
Dimethyltryptamine (DMT)
5-Methoxytryptamine
5-Methoxydimethyltryptamine
5-Hydroxydimethyltryptamine (bufotenin)
Tryptolines
ATP
Acetylcholine
Carnosine
Histamine
Gamma - aminobutyric acid (GABA)
Gamma - hydroxybutyric acid (GHB)
Glycine
Taurine
Purine
Aspartate
Glutamate
Prostaglandins
Corticosteroids
Oestrogens
Testosterone
Thyroid hormone
Bombesin
Cholecystokinin (CCK)
β-Endorphin
Gastrin
Neurotensin
Proctolin
Prolactin
Oxytocin
Substance P
Somatostatin
Angiotensin
Luteinizing hormone releasing hormone (LHRH)
Vasopressin
Vasoactive intestinal polypeptide (VIP)
Adrenocorticotropic hormone (ACTH)
Thyrotropic releasing hormone (TRH)
Sleep factor delta

of them has its own vesicles. Presumably, the general view is that those neurons excited or inhibited by a particular putative neurotransmitter have in their synapses vesicles containing 'packets' of their own transmitter. The transmitter hypothesis has only been worked out in detail for acetylcholine and some catecholamines, (see pages 226-230).

DENDRITES

Each neuron has up to about 20 primary dendrites and their number increases with old age (Golgi, 1879; Conel, 1939-1963; Sholl, 1956; Nauta and Ebbeson, 1970). It should be noted that there are relatively few primary dendrites and that they divide into many secondary ones (Figures 50 and 51). The dendritic tree appears like a sapling in winter; that is to say, each neuron has probably one main axon and three to six larger primary dendrites, each giving out few branches.

In our view the dendrites radiate out into the syncytium and make no contact whatsoever with other dendrites, synapses or cell somas. This observation was first made by Ramon y Cajal (page 12). The morphology of the dendrites is probably similar to that of the free nerve endings found in the cornea, skin, blood vessels and viscera (Boeke, 1932; Kuntz and Hamilton, 1938; Tower, 1940; Woollard, Weddell and Harpman, 1940).

The ideas that the axons can be distinguished from the dendrites by any criteria other than their size, or that each neuron has only one or two axons, are both assumptions.

The surfaces of the dendrites in tissue culture appear much smoother than they do in histological sections, probably because they shrink and their surfaces become granular during preparation; heavy metal stains and intracellular components precipitate during dehydration.

If, as is proposed here (page 92), the cells generally described as astrocytes and oligodendrocytes are neurons, it follows that all neuroglial processes and fibres are dendrites.

NEUROMUSCULAR JUNCTIONS

In mammals neuromuscular junctions can be seen unfixed, or stained by light microscopy, and by electron microscopy (for reviews, see Cole, 1955, 1957; Couteaux, 1960; Zacks, 1964; Coers, 1967; Cold Spring Harbor Symposium, 1975; Gauthier, 1976); they are sometimes visualized by the presence of cholinesterases (Koelle and Friedenwald, 1949; Karnovsky and Roots, 1964). By light microscopy they appear as tubular strands ending in muscle (Figure 56), or they may sport knobs at the ends of them (see, for example, McMahan, Spitzer and Pepeu, 1972). Sometimes there appears to be one junction for a group of muscle fibres (Figure 56), sometimes one for each muscle fibre, and sometimes several for each muscle fibre (Vrbova, Gordon and Jones, 1978); even in the latter case, a single neuromuscular junction services many sarcomeres. This means that excitation spreads across the sarcomeres, a long way from the end-plate, visible by light or electron microscopy.

By electron microscopy one usually sees between the axon and the muscle invaginations

or junctional folds; these may branch and their appearances are highly variable (op. cit.). The axonal end contains a large number of vesicles, which should be considered in the same light as synaptic vesicles, and are probably artifacts (page 154). The junctional folds themselves have a dangerous proclivity to lie nearly always in the plane of the electron micrographs. Their dimensions are such that they or the space believed to be occupied by them should be visible by light microscopy. Perhaps micrographs showing these have not yet reached the literature, but I would be grateful if anyone would send me a light micrograph showing them, or draw my attention to a publication in which they feature.

CYTOPLASM OF NEURONS

In life, the cytoplasm is a liquid of low viscosity permitting diffusion, Brownian movement and the movement of mitochondria; pinocytosis may also occur. The mitochondria are filamentous structures (Figures 24 and 42) with single-layered membranes and they contain a liquid mitochondrioplasm. They are the only identifiable structures in the cytoplasm, although other granules - which are difficult to classify, name or analyse chemically - are sometimes also present there (Figure 44, pages 128-130). In tissue cultures, the cytoplasm is of low refractive index and is translucent. In living cells there are no Golgi apparatuses, lysosomes, fibrils, filaments, trabeculae, reticula or ribosomes (Table 17).

The intracelluar movements would be accelerated by heat released as a consequence of metabolism, by pressure changes in the tissue due to changes in posture and muscle action, and by changes in the chemistry of the extracellular fluid, cytoplasm, nucleoplasm, mitochondrioplasm or pars amorpha; the chemical constitution of fluid compartments would change continuously as a consequence of transport, secretion and changes in permeability of the membranes between the relevant compartments.

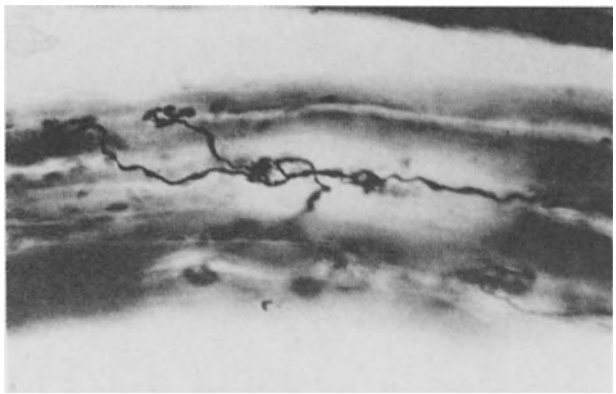


FIGURE 56 Neuromuscular junction of human being stained with Schofield's modification of Bielschowsky's silver stain. The bar is 100 μm. This figure is reproduced from Ralis, H.M., Beasley, R.A. and Ralis, Z.A. (1973) Techniques in Neurohistology (London: Butterworth), page 141, by kind permission of the authors and publishers. Please note the asymmetry of the entry of the nerve fibres and the rather few neuromuscular junctions

The questions have been raised as to what is the site of protein synthesis, if the ribosomes do not exist? Where do acid hydrolases occur, if not in the lysosomes? The answers to these two questions are the same. These activities occur in life in solution or in suspension in the cytoplasm. Where do the enzymes for oxidative phosphorylation sit, if not in lines along the cristae? The answer is in the mitochondrioplasm. Moreover, posing these raises several other pertinent questions. What is the site of protein synthesis, when ribosomes cannot be seen? Are 'free ribosomes' the name given to any granules, which must be presumed to be the sites of protein synthesis whenever ribosomes cannot be seen lining the endoplasmic reticulum? Similarly, where do acid hydrolases occur in those cells such as neurons which do not seem to contain lysosomes? Is there any evidence that the enzymes of oxidative phosphorylation do line the cristae, other than that they provide greater surface area (greater than there would be without them)? Does not the concept of 'pools' imply the existence of biochemical compartments which are not physical compartments? Why is the intracellular viscosity so low if the cytoplasm is full of 'endoskeleton' and their dependent granules? Finally, if prokaryotes can pack all their metabolism and genes in one compartment, why cannot eukaryotes do likewise?

It is universally agreed among all biologists that living cells contain hundreds to thousands of enzymes or enzyme activities. The subcellular biochemists believe in the existence of the following 13 compartments or phases: extracellular space; intramembrane space; cytoplasm; cisternae; lysosomes; mitochondrial intramembrane space; intramitochondrial space; intramembrane nuclear space; Golgi body space; ribosomes; nucleoplasm; nucleolonema; pars amorpha, of which we accept the existence of the six compartments or particles underlined. It is clear that our housing problem for the thousands of enzymes is serious, but the same problem is shared to a lesser extent by those who would multiply the number of compartments by 13/6, i.e. 2.2. Neither of us has anything like the problem of the prokaryotes, who have to pack all their biochemistry into one compartment. The biochemists cannot solve this problem because so far they have concentrated on finding out where the enzymes end up in the subcellular fractions in vitro, and not where they are located undisturbed in vivo (Hillman, 1972).

THE NUCLEAR MEMBRANE OF THE NEURONS

The nucleus is spherical or oval. It is surrounded by an imperforate membrane which is one layer thick. The chemical composition of the nuclear membrane is unknown, since it cannot be isolated and identified unequivocally in a fraction which has been demonstrated to contain no significant concentration of other cellular elements, or to have lost major components during preparation. Nevertheless, it is widely believed to have the same chemical composition as that proposed for the cell membrane by Davson and Danielli or Singer and Nicholson.

NUCLEAR PORES

Nuclear pores can be seen by electron microscopy (see, for example, Peters, Palay and Webster, 1976, page 55). We have concluded that they are artifacts and suggested

a number of ways in which they may arise (Hillman and Sartory, 1980, pages 68-75). At present, the best explanation for the appearance on electron micrographs of granules passing across the pores is as follows. Any particle which happens to be present on the nuclear membrane, either during the deposit of the heavy metal stain, or after microtomy, or at the time when the electrons bombard the membrane in the electron microscope, will either prevent the heavy metal stain being deposited on the nuclear membrane, or prevent access of the electrons to that part of the membrane. This will create the appearance of a pore in the membrane, especially if the particle explodes. The same explanation seems likely for apparent endocytosis and exocytosis at synapses.

It has also been pointed out elsewhere that the connections of the nuclear membrane to the endoplasmic reticulum are seen frequently in diagrams (Hillman and Sartory, 1980, pages 102-103), but rarely in electron micrographs.

NUCLEOPLASM

In tissue culture the nucleoplasm appears clear and translucent (Table 20) and contains the nucleolus. In stained tissues it appears as a granular uniform precipitate.

NUCLEOLI OF NEURONS

The nucleoli of unfixed neurons of rat, rabbit and guinea pig, can be seen by phase-contrast microscopy to be surrounded by a relatively thick membrane (Figures 3, 18 and 44) (Hussain, Hillman and Sartory, 1974). If cells are stained, the membrane can no longer be seen, because the contents of the nucleoli precipitate (Hillman, Hussain and Sartory, 1976). Nothing is known about the chemistry of the membrane, except that it can only be seen in unfixed cells if the neurons are isolated in saline, since sucrose also precipitates the contents of the nucleoli (op. cit.).

By light microscopy one can also see the nucleolonema, which is in a state of movement in unfixed cells (Figure 44), but stops after the addition of phenol (Sartory, Fasham and Hillman, 1971). Some of the shapes of the nucleolonema present in the nucleoli resemble rosettes, stars or umbrellas, as they move, change, and go in and out of focus continuously (Figure 57). Very little attention has been given in the literature to the nucleolonema, its movement and its structure, probably because neurons, even in cultures, are usually examined in fixed tissues, in which this movement does not occur. Furthermore, by electron microscopy, the nucleolus looks like a granule with little structure (Figures 32 and 33).

ASTROCYTES, OLIGODENDROCYTES AND MICROGLIA

A few classical views of these kinds of cells are shown (Figures 58-62), and the difficulties of the classification have been illustrated in Tables 2-12 and Appendices 1 and 2.

All light and electron microscopists have accepted the view of Golgi (1885) that astrocytes are characterized by their attachment to blood vessels. Del Rio Hortega (1928) was also of the view that they were all attached to blood vessels or pia mater. However, Penfield (1932, page 424) says that this is 'a sweeping statement to which we cannot at present subscribe'. Nevertheless, the view is widely held at present,

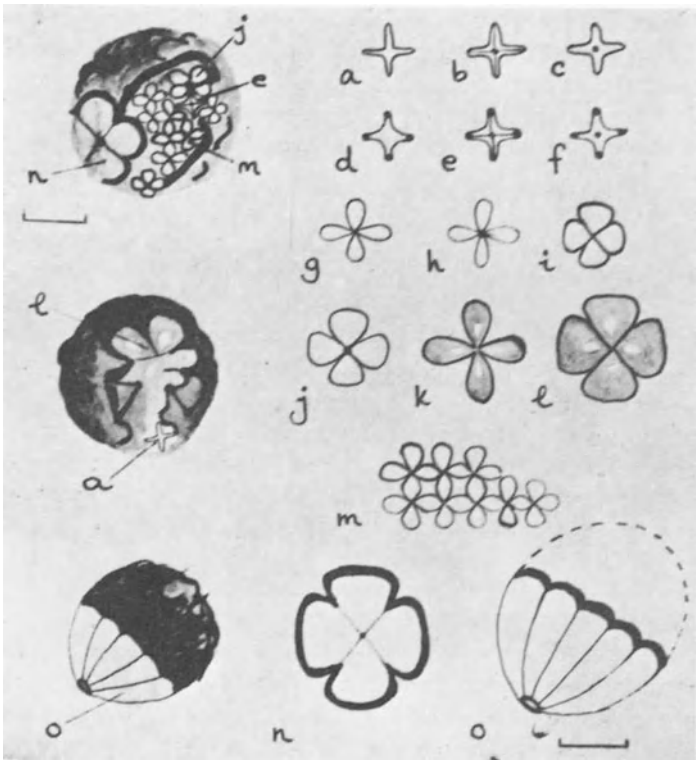


FIGURE 57 The fine structure of the nucleoli of rabbit neurons, from the Deiters' nucleus, as seen by phase-contrast microscopy. Various shapes could be detected in the nucleolonema which was changing continuously. The neurons were isolated in 0.25 mol/l sucrose, which does not permit one to see the nucleolar membrane clearly. The movements stopped on the addition of phenol. The same structures were also seen in neuronal nucleoli from rat, guinea pig and frog. The bar is 1 μm long. This illustration is from Sartory, P., Fasham, J. and Hillman, H. (1971) Microscopical observations of the nucleoli in unfixed rabbit Deiters' neurons. Microscopy, 32, 92-100. These drawings are reproduced by kind permission of the Quekett Microscopical Club

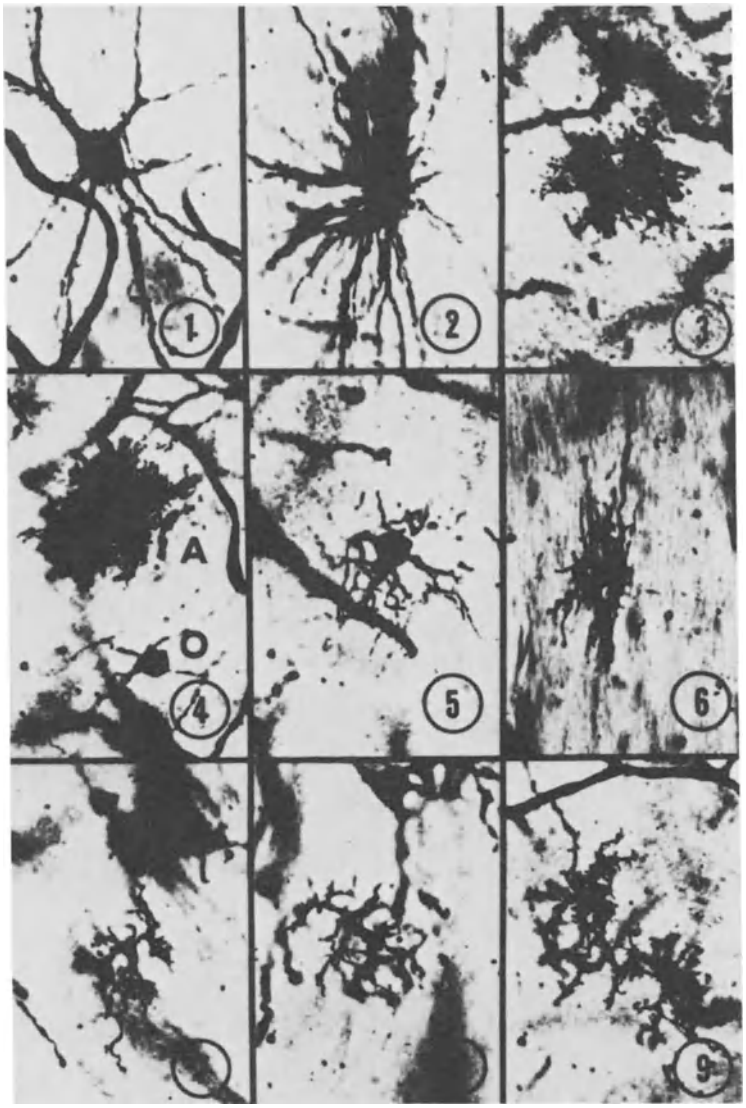


FIGURE 58 Different cells in the cat cortex stained by Rio del Hortega's modification of the Golgi method, x160: 1 neuron; 2 fibrous astrocyte; 3 protoplasmic astrocyte; 4 above, protoplasmic astrocyte, below, oligodendrocyte; 5 oligodendrocyte; 6 transitional oligodendrocyte; 7,8,9, microglial cells. These identifications are due to the author. This illustration is from Ramon-Moliner, E. (1968) The morphology of dendrites. In The Structure and Function of Nervous Tissue, ed. Bourne, C.H., Vol. 1, (New York: Academic Press), pages 205-267, and is reproduced by kind permission of the author and Academic Press. We regard 1-6 as being neurons (page 189)

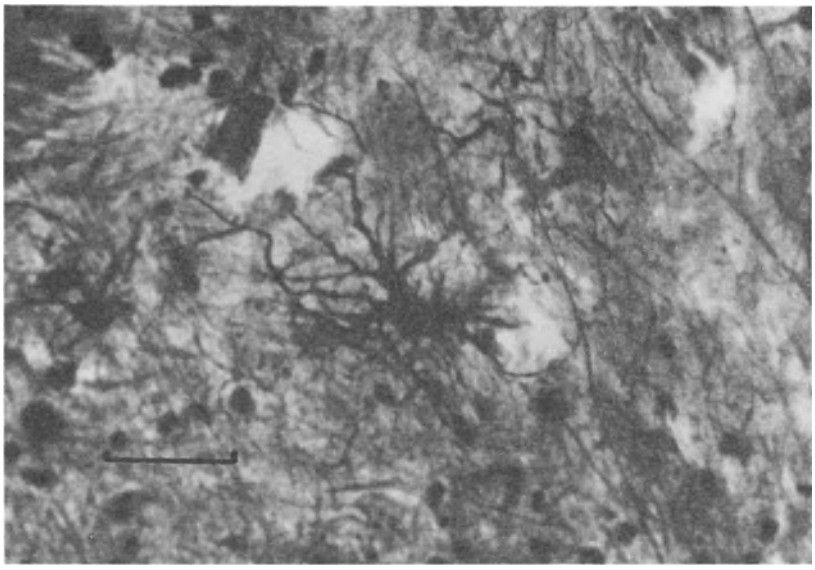


FIGURE 59 A human astrocyte from the cerebral cortex stained with Cajal's gold sublimate. The slide was kindly supplied by Dr C. Treip of Cambridge University. The large astrocyte is probably of the reactive type. The bar is 50 μm

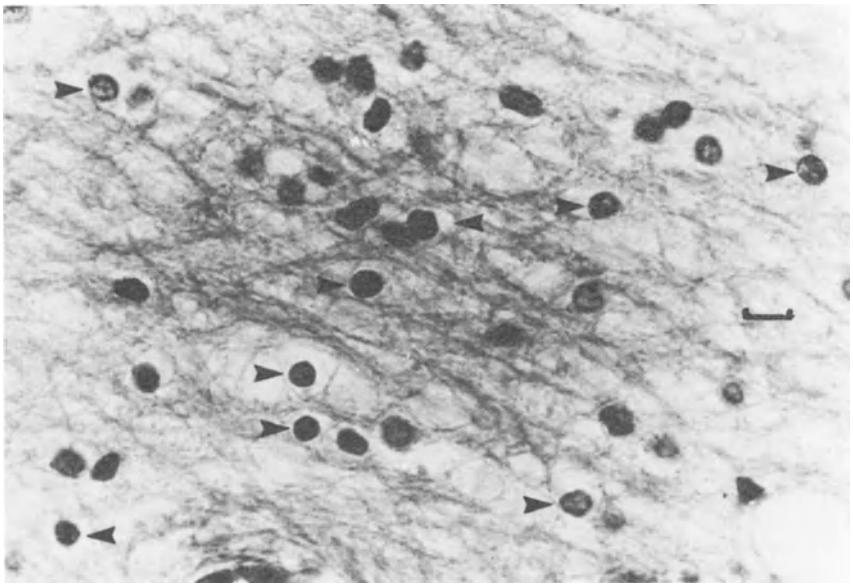


FIGURE 60 Cells which are identified by arrowheads as oligodendroglia in white matter. The original legend reads: 'They are not always readily distinguishable from astrocytes'. This section is stained with haematoxylin and eosin, and the bar is 10 μm long. From Lewis, A.J. (1976) Mechanisms of Neurological Disease (Boston: Little, Brown and Co.), page 44, by kind permission of the author and publishers

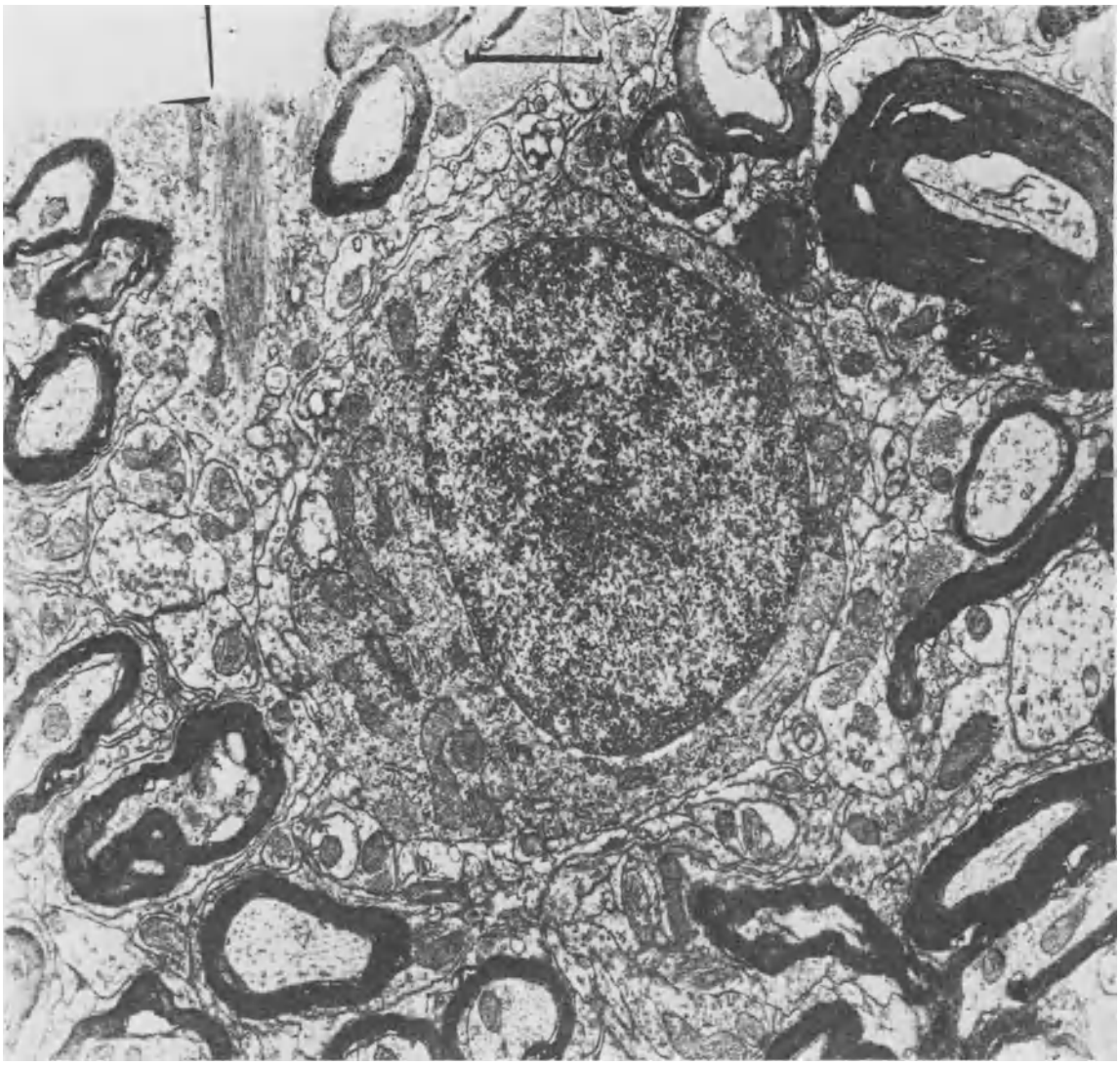


FIGURE 61 Electron micrograph of a cell from rat lateral geniculate body identified by Professor Paul Glees, who kindly provided it; the cell is believed to be an oligodendrocyte because of its elongate nucleus, dark cytoplasm and clear mitochondria. Please note that the cell membrane is visible all round the cytoplasm. The bar is 10 μm. We would classify this as a neuron, as it has a discernible membrane

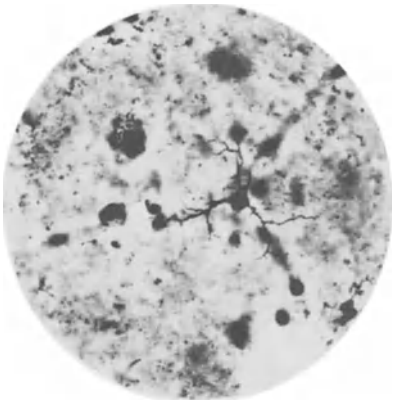


FIGURE 62 A microglial cell stained with Del Rio Hortega's stain. At this scale the diameter of the circle would be approximately 170 μm. Please note that the cell in the centre which the authors call a microglial cell has processes, and there are other spherical bodies, probably neuroglial nuclei without such processes, for example, between 1 o'clock and 2 o'clock. From Bouman, L. and Bok, S.T. (1932) Histopathology of The Central Nervous System (Utrecht: Oosthoek's Publishing Comp.), plate 2. We would classify this as a neuron.

both by light and by electron microscopists.

Golgi staining procedures require thick sections, sometimes as much as 150 μm (Sholl, 1953), so that one should be able to see a great deal more of the three-dimensional structure of cells than is possible with the usual thinner sections. The earlier electron microscopists cut 'thick' sections of 0.5-1 μm, and modern practice is to cut them 10-50 nm thick. Even the use of the thickest of these sections creates difficulties in identifying astrocytes by their 'feet'.

A micrograph is taken of a fibre ending on a capillary and its cell of origin is then dubbed an astrocyte. If such a foot is not seen, but it is believed that that particular cell is an astrocyte, other morphological criteria are used to identify it, or it is supposed that the particular foot would be seen in another serial section of the same cell.

As far as I am aware, no-one examining sections, say 100-150 μm thick by a Golgi-Cox procedure, has shown that processes of the cells which they identify as astrocytes are attached to cerebral capillaries and blood vessels at a greater incidence than are processes originating from neurons or oligodendrocytes. The alleged high incidence of relationship between astrocytic 'feet' and capillaries should not be accepted without comprehensive calculations being made, and several positive and statistically significant studies being carried out at several different neurobiology research centres. One would also have to show that capillaries are associated with astrocytic feet with a significantly greater incidence than they are associated with oligodendrocytes, microglia and neurons.

The other difficulty is that both the neurons and neuroglia with their processes - as well as the capillaries - are widely distributed in three dimensions in the central nervous system, so that it is very likely that many of them would appear to be in contact at low magnification, when virtually the whole thickness of the section is in focus.

The astrocytic 'feet' appear to electron microscopists as spaces between cells (Figure 63). The space stains lightly and contains relatively few particles. Close examination reveals that this 'foot' could not be an extension of a cell. It simply has no membrane around it. If one looks at this region on an electron micrograph at relatively low power electron microscopy, one sees the membrane of the cells adjacent to the foot as a single line. If one views it at higher power these cells are seen to have membranes appearing as the 'unit' membrane of Robertson. Unfortunately, the astrocytic foot itself completely lacks membrane appearing either as one or as two lines around it. It seems to 'share' the membrane of the adjacent cells. I would like to invite any electron microscopist to send me micrographs showing the expected adjacent membranes represented in our diagram (Figure 64). It has been argued that the membranes are only seen when they are almost normal to the plane of section, but this would not explain the absence of a membrane visible around the 'foot', when it is nearly always clearly present along most of its route around the adjacent cells, as well as, of course, around the nuclei and the mitochondria. The simplest explanation for the appearance of the 'foot' by electron microscopy, the sparsity of its contents, and its lack of personal membrane, is that it is due to shrinkage of two adjacent neurons, at the end of which there is a space containing a naked nucleus.

Astrocytes and oligodendrocytes are difficult to demonstrate by light microscopy in normal tissues, and some authors deny the existence of microglia. Clear light micrographs of astrocytes and oligodendrocytes - as opposed to diagrams - are hard to come across in the literature.

Usually when a nucleus is seen near an axon, the adjacent cell is called an oligodendrocyte. The rationale behind this is not at all clear. One may surmise that it is because most histologists regard any nucleus with little cytoplasm evident around it as an oligodendrocyte. In this monograph it is suggested that the nuclei of Schwann cells are the nuclei of adjacent connective tissue (Figure 65). In this location we would regard these oligodendrocytes as naked nuclei, and not neurons.

A few 'classical' views of these kinds of cells are shown (Figures 58-62), but a serious effort needs to be made to resolve the contradictions and uncertainties about the different kinds of allegedly different neuroglial cells exhibited in Tables 2-12 and Appendices 1 and 2. I would suggest that all the following cells with processes are neurons: astrocytes other than the reactive ones; oligodendrocytes and microglia, when they are described as having processes. Cells which have no processes and no cell membranes hard by are naked nuclei; reactive astrocytes; microglia; 'satellite' cells; oligodendrocytes, when they are described as having little cytoplasm. The latter group are probably identical with the original cells of Deiters' (1865), but they should not be confused with the neurons of the lateral vestibular nucleus of Deiters', which have been so extensively studied by Hyden and his school (Hyden, 1961, 1967).

