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The Relationship of Biochemical and Morphological Information in the Central Nervous System: The Problem of Sampling

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Abstract

The histology of the central nervous system (CNS) is complex, consisting of membranes derived from a number of cell types as well as specialized membranes associated with structural modifications of the neuron. The cell structure, and thus the membrane content, of the CNS varies with age and from area to area; in no instance is it possible to isolate a single cell type without special microdissection techniques. Neurological disease often involves specific areas of the CNS attacking one or all of the cellular elements in that region. In other instances, damage may be widespread, but at the histological level it may be restricted to a single cell type or even to a single membrane. The latter situation is particularly applicable to diseases that attack the myelin sheath.

The biochemical investigation of changes in the lipid content of the brain resulting from neurological disease is hampered not only by the morphological complexity of nervous tissue, but by the tendency of destructive processes to be accompanied by proliferation of other cellular elements, both during the acute phase of disease and during the process of repair, which may mask significant abnormalities. Thus, in order to decide which changes in the structural lipids of the diseased brain are meaningful, a knowledge of the histopathology of the disorder is essential. When the morphological changes that accompany a neurological disorder are known, one may choose an appropriate tissue sample for study. Isolation of specific structural or functional subunits of the CNS, dissection of small groups of cells, isolation of single cells and separation of specific membranes are techniques available to sample nervous tissue. These are discussed, and their applicability to the biochemical study of specific neurological diseases is evaluated.

Introduction

THE MAJOR BARRIER to evaluating information about the biochemical changes that accompany neurological disease is the complexity of the normal and pathological anatomy of the brain, which exceeds that of any other organ in the body. Cells that differ markedly in structure and function exist side by side in normal brain, and the cellular composition of nervous tissue differs throughout the neuraxis. The histological derangement that accompanies disease may vary in intensity and in rate of progression in adjacent areas of the brain, while within a single area, several morphological changes may be taking place simultaneously.

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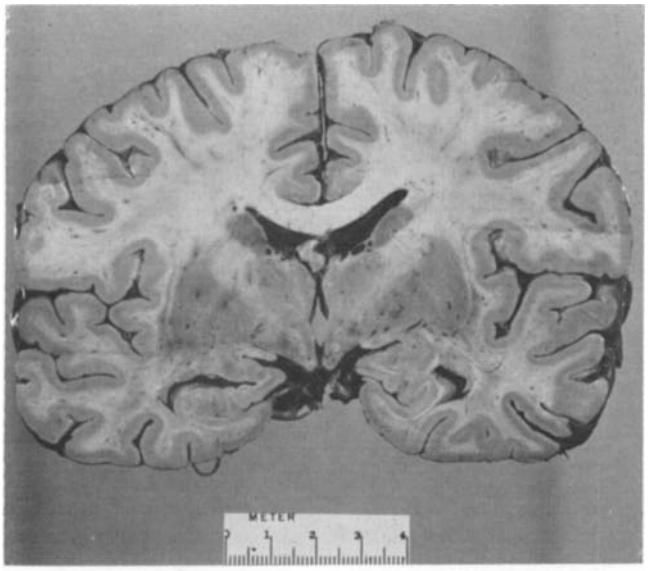

FIG. 1. A coronal section of the cerebral hemispheres at the level of the mammillary bodies showing cortex, white matter and several of the deep gray nuclei. In the adult brain these gray and white masses can be distinguished by color, and in the cerebral hemispheres, at least, their margins are usually sharp.

Lipids, which are primarily structural elements located in cell membranes, reflect these variations in cell composition; quantitative information about the lipid classes present in diseased tissue is meaningful only when related to the morphology of the sample from which it was derived. Small samples with fresh weights of 0.5 to 2 g are usually used for such analyses, and the results are then compared to similar samples taken from normal controls. This type of sample is simple to prepare, but it is of limited value since it emphasizes most of the morphological uncertainties inherent in brain tissue. Meticulous attention must be devoted to obtaining reproducible control data by reducing the variations that normally occur with the age of the patients and the area of brain sampled; there should be histological confirmation that the sample to be analyzed is severely diseased. Even if these criteria are met, and the lipid composition of a sample differs considerably from that of its controls, it is usually not possible to assign a biochemical abnormality to a single class of cells or to locate it in a specific membrane because of the morphological complexity of brain tissue. Thus, when dealing with this type of sample, only gross changes in lipid-class composition combined with a uniform pathological picture can be considered to have real meaning.