THE SYNCYTIUM

This pervasive tissue consists of a fluid probably of similar composition to that of the cerebrospinal fluid, containing naked nuclei, mitochondria and fibres. It would be of

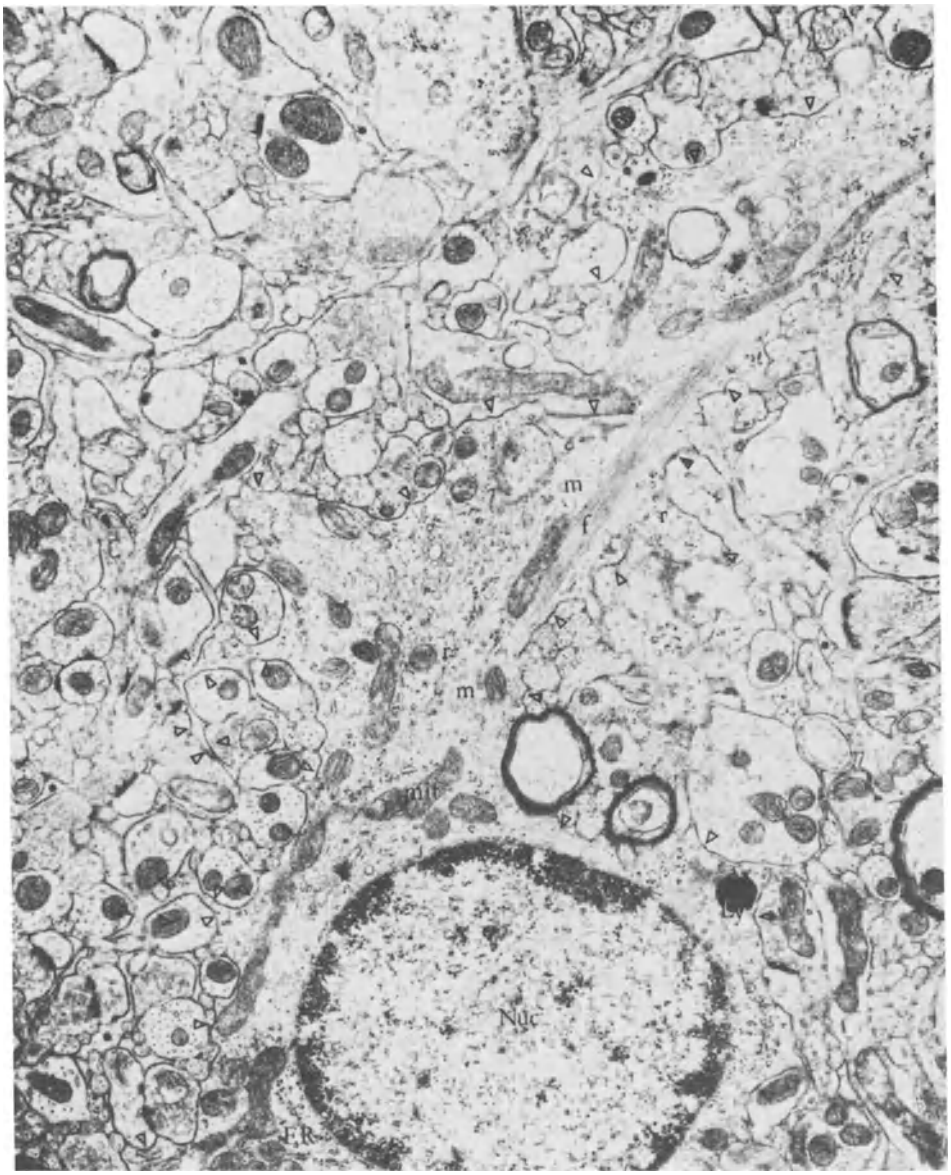


FIGURE 63 Electron micrograph of astrocytic foot from adult rat cerebral cortex. The original text reads: 'At the bottom of the field is the nucleus (Nuc) of a protoplasmic astrocyte. This shows condensations of karyoplasm beneath the nuclear envelope. Arising from the top of the cell body is a thick process that contains a bundle of filaments (f). Other organelles present in the cytoplasm include mitochondria (mit), microtubules (m), free ribosomes (r), rather short cisternae of the granular endoplasmic reticulum (ER), and two lysosomes (Ly). The outline of this astrocyte is very irregular and is partially picked out with triangles, which show that its contours follow those of the dendrites and axons in the surrounding neuropil. Sheet-like processes (asterisks) from neuroglial cells such as this one pervade the neuropil and are recognised by their irregular contours.' This figure is reproduced from Peters, A., Palay, S.L. and Webster, H. de F. (1976) The Fine Structure of the Nervous System (Philadelphia: W.B. Saunders Co.), page 237, by kind permission of the authors and publisher

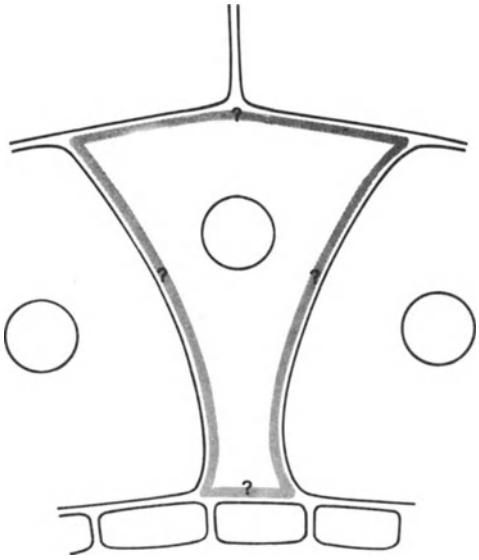


FIGURE 64 Diagram of an astrocytic 'foot' between four neurons, abutting on to a capillary as visualized by electron microscopy. The membrane around the neurons and capillary cells can be seen, but where is the membrane of the astrocyte itself(?) of which the foot is an outgrowth? The astrocytic foot should always be seen as having a membrane of its own, as well as those of the neurons between which it obtrudes. In electron micrographs the membranes from what are believed to be the neurons can be seen clearly along most of their courses, so that the suggestions that the absence of the astrocytic membrane can be explained by the orientation of section or by the lack of staining of oblique sections are not satisfactory for the usual absence of the membrane of the astrocytic foot.

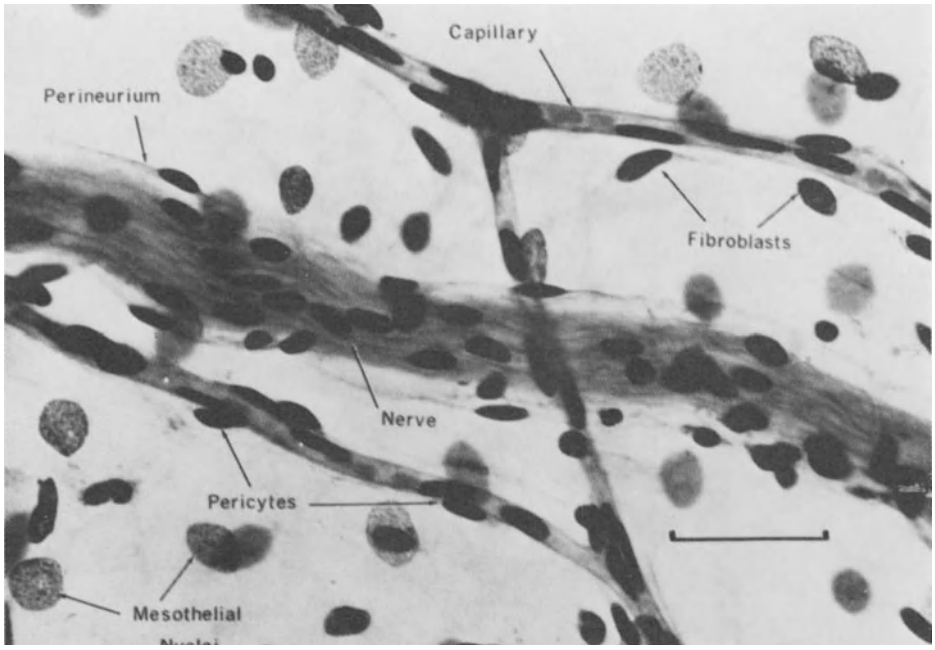


FIGURE 65 A whole mount of a small nerve in a thin spread of rat mesentery, showing the longitudinally orientated connective cells of the perineurium, and the nuclei of the Schwann cells in the interior of the nerve. May-Grunwald-Giemsa stain. The bar is 50 μm. This figure is reproduced from Bloom, W. and Fawcett, D.W. (1968) A Textbook of Histology, 9th edn (Philadelphia: Saunders), page 325, by kind permission of the authors and publisher. Please note the similarity of appearances and dimensions of fibroblast, pericyte and Schwann nuclei.

low viscosity were it not for the latter three components, which have the effect of stiffening it. When neuroglial clumps are examined in 0.25 mol/l sucrose by vertical illumination, they glisten white, and they show autofluorescence (Hillman, Hussain and Sartory, 1973). The syncytium or 'ground substance' is translucent to transmitted light. It should not be confused with the intracellular 'ground substance' of the electron microscopists (see, for example, Wolosewick and Porter, 1979).

The syncytium contains two kinds of fibres with diameters of less than 2-3 μm, whose lumens are not visible even under high-power light microscopy; there are bundles of fibres constituting tracts and commissures. We have reproduced a diagram produced by Bonin in Bailey and Von Bonin (1951), to show the richness of intracortical connections which had already been shown by physiological and histopathological techniques by that date (Figure 66). Of course, there are now known to be many more. There is also a rich network of apparently single fibres going in every direction. These show up well with Palmgren's staining procedure (Figure 38). In the literature, little attention has been paid to the latter fibres, and no-one has speculated about what they do. One can easily imagine that a particular such fibre would be very difficult to trace histologically, and it would be even more difficult to find the similar fibre again in another animal or post mortem human specimen. Nevertheless, it would probably be of value to try to envisage experiments which could find out what these ubiquitous straight single fibres can do

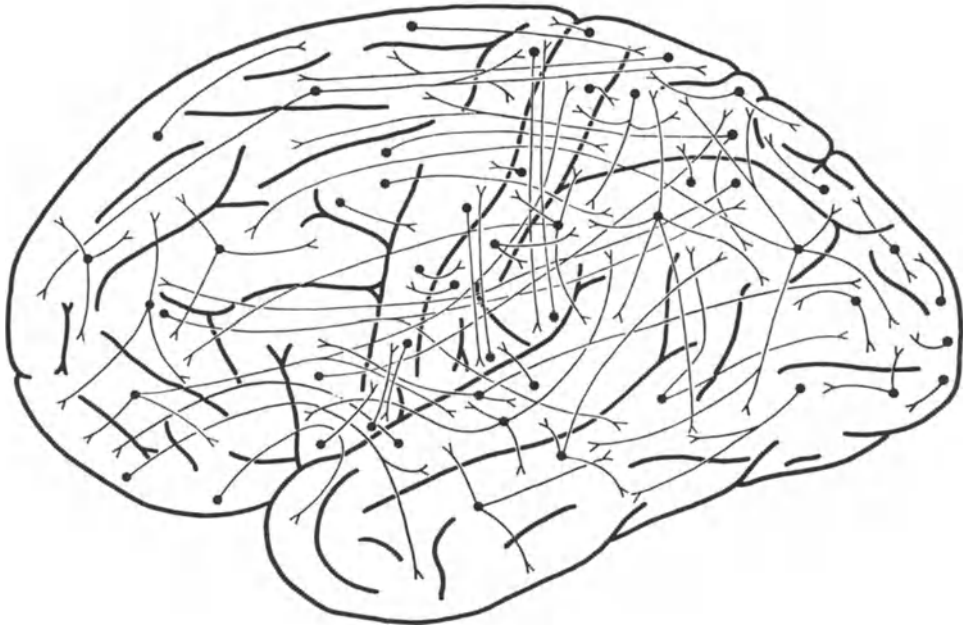


FIGURE 66 Cortico-cortical connections revealed by physiological neuronography in the chimpanzee (after Bonin). This diagram is reproduced by kind permission of the publishers, from Bailey, P. and von Bonin, G. (1951) The Isocortex of Man (Urbana: University of Illinois Press), page 239

physiologically. They do not appear to touch, to connect, or to synapse with, each other.

NAKED NUCLEI

The naked nuclei are ubiquitous in the central nervous system (Figures 25-36). Some can probably be identified as free nuclei in subcellular preparations (Figure 67). These nuclei are denoted as 'naked' here because they are devoid of cell membrane (although not of nuclear membranes); the term 'naked' here does not mean that they do not have cytoplasm, which we have concluded is the whole syncytium. Since one never sees nucleoli in these nuclei, it may be presumed that they do not possess them (Figures 60 and 62). Their nucleoplasm is granular after staining, but it seems very likely that in life it is a uniform fluid, and only becomes granular on dehydration.

In tissue culture active phagocytes can be seen and they are often identified as microglia. They are probably also naked nuclei.

WHITE MATTER

White matter can be seen in the cerebrum, the cerebellum and the spinal cord (Figures 38-40 and 68). The individual axons are of a thickness which permits them to be seen by light microscopy, especially when stained with silver salts. At low-power magnification, bundles of fibres can be seen in longitudinal section (Figure 40). In thick transverse sections of the spinal cord one can focus at high-power magnification through the depth of the section, and see that one is observing fibres which are truly longitudinal and three-dimensional.

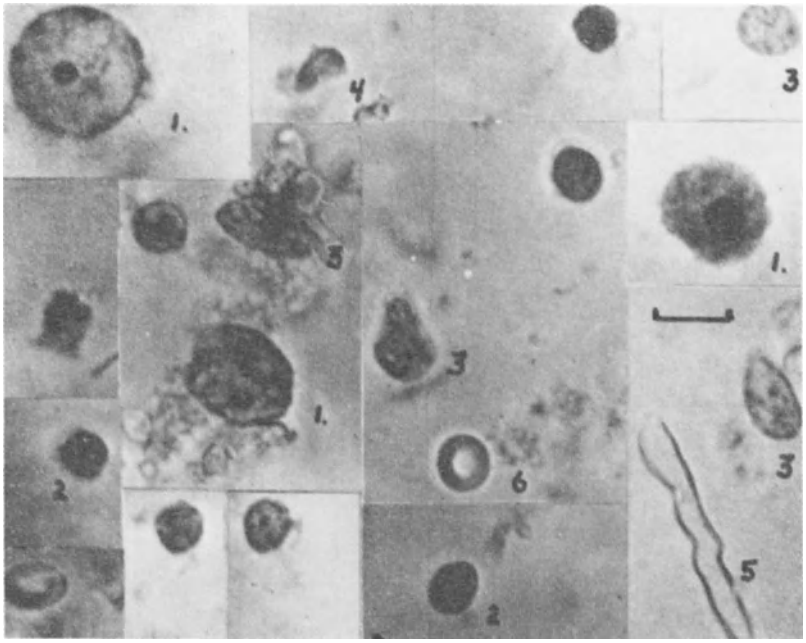


FIGURE 67 Light micrographs of free nuclei from rat brain liberated into 0.2 mol/l KCl by ball mill agitation. Nuclei are stained with the counting diluent. 0.1% methylene blue-chloride in buffered 0.2 mol/l KCl. 1, Nuclei of neurons from several neuronal types; note heavily stained prominent central nucleolus and pale chromatin network (except in folded and partly disintegrating nucleus at left centre). 2, Nuclei of glial elements, probably oligodendroglia; note dense, heavily stained chromatin mesh and dark multiple paracentral nucleoli; these nuclei are uniformly oval or round and are about the same size as an erythrocyte; in this medium they stain brilliantly and have an iridescent sheen. 3, Large oval to round pale nuclei with multiple paracentral nucleoli and diffuse chromatin mesh; these presumably derive from astrocytes. 4, Small, crescentic nucleus which may either be of microglial origin or from vascular endothelium; such nuclei are relatively common in cortical samples. 5, Myelin fragment. 6, Erythrocyte. All photo-micrographs on recorded Eastman ortho-press plates at a uniform magnification of x1,450. This figure is reproduced from Nurnberger, J.I. and Gordon, N.W. (1957) in Ultrastructure and Cellular Chemistry of Neural Tissue, ed. Waelsch, H. (London, Cassell), page 109, by kind permission of the authors and publisher. Please compare these nuclei with those of oligodendrocytes (Figure 60)

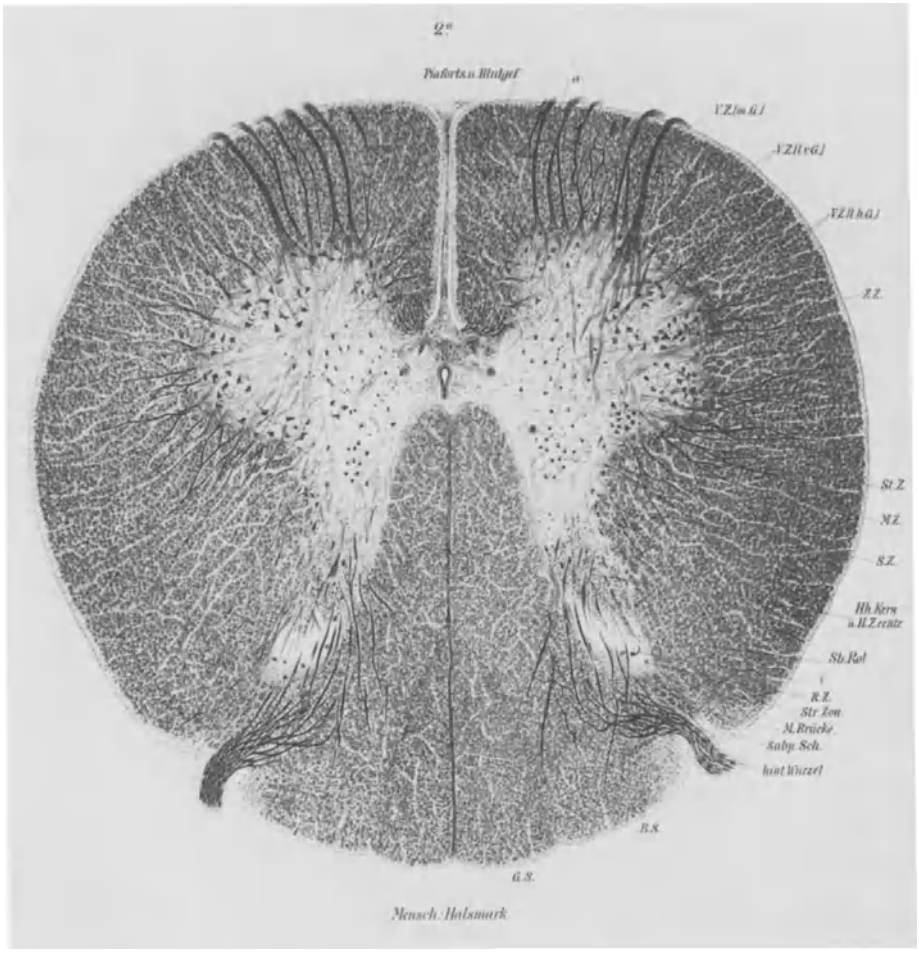


FIGURE 68 Drawing of a transverse section of a gorilla spinal cord, with the ventral surface above, and the dorsal surface below. Fibres can be seen in the ventral and dorsal horns by light microscopy, and their diameters are of the order of micrometres. From Waldeyer, H. (1888) Das Gorilla Ruckenmark. Akad. Deutsch. Wissenschaft, Phys. Abh. III, Table 13

It is generally held that, like peripheral nerves, the fibres of the white matter are myelinated, although they have neither nodes of Ranvier nor Schmidt-Lantermann clefts. The only evidence for their similarity is that they both appear as relatively thick concentric circles. It is hard to find electron micrographs in the literature of 'myelin lamellae' in white matter of the spinal cord, while they are always found in peripheral myelinated nerves (page 176).

GANGLION CELLS

Cells from dorsal and sympathetic ganglia are spherical or oval. They are best visualized by phase-contrast microscopy of hand isolated cells (Hillman, 1966;

Hillman and Khallawan, 1970). Under these conditions they can be seen to have extraordinarily few axons or dendrites, perhaps only one or two. The possibility that many dendrites are not seen, because they adhere to the cell body by surface tension after isolation, can be discounted, since one does not see such processes waving about when the cell bodies are manipulated in aqueous media - as, for example, cilia are observed in vitro on tracheal epithelia or on paramecia. The cytoplasm of the ganglion cells is granular, probably due to intracellular mitochondria. There are no end-feet or synapses apparent by light microscopy on the surfaces of the ganglion cells, despite the physiological evidence that synaptic activity as defined physiologically is a characteristic of some sympathetic ganglion cells.

The population of neurons in dorsal and sympathetic ganglia appears to be homogeneous, when viewed by light microscopy, either unfixed or stained (Figures 17, 18 and 37), although there appear to be approximately two intensities of staining. There is a fluid between the neurons, and the ganglion cells are surrounded by capsules - diamond-shaped in the case of the autonomic ganglia, and spherical in the case of the dorsal root ganglia. The capsules form a considerable proportion of the weight and volume of the whole ganglia.

EPENDYMAL CELLS AND ENDOTHELIAL CELLS

In the central nervous system the ventricles are lined by columnar or cubical ependymal cells (Figure 69). The capillaries are composed of the typical small narrow flattened endothelial cells lining capillaries elsewhere in the body, and no further description is necessary.

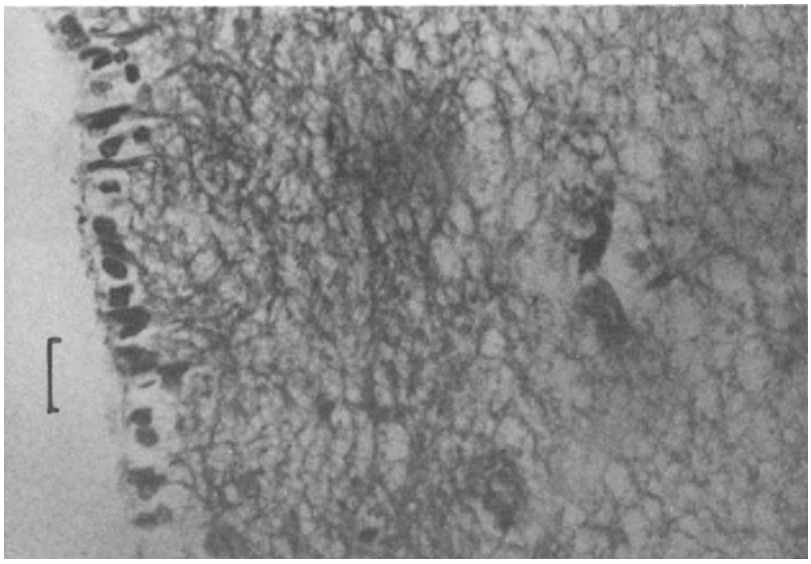


FIGURE 69 Anterior horn of lateral ventricle of rat stained with Holzer's stain showing the cubical to columnar ependymal cells, and syncytial fibres. The bar is 50 μm

MYELIN SHEATH

Much of the understanding of the structure of cell membrane is extrapolated from studies on myelinated nerve fibres into which the Geren model (1954) has been incorporated (Figure 70). Both the lamellar structure of the myelin sheath and the Geren model need careful examination. We will discuss the Schwann cell separately.

1. The myelin lamellae are virtually always seen perfectly transversely or as equally spaced parallel lines (Figure 71). Oblique views are hardly ever seen. If one looks at each of the illustrations in the references listed (Table 28), one can see many axons which have obviously been cut obliquely, since they appear oval instead of circular. Yet in none of these is there a significant incidence of oblique views of the individual lamellae. In general the width of the individual lamella as seen at the maximum diameter of the whole myelinated nerve should bear the same relationship to the width of the lamellae at its minimum diameter, as the whole nerve maximum and minimum diameters bear to each other. Examination of electron micrographs reveals a uniformity of spacing of the lamellae virtually independent of the orientation of the section.

This uniformity of spacing could occur if, for example, the nerve were compressed while it was being sectioned. However, these references were only chosen because in each of their illustrations nerves can be seen in several orientations. It would be inconceivable that compression, either during separation of the nerve or during section, would cause the myelin sheaths to be thinned in a random orientation relative to the source of compression, rather than clearly related to the direction from which it came. Of course, if an illustration contained only <u>one</u> myelinated nerve, compression could apparently cause the same spacing in the longer and shorter axis of the visible face of the section.

2. The lamellae show a constant spacing on longitudinal section. If one were to cut a Swiss roll (jelly roll) longitudinally through its maximum diameter, one would expect the layers to appear equally spaced. If one cut a longitudinal section <u>away</u> from the maximum diameter, one would expect the layers nearer the centre to appear further apart (Hillman and Sartory, 1980, page 37). Therefore, there are two geometrical constraints imposed on the myelin sheath as seen in longitudinal section. If the section has gone through the maximum diameter, firstly, the lamellae should appear equally spaced; secondly, the axon should be clearly visible; and thirdly, the sheath should be seen on <u>both</u> sides of the axon. These <u>three</u> geometrical imperatives rarely speak together.

If, on the other hand, one can <u>not</u> see the axon, it follows that the longitudinal section has <u>not</u> passed through the maximum diameter of the axon, and therefore the lamellae should again show splaying. It has proved impossible to find in the literature, or to coax from any electron microscopist, any micrograph showing the lamellae in <u>any spacing</u> other than equal in longitudinal or transverse section. In summary, one can say that the commonest expectations arising from geometry of longitudinal sections of real tubes are realized rarely by the electron microscopists, but, alas, more commonly by the graphic artists.

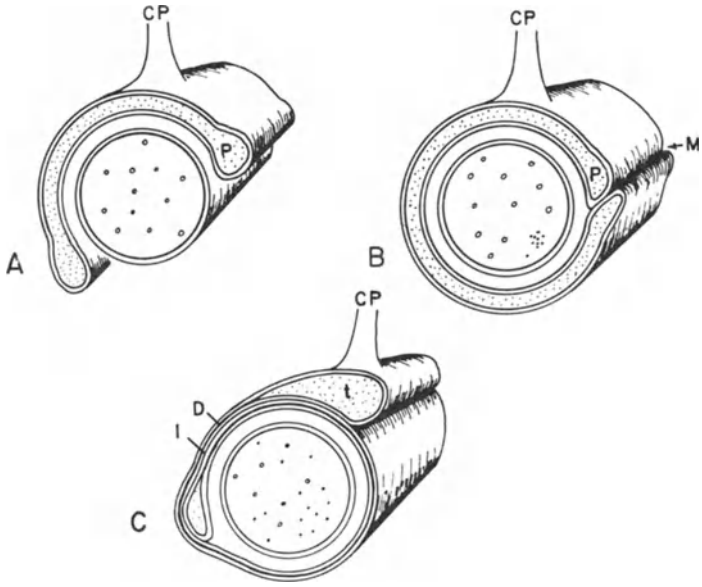


FIGURE 70 A diagram of how the Schwann cell is believed to ensheath the axon according to the Geren model. CP is the cell process, P is the myelin-forming process, M is the mesaxon, D is the major dense line, I is the intraperiod line, t is the external tongue process. This figure is reproduced by kind permission of the author and publishers, from Peters, A. (1968) The morphology of axons of the central nervous system. In The Structure and Function of Nervous Tissue, ed. Bourne, G.H., Vol. 1 (New York: Academic Press), page 170

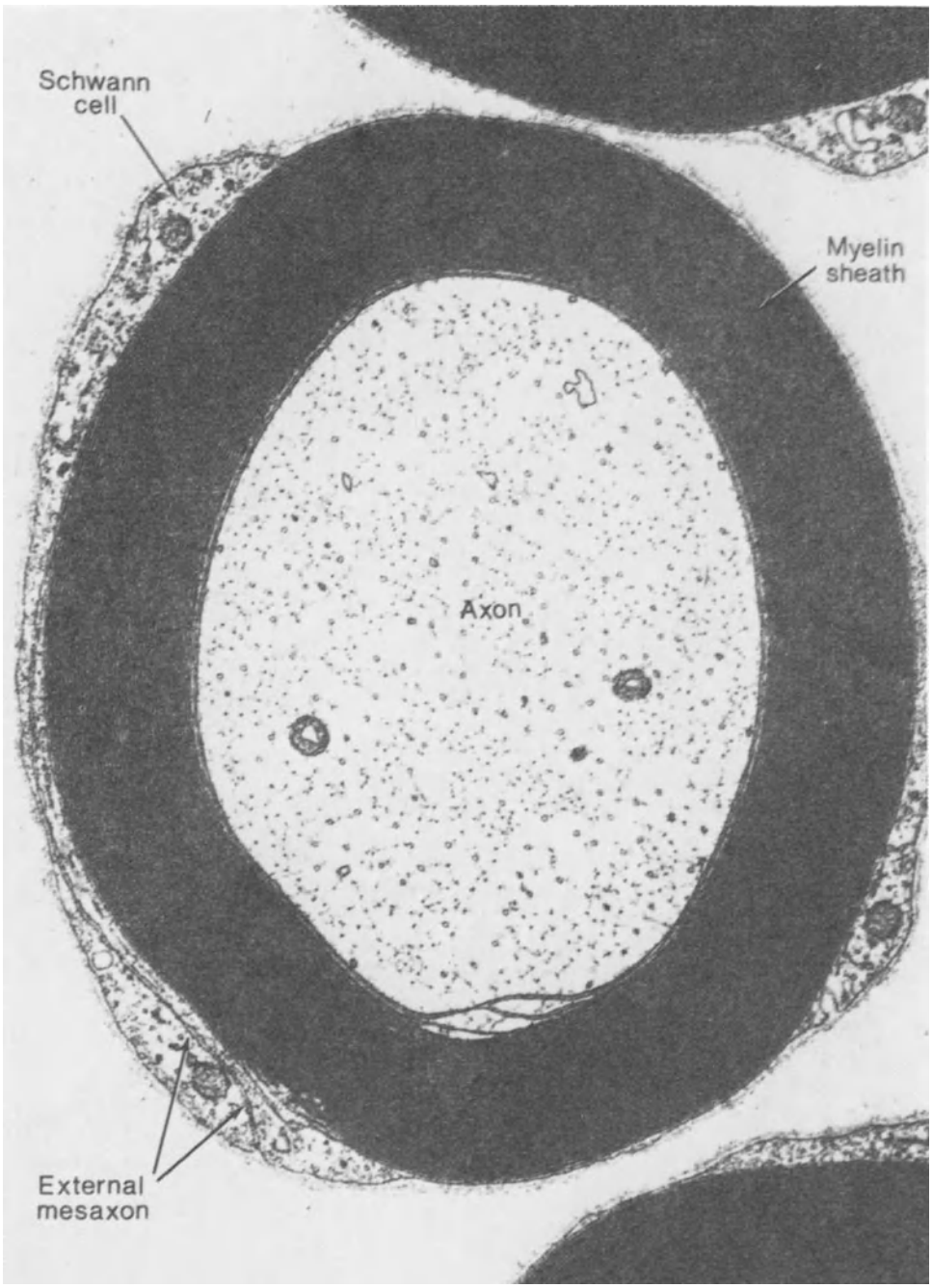


FIGURE 71 Electron micrograph of a transverse section of the cochlear nerve of a cat. This figure is reproduced by kind permission of the author, Mugnaini, E., and publishers in Fawcett, D.W. (1981) The Cell (Philadelphia: Saunders), page 31

TABLE 28 Publications showing myelin at several orientations in a single illustration, but in which the spacing of the lamellae do not appear to differ significantly in the various orientations seen in the individual illustration. Also there is no significant variation in spacing of lamellae either between several illustrations in the same publication, or between publications, although, obviously they would not have been sectioned all at the same orientations

Tissue	Authors
Cat skin nerve	Gasser (1958)
Rat spinal cord	De Robertis, Gerschenfeld and Wald (1960)
Rabbit peripheral nerve	Causey (1962)
Mouse sciatic nerve	Robertson (1962)
Rat sciatic nerve	Schade and Ford (1965)
Rat dorsal root ganglion	Novikoff (1967)
Monkey cerebellum	Glees and Meller (1968)
Rat sciatic nerve	Elfvin (1968)
Rat optic nerve	Peters (1968)
Rat optic nerve	Vaughn and Peters (1968)
Rat optic nerve	Hirano (1969)
Rat medullary pyramid	Curtis, Jacobson and Marcus (1972)
Rat sciatic nerve	Johnston and Roots (1972)
Calf cerebral cortex	Deutsch (1973)
Monkey lateral geniculate body	Glees (1973)
Guinea pig sciatic nerve	Bloom and Fawcett (1975)
Rat spinal cord	Rhodin (1975)
Baboon peripheral nerve	Landon and Hall (1976)
Baboon middle popliteal nerve	Ochoa (1976)
Cat medial gastrocnemius nerve	Aoki and Munoz-Martinez (1981)
Rat optic nerve	Leeson and Leeson (1981)

3. The lamellae appear close together by electron microscopy. Although each individual lamella is below the resolution of the light microscope, together the whole sheath should be thick enough to appear solid on light microscopy. Although the Schwann cell membrane can be seen or detected by light microscopy, the lamellae of which it is supposed to be composed - according to the generally accepted Geren view - cannot be seen in longitudinal or transverse sections of unstained peripheral nerves (Figures 19,41,73).

4. By high-power phase-contrast microscopy, Brownian movement can be seen in the cytoplasm of the Schwann cell. This is well illustrated in the beautiful time-lapse films of Costero and Pomerat (1951).

5. 'Myelin figures' are seen in normal kidney (Figure 72) and more frequently in liver. In a number of toxic conditions it has been shown by electron microscopy that the

number of 'myelin figures' increases in mammalian liver (Emmelot and Benedetti, 1960, 1961; Albot and Jezequel, 1962; Herman, Eber and Fitzgerald, 1962; Rouiller and Simon, 1962; Salomon, 1962; Steiner and Baglio, 1963; Mikata and Luse, 1964; Steiner, Mujai and Phillips, 1964; Stenger, 1964; Herdson and Kaltenbach, 1965; Campbell, 1967). It is hardly likely that there are occasional pieces of real myelin (as claimed to be present around peripheral axons) in the liver and kidney; secondly, it seems difficult to believe that toxic agents, such as dimethyl nitrosamine, hepatitis virus, ethionine, thioacetamide, naphthylisocyanate, N-2-fluoronyldiacetamide, carbon tetrachloride or thiohydantoin, would themselves cause myelination. Many toxins like lead, mercury and diphtheria cause demyelination. It is interesting that Emmelot and Benedetti (1961) talked of 'parallel-packed smooth membranes, resembling myelin sheaths'. Others have talked of 'myelin figures' or 'myelin-like figures'.

THE GEREN MODEL

6. The Geren model is illustrated in Figure 70. If the Schwann nucleus did wrap around the axon, as the Geren 'model' proposed, the nucleus would be expected to appear as if it were at the apex of the wrapping cell, i.e. in the middle of the angle subtended by it at the anti-node, rather than the way which it is drawn in diagrams of light and electron micrographs as the occupant of a concentric tube of Schwann cell cytoplasm parallel to the axon.

7. The Geren model shows the unfolding of the double-layered myelin sheath externally, which has been named the external mesaxon, and their parting at the internal mesaxon. The joining or separation of two double layers should, of course, make a four-layered membrane, which should thus be double the thickness on low-angle diffraction of the cell membrane. A four-layered mesaxon is simply not seen. One could postulate a mechanism whereby the two double membranes would 'fuse' together, but this would require very active proteolytic and lipolytic enzymes, which would also have the remarkable properties of knowing which two of the four membranes should be dissolved away, and when to stop before dissolving the other two. This problem would only be slightly simplified if our view that the membrane was of single thickness (page 133) were accepted.

8. Another requirement of the Geren model is that the Schwann cell cytoplasm is continuous between the two double-layered membranes investing the axon (Figure 70). Can any histologist, electron microscopist, or neurobiologist using tissue culture produce a convincing photograph of this?

9. In tissue cultures of myelinating fibres the movements of the swellings along the axon were observed (Figure 73); electron microscopy of myelinated axons showed lamellae around the axons. These findings led to the 'Geren model', which was a hypothesis that the bulges on the axon were the nuclei of Schwann cells, and that they rotated around the axon with their cytoplasm to produce the lamellae; implicitly, this would involve rotation around the axon in a transverse plane, since diagonal rotation would produce lamellae of characteristic but totally different orientation.

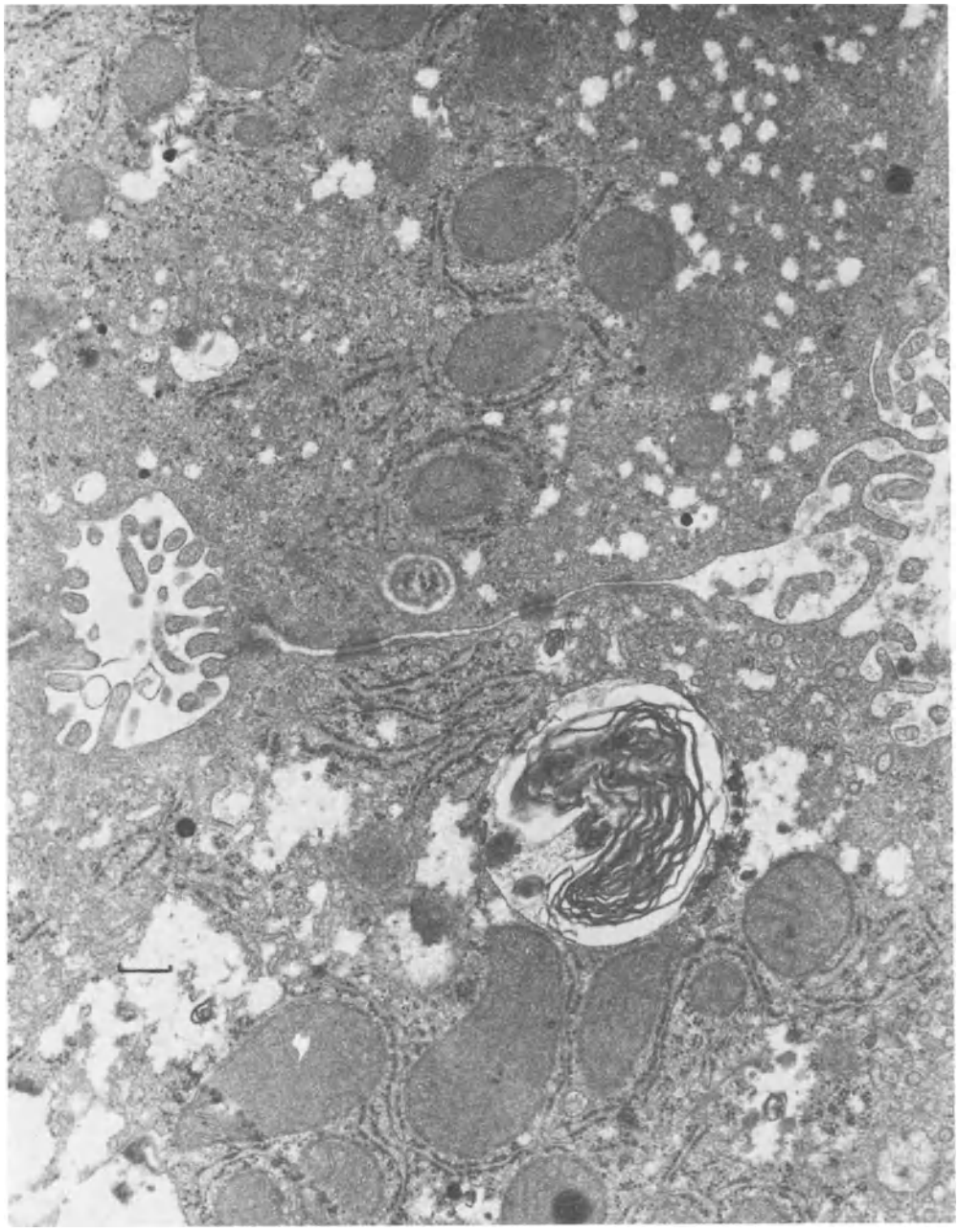


FIGURE 72 Electron micrograph of rabbit kidney fixed with glutaraldehyde and osmium tetroxide (2%), and stained with lead citrate and uranyl acetate. Please note the large 'myelin figure' in the centre. The bar is 1 μm

However, to my knowledge no-one has demonstrated the two main findings which would be required by, and would justify, the hypothesis; viz: firstly, by time-lapse photography of tissue cultures one should be able to take successive views of the nucleus rotating radially around the axon, not just appearing in different positions diagonal to each other; secondly, one should be able to make histological and electron microscopic preparations of successive stages of the invagination and rotation of the Schwann nucleus, including views of the membranes before they have fused together. Until these findings have been made positively, the 'Geren model'

must remain a hypothesis. I think that we have here a clear example of the trans-substantiation of a much-repeated hypothesis into a hallowed finding.

The explanation for the appearance of the lamellae is that they represent deposits of the intracellular fluid of the narrow tubular sheath around the axon, when it is dried out by subjection to the high vacuum and the bombardment by the electrons in the electron microscope. In longitudinal section the salty solution would dry out as equally spaced lines parallel to the walls of the sheath in which it was enclosed; in transverse section it would deposit as the fluid within a thin vertical tube as parallel concentric circles. This explanation is similar to that given for the appearance of the endo-skeleton in the axon.

It should be noted that although the electron microscopists claim that they are looking at membranes largely consisting of lipids and proteins, the thin sections have probably been prepared with at least one of the following agents, which can extract lipids: ethanol, glutaraldehyde, propylene oxide or xylene (Hopwood, 1969, 1970; Vanha-Pertulla and Grimley, 1970). In addition, water-soluble proteins must be extracted by all aqueous reagents.

The myelin sheath is usually regarded as the insulation of the axon. The staining of the sheath by such reagents as osmium salts and Sudan III makes it likely that the liquid within the sheath contains a high concentration of lipids and/or proteins, which would impart a high resistance to the sheath, and would render the antinodal region more resistant than the region of the node of Ranvier. Thus the simpler concept of the 'myelinated' nerve presented here also fits in with saltatory conduction (Stampfli, 1954).

If the lamellae are an artifact of preparation, they cannot represent a useful model for study of individual cell membranes.

It should be emphasized that, for the reasons presented above, we do not believe that the lamellae occur either in the living peripheral or central nervous systems. The cytoplasm appearing to be present in the Schwann cells is simply the cytoplasm of the connective tissue between the axons.

Thus the myelinated and unmyelinated nerves have a simpler structure than the electron microscopists ascribe to them. The myelinated nerve consists of two concentric tubes containing clear fluids in which intracellular movements occur in life; the tubes are interrupted by nodes of Ranvier and incised partially by Schmidt-Lantermann clefts. The unmyelinated nerves exhibit the elegance of a narrow capillary tube made of the finest bohemian glass.

Furthermore, it must be stressed that the chemistry of extracts of neural tissue does not necessarily provide any direct information about the structure of the cells in the intact living animals.

THE SCHWANN CELL

The Schwann cell represents an interesting problem. Schwann's (1838) original diagram shows what appears to be a nucleus lying on the outside of the axon (Figure 11). Of course, his drawing was made before photomicrographs had become a practical proposition. They had only been developed by J.B. Reade in 1836 (please see Henderson,

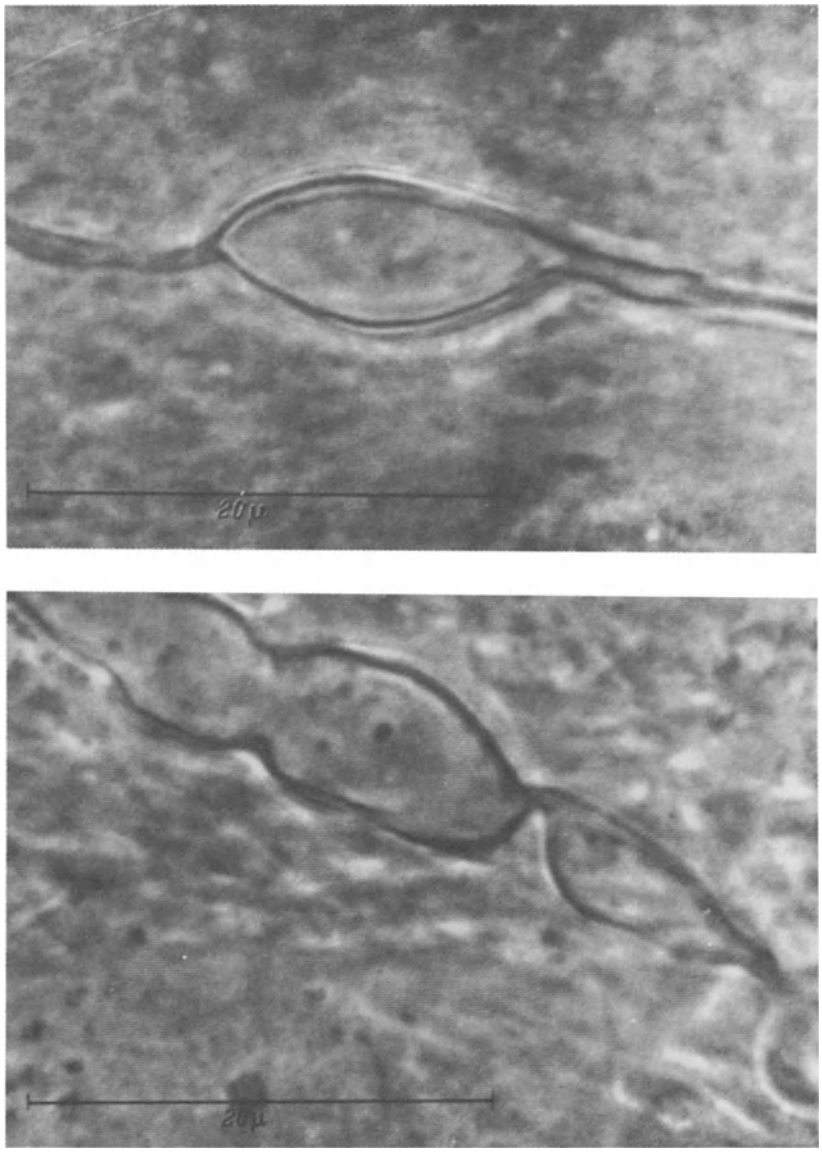


FIGURE 73 Living cultures of kitten cerebellum. (Upper) 43-day-old culture; single swelling with maximum thickness of 6.32 μm in nerve fibre of 1.3 μm average thickness. Phase-contrast (lower) 34-day-old culture; multiple irregularities in myelinated fibre. Phase-contrast from Hild (1959), Myelin formation in cultures of mammalian central nervous tissue. In The Biology of Myelin, ed. Korey, S.R. (London: Cassell), page 195, reproduced by kind permission of the author and publishers

1978) and demonstrated before an audience by John Dancer in 1840. However, the following authoritative works - including Causey's monograph entirely devoted to the Schwann cell - show diagrams of the Schwann cell, rather than photomicrographs (Ramon y Cajal, 1909; Schmitt, 1958; Causey, 1960; Wechsler, 1970; Peters, Palay and Webster, 1976; Landon, 1976; Sunderland, 1978). This curious lacuna in the literature should be a matter of concern. The diagrams in the works cited generally show the Swiss, or jelly roll, tube, surrounded by an apparently further tube containing a nucleus; the latter is believed to be the nucleus of the Schwann cell. In the rare

circumstance of the nuclei being seen by light microscopy, it may appear as:

1. A granule within the myelin sheath (Nageotte, 1932). Nageotte, like some of the previous authors he cited, described it as the 'Schwann cell syncytium'. In a comprehensive review of the literature, Sunderland (1978, page 14) gives the thickness of the myelin sheath as between 0.25 μm and 3 μm in various myelinated nerves. Thus, one would have to suppose that the diameters of the nuclei were of the same order as those values. These would make them hardly visible by light microscopy. In fact, if one views them carefully, they appear more as granules without clear membranes than as nuclei.

2. Another appearance by light microscopy, of which Bloom and Fawcett (1968, page 325) gave a beautiful illustration, is of nuclei lying on the surface of the axons (Figure 65). The cytoplasm around these nuclei is not evidently connecting them to the axon. Also, they are quite indistinguishable in appearance from the nuclei of capillaries or connective tissue.

3. The third appearance is well seen in the tissue culture of Hild (1957, 1959) reproduced here as Figure 73, as well as Pomerat, Hendleman, Raiborn and Massey (1967). These shots are taken from time-lapse motion films, similar to those of Costero and Pomerat (1951). They are particularly important, since they are regarded as being clear evidence for the Geren model, which has already been discussed. However, when one examines these pictures carefully, it becomes extremely doubtful whether they can be described as nuclei at all. Nuclei are characterized both in unfixed and stained cells of all species by having a different refractive index or staining more darkly in cells of all tissues. It seems quite inconceivable that they should appear transparent and structureless as they do in tissue culture. I would suggest that these 'Schwann cell nuclei' seen in culture are early swellings of the axoplasm, which subsequently enlarge and widen to give the classical appearance of the sheath.

Sometimes by electron microscopy large nuclei are seen adjacent to the lamellae, and these are called either Schwann cell nuclei or oligodendrocytes; the latter identification is based on their having similar appearances to those of cells called oligodendrocytes in the central nervous system, since they have scant cytoplasm (references in Table 28). It is noteworthy that the asymmetrical appearance of the nuclei in the Geren model, like the appearance of the mitochondria in the cytoplasm of the Schwann cell, is very rarely seen in electron micrographs.

One must then conclude: (1) that the granules seen sometimes by light microscopy within the antinodes are precipitates due to staining; (2) the larger nuclei on the surface of myelinated or unmyelinated axons by light or electron microscopy are the nuclei of connective tissue or endothelial cells outside the sheath; (3) the Schwann cells in tissue culture are concentric tubes originating from the axons (as was suggested by Hardesty in 1904); (4) the diagrams in the textbooks represent artists' views based upon a hypothesis stretching beyond the discipline of careful observation.

The real sheath probably contains high concentrations of lipids and proteins because it stains with osmium salts and Sudan III, although the transparency of the sheath and the Brownian movement within it make its contents liquid. We conclude that the Schwann cell nucleus is the nucleus of the connective tissue adjacent to the axon, and it may well be mobile (Figure 65).

8
Other Evidence Supporting these Conclusions

TISSUE BETWEEN NEURONS

The original observation of Virchow (1846) of a 'nerve glue' was widely accepted at the time. Bevan Lewis (1889) characterized the ground substance between the neurons as 'vastly preponderating over the cell and nuclear elements of the neuroglia'. Brierley (1957) quoted Bevan Lewis (1889), Obersteiner (1890), Taft and Ludlum (1929), Taft (1938) and Hogue (1946) describing it as a fine granular mass, and similar observations were made by Held (1909), Hess (1953) and Windle (1958). Brierley (1957) said that this ground substance was 'the main factor responsible for the blood brain barrier'.

Von Economo and Koskinas (1925) and von Economo alone (1929) stained human cerebral cortex probably with cresyl violet and toluidine blue (ibid., page 24). The latter author (1926) had calculated a grey matter-cell coefficient, which was the ratio of the mass of the grey matter of the cortex to the total cell mass. This turned out to be 20-27:1, giving the cells 4-5% of the weight of the grey matter. He suggested that their ratio could be used as an index of the stage of development of any brain, since higher animals had a higher proportion of cells. The cellular elements were found to be a small proportion of the tissue as we found (Tables 15 and 16). Of course, in both studies the proportion could have been depressed by the staining procedure not showing up all the elements, as Wyckoff and Young (1956) and Nauta and Feirtag (1979) among many others have asserted. Yet it is difficult to allege that some elements have <u>not</u> been stained without careful and comprehensive studies identifying <u>all</u> the cells, using independent 'specific' procedures, which have been demonstrated to show up <u>all of</u>, <u>and only</u>, the elements for which they are claimed to be specific.

Alzheimer (1910) and del Rio-Hortega (1919b) suggested that there were some amoeboid cells in the brain which could move, and they called them microglia. Movement of the cells in cerebral slices was seen directly by Dodgson (1948). He observed sections of mouse brain slightly stained with methylene blue; when he applied light pressure 'sluggish streaming movements', as well as Brownian movements of granules, occurred. His tissue was fresh, and he did not have to bathe it in any medium. Yet the movement <u>could</u> have resulted only from the damage when the slices were cut, or

from the pressure, and one cannot exclude the possibility of it being an artifact. He did not pursue these investigations (personal communication, 1978).

Cells migrate during the development of the central nervous system. Cells adjacent to the lumen of the neural tube divide first, and then migrate centrifugally through the tissue of the neural tube; this, therefore, could not be solid, and must be syncytial at that stage. Sauer (1935), like the many electron microscopists who have since denied the existence of a syncytium (please see Jacobson, 1978, page 32) nevertheless show the migration of single neuroepithelial germinal cells up columns of presumed cytoplasm, which are sometimes claimed to be attached to a basal membrane. However, these columnar cells stand like grenadier guardsman in sentry boxes with spaces containing no nuclei between them. Must not these spaces be dubbed as syncytial? It is extremely difficult to conceive of a column of cells fleeing centrifugally away from the lumen of the neural tube if the tube itself was solid with cells; there would have to be either a counterflow of cytoplasm, or radial growth of the whole neural tube at quite an explosive rate.

At a later stage of development, cells in the spinal cord, cerebellum and isocortex have been shown to migrate from the ependyma over a period of a few days (Figure 74). One cannot avoid the conclusion that at this stage, also, they must be migrating through a syncytium. Nevertheless during the whole of this period the cortex appears largely solid by electron microscopy (Voeller, Pappas and Purpura, 1963; Hassler and Stephan, 1966; Aghajanian and Bloom, 1967; Gruner and Zahnd, 1967; Meller, Briepohl and Glees, 1968a,b).

The existence of a syncytium in the brain during development is not, in itself, proof that it persists into adulthood. However, there are two other lines of evidence for this belief. Smart and Leblond (1961), using autoradiography, showed that new neuroglial cells could be formed in the adult brain. Microglia may surround injured brain as a result of proliferation of cells adjacent to the wound (Maxwell and Kruger, 1965; Cammermeyer, 1970; Vaughn, Hinds and Skoff, 1970; Stenwig, 1972; Korr, 1980). Or they may originate from leucocytes in the blood which escape into the region of injury (Konigsmark and Sidman, 1963; Oehmichen, Gruninger, Saebich and Narita, 1973; Matthews, 1974; Oehmichen, 1978). Whatever their origin, microglia or phagocytes - with apparent nuclear diameters of 5-20 μm in the stained preparations, and, therefore, larger in vivo - migrate in considerable numbers around the site of injury, inflammation or infection, starting as early as 12 hours after (Del Rio Hortega, 1932, pages 520-521, quotes 29 references). These movements are comparatively rapid, of the same order as occurs in 'axonal flow'. It seems extremely unlikely that the cells or nuclei could move at such a rate if, indeed, the central nervous system was composed of a testudinous block of cells with an extracellular space of between 4% and 25%; this would give linear extracellular spaces of approximately 1-10%. Since the diameter of the nuclei is much larger than 1-10% of the maximum diameter of the neuronal somas, it could not be supposed that microglia or phagocytes could interpolate themselves between what are considered to be the packed neurons and neuroglia. Furthermore, if they were, they should be caught in the act at least occasionally by zealous microphotographers.

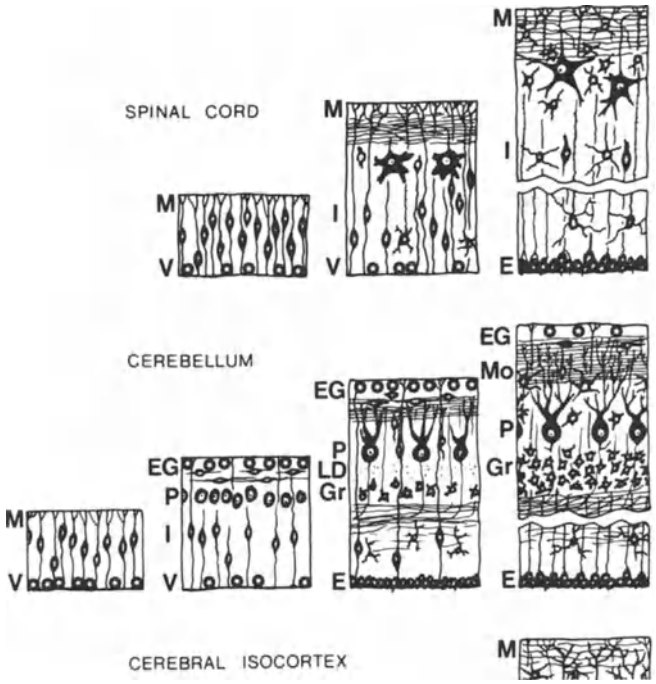


FIGURE 74 Germinal and other cellular zones and layers in the developing cerebral isocortex, spinal cord and cerebellum of mammals. Progressively later stages of development are shown from left to right. CP, Cortical plate; E, ependymal layer; EG, external granule layer; Gr, granule layer; I, intermediate zone; LD, lamina dissecans; M, marginal zone; Mo, molecular layer; P, Purkinje cell layer; S, subventricular zone (also called subependymal zone); V, ventricular germinal zone. Mitosis can sometimes be seen. This figure is reproduced from Jacobson, M. (1978) Developmental Neurobiology, 2nd edn, (New York: Plenum Press), page 62, by kind permission of the author and publishers. Note the cell migration.

In a similar fashion, many large molecules like inulin, serum proteins, ferritin and horseradish peroxidase enter the parenchyma after injection from the ventricles, the choroid plexus or the subarachnoid space (Klestadt, 1915; Bowsher, 1957; Lee and Olszewski, 1960; Pappenheimer, Hersey, Jordan and Downer, 1962; Klatzo, Miquel, Ferris, Prockop and Smith, 1964; Rall, 1964; Brightman, 1965a,b; Kitai and Bishop, 1981). It is quite possible that the damage due to the manipulation of the brain or pressure on it adds to the spread of these large molecules, or their micelles, but there is too much evidence of such 'pinocytosis' in the central nervous system, to explain away all the findings describing the passage of these large particles. Obviously, the movement of particles, cells or nuclei implies that the resistance to them is low.

Conel (1939-1963) made a monumental six-volume study of the human cerebral cortex from babies of 1 month to 2 years old, and we have selected drawings from the two extremes of age, which illustrate the following points (Figure 51). With increasing age, there is an increase in (a) the number of neurons, (b) their density, (c) their arborization, and (d) the overlap of these fibres. He did not show any microglia or naked nuclei. Dendrites are generally believed to connect with the hundreds to thousands of synapses which most authors believe inhabit the surfaces of neurons (Tables 22 and 23). In the presence of such a mass of processes radiating

from the soma in all directions, it is difficult to imagine how the same space of the neuropile could also contain neuroglial cells of the form shown (Figure 50; see also Conel, ibid.; Kitai and Bishop, 1981). The only circumstance in which they could coexist as close neighbours to each other would be if the neuroglia contained holes like an Emmentaler cheese, through which the processes of the somas could pass. I do not think that anyone has described this caseous appearance, nor have I seen it myself in histological sections or in micrographs.

RESISTANCE AND IMPEDANCE OF BRAIN AND SPINAL CORD

Many measurements have been made of the resistance and impedance of cerebrospinal fluid, cerebral cortex, white matter and spinal cord, of rabbits and cats (Crile, Hosmer and Rowland, 1922; Freygang and Landau, 1955; Ranck, 1963a,b; van Harreveld, Murphy and Nobel, 1963; Li, Bak and Parker, 1968). These authors found values in the range 100-1200 Ωcm for either of these parameters. However, the resistance of the membranes of individual excitable cells measured using intracellular microelectrodes was 4-43 MΩ (Coombs, Eccles and Fatt, 1955; Frank and Fuortes, 1956; Lux and Pollen, 1966; Nelson and Lux, 1970; Li, Okujava and Bak, 1971). Neuroglial cells in tissue culture (Hild and Tasaki, 1962) were calculated to have resistance of 2 MΩ when the processes were taken into account (Li, Okujava and Bak, 1971). In reviewing these measurements it is quite clear that the values of resistance and impedance of excitable cells, and also cells identified by the authors as neuroglia, were very high relative to the values for the tissues as a whole, and it was concluded that 'the low impedance of gray matter and of white matter measured in the fibre direction is consonant with the presence of an appreciable extracellular space in central nervous tissue, and can be reconciled only with difficulty with the paucity of extracellular space shown in many investigations of the tissue with the electron microscope' (Van Harreveld, 1966, page 69). Thus, all the findings agree that tissue impedance is low. It is usually assumed that this is due to shunting in the extracellular space, but it also fits in well with the high-resistance and impedance neurons being surrounded by a low-resistance syncytium.

PROPORTION OF BRAIN IN WHICH ABRUPT DIRECT CURRENT POTENTIALS CAN BE RECORDED

When an intracellular electrode penetrates a living tissue the sudden change in direct current voltage which is recorded is normally interpreted as penetration of the membrane of a cell. If the central nervous tissue is then stimulated by electric current, transmitters, hypoxia, or potassium ions, and an action potential results, this is taken to mean that the cell was a neuron; if not, the cell penetrated is believed to be a neuroglial cell.

All living cells exhibit transmembrane potential differences so that a measurement of the proportion of the distance in which an electrode tip, say, less than 1 μm in diameter, records a voltage compared with the total distance which the electrode travels gives one an assessment of the closeness of the packing of the cells in the tissue; the measurements are not precise, because the tissues are elastic and the electrodes may bend. Very often the electrode does not record the maximum voltage

across the cell membrane, because the larger the diameter of the tip of the electrode relative to that of the soma, the smaller the voltage it records; also, the electrode may become blocked (Adrian, 1956), or it may penetrate the soma or its processes obliquely (Woodbury, Hecht and Christopherson, 1951; Frank and Fuortes, 1955; Haapanen, Kolmodin and Skoglund, 1958). However, if one simply adds up total distances during which penetrations record voltages of any value, one derives a measure of cell density. This proportion was only 22% in cerebral slices in vitro (Figure 75; Hillman, Campbell and McIlwain, 1963). Even allowing for a 15-50% increase in volume of cerebral slices after incubation in vitro, it is evident that only a minority of the tissue consists of cells with membranes. In muscle, the proportion of distance of travel of the electrode without recording a voltage is much smaller, probably 5-10% (H. Hillman, 1962, unpublished).

VISCOSITY OF THE BRAIN AND SPINAL CORD

Anyone who has operated on, or assisted in an operation on, the brain or spinal cord of a living mammal, has the experience that the living tissues are extremely soft compared with other solid organs, such as the liver or muscle. This, of course, is an anecdotal observation, which one would like to have made more scientifically respectable by measuring such parameters as viscosity, compressibility, impedance to sound, or elasticity. Unfortunately the tissue is heterogeneous, and measurements one would make would be likely to be operational. Measurements on dead tissue, or tissue suffering from ischaemia, acidosis, autolysis, cooling or substrate deprivation would probably not be wholly relevant.

ASTROCYTES AND OLIGODENDROCYTES

Many neurohistologists, including Purkinje, Deiters, Hyden, Giacobini, Pavlin, Hertz and Hamberger, have dissected out single neurons by hand, but none of them have ever claimed to separate single astrocytes or oligodendrocytes, either deliberately or by accident. Hyden and Pigon (1960) reported that the neuroglial clumps mainly consisted of oligodendrocytes, with some astrocytes adjacent to blood vessels. Hamberger (1963) said that his neuronal glia were 90% oligodendrocytes and 10% astrocytes, and capillary glia were 70% oligodendrocytes and 30% astrocytes, but nevertheless these cell types were never isolated as individual bodies from the neuroglial clumps. Of course, the fact of not having found particular evidence is not as cogent as positive evidence; this is especially true when it is not clear if the research workers mentioned actively sought to isolate astrocytes and oligodendrocytes, and failed, or if they did not try to do so. I, myself, have never had difficulty in isolating the characteristically shaped 'neurons', and I could also point to the naked nucleus in a neuroglial clump. Following Cummins and Hyden (1962), I could dissect out the nucleus from a neuron, but I, also, have never seen any other recognizable shape of a whole cell in a clump.

Throughout the literature on cell types in the nervous system, there are references to 'nerve glue', 'nutritive' cells, 'satellite' cells, and 'supporting' cells. These terms indicate both ignorance about what they do, and an albeit subconscious feeling that they represent a less worthy class of cell in the architectonic pantheon.

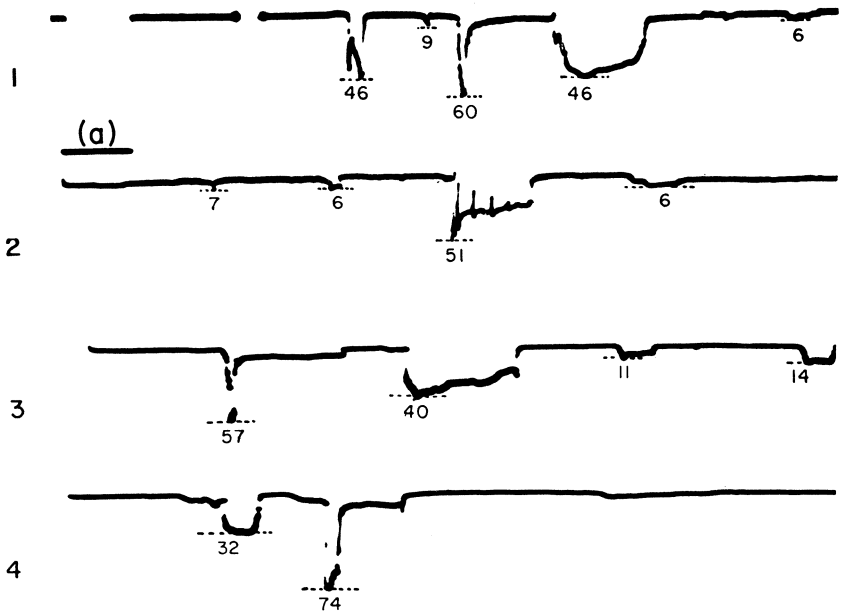


FIGURE 75 Four photographs of typical changes in potential differences during penetrations of 310 μm, from the surface into slices of guinea pig cerebral cortex in vitro. At (a) the vertical displacement is 100 mV and the horizontal displacement 0.5 ms. In cerebral slices whose volume increases about 25% on incubation, the tipss of the electrodes recorded a voltage for only a mean of 28% of their total excursions. As can be seen, many of the voltages recorded were very low, indicating very few cellular elements with large voltages. This recording is reproduced from Hillman, H., Campbell, W.J. and McIlwain, H. (1963) Membrane potentials in isolated and electrically stimulated mammalian cerebral cortex slices. J. Neurochem., 10, 325-339, by kind permission of Pergamon Press

This may not be a trivial matter, because it preconditions the belief that the total volume of the brain is largely occupied by the 'more important' neurons. This seemingly semantic difficulty is compounded by the fact that 'satellite' cells and microglia being apparently smaller than neurons would be much more difficult to penetrate with an intracellular electrode to study their membrane properties, for example by iontophoresis. In general the electrophysiology of smaller cells is more difficult to study, and so less is known about them in the central nervous system.

9
Summary of Conclusions about Nervous Tissue in *Living* Mammals

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1. The central nervous system contains neurons, naked nuclei, fibres, capillaries, ependymal cells and a syncytium, which is the largest component by volume.
2. The syncytium is a viscous jelly, in which naked nuclei move slowly and mitochondria move rapidly. The mechanical, physical, biochemical and pharmacological properties of the central nervous system are dominated by the properties of the syncytial ground substance.
3. All cells with processes are neurons. The only other cells are the naked nuclei, the ependyma and the capillary endothelium.
4. Cells in the central nervous system are not in contact with each other, even when their processes are close in rare locations like the Purkinje and the basket cells of the cerebellum.
5. Neuron somas are almost spherical in shape, and are invested in solid membranes. All the membranes are one layer thick. (Synaptic knobs do not exist in life, although the physiological properties generally attributed to synapses are real. Synaptic vesicles do not exist.) Each neuron has probably only one axon, and usually very few primary dendrites (Figure 76).
6. The cytoplasm of the neurons is a fluid of low viscosity, in which mitochondria and a few other unidentifiable particles move continuously. (The Golgi body, the endoplasmic reticulum, the lysosomes, the tubules, fibrils, filaments and trabeculae do not exist in living cells.)
7. The nuclear membranes of neurons and naked nuclei are imperforate.
8. The mitochondria both in the neurons and in the syncytium contain a clear fluid, the mitochondrioplasm. (The cristae do not exist in life.)
9. The nucleoli of neurons are surrounded by single-layered membranes. They contain a skein-like nucleolonema.
10. Naked nuclei are spherical and contain a uniform translucent liquid nucleoplasm; they do not have nucleoli.
11. Ganglia contain only spherical or oval neurons; synaptic knobs are not seen there.
12. Unmyelinated axons and nerve fibres are tubes. The axoplasm is a clear fluid of low viscosity. (It does not contain an endoplasmic reticulum, neurotubules, neurofibrils, neurofilaments or microtrabeculae.)

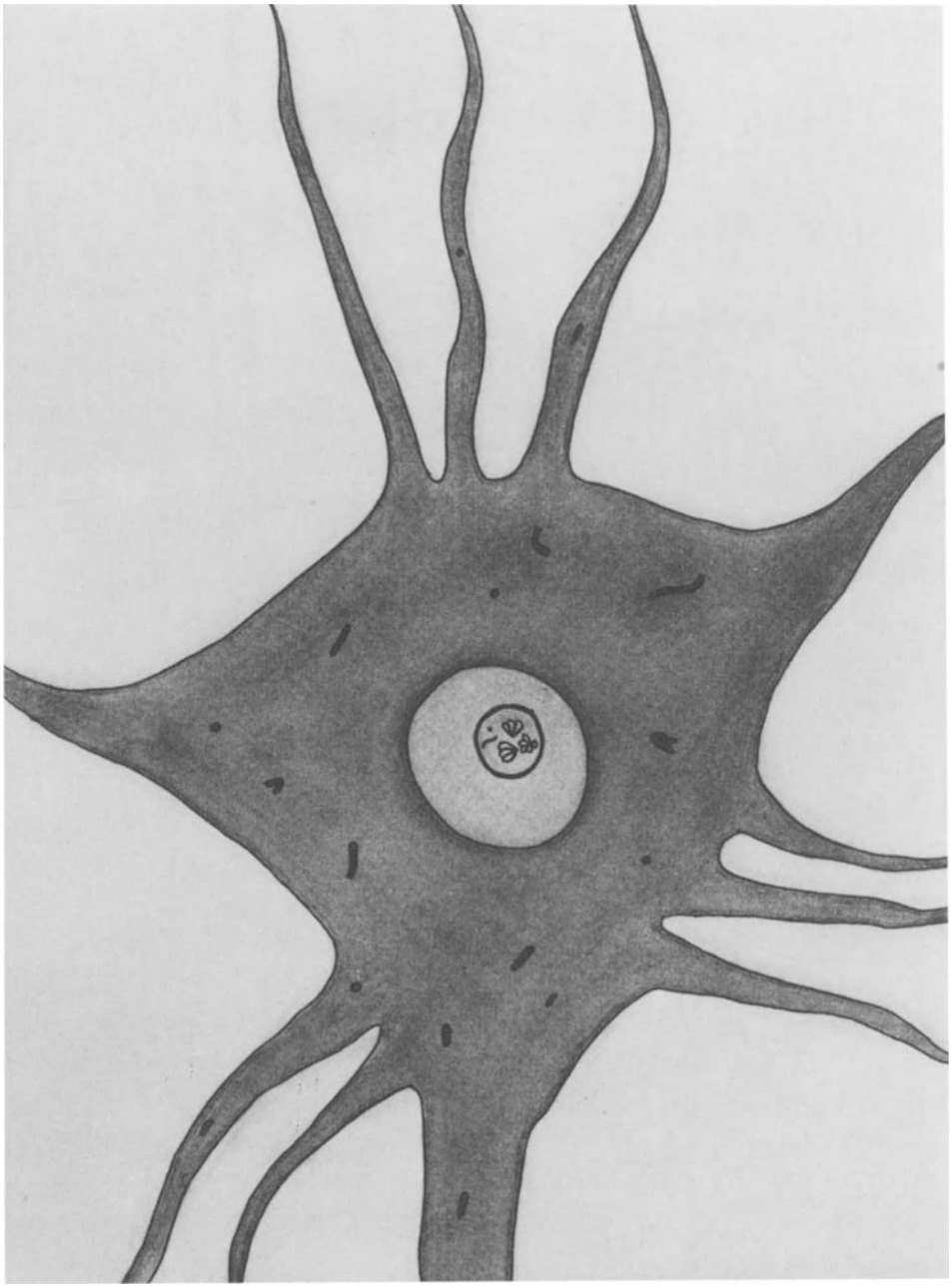


FIGURE 76 A diagram of a high-power view of a live neuron. Please note the absence of an endoplasmic reticulum, Golgi bodies, synapses, nuclear pores, endoskeleton or mitochondrial cristae. The cytoplasm, the nucleoplasm and the mitochondrioplasm are of low viscosity and each is uniform. Please compare with Figure 44

13. The myelin sheaths around peripheral axons are concentric tubes containing a viscous fluid. Nodes of Ranvier and Schmidt-Lantermann clefts are spaced along its length. (The Schwann cell does not exist.) The Schwann nucleus is the nucleus of a cell in the adjacent connective tissue. (The myelin lamellae do not exist in life.)
14. The fibres of the tracts in the white matter of the spinal cord are smaller in diameter than those in peripheral myelinated nerves.
15. Transmission is mainly electrical, but the excitability of the cell membranes can be conditioned by the chemical nature of the syncytium, including the presence and interactions of substances believed to be transmitters.
16. The syncytium and the choroid plexus are the blood-brain barrier.
17. The Geren model does not describe the events during the myelination of the axon.
18. Axoplasmic flow is a combination of diffusion, Brownian movement, streaming, laminar flow and convection.

Our views on the cellular structure of the brain are summarized in Figures 77 and 78.

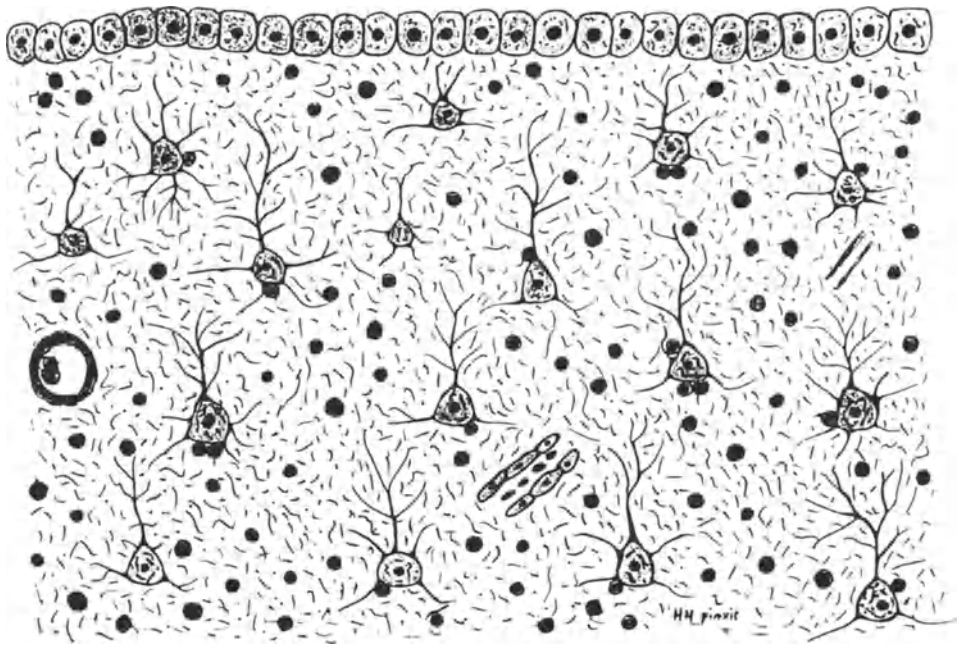


FIGURE 77 Diagram of a low-power light microscopic view of brain adjacent to the ventricle, according to conclusions arrived at here. In addition to the ependymal cells at the upper margin, and two capillaries depicted, the other elements are neurons, naked nuclei and syncytium. Please note: there are no synaptic connections between any of the dendrites or cell bodies, nor are there any synaptic knobs. The processes are much straighter than appear in fixed dehydrated sections. In life, the brain is very soft. This figure should be compared with Figures 50 and 51

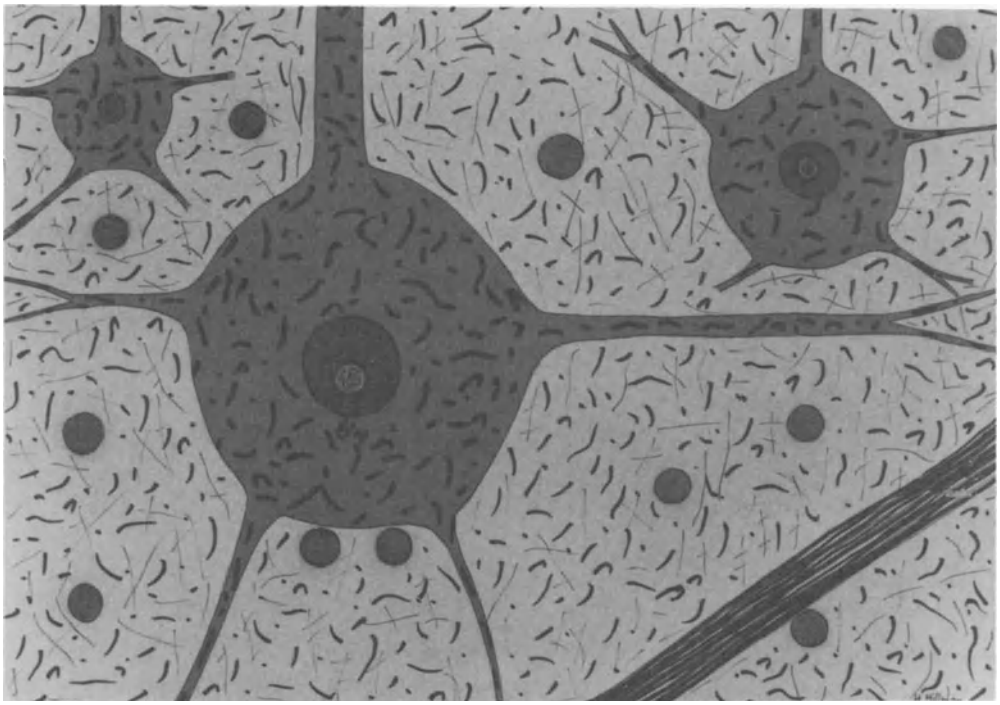


FIGURE 78 Diagram of a high-power view of the central nervous system of the mammal as it would be seen in life. A nerve tract traverses the right-hand corner. Please note the mitochondria inside and outside the neurons (page 122); they appear in all orientations. The neuron cell bodies are rounder than they usually appear in dehydrated sections (page 35). The neuronal nuclei contain nucleoli, which are surrounded by a membrane (fig. 18); the nucleolonema is seen in the nucleoli (page 160). In life the mitochondria, the naked nuclei and the nucleolonema are in constant motion. In the syncytium one can see fibrils much finer than the mitochondria (fig. 38). Nucleoli are not seen in the naked nuclei, whose nucleoplasm is uniform

10
Problems Arising from the Latter Conclusions

The first question which arises is, 'Have these views been held before, and, if not, why not?'

During the nineteenth century it was generally held that the central nervous system consisted of ganglion cells (neurons), Deiters' cells (microglia or naked neurons) and neuroglial material (pages 11-13). This view is similar to our own. In the twentieth century the different kinds of cells were identified by their being stained by newly described procedures. The idea then evolved, rather than originated, that if all the different kinds of cells characterized thus could all be stained in the same preparation, the whole tissue - with the exception of a small extracellular space - would be seen to be filled with these named elements. This was most recently stated by Nauta and Feirtag (1979). This belief was based on the acceptance by neuropathologists of the specificities of the staining procedures which their original authors had neither tested nor even alleged. Unless one examines the original papers (Table 10), one would naturally assume that the specificities and non-specificities of each staining procedure would have been rigorously tested. The reasons for the early failure to do so have been discussed briefly, viz: respect for the authority of the early neurohistologists; the fact that the concepts of control experiments and specificity were not considered important by then; the failure to appreciate that different procedures distort cells differently. We would not wish to encourage disrespect for authority, but would urge that experiment should always be more persuasive than the power or pelf of its protagonists. By and large the relevant control experiments are still waiting to be done (page 242), and specificity is better known for its assertion than its demonstration. History may well view even more unkindly the premature derivation of conclusions from these incomplete experiments, than the fact that the experiments themselves have not yet been completed - albeit after more than a busy half-a-century.

In the late 1940s and early 1950s the electron microscopists looked at the central nervous system and found it full of nuclei and mitochondria. They were very good at seeing nuclear and mitochondrial membranes but did not ask themselves why they could see the latter membranes so clearly and along most of their routes, but could not see the cell membrane so well around 'astrocytic feet' or cells they characterized

as oligodendrocytes (Figures 61 and 63). Whatever optical or geometrical explanations they could produce for their failure to see the latter as frequently as expected (Ham, 1974; Horne and Harris, 1981; Michell, Finean and Coleman, 1982) were rapidly shown to be irrelevant by the high frequency with which they could see the nuclear and mitochondrial membranes (Figures 31-33, 61 and 79).

The belief that the whole central nervous system consisted of neurons, neuroglial cells and a small extracellular space naturally led to the supposition that the pervasive mitochondria outside neurons seen by electron microscopists were located in the neuroglial cells thought to be present between the neurons. When they could not see the membranes which they expected to surround the neuroglial cells, they looked for geometrical explanations to explain the invisibility.

A number of possible educational and sociological factors which have predisposed to uncritical acceptance of the validity of the findings of electron microscopy have been discussed elsewhere (Hillman and Sartory, 1980b).

A further fundamental point must be made here. Observers use their brains as well as their microscopes. There is a mind in the eye of the observer, as well as an eye in the mind of the microscope. One is conditioned by expectations which have been implanted by education. Two observers see different parts of the same image, attach different degrees of importance to each part, and often interpret observations of the same image differently. For this reason I would strongly advocate the fabrication of three-dimensional models of any alleged structure (page 126).

IDENTIFICATION OF ASTROCYTES

The Golgi stain requires thick sections (up to 150 μm; Sholl, 1953), so that one should be able to see a great deal more of the three-dimensional structure of sections than is possible with the usual thinner sections; modern histologists cut 4-6 μm, the earlier electron microscopists cut 'thick' sections of 0.5-1 μm, and modern practice is to cut sections 10-100 nm thick. Even the use of the thickest of these sections creates difficulties in identifying astrocytes by their 'feet'. If one cannot see a 'foot' it may be either because the cell is not an astrocyte, or because no 'foot' happens to be present in the plane of section, or because 'feet' do not exist. Thus serial sections have to be examined to identify with certainty a particular cell as an astrocyte by this criterion.

The lack of membrane of the astrocytic foot has been discussed (page 167). The simplest explanation for the appearance of the 'foot' by electron microscopy, the sparsity of its contents, and its lack of personal membrane, is that it is a shrinkage artifact between two neurons, at the end of which there is a lightly staining space containing a naked nucleus. Thus, in our view, the astrocytes are the nuclei of the syncytium, which are identical with microglia.

SYNAPSES AND TRANSMISSION

It has been shown that the vesicle hypothesis of transmission embraces a number of unlikely assumptions and findings which have not yet been demonstrated (pages 226-230). Nevertheless, there is a vast bank of data on the iontophoretic application of transmitters



FIGURE 79 Electron micrograph of layers I (above) and II (below) of cat cerebral cortex. The original legend of Jones and Powell's paper (1970) shows 'the bifurcating (ringed arrows) apical dendrite of a pyramidal neuron passing between two other cells to reach layer I. Note the densely packed neuropil of layer II with many small axon terminals, dendrites and dentritic spines; many of the spines can be seen to be derived from the small dendrites of the layer (arrow)'. We show this micrograph to indicate the appearance, at 2 o'clock and 9 o'clock, of two neurons with their membranes and, at 5 o'clock of one nucleus, with no apparent membrane around it. This electron micrograph is reproduced from Jones, E.G. and Powell, T.P.S. (1970) Electron microscopy of the somatic sensory cortex of the cat. II. The fine structure of Layers I and II. Phil. Trans. Roy. Soc., B, 257, 13-21, plate 17, by kind permission of the authors and the Royal Society.

and 'putative' transmitters (Table 27) to cells in the brain and spinal cord presumed to be neurons (Eccles, 1961, 1964; Triggle, 1967; Katz, 1969; Berl, Clarke and Schneider, 1975; Iversen, 1975; Fonnum, 1978; Pepeu and Kuhar, 1980; Bradford, 1982). The injection of each of these substances on the surface of particular cells either excites them, inhibits them or has no effect on them. Such findings have traditionally been interpreted in relation to the existence of synapses and the vesicle hypothesis, but they can also be interpreted simply without the necessity of belief in these structures. The experiments tell us quite clearly that some cells react to some naturally occurring substances in the brain, but not to others. Sometimes they are injected at rather high concentrations, since it is difficult to know precisely the concentrations at different distances away from the micropipettes, and it is also virtually impossible to know the relevant natural tissue concentration in vivo. Furthermore, the assumption has to be made that the current used to inject the transmitters does not itself have any significant effect on the permeability or excitability of the membranes of the cells being examined; this assumption has usually been tested.

Despite these reservations the abundant evidence of iontophoretic experiments tells us only that different cell membranes are sensitive at low concentrations to different naturally occurring substances. It does not prove that the loci of these sensitivities are the knobs on the surface of the neurons. Nevertheless, this does pose a considerable question. If the neurons are not connected at synapses, how is an action potential from one cell communicated to another? For the time being I must put forward the following hypothesis: the action potential in one cell body is conducted (in an electrical sense) through the syncytium in all directions, and decrements according to its space constant. The nearest membrane is excited if the current reaching it is sufficiently high. The sensitivity of the membrane of the 'receiving' cell mainly determines if the cell fires. The various neurotransmitters, chelating agents, labilizers, stabilizers, depolarizing agents and toxins, all determine the sensitivity to incoming current of particular cell membranes. It should be noted that it may be difficult to devise experiments which could distinguish unequivocally between the direct effect of a substance on the membrane and a 'modulating' effect on its sensitivity. For example, a neuron may fire because it is depolarized by acetylcholine, made hypoxic by diminishing its oxygen supply, 'labilized' by a low concentration of calcium ions in its immediate environment, or deprived of substrate during dying.

The scarcity of cogent evidence for the existence of structural synapses induces one not to accept their reality in life. I have therefore felt obliged to propose an alternative explanation to the widely held transmitter and vesicle hypotheses, which are partly based on microscopical findings. I believe that I have brought sufficient evidence against the synapses that they should be regarded as artifacts unless new data be produced about them. Logically, of course, if the hypothesis which I have outlined briefly above should prove to be wrong, this would in no way invalidate the evidence that the synapses are artifacts. Rather, it would oblige me to propose another hypothesis for transmission which would also not require the existence of synapses.

It should be made clear that the term 'synapse' is being used here in the sense of a nerve-nerve connection, and not as a neuromuscular junction.

I would propose that excitability in the nervous system spreads across the syncytium by electrical conduction. This suggestion is not difficult to accept since it is evident that when electrical activity is recorded with extracellular electrodes, such as fine metal microelectrodes, or coarser electrodes used in electrocorticography, or in electroencephalography, or in class experiments recording action potentials in sciatic nerves, excitability has passed through the bulk nervous tissue, without necessarily involving synapses. Electrical conduction could provide a starting point for an attempt to synthesize a general theory, involving nerve conduction (Hodgkin, 1951), the transmitter hypothesis (Katz, 1969) and the chemical hypothesis of nerve conduction (Nachmansohn and Neumann, 1975). Such a synthesis would have the effect of making the hypotheses much less incompatible.

In summary, then, having shown difficulties of accepting the existence of synapses, I wish only to initiate discussion to encourage physiologists, biochemists, pharmacologists and biophysicists to re-examine their views on transmission in so far as they are linked in their minds with the current view of the structures they call synapses.

THE NATURE OF THE STRUCTURES DESCRIBED AS SYNAPSES SEEN BY LIGHT AND BY ELECTRON MICROSCOPY

We have shown that the granules seen when examining unfixed neurons (Figure 54) are very likely to be intracellular, and, therefore, they are probably mitochondria (page 148). We have further shown that the granules seen on the surfaces of cell somas in stained sections of spinal cord and brain are not connected with any preterminal fibres, teloglia, telodendroglia, dendrites, fibrils or cell bodies (pages 141-144). Their dimensions and their incidence of occurrence as seen by light microscopy hardly overlap the same parameters seen by electron microscopy (Tables 22-25). The stains used in histology are generally silver salts, which are reduced, react with, and precipitate proteins and lipids. This gives a clue as to the nature of the appearance of granules described as synapses. They are most likely to be silver precipitates on tissue, bearing in mind that the silver salts are reduced and the silver-tissue reaction product is dehydrated subsequently. That this explanation is likely is demonstrated by the following experiment. A smear of egg albumin was stained with Weil and Davenport's stain, and another with haematoxylin and eosin. It is quite clear that all sorts of artifactual appearances similar to granules, fibres and fibrils can result (Figure 10).

The electron microscopic image of the synaptic cleft appears as two parallel lines far too frequently than solid geometry would permit, and is, therefore, a two-dimensional deposit (Figure 53, page 145). Please see references in Tables 21-26.

The most likely explanation for both the pre- and the post-synaptic thickenings is as follows. A certain proportion of granules of osmium salts deposits in the region of the membrane of the neuron. When the chamber of the electron microscope is evacuated and the specimen is subjected to electron bombardment, X-radiation and high

temperature, the underlying membranes evaporate beneath the osmium granule and crack it.

However, irrespective of the latter unproven explanation, I would like to stress that the inspired hypothesis of synaptic chemical transmission could never be proved unequivocally with respect to mammalian neurons, since one cannot localize the tip of a relatively large iontophoretic micropipette sufficiently accurately to be certain that the transmitter was actually being applied to a single very small synapse (pages 153-154). It should also be noted that - whereas in autonomic ganglia one can distinguish between the pre- and the post-synaptic fibres in mammals - it is also difficult to know for certain the precise localization of applied drugs in this sense as well. The use of the terms pre- and post-synaptic is often based on pharmacological criteria alone.

'UNIT' MEMBRANES

The 'trilaminar' appearance of various cell membranes can be seen in any electron micrograph and all textbook diagrams, except our own (Hillman and Sartory, 1980). We have shown the trilaminar appearance - as opposed to the cell membrane - to be an artifact, and we would proffer the following explanation for its genesis. Since it is two-dimensional, and the fixatives and heavy metals are added to the tissue before section, it follows that the 'trilaminar' appearance must have arisen after the sections were cut. The focal plane of the electron microscope is very deep, but the image of the membrane is usually only on the surface (Ibid, page 41). Therefore, the 'trilaminar' appearance must arise on the surface as a consequence of the exposure of the heavy metal covering the membrane to the vacuum, heat and irradiation. This must cause the membrane beneath the heavy metal to expand, vaporize and probably explode, leaving a groove lined by heavy metal on both sides (Figure 45). Please see also Appendices 4 and 5.

CYTOSKELETON OR ENDOSKELETON

We have already concluded that all the elements described by this term (Table 17) are artifacts - that is, they do not exist as structures in living cells (for detailed discussion, please see Hillman and Sartory, 1980, pages 44-57). In addition to the geometrical objections to its existence in life, we have pointed to the several different kinds of movements within living cells, which would be incompatible with any network or large body within the cytoplasm.

Cytologists have accepted that there is a problem here, and there is a considerable literature on cell motility. They see the endoskeleton by electron and light microscopy (Allen and Kamiya, 1964; Goldman, Pollard and Rosenbaum, 1976a,b,c; Dustin, 1978; Roberts and Hyams, 1979; De Brabander and De May, 1980). The ability to extract actin and myosin from many cells has led to the suggestion that they can cause the movements, as actin and myosin are claimed to do in muscle. This has been regarded as the answer to the question as to how the cytoskeleton would permit intracellular movement. This view is based on the following evidence: firstly, the ability to extract actin and myosin chemically; secondly, the appearance by electron microscopy of largely two-dimensional strands said to be of the expected

size of actin and myosin molecules; thirdly, the antigen reactions which are reported to occur only after cells have been fixed and dehydrated; fourthly, the strands visible by light microscopy (page 131). All of this evidence together does not not prove the hypotheses either that the fibres do consist of actin or myosin, nor that if they are of this chemical nature, they do indeed cause intracellular movements. At this point I would like to reiterate my view that we cannot be agnostic on this or other hypotheses (page 4); either there is enough cogent evidence to prove the matter beyond reasonable doubt - or we cannot accept it. The fact that an increasing number of people believe the hypotheses encourages us to examine the evidence carefully, but it is not itself evidence of the validity of the hypotheses. The validity of a viewpoint cannot be measured by the number of its adherents.

Perhaps the hiatuses in the thinking should be spelled out precisely. The apparent similarity of dimensions of some fibrils to those of actin and myosin is quite insufficient proof that these fibrils are of this chemical nature. The demonstration of the presence of markers on fixed, dehydrated and sectioned tissue - often with added fluorochromes - is quite insufficient evidence of true specific localization of antigens unless and until the antigen-antibody reaction has been shown to occur in unfixed, fully hydrated sections. The failure to carry out or to publish the latter experiments so far should only serve as a stimulus for anyone claiming localization by these techniques to proceed with these definitive experiments without delay; they should not continue using quite incomplete experiments distant from natural conditions, as sources for substantive findings. Thus, I would suggest that if these reactions claiming to show localizations cannot be demonstrated in fresh tissue to which no fixatives or alcohols - which might reasonably be expected to alter antigen-antibody reactions - have been added, then we should cease believing in these results, rather than abstain from doing the relevant crucial experiments or accepting hypotheses with their major assumptions unproved.

There are further reasons which make it unlikely that contraction of acto-myosin filaments or of other contractile proteins could cause intracellular movements, like streaming, Brownian movement, pinocytosis, phagocytosis, mitochondrial movements, etc.

Firstly, if, for example, mitochondria were to be moved by acto-myosin filaments, the filaments would have to be attached not only at the mitochondrial end, but also to the cell membrane, or to the nuclear membrane, in the direction of which the mitochondrion was about to move. However, contraction of a filament at the east end of a cell would stretch the opposite filament at the west end of the cell. After a finite contraction the filament at the east end would be at its shortest length and the one at the west end would break. That is to say, a one-step movement could occur, but not a continuous movement, like streaming or pinocytosis, nor any reversible movement.

Furthermore, we would ask for evidence that: the filaments are attached to another firm structure; they are not randomly orientated; they are seen both in the contracted and in the relaxed state; and, of course, that they are composed of actin and myosin.

The weakness of the suggestion that acto-myosin filaments could initiate or

accelerate movements of mitochondria, Golgi bodies or lysosomes, can be illustrated by an analogy. If a crowd is trying to move along in a packed closed underground train, under no circumstances can the introduction into the carriage of any persons, animals, machinery or appliances, increase the rate at which the passengers could change places in the compartment. It could only slow down their movement.

It is also noteworthy that during the last century there were many publications on 'colpoids' which were mixtures of salts, oils, granules and coloured reagents, whose microscopic properties imitated those of cells (Herrera, 1869-1942; Butschli, 1894; Hardy, 1899; Bastian, 1913). Suitable mixtures showed streaming, Brownian movement, laminar flow, and coalescence of globules. (Nowadays, the latter would probably be called 'endocytosis'.) Of course, the colpoids possessed no metabolic source of energy, acto-myosin filaments or 'cytoskeleton'.

We have concluded that the whole 'cytoskeleton' is a precipitate of the cytoplasmic constituents, with heavy metals, after having been bombarded by electrons under conditions of very low pressure. Unpalatable as it may be, one must insist that direct evidence of intracellular movement can never be derived from examination of fixed, dehydrated, frozen or dead tissues, or of electron micrographs.

One reason for the difficulty in relating movements to chemical phenomena arises from the use of the word 'movement' to cover: intracellular movements; movements of whole cells such as phagocytosis; putting out pseudopodia; flagellar movement; muscle contraction.

If the ribosomes and lysosomes do not exist in the living cells, where does protein synthesis and acid hydrolysis occur in the living cell? We have shown that the biochemical compartmentation is unknowable by current destructive techniques (Hillman, 1972). Protein synthesis and acid hydrolysis occur in virtually all cells, yet ribosomes and lysosomes are not seen as structures in all tissues. (For example, they do not appear in muscle or kidney cells.) If one admits that either the biochemical activity can be present in cells which do not appear to have the granules at all, or that it may be present in compartments which are neither ribosomes nor lysosomes, one finds oneself in the difficult position of describing 'free ribosomes' or 'free lysosomal activity'. The implication of this is that one is admitting that these particles are not necessary to house the biochemical activities, or the biochemical activities must be in particles one cannot identify. The first of these alternatives admits that the biochemical activities do not require these locations; the second involves one in the difficult logical position of attempting to use supposition as evidence.

Of course, denial of the existence of the cytoskeleton in no way means denial of the existence of the biochemical activities alleged to be in it, although this should not be taken as implying that we accept that the locations of the activities found by subcellular fractionation techniques are true representations of their locations in intact living tissues.

THE INTEGRITY OF THE BRAIN

We have concluded that the brain is composed of neurons with their axons and dendrites within an embracing fluid syncytium. I should like to stress that we regard the neurons

and all their processes to be in fixed positions. The rest of the central nervous system, which is the majority, is a fairly viscous syncytium in which the naked nuclei may move and they may act as phagocytes. Neurosurgeons taking biopsies invariably find that the texture of the living brain and spinal cord is extremely soft.

The question then is raised as to what the central nervous system owes its mechanical integrity. The tissue itself is probably bound together by the axons, the processes, the blood vessels and the capillaries, as concrete is reinforced by steel rods. On a larger scale, the processes are seen as commissures, tracts, and projections. The ventricular system and the central canal is lined by an ependymal epithelium internally (Figures 6 and 77), and by the meninges externally. The pia mater, of course, embraces the central nervous system most intimately. I would suggest that the system has sufficiently strong covering and internal reinforcement to account for its mechanical integrity, without requiring all the cells to adhere to each other.

THE CENTRAL NERVOUS SYSTEM AS A SYNCYTIUM

There have been three different concepts of the syncytium (pages 11-13). The reticularists, like Gerlach (1872) and Golgi (1883), believed that all the neurons were connected by a continuous fine meshwork of fibrils. Bauer (1953) had a different view of the syncytium - that the fibrils within the neurons connected through the membrane to dendrites outside in the syncytium (Figure 7). A third view - to which we adhere - is that the neuroglial tissue between the neurons only is a syncytium; this is illustrated in our generalized diagram of the central nervous system (Figure 78).

In denying the existence of connections to synapses, we have implicity ruled out the first possibility (pages 136-146). Our conclusion that there are no intracellular filaments in the cytoplasm other than mitochondria would require us to deny the possibility of any connections between extracellular or syncytial fibres, on the one hand, and any intracellular fibres, on the other; obviously, we would hold it as extremely unlikely that dendrites are attached to mitochondria, when the latter are moving continuously in life. Furthermore, under the microscope, one simply does not observe such fibres passing through the neuronal membrane. Our explanation for the belief in such an apparently continuous syncytium is that the mitochondria are seen in the syncytium between the neurons, and are also seen in the neuronal cytoplasm; a small indulgence in unconscious extrapolation (Cook, 1983) has joined them together in the minds of some observers.

EVIDENCE OTHER THAN STANDARD TRANSMISSION ELECTRON MICROSCOPY

We have denied the existence in life of a wide range of structures, all - except the Golgi apparatus - originally seen by electron microscopy (Table 17). Horne and Harris (1981) talk about the 'enormous range of structures which have been meticulously investigated by independent measures', and Michell, Finean and Coleman (1982) stress particularly that freezing techniques are productive sources of independent data.
A few comments might be apposite here:

1. Biochemical evidence cannot be used in support of, or to deny, morphological findings. They are different epistemologically, often different in dimensions by several orders, and their interpretation has usually been based on certain conceptual models of the structure.
2. Low-angle diffraction studies are usually made on partially or completely dehydrated tissues - except for the studies on frog peripheral nerves (Finean, 1954, 1960). Obviously, unless one attempts to examine, calculate and correct for, the distortions and shrinkages due to the reagents, the dehydrations during preparation, and the effects of sectioning and the electron bombardment, conclusions about shapes and sizes of organelles or the distances between them must be taken magno cum grano salis. Nevertheless - as we have stressed frequently - the desire to derive meaningful conclusions obliges one to attempt to quantitate the latter changes in every tissue whose microscopic structure one wishes to elucidate. Furthermore, it is not good enough to pay lip service to the known or suspected changes during tissue preparation, without using them to correct all one's measurements and calculations (Hillman, 1983).
3. Freezing procedures used with electron microscopy are said to provide independent evidence for beliefs in some of the structures seen by standard transmission electron microscopy. This view is not tenable because:
   (a) Like biochemical experiments, the results of experiments involving freezing are interpreted in relation to beliefs derived from the earlier techniques.
   (b) Freezing is only used to fix tissues, which are subsequently stained with heavy metals, dehydrated and subjected to vacuum and electron bombardment, just as it is with other techniques of electron microscopy (Stolinski and Breathnach, 1975; Rash and Hudson, 1979; Sleytr and Robards, 1982). Since we have concluded that the artifacts of electron microscopy originated after fixation of the tissue, in our view, evidence derived by the application of freezing techniques could only be regarded as independent of that of standard electron microscopy, if the two techniques did not share the same steps during which the artifacts may arise.
   (c) Nowadays, it is considered that tissue should be frozen as rapidly as possible to minimize the size of ice crystals. Unfortunately, the greater the rapidity of cooling, the less time the bubbles of gas coming out of solution at the lower temperatures have to escape from the tissue.
   (d) Although it is widely believed that vitrification occurs when cooling is very rapid, specific investigations of the simple systems of water and dilute aqueous solutions have demonstrated how difficult it is to achieve (Bruggeller and Mayer, 1980). The belief implied here is that if vitrification occurs, the tissue is not dehydrated or deformed. The technique called 'critical-point drying' is widely used in the belief that it preserves the structure of tissues. The term is misleading, since at the critical point, the solid, the liquid and the vapour phases are in equilibrium, and no drying would occur. Drying takes place only when one moves away from the critical point by raising the temperature or lowering the pressure. It is intended that sublimation of the

ice to vapour will avoid the surface tension and electrostatic effects in the tissue, which may distort the cellular structure. However, I am not aware of any studies of specific cells before and after the application of freezing techniques, showing that shrinkage and deformation do not occur. Sleytr and Robards have made very important contributions to the study of artifacts due to freezing techniques (Sleytr and Robards, 1977a,b, 1982). In the latter review they have listed 11 main agents during freeze-fracture, which have been shown to cause 30 different kinds of artifact during various procedures. They continue to use the techniques although they concluded that 'it is possible to assert that early hopes that freeze-fracture and etching techniques might provide artifact-free images because chemical fixation can be replaced by (undamaging) cryofixation have not been fulfilled.... It is now abundantly clear that each of the factors involved in the formation of the final image can either enhance, or compensate for, each other.'

(e) Since the images produced by freezing techniques appear as two-dimensional as those by the earlier techniques, those who practise them must produce their own explanations for these geometrical difficulties. They cannot use the shared problems as evidence that the problems do not exist.

GLIOSIS

The reaction of central nervous tissue to infection has a cellular and a fibrillary component. The cell bodies may have little or no cytoplasm and pathologists call them variously, reactive astrocytes, oligodendroglia, microglia, phagocytes or histiocytes. The pathologists assign the names partly on account of their knowledge of the clinical conditions which afflicted the patients (Zulch, 1979). It is generally agreed that the cells can be mobile. They may arise acutely by migration from other parts of the central nervous system, by division locally, or by diapedesis from blood vessels (for excellent modern reviews, see Jacobson, 1978; Oehmichen, 1978; Korr, 1980). We would characterize these cells on the basis of their lack of cytoplasm or obvious processes, as 'naked nuclei'.

The increase of 'protoplasmic' or 'fibrillary' astrocytes, with processes which occur as a consequence of inflammation (see, for example, Glees, 1955; Windle, 1958; Nakai, 1963; Greenfield, 1976; Treip, 1978) raises a difficulty for us, since we have put forward the hypothesis that, in normal central nervous systems, all cells with processes are neurons. If, then, these cells occur in normal tissue, they must be neurons. If they only occur in ill tissue - of which most slides and micrographs appear to be representations - we must ask if neurons can proliferate in these conditions. We cannot answer this question at the moment but would list the kinds of ways fibrils can arise in living tissues, or precipitates imitating them can appear as a result of the staining procedures:

1. Sprouting of 'neurites' has been shown to occur in cultures of sympathetic and sensory ganglia if nerve growth factor is added to them (Levi-Montalcini, Meyer and Hamburger, 1954; Symposium, 1964). In young rats, neurons isolated from the medulla, as well as from sympathetic and sensory ganglia, put out these fine

processes, and the addition of nerve growth factor increased the frequency with which they did (Hillman and Sheikh, 1968; Hillman and Khallawan, 1970). These fibrils did not develop into axons or dendrites in our cultures, and such fibrils are seen in cultures of growing cells, irrespective of the tissues from which they originate.

2. Fibrils have been shown to grow out of the proximal end of a transected peripheral axon, when it begins to regrow towards the distal end (Weddell and Glees, 1941; Guth, 1956).
3. If the protoplasmic or fibrillary astrocyte were really a neuron, and if it had been demonstrated rigorously that the number of processes increased due to inflammation, then one could suppose that real new dendrites could grow out of the cell somas in response to pathological conditions.
4. Inspissated pus may appear as fibres after precipitation by drying, and after staining - as egg albumin may do (Figure 10).
5. The fibrils could be a staining artifact arising from a change in local chemistry of the central nervous system, which would make the silver-tissue complexes less soluble, and thus precipitate out in rows. Although it may be difficult in micrographs to distinguish between precipitates and dendrites, no such difficulty presents itself when one looks at the stained histology slide using oil-immersion objectives, and can follow them through several focal planes. Precipitates appear two-dimensional, whereas dendrites appear in three dimensions.
6. 'Non-specific' fibrils are found in many tissues, such as fibrous tissue, mesenteries, ligaments and tendons. There seems to be no prima facie reason why they should not occur in the connective tissue of the central nervous system, nor why they should not increase following inflammation, especially if it is chronic.

We should reserve judgement upon which is likely to be the true explanation for the apparent increase in fibrils after inflammation, until it has been shown positively: that the number of fibrils does increase; that astrocytes are not neurons; that the affinity and specificity of the staining procedures has not changed significantly as a consequence of the pathological conditions; that clots containing fibrin or dried inspissated pus can be differentiated histologically from dendrites or glial fibres; that one can distinguish histologically between dendrites and neuroglial fibres.

11
Properties of the Nervous System in the Light of Present Conclusions

CLASSIFICATION OF CELL TYPES, NORMAL AND PATHOLOGICAL

We have characterized all those cells which have membranes and processes projecting from them as neurons; these include cells currently designated as neurons and astrocytes. Cells without membranes around each nucleus and without nucleoli are naked nuclei, which are sometimes called oligodendrocytes, astrocytes or microglia. Capillary cells and ependymal cells are also present in the central nervous system. The simpler classification would solve the major problems of disagreements about their characteristic structures by light microscopy, by electron microscopy, and in tissue culture (Tables 2-11). It would also solve the problem of the multiplicity of views about the specific or recommended staining systems appropriate to each kind of cell (Table 10). It would clearly imply the necessity to carry out control experiments on all staining systems used in pathology and histology, in an attempt to define the real specificity of each staining system, and whether there are morphologically recognizable sub-populations of neurons and neuroglia.

Different populations of cells are, indeed, defined by their immuno-chemical properties. Some neurons show up after complex histochemical procedures intended to indicate the presence of, say, catecholamines. As I have indicated previously (Hillman, 1972, pages 41-53; Hillman, 1983; and in this volume, pages 198-199). I do not think that one can accept that substances - mostly transmitters - are necessarily found in the same locations after fixation, embedding, sectioning and processing as they were in the living tissues. However, since the cells in a particular region after such processing do indicate different chemical and microscopical properties, which are constant (Adams, 1965; Ungerstedt, 1971; Pearse, 1972, 1980; Moore and Bloom, 1978, 1979; Pickel, 1981), one must conclude that there are, indeed, different biochemical sub-populations.

It is curious that, in general, histochemists find that most transmitters are located on neurons. This is probably because they believe that the transmitters nearly always arise in or near neurons, therefore any cells which apparently show the presence of a widely accepted or putative transmitter by that token define themselves as neurons. Alternatively, histochemists may have carried out careful examinations of cells they believe to be neuroglia and concluded that such cells independently identified do not

contain the transmitters. However, gamma-aminobutyric acid, adenosine, acetylcholine, and dopamine in rather few systems have been reported in neuroglial cells (Table 12).

Whereas the classification of cell types may be considered an academic exercise in histology, it has an important relevance in neuropathology. There are various classifications of tumours: Bailey and Cushing (1926) listed 14 types of gliomas; Zulch (1965, 1979) listed 30 different brain tumours and other space-occupying lesions; the Unio Internationalis Contra Cancrum in 1958 (see Zulch, 1965, page 37) classified 30 different tumours mainly on the basis of tissue of origin (see also Kernohan and Sayre, 1952). The classifications take into account (1) clinical history of the patient, age of onset of the diseases and rapidity of spread of the tumours; (2) whether the tumours arise in the more primitive cells, such as pineoblasts or astroblasts, or the later cells, the pineocytes or astrocytes; (3) whether they are believed to arise from neuroepithelium, mesoderm, ectoderm, the whole embryo or the vasculature; (4) their tissue of origin, such as the medulla, meninges or retina; (5) the cell type from which they are believed to arise, such as the astrocyte, oligodendrocyte or ependymal cell. Most authors are rather uncertain as to whether there are tumours in true adult neurons of the cerebrum, cerebellum or spinal cord. However, there is general agreement that in all tissues, tumours of differentiated cells and tissues are less malignant than those of undifferentiated cells; the degree of malignancy is assessed by the incidence of the latter cells, the degree of anaplasia, the frequency of mitotic figures, the amount of cellularity, the involvement of endothelial cells and the extent of necrosis (Kernohan and Sayre, 1952). I would suggest that the naked nuclei are the cells of origin of all undifferentiated tumours of the central nervous system; when they become pleiomorphic their precise identification becomes difficult, but the failure to see a membrane around the cytoplasm in histological sections of these tumours, and in the illustrations in all the publications cited, is really quite striking. Nishii (1929) characterized as sarcomas the undifferentiated tumours in the brain which contained cells reminiscent of our naked nuclei, but this idea is not generally accepted. However, the high grade of malignancy of undifferentiated cells, their invasiveness, the uncertainty about from which kind of glial cell types particular tumours originate, and the similarity of these undifferentiated nerve cells, all taken together, encourage one to attempt to simplify the cellular classification of tumours into two classes only - those showing malignant cells with fibres or processes, and those showing only naked nuclei. There are two possibilities about the origin of the cells in these tumours. Firstly, the cells with fibres are tumours of neurons and the others are tumours of naked nuclei. A second hypothesis, which I favour slightly more, is that primary tumours of differentiated adult neurons are extremely rare or do not exist at all, and that all tumours arise from naked nuclei; the fibres of the first kind in this case would not be dendrites or axons. The latter explanation seems more likely since (1) there is a general disinclination to identify, in adult animals, tumours originating from neurons in the central nervous system, as opposed to their common occurrence in the peripheral neurons (Russell and Rubinstein, 1977, page 260; Treip, 1978); (2) there do not appear to be reports of mitoses having been seen in neurons of adult mammals; (3) I have rarely seen any cell in a tumour, which had recognizably originated from a neuron,

and, for example, had the normal complement of processes attached to its soma, but had clear signs of being malignant. Russell and Rubinstein (1977, pages 260-269) devote a section to ganglioneuromas and gangliogliomas, and indicate that such tumours contain considerable glial elements. However, with the exception of one bizarre ganglioglioma with gemistocytic astrocytes (their Figure 202, page 263), most of the neurons themselves have appearances which are not grossly different from those found in normal tissue. Cavanaugh (1970) fixed cerebral tissues rapidly after he made lesions in the brain with needles, and he was able to observe many mitoses in reactive astrocytes around the lesions. Thus, we know that such cells (naked nuclei) may divide in adult animals. This would make them obvious candidates for malignancy. Their location in a syncytium, and their ability to move within it, may explain the relatively rapid spread and unclear boundaries of these malignant tumours of the central nervous system (Figure 80).

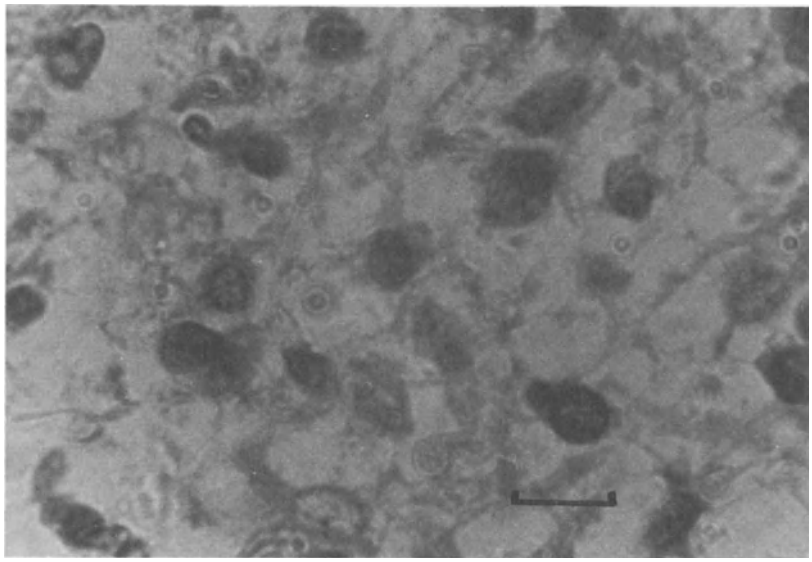


FIGURE 80 Human cerebral astrocytoma stained with haematoxylin and eosin. Please note that the nuclei have little or no adherent cytoplasm and no cell membrane. We would classify these as naked nuclei. The bar is 20 μm

MEMBRANE TRANSPORT

This term is used to mean transport both <u>across</u> a membrane, and <u>by</u> a membrane. Transport, itself, is a portmanteau word, since it encompasses a number of different mechanisms characterized by their individual kinetic characteristics (Bayliss, 1959; Wilbrandt and Rosenberg, 1961; Stein, 1967; Harris, 1972). The close use of Occam's Razor conditions us to recall that the simplest form of transport is diffusion, and that substances distribute themselves in different phases according to their different affinities for all the chemical species in each of the phases (see, for example, Goldstein, Aronow and Kalman, 1968). Therefore, unless one shows that any movement occurs at a significantly different rate than diffusion, one must assume that this simplest mechanism alone is operative. Indeed, in order to stop it, a tissue or reaction mixture would have to be cooled to absolute zero temperature, although it is drastically slowed if the

whole system is solidified.

In order to demonstrate that a movement of molecules is due to 'active' transport or is dependent upon metabolism, it is usual to show that subjection of the tissue to hypoxia or poisons, or deprivation of substrate, causes a massive redistribution of the components of the system in the direction of the apparent electrochemical gradients. Unfortunately, we are using crude measures here, since none of these experiments demonstrates that metabolism is directly involved. For example, the effects seen may result from increased lactate or lowered hydrogen ion concentration, and may not be a simple consequence of interference with metabolism. Indeed, it is very difficult to design experiments which can show unequivocally that the rates of particular reactions or movements require specifically biological mechanisms. Even the measurements of temperature dependence or oxygen uptake may show clearly simultaneous changes, whose relationship is not necessarily causative. Perhaps we are peering over the battlements at the joust between the vitalists and the mechanists, whose outcome it is probably several thousand years too early to predict.

One must distinguish between the evidence for the membrane as an interface between two different compartments, and the evidence that the mechanisms which regulate the composition of the phases on either side of the membrane are located within it. It is both a widespread finding and a reasonable expectation that any thin membrane - biological or artificial - permits different small molecules to cross it at different rates (Mueller and Rudin, 1968; Chang, 1972), so that the simplest model of the biological membrane is one composed of curious chemistry, which could account for most of the differences in chemical composition between - on the one side - the syncytium or extracellular fluid and - on the other - the cytoplasm.

I believe that cytologists should not fall into the trap of believing that because one can detect the difference of composition of the fluids on either side of a membrane, the mechanisms causing the electrochemical gradients are themselves located within the membranes (see, for example, Harris, 1972; Andreoli, Hoffman and Fanestil, 1978). (No-one believes that the heat for Montgolfier's airship was generated within the fabric of the balloon.) However, I believe that the gradual acceptance of this non-sequitur has conditioned cytologists to seek other evidence supporting, or compatible with, the location of the regulatory mechanism within the thickness of the membrane itself. The main sources of this belief are as follows:

1. Penetration of cells with intracellular electrodes results in the abrupt recording of a voltage.
2. Injection of adenosine triphosphate into poisoned squid and cuttlefish giant axons increases efflux of sodium ions (Hodgkin and Keynes, 1955; Caldwell, Hodgkin, Keynes and Shaw, 1960).
3. The finding of sodium/potassium ion activated ATPases in fractions believed to contain membranes (Skou, 1957, 1975).
4. The recording of resting and action potentials of the expected values in squid giant axons, whose axoplasm had been extruded and replaced by known potassium chloride solutions (Baker, Hodgkin and Shaw, 1962a,b).

5. The finding of acetylcholine, choline acetylase and cholinesterase in high concentrations 'in all types of excitable tissues investigated, in so-called "cholinergic", and "adrenergic", in motor and sensory, in peripheral and central fibres, in invertebrates and vertebrates, and in all types of muscle fibres' (Nachmansohn and Neumann, 1975, page 253). (Please see also Schlaepfer and Torack, 1966.) Subsequently, the enzyme was located mostly in invertebrate species in neuromuscular junctions, which are usually considered to be similar to synapses (Nachmansohn and Neumann, 1975, pages 253-259).
6. Examination of the chemistry, electron microscopy and low angle diffraction, of both myelin sheaths and extracted myelin, as models of the properties of concentrated samples of membranes (Schmitt, Bear and Clark, 1935; Fernandez-Moran, 1952; Finean, 1961; Stoeckenius, 1962; Autilio, Norton and Terry, 1964; Landon, 1976).
7. The chemical properties, especially the enzymology, of 'microsomal' fractions, are usually assumed to reflect the properties mainly of cell membranes and endoplasmic reticulum (Table 12). The presence in these fractions of the enzymes believed to be involved in transport is regarded as evidence that these enzymes are located in the membranes around the cell. Unfortunately, one cannot accept without reserve evidence from subcellular fractionation at present because: (a) the effects of the preparative procedures on the distribution of the enzymes have never been tested (Hillman, 1972); (b) it has yet to be shown quantitatively and rigorously that the microsomal fraction mainly or wholly consists of cell membranes and endoplasmic reticulum; (c) we have shown that the endoplasmic reticulum does not exist in living cells (Hillman and Sartory, 1980, pages 44-57).
8. Kinetic studies of transport of ions, sugars and amino acids into mammalian red cell 'ghosts', ascites cells, yeast, mitochondria, frog skin, toad bladder, slices of liver and kidney, and isolated gut have been regarded as models for the transport across membranes (Harris, 1972). Red cell 'ghosts' have been prepared by immersion in hypotonic media, which permits the egress of haemoglobin, which is due to a gross modification of the normal permeability of the cell membranes. Frog skin, toad bladder and isolated gut each contain many layers composed of different cell types sandwiched by connective tissue. Their properties are of great interest in understanding the overall movements of small molecules across the whole tissues, as well as the potential differences recordable across their thicknesses, but it seems unlikely that they can be regarded as models of single neuronal membranes.

This is an incomplete list of studies which have been regarded as evidence that the membranes themselves contain the mechanisms of transport, as well as being the sites whose permeability may change during regulation. However, if one scrutinizes attentively the results of each of these various approaches, it seems that most of them have produced data which cannot distinguish between the mechanisms causing differences in chemical composition on the two sides of a membrane being located within the wall of the membrane, or within the cytoplasm. Subcellular fractionation itself could provide the answer, if control experiments had been done satisfactorily and comprehensively, and had, indeed,

shown the legitimacy of the relationship between the parent tissue and the subcellular offspring.

Thus, there are two schools of thought. Both regard the semipermeability of the membranes and any channels, charged or uncharged, to be one site of regulation; they agree that the semipermeability may be influenced by the chemistry of the fluids on both sides of the membrane. However, the difference between the two schools is that most membranologists believe that the site of the biochemical machinery of regulation is within the thickness of the membrane, while I would suggest that it is a property of both the permeability of the membrane at any particular time, and of the biochemistry of the cytoplasm, the syncytium, the mitochondrioplasm and the nucleoplasm.

It seems to me quite inconceivable that the cell membranes could house the whole arsenal of: structural lipids and proteins; carriers for ions, sugars and amino acids; transport enzymes (Figure 81); microsomal enzymes; receptors for approximately 50 transmitters (Table 27); receptors for innumerable drugs; receptors for hormones (Table 29); natural antigens and agglutinogens; receptors for bacterial and viral toxins; surface antibodies to a host of bacterial and viral diseases. The molecular diameters of these substances would vary from 0.4 nm to 20 nm, and many of them are found in microsomal fractions in considerable quantity.

A few comments about each of these groups of substances are apposite.

The structural lipids and proteins are believed to be the main components that occupy the thickness of the membrane. This view was enunciated before the use of the electron microscope, as a result of studies on red cells. Calculations were made which showed that the quantity of lipids and proteins which could be extracted was enough to provide the two lipid molecules sandwiched between the two protein molecules, which is now known as the Davson-Danielli model (Gorter and Grendel, 1925; Davson and Danielli, 1942). This model was subsequently generalized to all membranes. Yet the original calculations were made for red cells, which are not claimed to have an endoplasmic reticulum. As far as I can find, the calculation has never been made for the membranes of any other mammalian cells. One probable reason for this is that it would be extremely difficult to calculate the areas of membranes of other cells. Furthermore, there seems no prima facie reason for believing that there is little or no lipid or protein elsewhere in cells. Yet even in the red cells the interesting calculation that there would be enough lipid to make a bilayer does not prove that the membranes in life are a bilayer, nor, of course, that they are largely composed of lipids and proteins. That being said, it seems likely membranes do contain these components, although I would suggest that more analytical and comprehensive experiments are needed to confirm the beliefs in respect of red cells, to assess them quantitatively in membranes and to permit one to generalize them to all mammalian tissues, as well as other animals and plants. If one accepts our view that the lamellae of the myelin sheath peripheral nerves are artifacts, this would cast into greater doubt the apparent congruence of the results of experiments on red cell ghosts and on extracted myelin as evidence supporting the current view of membrane structure.

The membrane is said to contain carriers. The belief in their existence arises from kinetic considerations involving separation of the region of entry and exit of particles (Osterhout, 1935; Widdas, 1952; Wilbrandt and Rosenberg, 1961; Stein, 1967,

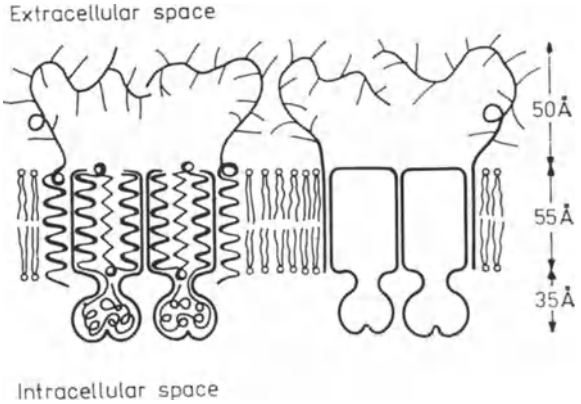


FIGURE 81 Schematic representation of the structure of the Na^+-K^+ transporting ATPase in the membrane. This figure is reproduced by kind permission of the author and publishers, Repke, K.R.H. (1980) Structural basis of the compartmentation functions of biomembranes. In Cell Compartmentation and Metabolic Channeling, ed. Nover, L., Lynen, F., and Mothes, K. (Leipzig: Fischer Verlag and Amsterdam: Elsevier), page 37. Please note that in this model, this enzyme molecule protrudes 50 Å from the surface of the membrane

pages 148-206). In nervous tissue their existence has been inferred from kinetic studies on sodium current in giant axons (Hodgkin and Huxley, 1952a,b) and of entry of monosaccharides and amino acids into brain in vivo and into cerebral slices (Crone, 1961, 1965; Fishman, 1964; Le Fevre and Peters, 1966; Gilbert, 1965; Blasberg and Lajtha, 1966; Joanny, Corriol and Hillman, 1969; Joanny, Barbosa, Hillman and Corriol, 1971). Yet, the binding of a substance carried to the carrier would considerably enlarge its size, so it seems highly unlikely that it could aid transport through pores. Although carriers have always been assumed to be located in membranes, such experiments are not evidence that they are, indeed, located there.

Microsomal enzymes are legion, and have been best characterized in liver (Dixon and Webb, 1964). Since the microsomal fraction is said to consist of membranes of both the cell and the endoplasmic reticulum - presumably in different proportions in different preparations - and both membranes appear to be trilaminar and narrow on electron microscopy, they both share accommodation problems for the enzymes.

Transmitters are believed to act by combining with 'specific' receptors. Several of the criteria for receptors, such as saturability, specificity and reversibility, are also met by 'non-specific' binding of non-biological materials (Cuatrecasas, Hollenberg, Chang and Bennett, 1975; Cuatrecasas and Hollenberg, 1975).

The acetylcholine receptor has been isolated from the Torpedo electric organ, and has a molecular weight of between 250 000 and 550000 (O'Brien, Eldefrawl and Eldefrawl, 1972; Moore, Holladay, Purett and Brady, 1974). If other neurotransmitters are of this size there would certainly be great difficulty in accommodating 'specific' receptors to each of them. The problem is gradually being diminished as the older belief in one neuron or one synapse for each transmitter is giving ground to the view that several transmitters, and therefore, presumably their 'specific' receptors, can

coexist in one soma or one synapse. More than one receptor has been isolated for nearly every transmitter so far examined, such as acetylcholine, adrenaline and dopamine. (Vizi and Magyar, 1984).

Three important points are worth making here. Firstly, the properties of apparent 'specificity' of binding by talc, alumina powder, microsilica, glass and cellulose acetate filters by Cuatrecasas and his colleagues (1975) bear a very important lesson - namely that 'specific' receptors are not necessary either as biological or as chemical entities. In other words, any biological mixture with its rich pot-pourri of chemicals and bonds represents a range of attractions to such a diverse population as precursors, hormones and transmitters. Secondly, we are not convinced either that 'specific' receptors exist as structures or as chemical compounds, and so we are not surprised that they are not seen. Thirdly, there is currently no consensus about whether every transmitter has one or more 'specific' receptors.

Virtually all the comments about receptors for transmitters apply equally to drug receptors. Indeed, both types of alleged chemicals are discussed together in pharmacology textbooks. The only difference is that whereas it is conceivable that cell membranes would have 'specific' receptors for neurotransmitters which occur naturally, it is extremely unlikely that they would have 'specific' receptors for any drug to which they may have been exposed for the first time, especially if it is rapidly acting. Furthermore, one must raise the question as to whether any individual really has one or more 'specific' receptors for every single effective drug in any pharmacopoeia past, present, forgotten, future and undiscovered.

The membranes are also supposed to be covered by a rich outcrop of natural antigens and agglutinogens, as well as receptors to bacterial toxins and to viruses, and antibodies to both of the latter. The antibodies for common exanthemata take a period of time of the order of days to be synthesized by the body, and may circulate in the blood as well as remain on the cell surfaces. Their effective lives in circulation vary from the very few days for the viruses for cold or influenza to the years for diphtheria and poliomyelitis.

When one assembles all these compounds which are believed to reside in or near the membrane together (Table 29), one would be led to the expectation that electron microscopy would show the surfaces of membranes to be populated by a rich tundra of mounds of a large range of sizes.

There would be little disagreement that in freeze-fracture-etch scanning electron microscopy, one usually sees an outcrop of knobs of uniform size. Nowadays these are usually attributed to the proteins of the Singer-Nicholson model (Figure 13), but where are all the other presumably protein receptors? Why can they not all be seen on scanning electron microscopy? It should be noted, en passant, that there is no direct or unequivocal evidence identifying these knobs as being due to the structural proteins such as phosphoproteins, glycoproteins or any 'extrinsic' or 'intrinsic' proteins (Guidotti, 1972; Singer, 1974) or any other specific molecules. When the molecular dimensions apparently identify a particular chemical species, the calculations have not usually taken into account the hydration of the chemical and the dehydration during preparation for electron microscopy. However, even assuming that the identification were correct, it would account for only one size of surface molecules of all those

TABLE 29 Large molecules reported to be present within, or attached to, the membranes of mammalian neural tissue

Substance	Tissue	Molecular weight (d) or diameter (nm)	Key references
Proteins			
Phosphatidopeptides	Microsomal fraction of mammalian cerebral myelin		Le Baron and Folch (1956)
Encephalitogenic basic protein	Guinea-pig central nervous system		Kier, Murphy and Alford (1961)
Myelin protein	Rat sciatic nerve	8.5 nm	Adams and Bayliss (1968)
Wolfgram protein	Ox sciatic myelin		Wolfgram (1966); Eng, Chao, Gerstl, Pratt and Tavastjerna (1968)
Proteolipid apoprotein	Ox and guinea-pig cerebral myelin	34 000 d	Thorun and Mehl (1968)
Basic protein	Man, ox, rabbit, guinea-pig cerebral myelin	25 000 d	Thorun and Mehl (1968) Mehl and Halaris (1970)
Proteolipid protein	Man, ox, rabbit, guinea-pig cerebral myelin	35 000 d	Mehl and Halaris (1970)
Rhodopsin	Mammalian retina	7.5 nm x 3.5 nm, 37 000 d	Wu and Stryer (1972) Lewis, Krieg and Kirk (1974)
Periodic-acid Schiff positive protein	Microsomal fraction of rat cerebral myelin	110 000 d	Quarles, Everly and Brady (1973)
S-100	Mammalian nervous system	19 5000 d	Calissano and Bangham (1971)
Enzymes			
NAD nucleotidase	Microsomal fraction of vertebrate brain	36 000 d	Jacobson and Kaplan (1957)
Cholinesterase	Microsomal fraction of vertebrate brain	315 000 d	Aldridge and Johnston (1959)
Arylesterase	Microsomal fraction of vertebrate brain	223 000 d	Aldridge and Johnston (1959)
$NADPH_2$-cytochrome c reductase	Microsomal fraction of vertebrate brain	80 000 d	Guiditta and Strecker (1959)

Substance	Tissue	Molecular weight (d) or diameter (nm)	Key references
Alkaline phosphatase	Microsomal fraction of vertebrate brain	120 000 d	La Bella and Brown (1959)
Alkaline protease	Microsomal fraction of vertebrate brain		La Bella and Brown (1959)
Glutamine synthetase	Microsomal fraction of vertebrate brain	450 000-525,000 d	Sellinger and de Balbian Verster (1962)
Na^+, K^+, Mg^{2+} activated ATPase	Microsomal fraction of ox and guinea-pig brain	360 000 d 259 000 d 250 000 d 8-10 nm	Schwartz, Bachelard and McIlwain (1962) Hosie (1965) Tanaka and Strickland (1965) Maunsbach, Skriver and Jorgensen (1979)
	Synaptosomes of ox brain	280 000 d or 8.5 nm	Uesugi, Dulak, Dixon, Hexum, Dahl Perdue and Hokin (1971)
Ca^{2+} activated ATPase	Microsomal fraction of mammalian brain	102 000 d or 9.0 nm	McLennan, Seeman, Iles and Yip (1971)
Acetylcholinesterase	Microsomal fraction of guinea-pig Rat sciatic nerve	280 000 d	Schwartz, Bachelard and McIlwain (1962) Hosie (1965) Kasa (1971)
Neutral proteinase	Myelin in vertebrate central nervous system		Marks and Lajtha (1963)
Leucine aminopeptidase	Rabbit sciatic nerve Microsomal fraction of rat brain	78 000-80 000 d	Adams, Davison and Gregson (1963) Banik and Davison (1969)
Phosphatidoinositol kinase	Microsomal fraction of vertebrate brain		Colodzin and Kennedy (1964)
ADPase	Rabbit brain		Goldfischer, Essner and Novikoff (1964)
NADH-dehydrogenase	Rat sciatic nerve		Adams (1965)
Peptidase	Myelin of microsomal fraction of mammalian brain	340 000 d	Adams and Davison (1965)
	Human nervous system	340 000 d	Hallpike (1972)
2'3' cyclic nucleotide 3-phosphohydrolase	Microsomal fraction of brain	68 000 d	Kurihara and Tsukada (1967)

Substance	Tissue	Molecular weight (d) or diameter (nm)	Key references
5' nucleotidase	Mouse brain	150 000 d	Scott (1967)
Choline acetylase	Rat sciatic nerve	68 000 d	Adams and Bayliss (1968)
Succinate dehydrogenase	Microsomal fraction of rat brain	97 000 d	Banik and Davison (1969)
Phospho-diesterase	Mammalian brain		Loten and Sneyd (1970)
Inorganic pyrophosphatase	Human nervous system	63 000-120 000 d	Hallpike (1971)
Cholesterol ester hydrolase	Myelin of microsomal fraction of rat brain	110 000 d	Eto and Suzuki (1973) Quarles, Everly and Brady (1973)
Guanylate cyclase	Mammalian brain		Illiano, Tell, Siegel and Cuatrecasas (1973)
Cytochrome c oxidase	Microsomal fraction of rat brain	200 000 d, 6.7 nm	Norton and Poduslo (1973) Grey and Chan (1980)
NADH cytochrome c reductase	Microsomal fraction of rat brain	75 000 d	Norton and Poduslo (1973)
N-acetylglucosaminyl-transferase	Ox brain myelin		Evans (1974) Harford, Waechter, Saul and De Vries (1979)
Mannosylphosphoryl-transferase	Ox brain myelin		Struck and Lennarz (1976) Harford, Waechter, Saul and De Vries (1979)
Adenosine 3'-phosphate, 5' sulphatophosphate-galactosyldiacyl glycerol sulphotransferase	Microsomal fraction of rat brain	440 000 d	Pieringer, Subbha Rao, Mandel and Pieringer (1977)
Glyceraldehyde 3-phosphate dehydrogenase	Microsomal fraction of human brain	6.9 nm	Solomon (1977)
Monophosphoglycerate mutase	Microsomal fraction of human brain	5.0 nm	Solomon (1977)
Phosphoglycerate kinase	Microsomal fraction of human brain	4.76 nm	Solomon (1977)
UDP-galactose-ceramide galactosyltransferase	Rat brain		Constantino-Ceccarini, Castelli and De Vries (1979)

Substance	Tissue	Molecular weight (d) or diameter (nm)	Key references
Cerebroside sulpho-transferase	Microsomal fraction of rat brain		Koul and Jungalwala (1981)
UDP-galactose: ceramide galactosyltransferase	Microsomal fraction of rat brain		Koul and Jungalwala (1981)
Nucleotide			
RNA	Ribosomes of rodent brains	10 000 - 2 000 000 d	Jacob, Samec, Stevenin and Mandel (1967)

The Table does not include receptors for all known transmitters, antibodies, toxins and drugs, as well as those undiscovered, those to be discovered in the future, those forgotten and those buried in the literature. This list could be lengthened in proportion to the time spent on a literature search. Some of the values of molecular weights or dimensions were not necessarily derived from the substance in the same tissues as cited; many are from Dartnall and Klotz (1976). One would expect that in view of the dimensions of the molecules listed, they should be seen by freezing, scanning and transmission electron microscopy as a large outcrop of knobs of a vast range of heights, reflecting the heterogeneity of their dimensions and configurations

listed in Table 29.

The situation is one molecule worse when one considers transmission electron microscopy. There the image of Robertson's trilaminar membrane is ubiquitous around every cell, nucleus and mitochondrion, and in the endoplasmic reticulum, cristae and Golgi membranes. It appears 6-8 nm thick. There is not even space for one protein molecule in addition to the protein layers enclosing the two molecules of lipid of Davson and Danielli's sandwich. Why does one not see Singer's mobile proteins on transmission electron microscopy?

One attempt has been made in the plant literature to explain this difficulty. Muhlethaler, Moor and Szarkowski (1965) concluded that the appearance of Robertson's unit membrane is due to uncoiling and flattening of protein molecules during fixation.

The question should be pressed as to how the vast majority of 'specific' carriers, receptors and antibodies have evaded capture and identification by the biochemists. Probably William of Occam would have explored the simpler hypothesis that one would expect the complex mixture that is biological tissue to exhibit a vast range of affinities for ions, small molecules, precursors, foreign proteins and drugs, to which they had not been previously exposed, without even extra machinery for doing so. We would thus imagine a hierarchy of affinities of the tissue from weak to strong, as follows:

diffusion
affinity for any cellular elements
involvement in biosynthesis
binding to tissues reversibly
binding to tissues irreversibly
having a pharmacological effect
inducing enzymes
producing antibodies.

It should be noted that there is no implication here that the affinity has to be for the cell membrane exclusively or any other organelle or its contents.

It would be important that all these affinities should be measured in vivo, or at least in isolated organs; it would be reasonable to expect that fixation, freezing and homogenization, would each affect all these kinds of affinity, and therefore affinities should only be measured in fresh tissue. In discussion, when I have questioned the belief that all these large molecules are located <u>within</u> the membrane (Table 29), some of my colleagues have conceded that the molecules may not actually be located <u>within</u> its thickness, but rather on the inside or the outside wall of the membrane. This too is unacceptable, for a similar reason as is the Singer-Nicholson model (page 132); if the molecules believed to be on either side of the membrane are each individually large enough to be detected by transmission electron microscopy, they should all be shown up by that technique.

Let us now harvest the crop of hypotheses about cell membranes, and attempt to assess them in relationship to each other.

It seems extremely unlikely that all the molecules listed in Table 29 could coexist

in the membrane together without being visible by electron microscopy. The following are possible explanations for failure to see them there.

1. They are washed off during subcellular fractionation and/or preparation for electron microscopy. This is unlikely because both these techniques are used to prepare, and often to identify, the proteins, enzymes, receptors etc.
2. There are many fewer (or no) such 'specific' proteins in or near the membrane. We believe that this is very likely because the total number of such 'specific' substances already shown, or believed to be capable of demonstration, or capable of being generated, is so large. It is worth recalling that the idea that each transmitter and drug has its own 'specific' receptor, and that it cannot have an action until it has combined with such a receptor, is a general hypothesis which should be tested in every particular case. However, if the binding were unstable, it might be impossible to isolate them and characterize them chemically, although many other unstable compounds, such as adenosine triphosphate or creatine phosphate have been extracted.
3. The localizations of some or all of these molecules on the membrane are wrong. It has been stressed repeatedly (page 48) that the localization of any molecule in a subcellular fraction believed to consist mainly of membranes cannot itself be regarded as certain proof that the particular molecule is in the same site in the intact tissue. The relevant and necessary experiments have not yet been done of the effects of the preparative techniques in subcellular fractionation, histochemistry and electron microscopy on the distribution of molecules (Hillman, 1972, 1983). There seems to be considerable reluctance to carry out these overdue but crucial experiments. Therefore, at present, we should not accept the alleged localizations of these substances listed as being present in the cell membranes.
4. It is likely that the usual thickness of the membrane as measured from electron micrographs is a gross underestimate due to shrinkage. It is possible that the real thickness is several times more in life than it appears from the measurements in red cells by the leptoscope (Gorter and Grendel, 1925; Fricke, 1925; Waugh and Schmitt, 1940), in fresh and fixed peripheral nerves by low angle diffraction and polarized light microscopy (Fernandez-Moran and Finean, 1957; Finean, 1960, 1961; Fernandez-Moran, 1962) and in dehydrated neural tissues by electron microscopy (for references, please see Sunderland, 1976, page 10; Watson, 1976, pages 102-114). If one knew the real thickness of the membrane, one might well then decide if it was, indeed, possible for it to accommodate the cohorts of molecules, which are believed to reside there.
5. It would be possible that only the enzymes and carriers believed to be involved in ion and other transport are lodged in the membrane. If this could be shown by non-disruptive procedures using reagents which one would not expect themselves to alter permeability, one would have advanced a long way on the road to showing that the membrane itself could regulate. In the classical experiments of Baker, Hodgkin and Shaw (1962a,b), they showed that placing squid axons between solutions of potassium sulphate, potassium chloride, sodium sulphate, sodium chloride or sea water, in equal concentrations on either side, produced a potential

difference across the axon of less than 1 millivolt. If the transport system were indeed within the axon, it should have started excluding the sodium ion and concentrating the potassium ion, as does the frog skin. The only reason for it not to have done so is that the prolonged perfusion may have washed the transport enzymes out of the axon wall, or energy supplied by axoplasm is 'required' for the sodium ion pump. It would seem easier to suggest that the biochemical mechanisms which cause the asymmetrical distribution of the cations in life are located in the axoplasm. In experiments, if one washed them out, one then has to provide the chemical gradient to produce the potential difference, or the potential difference to redistribute the ions.

Hillman (1966), in reviewing these experiments, suggested that the effect of the biochemical activity in the axoplasm was to maintain the semipermeability and the excitability of the membrane.

Frog skin is frequently used as a model of active transport of sodium ions (Ussing, 1949). When immersed in water with very low concentrations of these ions, it actively moves these from a low to a high concentration. I am not aware of any such demonstration for squid and cuttlefish axons, although red cells do exclude sodium ions and exclude potassium ions against the apparent electrochemical gradients (Danowski, 1941; Harris, 1941; Flynn and Maizels, 1949; Whittam, 1958). However, these would be in a relatively natural environment in experimental conditions.

6. It is possible that the molecules are indeed present in the cell membrane, but that they do not stain by transmission electron microscopy. Nearly all protein molecules of the molecular weight of dimensions indicated in Table 29 would be within the resolution of the electron microscope, which has, of course, been used for looking at individual molecules (Cold Spring Harbor Symposium, 1972; Hayat, 1973-1977; Wisse, Daems, Molenaar and Van Duijn, 1974; Baumeister, 1978; Baumeister and Vogell, 1980).

If the regulation of the ions is not carried out by the cell membrane itself, it is possible that it is due to the cytoplasm having a high affinity for potassium ions; that is, acting as a cation exchange for sodium ions. This view was put forward as the sorption hypothesis (Shaw, Simon, Johnstone and Holman, 1956; Troshin, 1961; Kurella, 1961; Ling, 1962, 1984).

Conway (1957), citing mainly studies on frog muscle, strongly attacked the latter view on the following grounds:

(a) the virtually complete exchangeability of potassium ions in frog muscle was indicated by influx and efflux measurements in frog muscle (Noonan, Fenn and Haege, 1941; Keynes, 1954) and in rat diaphragm (Calkins, Taylor and Hastings, 1954);

(b) carboxylic exchange resins with fixed anionic charges should prefer potassium ions, as should powdered keratin, but they did not (Conway, 1955);

(c) there was inadequate protein to account for ion binding;

(d) if potassium ions, carnosine, phosphate esters and phosphocreatine are all absorbed on to protein in the cell, how could it be isosmolar with the plasma. It was better explained if they were not;

(e) Ling's hypothesis (1952, 1955), did not explain the Donnan relation of potassium and chloride ions;

(f) the latter did not explain the agreement found between the potential difference across the cell membrane and the potassium ion gradient;

(g) it did not explain the accumulation of potassium and chloride ions when they were increased in the external fluid, nor the water entrance when these ions were accumulated from isotonic mixtures;

(h) Conway (1957) disputed Ling's assertion that $^{24}Na^{+}$ entered fibres freely at $0^{\circ}C$ when the potassium ion concentration was at or above threshold, and that metabolic inhibitors did not affect the high potassium, low sodium ion content of fibres.

Conway (1957) regarded the central fallacy of Ling as being the assumption that the smaller potassium ion could enter a zone of reduced dielectric around fixed ionic charges on protein chains farther than could the sodium ion, and therefore accumulate more in the muscle fibres. He believed that Ling underestimated the radii of the hydrated potassium and sodium ions. He also noted that Ling's calculations of radius of reduced dielectric around the ions were not as accurate as Ling believed.

Glynn (1957) added evidence from red cells against ion binding in the cytoplasm:

(a) the distribution ratios of chloride, bicarbonate and hydroxyl ions were the same on both sides of the membrane, indicating that they would have to be bound to the same extent;

(b) ion binding would explain the high concentration of potassium ions in cells, but not the low sodium ion concentration unless much of the cell water was bound;

(c) freezing point determination showed osmotic equilibrium between red cells and plasma, indicating that major electrolytes must be free in solution;

(d) Fricke and Morse (1925) had found that the specific resistance of the cell interior was 3.5 times that of the serum;

(e) the sorption hypothesis could not explain why sodium ion efflux was increased when potassium ion concentration outside the cell was increased;

(f) haemoglobin did not bind sodium ions;

(g) Hodgkin and Keynes (1953) had found that $^{42}K^{+}$ moved inside squid axons, and Harris (1954) had found that they moved in frog muscle at the same rate as they did in extracellular fluids.

If these findings - mainly from red cells, frog muscle, and squid and cuttlefish axons - are shown to be applicable in details to mammalian neurons, then one would have to conclude that the cytoplasm could not act as a cation exchanger.

However, if they are not, this still leaves two possible mechanisms which could explain the asymmetry of the distribution of ions and small soluble molecules on either side of a membrane.

The first one has already been mentioned (page 219). It is that the biochemical reactions going on in the cytoplasm could determine the nature of the semipermeability of the cell membrane. The permeability would also reflect sooner or later changes in the

chemical constitution, and chemical reactions going on at a particular time, in the syncytium, the mitochondrioplasm and the pars amorpha of the nucleolus.

Secondly, one should not view the idea of the cytoplasm as a cation exchange resin too narrowly. Cytoplasm is a rich mixture of chemical species and bonds - much more complex chemically even than a mixture of a few pure resins. Thus, one can think of the fluxes and concentrations of all the ions and small molecules as being totally interdependent; the concentration of each of them is determined continuously by the complete environment in which it finds itself at that instant.

The two possible mechanisms may coexist, but the first seems more likely because it would react much more quickly, and it is known that action potentials are generated and ion fluxes change rapidly. However, it should be stressed that the extremely significant experiments of Conway on muscle, Glynn on red cells, and Keynes, Hodgkin and Huxley, and all their collaborators on giant axons, show that ionic regulation occurs across cell membranes, and do not provide any evidence that the mechanism itself for the regulation is located within the cell membrane.

The pioneer work on thin films by Bangham and his colleagues, and on artificial membranes by Mueller and Rudin, have indicated the properties which may be found in artificial membranes, and may be common to biological membranes (Bangham and Horne, 1964; Mueller and Rudin, 1968; Chang, 1972; Finkelstein, 1978). The electrical properties of mixtures of natural lipids and proteins - to which antibiotics have sometimes been added - have been found to be remarkably similar to those of cell membranes examined with intracellular microelectrodes (for comparison, please see Henn and Thompson, 1969). The only constant and significant difference found was that the natural membranes had higher resistances, by two or three orders, than did the artificial membranes. Such elegant experiments clearly demonstrate what a rich variety of behaviour such 'simple' membranes have, and they should act as a constant reminder of what chemistry can do without the aid of biology.

THE EXTRACELLULAR SPACE

The extracellular fluid of the central nervous system was first measured by the use of markers, such as Na^+, Cl^-, thiocyanate, sucrose, sulphate, inulin and ruthenium red. The use of such markers implied the assumptions that they do not enter the cells, that they do not react with the tissue, that they are not broken down, that they themselves do not affect the size of the extracellular space, that they are recovered rapidly and completely and that they themselves do not affect the biochemistry of the tissue. Na^+ and Cl^- are present inside cells; the uptake of thiocyanate depends upon the plasma level (Lajtha, 1957; Streicher, 1961). Inulin drains away into the cerebrospinal fluid (Woodward, Reed and Woodbury, 1967); it leaves tissue very slowly (Varon and McIlwain, 1961), as does radiosulphate (Woodbury, Timiras, Koch and Ballard, 1956); the efflux of inulin can be resolved into three kinetic curves, only one of which is likely to be due to passage through the extracellular space (Lund-Andersen, 1974; Lund-Andersen and Kjeldsen, 1976). Of the assumptions listed for all these markers, the beliefs that they remain extracellular and that they do not react with tissue, are both quite crucial and have rarely, if ever, been tested. The inulin concentration in the brain of a dog after it had been perfused, went up after

death (Rall, Oppelt and Patlak, 1962). The values of the spaces measured by these markers were from 2% to 40% in the brains of mammals under various conditions (Table 30).

Several authors have measured similar spaces in cerebral slices (Allen, 1955; Davson and Spaziani, 1959; Pappius and Elliott, 1956; Bourke and Tower, 1966a,b; Joanny and Hillman, 1963, 1964; Schousboe and Hertz, 1971; Cohen, 1974a,b; Lund-Andersen, 1974; Rodnight, 1975; Lund-Andersen and Kjeldsen, 1976). Unfortunately, the use of these preparations in vitro implies all the assumptions about the markers listed above. It is also complicated by the increase of weight of slices on incubation, which was usually 15-40% in the experiments of the authors cited above. This increase consisted of 'adherent' fluid and swelling fluid. The exact value of the change of volume depends upon whether initially the slice had been cut dry and weighed, or had been temporarily immersed in a Petri dish of cold incubating medium before weighing. It also depends upon the length of time for which adherent fluid is wiped off the slice (for review, please see Hillman and Wraae, 1981). For these reasons, measurements of extracellular space by markers are even less reliable in vitro than they are in the whole animal.

Wyckoff and Young (1954, 1956) were the first to try to assess the extracellular space by electron microscopy. They perfused spinal cords with osmium tetroxide, and concluded that there was no large extracellular space. This question was subsequently taken up by many groups including those around Luse, Gray, Horstmann, Palay, Sjostrand and van Harreveld (for reviews see van Harreveld, 1966, pages 127-158; Johnston and Roots, 1972, pages 76-92). They all saw the extracellular spaces of 10-25 nm wide, which could generally be calculated to be less than 10% of the volume. The actual values found depend upon: the species, the tissue, the age of the animal, the fixative used and its osmolarity, the pressure and temperature of the fixative, whether the tissue was frozen rapidly or slowly, and assumptions about the areas of the cell membranes and the likely numbers of cells in the tissue. However, in their calculations, authors have not taken into account the shrinkage of tissue during preparation for electron microscopy (Boyde, Bailey, Jones and Tamarin, 1977; Sleytr and Robards, 1977a,b; 1981), probably because they had no accurate way of doing so. Furthermore, in examining a particular minute sample of the central nervous system composed of cells of irregular shapes, assessments of the total areas of the membranes in that region - and, a fortiori, in the whole nervous system - are extremely difficult.

The volume of the extracellular space has also been calculated from impedance measurements, mainly by van Harreveld and his school (1966) (please see page 186).

Regrettably, one has to conclude that there is at the moment no satisfactory way of measuring the extracellular space. In order to do so one would have to find a marker, which would have to be shown minimally, to remain extracellular, not to react with tissues, which could be extracted completely from them, and whose measurement would be unaffected by the presence of the tissue.

However, the conclusion here that most of the central nervous system is a syncytium largely circumvents the problems of the extracellular space. The fluid bathing

TABLE 30 Early measurements of the extracellular space in intact animals by markers

Marker	Author	Percentage
Na^+, Cl^-	Manery and Hastings (1939)	33-40
$^{35}SO_4$	Woodbury, Timiras, Koch and Ballard (1956)	4-5
Cl^-	Woodbury, Timiras, Koch and Ballard (1956)	25
Inulin	Morrison (1959)	2
$^{35}SO_4$	Barlow, Domek, Goldberg and Roth (1961)	2-5
Thiocyanate	Streicher (1961)	5
Inulin	Rall, Oppelt and Patlak (1962)	12
$^{35}SO_4$	Kibler, O'Neill and Robin (1964)	2-6
^{14}C, sucrose, inulin and thiocyanate	Bourke, Greenberg and Tower (1965)	19-40
Inulin	Vernadakis and Woodbury (1965)	5
^{131}Iodine	Davson (1967)	20
Sucrose	Oldendorf and Davson (1967)	10

the neurons and the naked nuclei is the syncytial jelly, which acts as the extracellular environment of the neurons, the naked nuclei, the axons, the fibres, the capillary cells and the ependymal cells. However it is, in fact, an intracellular fluid. If we now go back to Virchow's concept of the neuroglia as a nerve-glue, we may regard the neuron-glial relationship in an old light, illuminated with the new attitude. The all-pervasive large volume of the syncytium may be seen as acting as a barrier between the ventricles and the capillaries on the one hand, and the neurons and naked nuclei on the other. Thus the blood-brain barrier is located partly in the syncytium and partly in the choroid plexus.

It has been repeatedly stressed that histological and electron microscopic techniques cannot be used for measuring the volumes of any tissues, which are composed in life of 60-80% water. Therefore, although our histological measurements indicate that most of the volume of the central nervous system is occupied by the syncytium, they can not give us precise assessment of the relative volumes of the syncytium, the neurons, the naked nuclei, the fibres, the ependymal cells, and the capillary cells. At present there appears to be no way of measuring any of these volumes in living people or animals, although computed axial tomography and ultrasound can measure total tissue and ventricular volumes in the living intact nervous system.

CEREBRAL OEDEMA

In the living patient or animal a measured rise of intraventricular pressure is the clearest evidence of cerebral oedema; the pressure of the cerebrospinal fluid may also rise, and papilloedema may also be seen in the fundi. Cerebral oedema may be observed at operation or at post mortem. At a cellular level it must be very hazardous to diagnose oedema in either histological or electron microscopic sections, since oedema represents the state of hydration, and embedded sections have been completely dehydrated. However, chronic oedema has been produced by tumours (Williams and Lennox, 1939; Chou, 1961; Aleu, Edelman, Katzman and Scheinberg, 1964), by cold (Hass and Taylor, 1953; Klatzo, Piraux and Laskowski, 1958; Clasen, Cooke, Tyler and Pandolfi, 1960; Bakay and Hague, 1964; Blinderman and Markham, 1965), poisoning by triethyl-tin (Torack, Terry and Zimmerman, 1959; Aleu, Katzman and Terry, 1963), and several other pathological and experimental agents. Studies of swelling in the brain by electron microscopy show that the main swelling appears to occur in the astrocytes (see, for example, Gerschenfield, Wald, Zadunaisky and de Robertis, 1959; Torack, Terry and Zimmerman, 1959, 1960; Luse, 1961). Our view of the astrocytes is that their nuclei are naked nuclei, and their feet are syncytial cytoplasm (page 194). If the location of oedema in the brain is truly in the 'astrocytes', we would place it in the syncytium.

It is currently believed that membranes regulate the ions inside cells, not only by their selective permeability but by 'active transport'. Living neurons in vivo and in vitro maintain resting transmembrane potential differences, which can be altered by changing the K^+, oxygen tension and substrate, in the medium bathing them (for reviews, see Eccles, 1957; Hillman and Hyden, 1965). Since a syncytium would contain

no barriers in its cytoplasm, it could not easily regulate the electrolytes between one part and another. Furthermore, all agents would act on the syncytium before they reached the neurons. Therefore, oedema would be more likely to affect the syncytium before it became manifest in the neurons.

Nevertheless, it is confidently expected that in the future non-invasive radiographic techniques will indicate in what elements of the central nervous system oedema occurs in living intact animals. It may well be that oedema causes the syncytium to take up less stain than healthy tissue does. This is a plausible alternative explanation to the current view that astrocytes take up the oedema fluid - a finding which one might have expected the light microscopists to have detected before or since the electron microscopists did. Our explanation for the failure of the light microscopists to do so is firstly, that the astrocytes are difficult to identify by histological techniques, and secondly, the two kinds of microscopists are both looking at different structures when they use the term 'astrocyte'.

BLOOD-BRAIN BARRIER

There has been a long controversy as to whether the plasma or the cerebrospinal fluid is the extracellular fluid of the nervous system (van Harreveld, 1966; Davson, 1967). The identification of the syncytium as the immediate environment of the neurons diminishes the power of the controversy. Substances may travel across the blood to the neurons by two routes: either the substances in the blood in the cerebral and spinal capillaries pass through the syncytium to the neurons, or they proceed to the choroid plexus to the ventricles then cross the ependyma to the syncytium, and then reach the neurons. Obviously, the first is much quicker, and is probably the route taken by rapidly acting intravenous anaesthetics, by volatile anaesthetics, indeed by all intravenously administered drugs which act on the central nervous system soon after the few seconds that the circulation brings it to its site of action. Both of the two routes would constitute a blood-brain barrier, although the slower one is traditionally thought of as the 'blood-brain barrier' that elapses between the arrival of the substances in the blood and their penetration into the brain.

THE SYNCYTIUM AND THE EXTRACELLULAR FLUID

We have concluded that the neuroglia is a syncytium in the sense of Virchow (1854, 1871). This facilitates the understanding or explanation of several other phenomena:

1. The longstanding problem of whether the blood or the cerebrospinal fluid is the effective extracellular fluid of neurons is resolved, if the neuroglial syncytium is the environment of neurons throughout the central nervous system.
2. The tissue between neurons and microglia (naked nuclei) is very resistant to stains used in neurohistology.
3. Reactive glia (naked nuclei) migrate very rapidly to sites of injury because they do not have to pass between a solid phalanx of cells.
4. The relative rapidity of spread of undifferentiated gliomas, which are also very malignant. They are naked nuclei and can migrate without difficulty through the frontier-less syncytium.

5. Sherrington (1925) proposed that the interaction of reflex pathways and the long duration of central excitation could best be explained by the concept of a neuronal pool; this was involved in occlusion and spatial summation. The central excitatory and inhibitory states represented a prolonged residue in the central nervous system resulting from the much briefer excitatory and inhibitory stimuli. I would suggest that the stimulus engenders 'slow' changes in the chemistry of the syncytium, which could be the site of the central excitatory and inhibitory states in the subliminal pool. This would provide a very adequate explanation for the neurophysiological phenomena.
6. The finding that the intellect and neurophysiological function of some patients with advanced hydrocephalus are not necessarily impaired (Lorber, 1980; Lorber and Priestly, 1981) would be understandable if 80-90% of the brain is a syncytial jelly, and the neurons are not connected by synapses. The hydrocephalus compresses the brain, but the neurons would be displaced (Figure 82). This suggestion assumes that thought takes place in the cerebral cortex; which seems very likely, although it has not been proved conclusively. Of course, there is abundant evidence that many neurophysiological functions reside there.

THE VESICLE HYPOTHESIS OF TRANSMISSION

From the early ideas of transmitters and some seminal experiments of Fatt and Katz (1952) on frog nerve-muscle preparations, del Castillo and Katz (1956) proposed the vesicle hypothesis for neuromuscular transmission (for summary, please see Katz, 1969). It is interesting to note that in the latter publication 13 years after the original concept, he wrote 'I should like to introduce a working hypothesis which my colleagues and I have found useful in planning our experiments. We assume that each unit packet of acetylcholine (whose release produces a miniature potential) is pre-formed within a synaptic vesicle in the nerve terminal. The vesicle may be supposed to accumulate the transmitter substance actively.'

This short section contains the following hypotheses: (a) the synaptic vesicles exist in life; (b) each contains acetylcholine; (c) each contains approximately equal quantities of acetylcholine. Item (a) cannot be proved; (b) has not been shown; (c) indirect evidence compatible with it has been brought forward, but there is no direct way of showing it.

This hypothesis has gradually been accepted as being applicable to central nervous system synapses, and also as being substantial supportive evidence for interpreting electron microscopic findings about synapses and synaptic vesicles. The criteria for transmitters have been enumerated frequently (see, for example, Hillarp, Fuxe and Dahlstrom, 1966; Werman, 1966; Barchas, Akil, Elliott, Holman and Watson, 1978).

The modern concept of transmission has been synthesized by combing evidence from the physiology of the neuromuscular junction and of the synapse, the pharmacology of the transmitters and the junctional tissues, the electron microscopy of neuromuscular junctions and synapses, and the electron microscopy and biochemistry of synaptosomes.

The large accumulation of data of varying degrees of cogency which each appear to reinforce the overall view could be dangerous if any large component contributing to

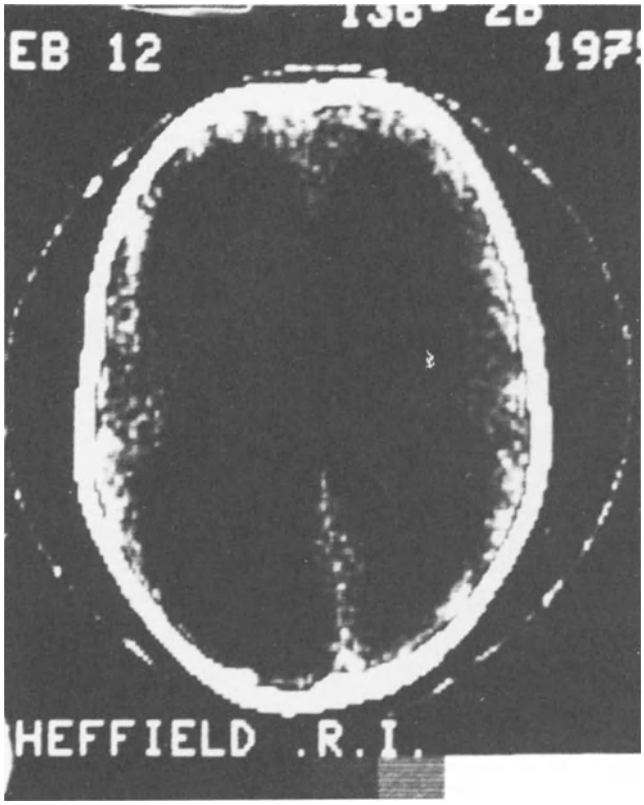


FIGURE 82 Computed axial tomogram of a young man with an intellitence quotient of 126, who had a first-class honours degree in mathematics, economics and computer studies. It shows gross hydrocephalus and an extremely narrow cerebral cortex. Published in World Medicine (1980) 15, page 21, and reproduced by kind permission of Professor John Lorber of Sheffield Royal Infirmary and the publishers

the resulting consensus should not be firmly based.

The vesicle hypothesis has been seriously questioned. Marchbanks (1975, 1978) has pointed out that synaptic clefts are rarely seen fused with the axolemma, as the hypothesis would require; depletion by prolonged stimulation is difficult to produce; the acetylcholine has not been shown to be present exclusively in the vesicles; newly synthesized acetylcholine is preferentially released before the 'stored' acetylcholine; the specific activity of the whole preparation is different from that of the isolated vesicles; it has been estimated that a 'quantum' of acetylcholine is seven times the quantity estimated to be present in the vesicle; isolated synaptic vesicles exchange acetylcholine slowly.

The incorporation of the synaptic vesicle into the pre-synaptic membrane is crucial to the vesicle hypothesis. Preparations of fractions of what are believed by the authors to be the two membranes, and examination of their chemistry, has revealed gross differences between the preparations with respect to lipids, ATPases, acetylcholinesterase, ganglioside and cholesterol, and turnover of proteins (Eichberg, Whittaker and Dawson, 1964; Hosie, 1965; Whittaker, 1970; Rodriguez de Lores Arnaiz, Alberici de Canal and De Robertis, 1971). One has to be extremely cautious in

comparing the chemistry of any subcellular fractions when they have been prepared using grossly different reagents and physical conditions, but the differences between these two fractions seem to be so gross that it is unlikely to be explicable solely on these grounds.

In addition, attempts to demonstrate that 'vesicle depletion' occurs by 'stimulating' preparations using potassium chloride, electrical stimulation and bungarotoxin, have been made in cat brain and superior cervical ganglion, rat superior cervical ganglion, rat and mouse neuromuscular junction, frog brain and frog neuromuscular junction. Results have varied from no change to complete depletion; sometimes the depletion has been accompanied by mitochondrial changes, sometimes the vesicles have become more or less coated, and often the changes have not been reversible (for review see Ceccarelli and Hurlbut, 1980).

Further implications of the vesicle hypothesis, as summarized by Katz (1969) do not seem to have been recognized. Firstly, no-one has shown directly that acetylcholine is present in the synaptic vesicles in vivo or in vitro, and it is difficult to conceive of experiments which could demonstrate the proposed location. Secondly, the vesicles are said to discharge the acetylcholine only when they happen to hit the pre-synaptic membrane, but not any other part of the inner membrane of the synapse. This implies that the following minimum number of events occur:

1. The vesicle hits the pre-synaptic membrane.
2. The vesicle differentiates between the inner surface of the pre-synaptic thickening and other parts of the inner surface of the membrane of the synapse; that is, the chemicals of the membrane around the synaptic vesicle must react chemically or electrostatically with constituents of the inner surface of the pre-synaptic membrane. Therefore the chemistry of the inner surface of the pre-synaptic and of the rest of the inner surface of the synaptic membrane must be different.
3. The vesicle releases the transmitter.
4. The transmitter reacts with the inner surface of the pre-synaptic membrane.
5. The membrane of the synaptic vesicle dissolves in the axoplasm or loses its ability to take up stain or is incorporated into the pre-synaptic thickening. Any of these events would occur in circumstances in which synaptic 'depletion' occurs. However, if the synaptic vesicles are believed to lose acetylcholine, which does not itself show up in electron micrographs, then the ejection of transmitter from the synaptic vesicles would <u>not</u> be expected to result in any change in <u>number</u> of vesicles, but would result either in them appearing at high magnification to have holes on their pre-synaptic aspects or to be part of 'exocytotic vesicles' in the pre-synaptic membrane. The disingenuous suggestion has been made that the synaptic vesicles pass through the pre-synaptic membrane so rapidly that they are not seen (Heuser and Reese, 1973; references given by Jones, 1975, page 192, not definitely agreed by him). Some workers have claimed to see 'pores' in the pre-synaptic membranes (Akert, Pfenninger, Sandri and Moor, 1972; Streit, Akert, Sandri, Livingstone and Moor, 1972). Others rather rarely see exocytotic vesicles (Andres, 1964; Westrum, 1965). I believe that they arise from the proximity of any

two granules at the time of deposition of the heavy metal, which is used to stain. The deposit will not fill in the regions at which the two bodies are in contact and will appear to surround them both.

The transmitter would have to activate an extraordinarily rapid and powerful mechanism - presumably enzymatic - which could render the thick insoluble pre-synaptic membrane (consisting as they believe of protein and lipid), highly permeable to the transmitter, and very soon after that to 'switch off' that permeability. It is a central supposition of the vesicle hypothesis that the transmitter is 'packaged' in the vesicles. If the calculations and assumptions which show that there is, on average, seven times as much acetylcholine released in a quantum as could be contained in a single vesicle are true, then the majority of that transmitter must be located outside the vesicles (Marchbanks, 1978). The calculations and assumptions need to be examined critically.

6. The discharge of the transmitter would induce a change in the membrane which would cause a miniature end-plate potential. (It should be noted that, although a strong case has been made for a statistical relationship between excitation and the incidence of these miniature potentials, the proposition that the discharge of transmitter causes or is caused by the electrical transient remains a hypothesis, which is difficult to prove.) The proposed mechanism is believed to be occurring in living tissues, while the evidence is sought in metal deposits subjected to high vacuum, electron bombardment and radiation.

   It should also be noted that the original relationships between the transmitter, the excitation and the miniature end-plate potentials in their physiological, pharmacological and biochemical aspects have been shown for acetylcholine and neuromuscular junctions. The evidence is rather sparse for any other transmitter or for the central nervous system. Most of the substances which have been claimed to be transmitters (Table 27) cause excitation or inhibition in neurons at low concentration, and other substances (mostly not naturally occurring) either increase or decrease the thresholds of excitation.

7. New synaptic vesicles would have to be made in the synapses, or they would become stainable again, or they would have to come from the cell body, or they would have to 'cycle' back from the synaptic cleft. When 'vesicles' are seen on either side of the synaptic membranes, and one calls them 'exocytotic' or 'endocytotic', there is no way of finding out in which direction they would have been moving if they were moving in life.
8. New transmitter has to be synthesized in the synaptic ending.
9. The transmitter has to be 'packaged', that is passed into the vesicle across the 'membrane' of the synaptic vesicle.

Considerable doubt about any calculations of the quantity of transmitter in the synaptic vesicles or any calculations based on them, or indeed the very existence in life of synaptic vesicles, must be entertained since the number of vesicles seen apparently depends upon the osmolarity of the buffer used in fixation (Bodian, 1970; Valdivia, 1971). (While these findings are of great significance empirically, it is difficult to know what role the buffering capacity can play, since the pH of the system

cannot be considered relevant if the tissue is to be dehydrated and impregnated with non-aqueous substances.) It has been shown that the pre- and post-synaptic thickenings are artifacts (please see page 145). A much simpler hypothesis has been proffered for the spread of excitation (please see page 197).

A GENERAL THEORY OF RECEPTORS

It is extremely unlikely that, in life, receptors for most transmitters, hormones, drugs, toxins and antibodies are found on the outside, within the 'trilaminar' membrane, or just on the inside of the cell membrane, for these reasons.

1. The cell membrane is probably solid and not trilaminar (Hillman and Sartory, 1980, page 37).
2. Many receptors for transmitters and drugs have been isolated, their molecular weights have been measured and they are physically large enough to be seen on the membranes by electron microscopy, but they are not seen by transmission electron microscopy at all; by scanning electron microscopy only, the popuation of protrusions from the membrane of uniform height could not possibly represent the rich repertoire of receptors of different molecular weights, dimensions and shapes claimed to be located there (page 210). If they cannot be seen because they do not stain with reagents used during the preparation for electron microscopy, then unstained pits or gaps should appear outside, within, or inside the membrane. These are not seen on scanning or transmission electron microscopy.

   If one were to suppose that they were present, although they could not be seen, one would have to make the additional assumption that they had been lost during the preparation for electron microscopy. It would also be dangerous to accept the validity of the current hypothesis about receptors on the basis of such an assumption which has not been proved, and may be both unprovable and unfalsifiable.
3. It is extremely unlikely that a membrane supposed to be 7-10 nm thick could contain not merely all these receptors, but also all the carriers for all the substances which are believed to be taken up by cells by 'active' transport and by facilitated diffusion. There is plenty of evidence that sodium ions, potassium ions, calcium ions, glucose and amino acids, among others, are in continual flux between the outside and the inside of the cell. The multiple biochemical reagents believed to be involved in this flux are large molecules, and are believed to be located in or near the membrane. However, if the current belief about membrane transport were correct, the gradients of ions and other molecules would have to be maintained continuously, not merely in the region of the membrane, but also in the whole bulk of the cytoplasm. This would have to apply to most cells, including neurons of mammalian cranial nuclei, Mauthner neurons of goldfish, supramedullary neurons of puffer fish, and squid giant axons. It would be generally agreed that the cytoplasm has a huge volume relative to that of the whole cell membrane, especially if one accepts the view that the trilaminar membrane is, indeed, only 7-10 nm thick. This consideration makes the sorption hypothesis of Ling much more likely than the membrane theory (please see Ling, 1984).

4. The fact that the addition of transmitters and many amino acids to the outside of the cell causes many neurons to be excited or inhibited, does not prove that the effect is produced in the membrane itself. It is known that these substances alter the excitability of the cells, but this could be due to any or all of the following: reaction with substances in the extracellular fluid; effects on the electrical properties of the membranes; effects on the chemical properties of the membranes; effects on the permeability of the membranes; alteration of gradients or fluxes across the membranes; effects on the cytoplasm. Although the effects of iontophoretic applications are fairly rapid, in my opinion, the temporal resolution of measuring systems currently in use is not sufficiently accurate to distinguish between which of these measurements contribute to the increase or decrease of activity, and to what extent each does. It is also highly likely that several of these effects would be interreactive, for example, that the biochemistry of the cytoplasm at any particular moment influences the properties of the cell membrane (Hillman, 1966b). Some of these possibilities could be explored in mammalian neural tissue, if one could obtain a pure neuronal membrane which one could be sure did not leak (Cummins and Hyden, 1962), or if one employed a patch clamp on such a preparation.
5. It is unlikely that rapidly acting drugs could generate receptors. If one accepts that all the receptors - if they occur in the profusion which modern pharmacologists and physiologists would have them - are unlikely to be housed comfortably in or around the membrane, one can then re-examine the concept of receptors in the nervous system. (Although this monograph is concerned only with the mammalian nervous system, the same considerations would apply to non-mammalian species, and also to tissues other than nervous systems, in which receptors are believed to play a role.)

I am not asserting that receptors may not occur on cell membranes, but that transmitters, hormones, drugs and toxins may act anywhere between the site of administration and the innermost sancta of the nervous system - the nucleolonema. We may share the common belief that drugs act near neuronal membranes. However, before they arrive there, they travel by a route with many obligatory and request stops.

1. Site of injection to plasma.
2. Plasma to plasma proteins, platelets, red or white cell membranes.
3. Blood cell membranes to their cytoplasm and organelles.
4. These membranes to capillary membranes.

5a. Capillary membranes to the cytoplasm and organelles of the capillary cells.

6a. Capillary cell membranes to the syncytium.

7. Syncytium to membranes of neurons or naked nuclei. Alternatively, they could go from 3 to 5b the membranes of the choroid plexus.

5b. Membranes of the choroid epithelium to their cytoplasm and organelles.

6b. Membranes of the cells of the choroid plexus to the cerebrospinal fluid.

7b. Cerebrospinal fluid to lymphocyte membranes and to proteins and other constituents of cerebrospinal fluid.

8b. Lymphocyte membranes to lymphocyte cytoplasm and organelles.
9b. The latter to membranes of the ependymal cells.
10b. The membranes to the cytoplasm and organelles of the ependymal cells.
11b. The membranes of the ependymal cells to the syncytium.
12b. Then becomes identical with 7.
8a. Neuronal membranes to cytoplasm, nuclear and mitochondrial membranes.
9a. Mitochondrial membranes to mitochondrioplasm and nuclear membranes to nucleoplasm.
10. Neuronal nucleoplasm to nucleolar membrane.
11. Nucleolar membranes to nucleolonema or pars amorpha.
Here the journey ends.

Obviously, the concentration at which any transmitter, hormone, drug or toxin arrives at any of the sites listed depends upon the following factors: its initial chemistry; its route of administration; the vehicle; the rate of administration; its affinity and reversibility of binding with each site; the precise chemistry of each site determined by the particular physiology and health of the organism reflected at that particular site, including agents which react with or degrade it enzymatically and non-enzymatically; similar properties of any conjugated or degradation products of the original substance reacting with any of these sites.

After the transmitter, hormone, drug or toxin arrives at each of these sites, it may affect the biochemical reactions of a pathway or cycle, or the electrical properties at that site, although at these molecular dimensions a distinction between biochemical and electrical properties may be purely academic.

Incidentally, I have always regarded the debate about whether learning and memory are electrical or biochemical properties of the central nervous system in rather the same light. It seems to me to be far too premature in the current state of knowledge about these phenomena even to pose this question. Any attempt to answer it would be tantamount to trying to forecast several decades or centuries ahead whether an electrophysiologist would be a more or less likely person than a molecular biochemist to unravel this particular mystery.

The multiplicity of sites and variables involved in the effects of transmitters, hormones, drugs and toxins on the central nervous system, makes it difficult to arrive at firm conclusions about these reactions, but it is worth trying to derive a few generalizations.

Firstly, anaesthetics and transmitters, such as thiopentone, nitrous oxide, adrenalin and curare, which act after delays hardly longer than the circulation time, probably act on sites near the beginning of the journey, the capillaries or the syncytium. Their effects may be on the local haemodynamics within the capillaries or on the chemistry of the syncytium, possibly its mitochondria. Since generally drugs administered intravenously react very rapidly, it follows that the capillaries and the syncytium are the first targets of those drugs, and their reactions to them merit investigation. It also follows that most of the delay of the action of drugs administered by routes other than intravenously is due to their failure to achieve sufficient concentrations in the nervous system to produce effects; this delay would be due to their diffusion from the

site of administration, their breakdown in the tissues, and their reaction en route with some elements of the tissue. The slower action of some drugs, when they are administered by mouth, intramuscularly or rectally, is either for the latter reasons, or because they take time to accumulate in sufficient concentrations in the syncytium, or because they reach the neurons via the choroid plexus. Since the blood-brain barrier is identified essentially by the delay of passage of substances between these two compartments (page 225), we must couple the syncytium and the choroid plexus as the two likely sites of this barrier.

Secondly, the fact that the addition of transmitters and some drugs to the outside of cells, results in changes in their excitability makes it possible, even likely, that the cell membranes are near the site of action, but does not prove conclusively that they are themselves, since the cells are so small that most of the substances added extracellularly could have penetrated to the cytoplasm within the time course that excitation or inhibition is seen to take place. On pages 231-232 we have already listed the other possible sites of action, which are not necessarily exclusive. It is an unprovable assumption that, for example, because any substance affects the permeability of a cell membrane, it might not also affect the biochemistry of the cytoplasm.

Thirdly, in contrasting the relative slowness of action, tens of minutes to hours, of many drugs in crossing the blood-brain barrier with their rapidity of action when injected intravenously, seconds to minutes (for review, see Davson, 1967, pages 82-92; Rapoport, 1976), I think one can conclude that many substances apparently act more quickly than studies of their passage across the blood-brain barrier would lead one to expect. This points to regions of high affinity, which may be too localized to detect in kinetic and dynamic curves of whole brain or spinal cord, and whose measurements may well be modified by the techniques used to make them, such as homogenization and extraction with powerful reagents.

Fourthly, substances such as thiourea, sulphaguanidine, sulphosalicylic acid, N-acetyl-4-amino-antipyrine, mecamylamine, barbital, and even Na^+ ions take tens of minutes to hours to reach a steady state in the cerebrospinal fluid (Brodie, Kurz and Schanker, 1960; Rall, Moore, Taylor and Zubrod, 1961; Davson and Pollay, 1963; Davson, 1967, page 67). On the one hand, intravenously injected thiopentone anaesthetizes patients usually within 10 seconds, and glucose takes slightly longer to revive hypoglycaemics from coma. On the other hand, passage from blood to the cerebrospinal fluid is obviously a slower process. Therefore, one may tentatively put forward the hypothesis that the rapid therapeutic effect is due to the substances taking the cerebral capillary-syncytial-neuronal route, while the slow passage across the blood-brain barrier travels via the choroid plexus-cerebrospinal circulation-ependymal-syncytial-neuronal route. The findings that many substances seem to accumulate in the brain before they do in the cerebrospinal fluid, and at higher concentrations (Davson, 1955; Rudolph and Olsen, 1956; Mayer, Maickel and Brodie, 1959; Wraae, 1980), leaves open the possibility that the substances normally travel from the blood into the brain, and from there into the cerebrospinal fluid, which is compatible with their taking the syncytial route.

It is difficult to pin-point particular sites, which play a more important role in the

uptake of substances into the nervous system, and maintain them on the basis of experiments which have not yet been carried out. However, on empirical grounds, we may suggest four of the list given on page 231. These would be the capillary membranes of the cerebral vessels and of the choroidal plexus, since these cells would be the first barriers from the blood. Then the next must be the syncytium, because it represents such a large volume, which molecules and ions must react with or pass through. The neuron with its membrane would be the fourth site, since iontophoretic and electrophysiological experiments show it to be so excitable. However, one must also bear in mind that the entry of particular substances into the neuronal cytoplasm would depend principally on their affinity for the syncytium, before they arrive at the membranes, and then on the permeability of membranes to them.

So far, the term 'receptor' has hardly been mentioned. I now wish to broaden the concept, while at the same time suggesting a solution to the accommodation problem for these apparently ubiquitous, populous, yet extraordinarily elusive, biochemical entities. Instead of regarding a particular neural receptor as being located exclusively on the surface of a neuronal membrane, we may regard it as the total of all the sites listed, which react with, or significantly delay the uptake, transport or action, of the corresponding transmitter, hormone, drug or toxin. The measured affinity of the particular substance for the receptor would be represented by the aggregate of the affinities for all of the main sites, depending upon the relative quantities of each. The delay before the substance acts would be due to the sum of the durations of all the reactions, both reversible and irreversible, which occur between administration and action.

If this hypothesis is correct, it would behove us to attempt to identify the reactions and delays at each of the component sites listed to see if we could thereby account for the total real delay both between the administration and arrival of any substance at its site of action, and also between the administration and the response of the tissue. For example, the rate of uptake and affinity of the components of blood for such a substance could be measured. The rate of passage through the choroid plexus of some substances is known. Neuroglial clumps could be used to measure approximately the rate of uptake and the affinity of the substance for the cerebral syncytium. Tracts from the spinal cord could be used to measure the same parameters.

In noting that the overall affinity of the substance for the nervous tissue is normally measured in homogenates, we must again draw attention to the total failure so far of those who study receptors to test the effect of homogenization on the affinity of substances for tissue or its effect on the rates of reactions within it. The truth of the supposition that the delay between administration and action of a substance is due to its reaction with different sites will only be demonstrated by showing that homogenization does not alter affinities of reactants or their rates significantly, that is, does not change the apparent number of receptors in the tissue under study. If homogenization does change them, the relevant corrections will have to be made.

If the view proposed here were accepted, one would explain the identification and extraction of receptors, for example, for acetylcholine, dopamine and noradrenalin, as extraction of the complex between these transmitters and the particular chemical components of the tissue with which they bind. However, the degree of binding could

depend grossly on the method of separation of the receptors, including the homogenization, addition of detergents, etc. These agents could increase the degree of binding, induce an unnatural binding which was not a result of the original reaction of substance bound and the receptor, weaken the binding, reverse it or bring other molecules into contact with the reaction, which would themselves modify the binding. Once more, one must urge that the control experiments to test the effects of powerful agents on the system under study are grossly overdue.

Obviously, the more active transmitters, hormones, drugs, antigens and toxins, have the greatest affinity for a particular site. Furthermore, of course, by spreading the receptors throughout the tissue, the hypothesis proposed goes some way to solving the accommodation problems of the large number of substances which act on the nervous system. Despite this, I think that one should utter two cautions. Firstly, the idea that each of the latter agents has its own individual complement of one or more receptors is a so far unproven proportion for the vast majority of substances in the above categories. Secondly, even the suggestion made here, that proposes that receptors may be distributed throughout a much broader range of sites, does not solve the accommodation problems completely for such a large category of different reagents. I would suggest that hundreds or even thousands of these agents could share one receptor. The alternative suggestion is that the reagents themselves could rapidly induce their own receptors. This view would not be exceptional to many in the field. It seems to be totally acceptable in respect of those antigens which take days to induce antibodies, but it seems to me to be very unlikely to occur when a tissue is subjected to an unnatural agent, which it has never encountered before, and which acts within a very few seconds or minutes.

DOES NEUROGLIA HAVE A HIGH SODIUM ION CONTENT?

When experimentally a micropipette is driven into brain or spinal cord in vivo or in vitro, no significant sustained voltage is recorded as one passes from the overlying medium into the bulk of the nervous tissue, until the sudden appearance of a direct current voltage of 60-80 mV, which is believed to signal penetration of a cell. Thus the syncytium would seem to be at the same voltage as the extracellular fluid, and itself to be of high conductivity. Extracellular fluids and incubating media normally contain high concentrations of sodium salts (142-152 mmol/l), which suggests that the syncytium is at a similar electrochemical potential.

Katzman (1961) proposed that neuroglial cells had high sodium ion contents on the basis of: firstly, the excess sodium ions in the whole brain; secondly, the high concentrations of sodium ions found in tissue cultures of tumours; thirdly, the finding of Torack, Terry and Zimmerman (1960) that triethyl-tin caused an increase of cerebral sodium ions and swelling of what they called astrocytes, as seen by electron microscopy. At the time, Katzman's view was questioned (Hillman, 1966b) on the grounds that:

1. measurements of tissue sodium ions made in vitro were unreliable since the ability of the tissue to exclude sodium ions is grossly impaired by the preparation procedure (Hillman, Stollery and Joanny, 1974);
2. evidence from tumours which are frequently hypoxic was weak, since hypoxia raises

tissue Na^+ (Hillman, 1961);

3. the fact that the triethyl-tin increased the sodium ion content in the brain at the same time as the astrocytes appeared swollen in Torack, Terry and Zimmerman's experiments (1960) was insufficient evidence either that the Na^+ went into the astrocytes or that normal astrocytes are high Na^+ cells;
4. when Katzman was trying to account for the relatively high sodium ion content of the brain, he had not considered the Na^+ in the blood and cerebrospinal fluid, which could account for up to 10% of the total Na^+ in the brain;
5. with the possible exception of the salt gland of the albatross, which has to exist in an inauspicious and inhospitable environment, to my knowledge no other cells with high concentrations of sodium ions have been reported in the literature (Hokin and Hokin, 1963).

Criticisms (1) to (4) still remain valid in asserting that there is insufficient cogent evidence to prove that in mammalian brains neuroglial cells are high sodium ion cells, yet one still has to explain the relatively high concentration of this cation in the whole unincubated brain.

The present hypothesis is only slightly different in asserting that the neuroglial syncytium, rather than the astrocytes, contains a high concentration of sodium ions. There are other findings which do not, however, add up to unequivocal evidence. Christensen and Hastings (1940) showed that extracted phosphatides had a high affinity for both sodium and potassium ions. Katzman and Wilson (1961) froze brain rapidly below the eutectic points of its main salts, and extracted lipids which contained a ratio of 1.6 of sodium to potassium ions compared with the overall ratio of 0.6. Ahmed and Hillman (1982) homogenized brain, dialysed and filtered out all the diffusible molecules, and found that their resultant fraction had a high affinity for sodium ions, and much less for potassium ions at the physiological range of the concentrations of these ions in the brain. This affinity was grossly affected by the presence of low concentrations of transmitters (to be published).

EPILEPSY

The generally accepted view of excitation of cells is that the arrival of an electrical stimulus or enough chemical transmitter at particular regions of the cell membrane increases permeability of the cell membrane, firstly to sodium ions, and shortly afterwards to potassium ions. Of course the cell membrane can be depolarized not only by a fall in potassium ions in the cytoplasm but also by a rise of potassium ion concentration in the extracellular fluid. Changes in the chemical composition of the syncytium, particularly a rise in potassium ions, or any other chemical change which would increase the permeability of the cell membrane, could trigger the firing. It has already been suggested that the argument between the proponents of chemical and electrical transmission could be resolved if the transmitters were regarded as conditioning agents; they may create an environment for the cell membrane in which it becomes more or less excitable (Hillman, 1961). This could occur as a consequence of changing the conductive or dielectric properties of the syncytium or the cell membrane.

In passing, a note of caution should be sounded here. The extremely important

work on the electrical properties of giant axons by Keynes, Hodgkin, Katz, Huxley and their collaborators, has been assumed by such distinguished figures as Eccles and Davson to describe properties which are shared with mammalian neurons. The majority of neurobiologists today accept the ionic hypothesis, and they interpret their findings from electrophysiological studies, in relation to their belief in this hypothesis. Similarly, the findings of Schmitt, Fernandez-Moran, Finean and their collaborators on the biochemistry and biophysics of myelin sheaths or extracted myelin have been universally accepted as reflecting the properties of the neuronal membrane, mainly as a consequence of the belief arising from the Geren model that the myelin sheath represents a scroll of neuronal membranes.

When one discusses transport in cell membranes of any kind, the observations of Glynn and his colleagues on red cell 'ghosts' have been regarded as generally applicable to all membranes; this is despite the knowledge that these preparations have been made by subjecting the red cells to hypotonic solutions which change their permeability - albeit reversibly. However, the fact that the red cells can subsequently regain their previous properties does not necessarily mean that the properties of the red cell ghosts at the time of studying them are unaffected by the hypotonic treatment. The failure to realize this is similar to that of some electron microscopists who deeply freeze their tissues in conditions which some cryobiologists have shown to produce reversible effects. However, this does not mean that the freezing has no effect on the hydration, structure, dimensions, or chemical composition of the tissue. The point I would like to stress here most cogently is that although the properties of the squid axon, extracted myelin, or red cell ghost, may be generally similar to those of mammalian neuronal membranes, the belief that they are definitely applicable is frequently based on extrapolation, rather than on comprehensive experiment or evaluation.

Changes in the chemical composition of the syncytium or of the cytoplasm would be relatively slow compared with electrical conduction. Therefore, one could suppose that slow changes in direct current potential differences are due to changes in the chemistry of the syncytium. A large number of different chemical agents can be applied to the brain to produce convulsions in vivo; for example, ouabain, strychnine, penicillin, pentylene tetrazol, cardiazol, carbachol, diisopropylfluorophosphate, barbiturates, acetylcholine, methionine sulphoxime, hydrazides, allylglycine, picrotoxin, alumina, cobalt and tungstic acid (for reviews see Ward, 1969; Prince, 1972). Many of these substances induce convulsions when given to animals experimentally by mouth (Stone, 1972). Also cortisol, thyroxine, triiodothyronine, oestradiol and insulin can increase the susceptibility to experimental seizures (Woodbury, 1969). Some of these substances would be so dilute when they arrived in the nervous system that it is unlikely that their effects would be a consequence of changes in the electrical properties of the syncytium; therefore they probably either act by causing gross secondary alterations in the biochemistry of the syncytium and cytoplasm - and consequently the conductivity of the latter two compartments - or they accumulate on the cell membranes and thus modify their properties. Substances which are applied to the surface of the brain or spinal cord, or driven by current or

injection, are usually present at relatively high concentrations at the tips of the micropipettes.

In addition to the large number of reagents which may cause seizures, they can also result from freezing lesions, hyperbaric oxygen, carbon dioxide, wounds, scars, abscesses or tumours. These findings led Tower (1960, 1969) and Glaser (1964) to propose that seizures resulted from movements of cations in the brain. Hillman (1970) correlated findings from depolarization of brain in spreading depression, cells in cerebral slices and isolated neurons. He suggested that focal epileptic attacks originated in an efflux of potassium ions in a localized region of the brain. This would depolarize neighbouring neurons and cause them to fire. The newly depolarized area would excite the adjacent region until the wave of excitation impinged on the motor area of the parietal cortex. After the whole area had been depolarized it would take some minutes to recover completely, as happens after a patient has an epileptic attack.

If the syncytium surrounds the neurons it would follow that depolarization could result from (a) long-term changes in the chemistry of the syncytium; (b) temporary hypoxia, hypoglycaemia or ischaemia, due to arterial spasm or pressure from tumours in the syncytium adjacent to neurons; (c) lowering the threshold of excitation by decreased calcium ion or increased potassium ion concentration in the syncytium; (d) direct effects of agents applied near the outside of neurons on the permeability of their membranes. This hypothesis would predict that any agents which could enter the syncytium and also hyperpolarize neurons might prevent attacks of epilepsy. Among the agents which could do this are high concentrations of oxygen or glucose in the tissue and, possibly, ganglioside, which has been shown to increase the transmembrane potential in cells in brain slices and in isolated neurons (Hillman, 1961; Hillman and Hyden, 1965).

MYELIN AND DEMYELINATING DISEASES

The term myelin is used in five different senses: firstly, the sheath observed by light microscopy as a swelling on an axon, or in tissue-culture of peripheral nerves, (Figure 73), secondly, the thickening seen in transverse sections of the spinal white matter (Figures 39 and 40); thirdly, the characteristic lamellae seen by electron microscopy in the peripheral nerves, in the spinal cord, and also sometimes in kidney (Figure 72) and liver; fourthly, the chemical mixture extracted by organic solvents from peripheral nerves, spinal cord and brain; fifthly, the nervous tissue, mainly in the central nervous system, which does not stain in demyelinating diseases, such as multiple sclerosis and Friedreich's ataxia, and is detected by classical neural stains.

Obviously the electron microscopist believes that he is viewing essentially the same structure as the light microscopist sees, but at a higher magnification. Does he believe that 'myelin figures' in kidney and liver are identical with those in the central nervous system?

The chemical mixture extracted as myelin (Laatsch, Kier, Gordon and Alvord, 1962; Hulcher, 1963; Autilio, Norton and Terry, 1964; Uyemura, Tobari, Hirano

and Tsukada, 1972) is an extract from a whole tissue. When dried out, it also appears lamellar with approximately the same periodicity as that seen around peripheral axons by electron microscopy. With the exception of those using phase-contrast microscopy of unfixed cells, microscopists use ethanol, xylene and propylene oxide, among other reagents, during dehydration, embedding and mounting; these are powerful chemicals which extract lipids, and yet the microscopists claim to be observing the repeating unit of lamellae high in lipids (please see page 173). The results of low angle diffraction studies of unfixed axons (Schmitt, Bear and Palmer, 1941; Fernandez-Moran and Finean, 1957; Finean, 1957, 1960) are believed to confirm and justify the studies of the histologists and electron microscopists. One might expect that the organic solvents would extract the lipids, and thus disrupt the lamellar appearance. A further problem is that the lamellae and the similarity of chemistry common to the electron microscopist and the biochemist, are the main criteria used to demonstrate the identity of the structure in the peripheral nerves and the whorls in the extracted myelin. Even if it had not been shown that the lamellae are unlikely to occur in living axons (pages 173-179), I think one should recognize the total inadequacy of the similar appearances as evidence that the biochemical properties of extracted myelin reflect those of peripheral nerves, and even less those of brain. The kind of evidence which would help one to do so would be to extract peripheral nerves with myelin extractants and subject them to powerful proteolytic and lipolytic enzymes, and then show that the lamellar appearance disappears. Another possible experiment would be to compare the chemistry of the lipids and other substances leaving the tissue during preparation for electron microscopy with those in myelin extracts, and also with those in the same tissue after preparation for electron microscopy. These experiments would be more persuasive than explanations of why the control experiments have not yet been published, or why the lamellae still appear after the tissue has been subjected to agents which extract lipids (Fleischer, Fleischer and Stoeckenius, 1967; Morowitz and Terry, 1969).

We have drawn attention to the lack of specificity of staining procedures (pages 63-91). All histopathological studies of demyelinating diseases show patches of tissue which do not stain (Blackwood, Dodds and Somerville, 1970; Greenfield, 1976; Treip, 1978). In the absence of staining, one cannot see which parts of the cells or their processes are affected. If these diseases really only affected the myelin sheaths, why are so few unmyelinated fibres, nuclei or nucleoli seen in plaques of multiple sclerosis? I would like to suggest that the term demyelinating disease is a misnomer, which has misled many neurobiologists into confining their attention to the electron microscopy of lamellae or to the chemistry of extracted myelin. The following are some more useful approaches currently in use:

1. Comparing the chemistry of plaques with that of adjacent and distant but similar unaffected tissues in the same subjects.
2. Comparing the chemistry of the blood and cerebrospinal fluid of patients with these diseases with those of matched patients without these diseases.
3. Comparing the biochemistry of metabolizing tissue slices of plaques with that of normal tissues in vitro (McIlwain, 1975).

I would also like to propose the following kinds of experiments, which to my knowledge have not yet been done:

4. Examination of unfixed plaques to see why they do not stain. Specifically, one would establish whether the structures in the plaques have degenerated and disappeared, or whether they have lost their staining properties with the procedures used. In the latter case, by comparing plaques and normal tissue at each stage of staining by bright-field and phase-contrast microscopy, as was done with neurons (Hillman, Hussain and Sartory, 1976), one could identify at which point they lost their ability to take up stain. This might well give a clue as to where to direct attention in studying the histopathology of these distressing diseases.
5. Teasing plaques under direct vision using dissecting microscopes and examining them by phase-contrast microscopy to attempt to define if any particular cells or parts of cells are affected by the conditions.
6. Comparing the findings in (1) to (4) with findings in animals from model conditions to see how accurately the models really imitate the diseases in human beings.
7. Comparing the plaques of regions of demyelination due to the different diseases by the techniques listed to see if there is, indeed, one common mechanism of demyelination, or if each disease has its unique biochemical pathology.

Of course, the use of traditional histopathological techniques for diagnosing the neurological conditions would not be affected in any way by the considerations in this section. Furthermore, I have used demyelinating diseases as an example of a histological approach to the study of neurological disease, but this approach need not necessarily be confined to this neurological condition.

BIOCHEMICAL STUDIES OF THE CENTRAL NERVOUS SYSTEM

The conclusion that most of the central nervous system consists of syncytium means that nearly all biochemical studies of whole brain or spinal cord have examined mainly the properties of the syncytium. True studies of the biochemistry of neurons have isolated these cells by hand dissection by the technique of Hyden (1959), or homogenized, filtered and subsequently picked out by the method of Roots and Johnston (1964). Since the volume of the naked nuclei is small compared with that of the total syncytium (Figures 60 and 62) one may say that study of the neuroglial clumps yields information mainly about the chemistry of the syncytium.

If the relevant control experiments had been carried out, so that one could be certain that oxidative phosphorylation occurs in mitochondria in the living intact animal, as it does in the subcellular fraction in vitro (Hillman, 1972, pages 39-40), one could attribute the high rate of cerebral oxygen uptake to the dense population of mitochondria, both in the neurons and in the syncytium. However, this would give us no clue as to what the central nervous system does with all the energy. A popular suggestion, that it is used to maintain the ionic gradients, is not adequate, since it is believed that all cells maintain the ionic gradients across the cell membranes by the

utilization of energy, and most tissues are composed of cells whose membranes are much more closely packed together. Action potentials occupy a minute proportion of the lifetimes of excitable cells, and very small quantities of ions move during and after them, so that the total extra energy due to the restoration of the electrochemical gradients across the membranes of the cells would add an insignificant sum to the high energy expenditure of the nervous system. Perhaps it is worth wondering whether the energy is in any way used in the storage or recall of information, but at the moment I cannot think of any experiments to test such a totally adventitious hypothesis.

Many research workers have devised bulk fractions of what they claim to be neurons, astrocytes and oligodendrocytes (but not microglia) (for references please see Table 7, taken from Pevzner, 1979). Originally they called their fractions by the names of the presumed cell types, but it was often difficult to be sure of the identification of the predominant cell in fractions, or that the fractions contained no substantial proportion either of other cell types or of 'debris' - which could affect the biochemistry of the fractions significantly. Therefore, more recently, the authors have talked about 'neuron-rich', 'astrocyte-rich' and 'oligodendrocyte-rich' fractions. This change of nomenclature cannot unfortunately circumvent the real problem of the ignorance of the contribution which the particular cell type makes to the biochemical properties of the fraction as a whole. The fractions have usually been 'washed' several times. The purity of the fractions is usually judged by the subjective impression of the light or electron microscopist, and it is illustrated in publications with selected electron micrographs which are intended to show fields typical of the fraction as a whole.

Furthermore, one is not entitled to assume that the homogenization, centrifugation, and the chemistry of the suspending media used, have no influence on the biochemistry of the fraction. This difficulty is compounded by the knowledge that the different cell-rich fractions are prepared by such different physical and chemical procedures that - even if the original cell types had all shared the same properties - they would be likely to exhibit different biochemical behaviour after having been subjected to such different regimes.

One is faced with an analogous situation when trying to compare the biochemical properties of the different cell types in culture (Crain, 1976; Fedoroff and Hertz, 1977; Pevzner, 1979; Giacobini, Vernadakis and Shahar, 1980). In this situation also, the presumed different cell types are separated in different chemical environments, and are grown in different culture conditions, both of which strongly influence the chemistry of the cells. Therefore one cannot simply compare the chemical properties of such cultured cells and assume that these properties accurately reflect those of the parent tissues. However, one can probably assume that those tissues such as retina, cerebellum and muscle, in which one can recognize cell types for some time after the tissue has been isolated and cultured, represent the best model to study. We must remind ourselves that tissues do change their biochemical and staining properties in culture (Hansson, 1982). One should really favour procedures in intact animals, and those that disrupt tissues minimally. (Hillman, 1976).

12
Practical Recommendations

Throughout this monograph, and in previous publications (Hillman, 1972; Hillman and Sartory, 1980), a wide variety of experiments to control the effects of technical procedures and the agents used in them have been urged. Neither the vastness of the task, nor the failure to execute them so long after conclusions have been drawn from them, can absolve the captains of the research industry of their duty to carry them out.

In addition, it would seem appropriate to make the following recommendations:

1. More studies should be made using non-invasive techniques, wherever possible, and on tissues which metabolize at rates comparable with those in vivo at the time of the examination (Hillman, 1983).
2. Hand isolation, micromanipulation and teasing of preparations using no or minimal powerful chemical reagents or physical agents are to be preferred (Gray, 1931; Chambers and Chambers, 1961).
3. The specificity of neurological staining procedures should be examined comprehensively, in an attempt to prescribe what few procedures should be most encouraged by virtue of their specificity and the smallness of degree of distortion they cause. It is widely believed that, if different staining procedures show up cells of different forms, this defines genuinely different populations of cells. The assumption inherent in this belief is that the 'specific' staining procedures all distort cells to approximately the same extent. Thus, we have two interrelated tasks - viz. to define the different populations, if the neuronal and naked nuclear populations can, indeed, be subdivided, and to specify the optimal staining procedure for all such populations so defined. The latter would result in a considerable saving on expensive colour reagents, perhaps to the detriment of the aesthetics of the histologists' art and the decorations on the walls of their laboratories.
4. Neuroglia should be subject to more intensive study. The simplest preparation is the neuroglial clump, but the samples are rather small. Many microtechniques have been developed for examining the chemistry of small samples (Neuhoff, 1973; Osborne, 1974). Unfortunately, there is no information on the electrical properties of

these neuroglial clumps, upon which studies would be rewarding, especially if the samples have been examined microscopically to ensure that they contain no neurons.

Further attempts should be made to study the physiology of nuclei isolated by hand dissection, as has been done with the nuclei of ganglion cells and of cells from Deiters' nucleus (Giacobini, 1957; Edstrom, 1958; Cummins and Hyden, 1962; Hillman and Sartory, 1980, page 77). The spherical nuclei are difficult to manipulate with a fine stainless steel wire, and their refractive index appears to be similar to that of the incubating medium.

5. Structures which have been shown not to exist (Table 17) should obviously not be studied by electron microscopic, biochemical or pharmacological techniques.
6. The nucleolar membrane (Hussain, Hillman and Sartory, 1974) and the nucleolonema of neurons (Estable and Sotelo, 1951; Sartory, Fasham and Hillman, 1971) should be more extensively studied by light microscopy, especially in tissue culture.
7. The structure of the membranes of the cell, nuclei and mitochondria needs to be re-examined, by non-disruptive techniques (Appendix 4, page 251).
8. The mechanism whereby particles cross cell membranes should be studied in unfixed tissues by non-disruptive techniques. It seems quite inappropriate to use histological, electron microscopic or disruptive methods, or dead tissue, to study the dynamics of a process which only occurs in living tissues. There is plenty of evidence that small molecules can diffuse into all cells, that large particles can be ingested by phagocytes, and that particles can enter the syncytium. However, knowledge is scant on the mechanisms of these movements.
9. The concepts and evidence for transport by the membrane, for transmission, and for receptors, stand in need of complete reassessment. These should start with a fundamental investigation into each element of the evidence for them. Neurobiologists should not regard the enormous volume of experiments carried out on the assumption of these concepts, as adequate evidence in support of them. I would suggest that the time has come to re-examine the validity of the concepts themselves. More vigorous attempts to falsify the hypotheses behind the interpretations should be pursued. Unfortunately, neither the number of adherents to a particular concept, nor the volume of uncritical publications to adumbrate it can be regarded as an adequate measure of its validity.
10. Intraventricular injection of antimitotic drugs must be effective against gliomas.
11. Controls for subcellular fractionation should be done urgently.
12. Receptors should be sought by light microscopy in unfixed cells.
13. Immunocytochemical experiments should be repeated in unfixed tissues.
14. Attempts should be made to find out if adult neurons become malignant.

13
Postscript

In this monograph I have tried to use only proper evidence and reasoning. The following elements are particularly important: the distinction between hypothesis and finding; the citation of the evidence of the best established authorities in a discipline (although not necessarily their interpretations of their own data); quotation of several authors, especially for dimensions; the deliberate practice of not selecting values from the literature to fit in with the hypothesis at hand; insistence on analysis of the original evidence for the hypothesis being discussed; unequivocal respect for geometry; identification of crucial control experiments; attention to the techniques and calibrations of measurements; total willingness to enter into dialogue anywhere in the world; total willingness to modify or abandon any hypotheses or conclusions as a result of such discussions.

The subject of this monograph has implications for other sciences. It represents a 'paradigm' (in the sense of Kuhn) for the way research is pursued. However, there are several other important aspects of this problem. These include what in my view are two other imperatives:

1. We have a duty to society to employ the expensive resources and skills placed at our disposal as efficiently and productively as possible. Study on apparent structures which have been shown to be artifacts, or the use of uncontrolled techniques, is an abuse of the trust society puts in research scientists, and of the esteem in which they are held.
2. Neurobiologists should be humble in relation to their relatively poor success to date. We are still far from understanding the normal physiology and biochemistry of memory and thinking, the pathology of the main psychoses, the immunology of the nervous system, the genesis of psychoses, and many other phenomena. The ill have a right to be impatient. We are hardly nearer solving these problems than Vesalius was in sixteenth-century Brussels, when he sliced off the crowns of executed criminals and looked down into their brains (please see front cover).

Appendices

APPENDIX 1. Comments by some authors on the specificity of staining procedures

(See also pages 63-91)

Authors	Page	Comments
Penfield (1924)	434	'That oligodendroglia is very difficult to stain completely has been well demonstrated by the history of these adendritic cells.' A much more complete description has been provided by Del Rio-Hortega, using ammoniacal silver carbonate. Even by this method the results are variable. Some undetermined factors in the preparation of the tissues for the silver bath at times cause only microglia to be stained, or microglia and classical neuroglia. When the tissue is in a more favourable condition oligodendroglia is stained, but there is also impregnation, though incomplete, of the two above elements. When the tissue is in the most favourable condition a completely selective stain of oligodendroglia is obtained and the cell prolongations are beautifully clear-cut and appear much ramified.'
Weil and Davenport (1933)	174	'Even trained technicians, however, do not always succeed in the preparation of the silver solution devised by Stern, and, besides, the use of a 10 per cent commercial formaldehyde solution in connection with 10 per cent ammoniacal silver nitrate frequently leads to the production of disturbing silver precipitates.'
Gray (1954)	401	'Nerve cells and processes are stained more easily by the dye-staining techniques, than they are by metal-staining techniques. It must be admitted that most of the techniques are not so specific, but they are certainly adequate for class demonstrations; and it must not be forgotten that much of the classical research was done by these methods rather than by metal stains.'
	403	'The literature of the hematoxylin staining techniques for nerve cells and their processes is almost as confused as the literature of the metal-staining techniques for the same purpose.'
	525	'It is difficult to decide, in the case of osmic acid, which of the staining formulas given should be regarded as fixatives.'

Authors	Page	Comment
	537	re: demonstration of protoplasmic neuroglia in the cerebral cortex by the method of Cajal (1916), 'The successful application of this technique depends on the ability to judge the exact shade of purple which indicates a satisfactory termination of the staining process.
	543	'Dichromate-silver method. The techniques are experimental, yielding the most brilliant results when a successful impregnation is obtained, but for which it is almost impossible to specify conditions leading to success. The length of time is entirely critical, but results cannot be predicted or even reproduced with certainty. Among the numerous pieces thus treated, one may be found which will show the required condition.'
Clark (1973)	74	'Methods that have been developed for staining a particular component are usually selective but may partially stain other components.'
	76	'Bubbles - but when small, they appear as holes 5-20 μm in diameter in cut sections and may show little compression of adjacent tissue. They may become filled (after aqueous formalin) with myelin derivatives and this material may even take a stain with basic dyes and be mistaken for degenerative changes of glia cells.'
Carleton and Drury (1962)	221	'The complexity and the intervention of factors as yet unknown make it often difficult to stain particular elements of the nervous system in the same way on different occasions.'
	232	'The Golgi methods are capricious, and it is difficult to get the same results in different species - even of the same tissue.'
	240	'When owing to post mortem change the silver methods fail, Cajal's gold technique (p. 245) is worth trying.'
Gray (1964)	411	Microglia - 'Neurons that have become dark and shrunken as a result of poor fixation or mechanical pressure may take on a similar appearance.'
McClung Jones (1967)	405	'In general the pathological forms of all three types of cells are more easily demonstrated by the metallic methods than are the normal forms. Oligodendroglia may be impregnated in the grey and white matter with equal facility. Microglia cells are studied more easily in the grey matter where they are much more numerous. When oligodendroglia is well stained microglia is usually stained also. But either cell group may be stained with exclusive selectivity. At times when using silver carbonate, astrocytes may be incompletely stained along with the two groups of smaller cells.'
Ralis, Beasley and Ralis (1973)	57	'Haematoxylin and eosin. This method has little to recommend it for demonstrating nervous tissue proper...'
Smith and Bruton (1977)	7	'The majority of "staining" techniques used today were conceived by accident: they were observed by the perceptive eye or resulted from inspired guesswork. Frequently the precise chemical constituents of the

Authors	Page	Comment
		tissue were, and are, unknown and the "theory" of the staining mechanism empirical. Techniques based on the known chemistry of the tissue reacting to a known and understood reaction are few in number.'
	9	'In practice it must be admitted that few techniques are specific.'
	11	'Haematoxylin and eosin sequences provide a good example of the difficulties in stating "correct" staining times and the correctly stained preparation.'
Jacobson (1978)	47	'A wide spectrum of cytoplasmic densities in developing oligodendrocytes has been seen. This may make it difficult to distinguish between young astrocytes and young oligodendrocytes. To add to the difficulty, the appearance of glial cells changes with their functional states. For example, the oligodendrocyte active in myelination has more extensive cytoplasmic processes, more abundant cytoplasmic organelles, and more dispersed nuclear chromatin than the mature, resting oligodendrocyte.'
	48	'The Golgi method and the silver methods are not selective for neuroglia . . . . However the staining method used to study oligodendrocytes is not completely selective and cannot always be depended on to show those glia cells.'
Mirsky, Wendon, Black, Stolkin and Bray (1978)	121	'The neurofilaments which can be seen with the light microscope in so-called neuroblasts, neurons, and astrocytes after silver impregnation, are probably artifacts caused by clumps of neurofilaments and neurotubules.'
	258	'There are no other satisfactory histological markers for neurons; even the reduced silver methods may stain glia or fail to stain neurones under certain conditions.'

APPENDIX 2. Comments by some authors on the specificity of markers

(See also pages 88-91)

Authors	Page	Comment
Uyeda, Eng and Bignami (1972)	86	'Thus the double band obtained with extracts of tissues containing few astrocytic fibers, such as the cerebral cortex and white matter, and the basal ganglia, was lightly stained.'
	88	'The GFA protein appears to be confined to the central nervous system, as indicated by Ouchterlony double diffusion technique. It was found in normal brain tissue, brain tissue with reactive fibrillary gliosis and fibrillary astrocytoma.'
Jacobson (1978)	116	'The identification of distinct neuronal and glial phenotypes has, until recently, required overt cyto-differentiation, and thus the cells could be recognised unambiguously only when they were approaching the final stages of differentiation. This often involved the subjective impressions of the observer, and was especially subject to error in cases where cells have similar appearances during their preterminal stages of differentiation, when transitional forms might arise, or when cell differentiation might be somewhat atypical, as in tissue culture.'
Mirsky, Wendon, Black, Stolkin and Bray (1978)	258	'It is not possible to prove rigorously the specificity of tetanus toxin in all situations.'
Varon (1978)	94	'Thus, a cell specific marker can be best defined as a property that is expressed uniquely or prevalently in a given cell class when correspondingly optimal conditions prevail. This definition requires that description of a marker include the set of extrinsic circumstances under which it has been observed, and eventually the set of extrinsic circumstances which condition its expression.'
	96	'S-100 is an example of a molecular marker in search of a function.'
Raff, Fields, Hakamori, Mirsky, Pruss and Winter (1979)	284	'The increasing use of in vitro techniques in neuro-biology has accentuated the need for cell-type specific markers, which would allow unambiguous identification of the different types of neural cells in culture.'
	290	'Although the GFAP+ cells in cultures of cerebellum, cerebral cortex, corpus callosum and meninges did not label with tetanus toxin, some process-bearing GFAP+ cells in cultures of optic nerve were weakly labelled.'
	306	'Although the use of cell-type specific markers has enabled us to study a number of properties of defined neural cell types in culture for the first time, it should be appreciated that the assignment of "positive" or "negative" for a particular property is operational and depends on the sensitivity of the assay used.'
Kennedy, Lisak and Raff (1980)	344	'Some of the GFAP+ cells were large and had large vesicular nuclei, and others were smaller and darker and had multiple branching processes. While it is likely that these were protoplasmic and fibrous astrocytes, respectively, many GFAP+ cells had an intermediate morphology and could not be so classified.

Authors	Page	Comment
	347	'It is still uncertain, however, whether all astrocytes are GFAP+.'
Raff, Brockes, Fields and Mirsky (1980)	18	'While it is clear that the great majority of neurones in cultures of peripheral and central nervous tissues in various species are labelled by tetanus toxin, it is not certain whether all neurones label with tetanus toxin. Therefore, one must be cautious in using tetanus toxin as a neuronal marker, particularly since some preparations of tetanus toxin label various cell types "non-specifically." '
	20	'Although the morphologies of some neural cell types are, at times, sufficiently distinctive to allow identification by this criterion alone, this is usually not the case.'
Bartlett, Noble, Pruss, Raff, Rattray and Williams (1981)	346	'All the latter cells could be identified as neurones by the fact that they bound tetanus toxin.'
	348	'Anti-Ran-2 antibodies. . . . They labelled astrocytes having diverse morphologies.'

Appendix 3 Typical counts made with the Quantimet particle counter in examining six slides of coronal sections of the frontal lobe of rat brain. The area x1000 is the number of Pix cells in that particular field, whose mean is given in column 2 for each staining procedure. The Selector A is the setting on the particle counter chosen for each field to give the maximum contrast between the stained tissue and other elements. The overall results are given in Tables 15 and 16.

Marsland, Glees & Erikson			Phosphotungstic acid haematoxylin			Weil-Davenport			Haematoxylin & eosin			Patay			Gallyas		
Area x1000	Mean	Selector A	Area x1000	Mean	Selector A	Area x1000	Mean	Selector A	Area x1000	Mean	Selector A	Area x1000	Mean	Selector A	Area x1000	Mean	Select A
17-20	18.5	15	36-37	36.5	35	21-24	22.5	30	44-47	45.5	52	36-38	37	25	29-30	29.5	34
5-6	5.5	15	25-27	26	34	16-18	17	32	28-30	29	52	19-20	19.5	25	28-29	28.5	34
15-18	16.5	17	8-10	9	34	16-18	17	31	18-20	19	53	20-22	21	25	40-41	40.5	34
5-6	5.5	17	28-30	29	34	22-24	23	31	13-14	13.5	51	32-35	33.5	26	25-26	25.5	34
7-8	7.5	17	23-25	24	36	27-31	29	33	17-18	17.5	51	30-31	30.5	25	27-29	28	34
15-16	15.5	34	48-49	48.5	37	24-25	24.5	32	26-28	27	52	27-28	27.5	24	30-31	30.5	29
19-20	19.5	35	37-39	38	35	12-13	12.5	31	35-37	36	52	21-23	22	24	40-41	40.5	29
17-19	18	34	25-27	26	37	17-18	17.5	32	38-39	38.5	52	33-38	35.5	23	28-30	29	29
9-12	10.5	35	25-26	25.5	37	22-23	22.5	32	22-23	22.5	52	41-44	42.5	25	29-31	30	29
7-8	7.5	36	42-44	43	37	17-18	17.5	32	26-27	26.5	52	32-33	32.5	24	26-28	27	29
4-5	4.5	35	35-36	35.5	38	41-43	42	34	19-21	20	53	33-35	34	26	32-33	32.5	28
9-10	9.5	35	20-22	21	38	23-25	24	34	38-41	39.5	53	33-35	34	25	50-52	51	29
11-12	11.3	35	38-39	38.5	36	30-33	31.5	34	32-33	32.5	52	38-41	39.5	24	42-43	42	28
14-15	14.5	35	13-14	13.5	36	27-32	29.5	31	34-36	35	52	43-44	43.5	23	38-39	38.5	28
20-21	20.5	35	38-39	38.5	36	18-19	18.5	34	9-10	9.5	52	26-27	26.5	22	42-43	42.5	28
10-11	10.5	36	23-24	23.5	34	40-42	41	35	29-31	30	52	47-49	48	22	28-30	29	28
19-20	19.5	35	26-28	27	36	14-16	15	35	29-31	30	53	32-34	33	23	26-27	26.5	28
19-21	20	35	39-40	39.5	37	26-29	27.5	35	23-26	24.5	53	52-55	53.5	23	17-19	18	28
21-24	22.5	37	18-19	18.5	35	26-27	26.5	34	19-21	20	52	50-53	51.5	23	40-41	40.5	28
13-14	13.5	36	38-39	38.5	37	34-36	35	34	22-23	22.5	52	41-42	41.5	21	51-54	52.5	28
Total mean	13.6			30			24.7			26.9			35.3			34.1	
SD±	5.7			10.3			8.2			9.2			9.6			8.6	

APPENDIX 4. Biological membranes and measurement of their thicknesses

An interface may be defined as a detectable region where two immiscible fluids meet. A biological membrane is a thin solid structure separating two different fluids.

The boundaries of vacuoles and of lipid droplets in milk are interfaces. Little more need be said about them, except that most microscopic techniques can not distinguish between an interface and a membrane.

Membranes were first detected by light microscopy, usually in stained tissues, and subsequently by electron microscopy. They may be detected by (1) a difference between them and the phases on both sides of them, or (2) inference from the fact that the phases on both sides appear different, which the observer interprets as being due to a membrane being present. The differences observed under both headings are due to dissimilarity with respect to:

(a) absorption of light of a particular wave length (colour);
(b) emission of light after exposure to light of a particular wave length, including excitation of fluorescent emission;
(c) diffraction at the edges (which does not permit measurement of thickness);
(d) intensity of colour - the commonly used difference in histology and electron microscopy;
(e) refractive index, enhanced in phase-contrast microscopy;
(f) any of these properties consequent upon the tissue being colour stained, and the membranes and phases on either side of the membranes, having different retention of all the reagents, used throughout the whole histological procedures;
(g) birefringence seen by polarization microscopy;
(h) reflectivity seen by dark-ground illumination and reflected light illumination;
(i) textures, especially with freezing techniques of electron microscopy;
(j) different structures being present on both sides of the apparent membrane, for example, the synaptic vesicles which are believed to be on only one side of the synapse;
(k) antibodies reacting with antigens present on, or adherent to, the cell membranes;
(l) deposit of salts of heavy metals, such as those of silver in light microscopy, and osmium, platinum or tungsten in electron microscopy;
(m) shrinkage artifacts around membranes.

There are several other techniques which have given more or less direct evidence for the existence of membranes:

(n) differences of impedance, which is much higher in tissues than would be accounted for by the composition of the extracellular and cytoplasmic fluids; also, the impedance can be measured between an electrode believed to be intracellular and one in the extracellular fluid or incubating medium (Coombs, Eccles and Fatt, 1955; Frank and Fuortes, 1956);
(o) low angle diffraction of X-rays, from which the repeating unit of the presumed membrane is calculated.

Other information about membranes is gleaned from:

(p) the considerable potential difference which is recorded abruptly when an electrode with a tip diameter of the order of microns is advanced into a tissue and which changes with the cation constitution of the extracellular fluid or incubating medium;
(q) calculations based on the quantity of lipids and proteins which could be extracted from red cells and the calculated areas of those cells (Gorter and Grendel, 1925);
(r) examination of reflections from dried erythrocytes compared with films of barium stearate using the 'leptoscope' (Waugh and Schmitt, 1940);
(s) the ability of lipid mixtures to make monomolecular layers on salt solutions (Bangham and Horne, 1964);
(t) the similarity of many of the physicochemical properties of well-characterized artificial membranes to those of biological membranes.

The presence of solid membranes around cells is attested by the ability with which neuron somas can be cut open (Cummins and Hyden, 1962), and the contents of squid axons can be squeezed out with a 'microsteamroller', and the axons can subsequently maintain their excitability on being refilled with potassium chloride solution (Baker, Hodgkin and Shaw, 1961). The fact that the contents of the cells of the sympathetic and dorsal root ganglia do not all flow away when the capsules are removed is further powerful, if indirect, evidence that the cells are each personally invested with a mechanical membrane, rather than a fluid interface.

Any skilled advocate knows that individual pieces of evidence vary in their cogency, and one must beware lest the quantity of evidence might be more persuasive than the quality of its individual elements.

The common belief nowadays based on electron microscopic observations and low angle diffraction measurements is that the 'unit' membrane is, say, 6-8 nm thick. It must be repeated that the electron micrographic 'trilaminar' membrane is a metal deposit on a structure which has been dehydrated, is believed to contain much lipids and has been extracted with reagents including alcohol and propylene oxide, and subjected to high vacuum, electron bombardment and X-irradiation. Many of the low angle diffraction measurements have been made on the myelin lamellae of peripheral nerves, which are shown to be artifacts (please see page 173-182).

The maximum resolution of the light microscope under optimal conditions is 20-25 nm, so that a membrane of 6-8 nm thickness could only be seen under the following circumstances:

(a) if a metal stain was deposited along the membrane in sufficient thickness;
(b) if an antigen-antibody reaction produced a precipitate of the required dimensions on the membrane;
(c) if there was a large difference of refractive index on both sides of the membrane;
(d) if the membrane was composed of multiple layers whose total thickness exceeded 20 nm.

One must state quite categorically that if the membranes in life did have a thickness of 6-8 nm, they could not be measured by any technique of microscopy.

We have already shown elsewhere that the 'unit' membrane is an artifact, because it occurs normally to the plane of section more frequently than geometry would permit (Hillman and Sartory, 1980, page 40). Therefore, we must seek further but indirect evidence about the dimensions and the existence or non-existence of the cell membrane from non-microscopic observations (h) to (t).

The high impedance between two electrodes which can be measured when a 'resting' potential attributed to penetration of the cell membrane is recorded - like the resting potential itself - is likely to be good evidence, but cannot be conclusive until compared precisely with the impedance and the zeta potential of interfaces of droplets of the same dimensions and similar chemical compositions. Nevertheless, resistances of 0.8 MΩ found across presumed neuronal membranes (Coombs, Eccles and Fatt, 1955; Frank and Fuortes, 1956) seem high for a relatively thin interface of any kind, just as a resting potential of 50-80 mV seems high compared with zeta potentials of up to 5 mV.

Calculations of membrane thickness based on the quantities of protein and lipid which may be extracted and the surface area of the cells studied depend crucially on the completeness of the extraction of the chemicals, the accuracy of measurements of the protein and lipid, the accuracy of measurement and calculation of the total membrane area of the tissue under study (usually red cells), and the belief that the particular chemicals are almost exclusively located in the cell membranes. All of these require a pretty interplay of assumption and interpretation whose total effect is not wholly reliable. However, it would be a reasonable approach if all these uncertainties and approximations were resolved. Analogous difficulties beset the use of the leptoscope and of low angle diffraction studies, which are usually carried out on partially or completely dehydrated tissues.

The ability of lipid and proteins to make monomolecular layers and to complex in thin layers, and all the artificial membranes which have been studied, represent elegant models which can explain the biophysical properties of membranes, and they are properly used for that purpose, but in logic they cannot and do not show that the model represents the structure of the cell membranes or their dimensions. Their fundamental value in this respect is a function of the accuracy to which their physicochemical conditions are designed to reflect the total milieu of the biological environment of the real cell membrane. I have urged the value of this experimental approach elsewhere (Hillman, 1983).

It is tempting to regard the dimpling and resistance at the tip of an advancing micropipette, at what appear to be the membranes of neurons and muscle cells, as further mechanical evidence for the existence of the membranes around the cells, but unfortunately similar phenomena can result from attempts to penetrate immiscible droplets using fine 'intracellular' micropipettes.

If we now return to the light microscope and direct our attention to an attempt to see the membranes in unstained or stained tissues, we realise that we do not see the membranes, we see differences of colour, texture, refraction, and intensity of staining

between the cytoplasm and the extracellular fluid (a) to (o); we are conditioned to believe, albeit subconsciously, that we are seeing membranes. In electron micrographs one is often inferring the existence of a membrane between two adjacent areas of different texture or intensity of staining.

The concept that the cell, nuclear or mitochondrial membranes might be liquid, fluid, quasi-liquid or quasi-fluid, is dealt with elsewhere (please see pages 25-26, 132-135).

We may therefore arrive at the following tentative conclusions. The mechanical and electrical properties present near cells make it very likely that they possess membranes, unless they are part of syncytia (please see page 108). The presence of a membrane is detected or inferred by techniques of light or electron microscopy, and confirmed by non-microscopical techniques, but so far attempts to measure membrane thickness representative of that in vivo have ignored known and expected effects of preparation on the measurements.

APPENDIX 5. The tissue in the electron microscope

In a preparation for electron microscopy the following types of material are present:

1. The tissue, consisting of water, proteins, lipids, carbohydrates, nucleotides, electrolytes, dissolved gases, inter alia.
2. The heavy metal stain, which consists of metals or salts of osmium, lead, uranium, tungsten or manganese.
3. The embedding media of epoxy resins, methacrylates, ice, polyesters or polyampholytes.
4. Other reagents, which have not evaporated, including fixatives, alcohols, propylene and solvents.

Each of these reagents will be grossly different in respect of the following parameters: volatility, stability, temperature coefficient of expansion or contraction, heat capacity, heat conductivity, reactivity with each of the other substances at each of the temperatures to which they are exposed, radiation emission, radiation absorption and electron density. During the preparation each of these parameters will be changing continuously due to changes in their chemical environments, temperature, electron bombardment and exposure to high pressures during freezing and low pressures in the electron microscope.

In view of the complexity of the system, it is unlikely that studies of simplified reactions like those between osmium salts and proteins or lipids can be accepted as sufficiently comprehensive models of what is occurring to tissue during preparation for electron microscopy.

The electron bombardment raises the temperature in the specimen to the equivalent of several hundred degrees (Reimer, 1965; Favard and Carasso, 1972; Grubb and Keller, 1972a,b). Such temperatures are within the range used to ash tissues for chemical measurements of metal ions, or in flame photometry to vaporize atoms. The tissue could not survive. Therefore it is a sad, if unpopular, fact of life, that the image seen through the electron microscope must be composed almost entirely of heavy metal.

It is often said that freezing techniques are independent evidence to confirm findings from transmission electron microscopy. The only step which fundamentally differs between the two techniques is that the initial fixation in the former technique is done by extreme cold, while in the latter a classical fixative such as osmic acid or glutaraldehyde is used. A large number of artifacts have been identified during the study of both biological systems and simpler systems of plastics, sucrose and even water (Sleytr and Robards, 1977, 1928a,b; Dubochet, Lepault, Freeman, Berriman and Homo, 1982; Chang, McDowell, Lepault, Freeman, Walker and Dubochet, 1983). However, until now, to our knowledge, no authors except ourselves have addressed themselves to the question of how much of what is generally regarded as the intracellular structure could be accounted for by these artifacts.

APPENDIX 6. Unsatisfactory terms

Two previous lists of terms, which in my view are misleading, loose, or meaningless, or they obscure real difficulties of interpretation, have been drawn up (Hillman, 1972, pages 115-120; Hillman and Sartory, 1980, page 99). I would respectfully refer the reader to these lists, as they are highly relevant to the considerations in this monograph. Many of the words are metaphors, and they have been taken over from other disciplines, where their meaning is often more precise and better understood. The semantic problem is a real one. How often has one heard students saying that particles enter cells by the mechanism of endocytosis, not appreciating that they are merely translating ignorance into crypto-classical Greek? Some of these terms are worthy of exposure. *An asterisk indicates a largely meaningless term.

1. *Particles are assembled or in an assembly; this means that they appear to be in a row, but the use of the term should not bear a teleological implication.
2. Concept is an idea, hypothesis, construct or fantasy, and cannot be used for a structure which is alleged to have material existence, such as a membrane. It can be used for a theoretical proposition about its properties, only until such a time as the hypothesis is tested. The longer the time that elapses before it is tested, the more urgent the testing of the hypothesis becomes.
3. *Cues are presumably movements of particles or molecules of biochemical changes which induce other changes. The term has a real meaning only in sensory physiology.
4. *Particles decorate a pore, granule or vesicle, because the author likes the look of them. They decorate, mainly because - like the mountain - they are there.
5. Endocytosis, exocytosis, pinocytosis and phagocytosis mean that particles or droplets go into and out of cells, but tell one nothing about the mechanisms causing these movements.
6. *One particle or reaction is implicated with another, if the author believes that they have a physical or biochemical relationship, but has not yet demonstrated it.
7. *Structural changes, physiological events or biochemical reactions should only be described as integrated if there is abundant evidence of their relationship in time, if there is a rationale for the alleged relationship, and if some control mechanism has been proposed or demonstrated for it.
8. A marker is a substance which should have been demonstrated to be located in a particular kind of cell, or part of a cell.
9. A model is either a physical object imitating a proposed structure and changes in it, or it is a biophysical or mathematical concept of a proposed structure or of biochemical reaction or changes in it. It is essentially a series of hypotheses whose totality is intended to mimic the structure or biochemical reaction as closely as possible. The term can not be used for the structures or the reactions themselves, since they are more closely related epistemologically to findings.
10. *Modulation means that a substance affects the excitability of a cell or the release or effect of a substance believed to be a transmitter. In using the term one should really wonder whether the modulation is special in that the modulator is being alleged to affect the reaction in a way which is more particular than simple chemistry would dictate.

11. *An organized structure appears to have a pattern which is believed not to be an artifact of preparation.
12. *A substance packaged or parcelled in a particle is a substance which is believed to be present in that particle, and to travel in it.
13. *A putative transmitter is a substance found in the nervous system, which some authors believe to be a transmitter, and they hope to accumulate enough evidence in the future to satisfy classical criteria that it is (please see page 4).
14. A neural receptor is a particular physical or biochemical part of a cell membrane, which is believed to react with a transmitter, hormone, drug or antibody before any of them can exert their action. Few receptors have been shown, and the demonstration of the effects of one of these agents is often believed to be sufficient evidence that a receptor for it must exist on the cell membrane. Nevertheless, there are several classical criteria which define a receptor, including high affinity, competition, saturation and simple numerical relationships of their materials with the transmitter, drug, hormone or antibody.
15. *A particle or substance is restricted if it always appears in the same part of the cell. It means that it is there, and the use of the term implies that it has not moved during preparation.
16. The role or function of a particle or chemical either means the effect it has, or it means the 'purpose' for which it is there. The latter meaning is either teleological or theological.
17. *A substance which is sequestered is believed to be at a particular site; that is, it is there, temporarily or permanently.
18. *A specialization is a feature which is seen to be constant and which is believed to be the site of a particular biochemical activity.
19. *The statement that a substance is stored means that it is there, usually as a granule.
20. A transmitter is a naturally occurring substance, which is believed to be released near a neuron or a neuromuscular junction, to have an effect at relatively low concentration, to have a high affinity for the membrane, and to increase or decrease its excitability. It is also defined as having enzymes to synthesize it near its site of action. Several transmitters are believed to be broken down by enzymes in the tissues adjacent to the neurons. There are also a wide range of unnatural compounds which in low concentrations either increase or decrease the effects of transmitters.

APPENDIX 7. A few naive but important questions

Why have no systematic attempts been made to control the effects of killing animals, and of homogenising and centrifuging tissues, on the form, biochemistry and pharmacological properties, of cells?

How is the existence of a cytoskeleton seen by electron microscopy compatible with intracellular movements seen in life by light microscopy?

Why does evidence cited in discussions on the structure of central synapses, and for the vesicle hypothesis, usually originate from findings on the neuromuscular junction?

Why do immunocytochemists not use unfixed, unembedded, unstained, tissues for localising cellular constituents?

Do homogenisation and centrifugation affect the affinity of substances for receptors?

What is the difference between a particle, granule or body, being assembled, decorating, being organized, packaged, parcelled, restricted, sequestered, occupying a specialized site, stored - or just being there?

What are the nuances between enhancing, implicating, mediating, being a second messenger, modulating - and just causing?

How does one differentiate between diffusing, flowing, migrating, partitioning, being transported - and just moving?

What are the criteria used to identify bodies, deposits, free ribosomes, lysosomes, particles, precipitates, secretory granules, vesicle-like bodies - and artifacts?

What fills a bubble, droplet, lumen, pinocytotic vesicle, pore, space, shrinkage artifact, vacuole - or just a hole?

What is life?

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