However, in recent years a number of other sampling techniques have been developed which, when applied to selected pathological problems, may yield more precise biochemical-morphological correlations. Some of these methods approach the ultimate goal of the chemical analysis of structural lipids in diseased tissue: to establish if qualitative or quantitative changes in the lipid composition of specific membranes may be causally related to the development of the disease.

An understanding of the need for such sophisticated sampling techniques, and of their limitations, requires an awareness of some of the unique features of the normal and pathological anatomy of the brain.

Variations in Structure of Different Areas of Brain

The normal adult brain is divided into areas of gray and white matter. The distinction is relevant since gray matter contains the cell bodies of neurons, while their axons, surrounded by a myelin sheath, account for the bulk of the white matter. Since myelin is the major lipid-containing membrane in the nervous system, the ability to distinguish areas of high and low concentration by visual inspection is a distinct advantage to the lipid chemist. Within the cerebral hemispheres, this differentiation can be made after the first months of postnatal life, and the cortical mantle as well as many of the deep gray nuclei such as thalamus, putamen and caudate can be sampled without difficulty (Fig. 1). However, at other levels of the neuraxis, particularly in the brain stem, no clear separation is possible (Fig. 2). White and gray matter break up into small, interdigitating

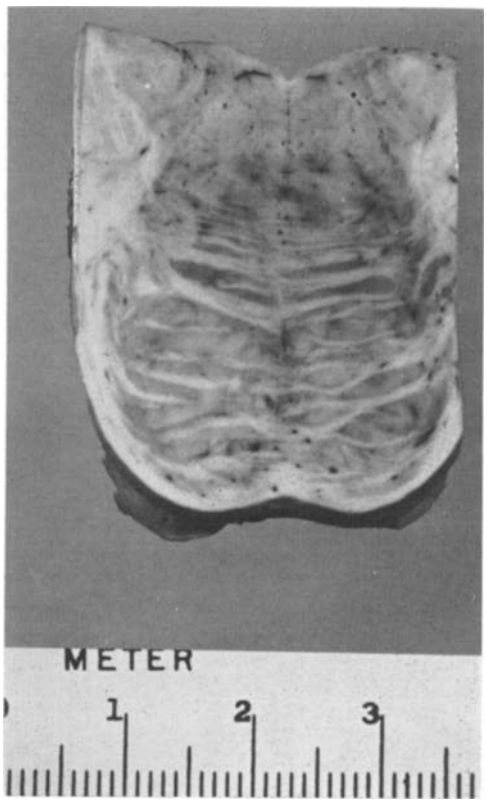

FIG. 2. A horizontal section of the brain stem at the level of the pons. Gray and white matter can be distinguished visually, but they intermingle to such a degree that it is not possible to separate them accurately by gross dissection.

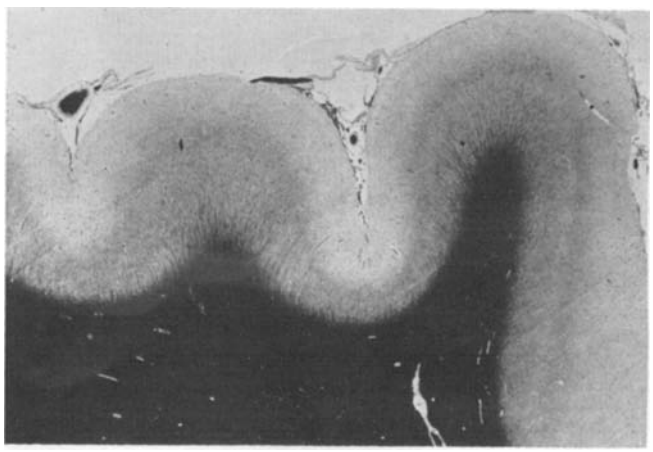

FIG. 3. A photomicrograph of adult cerebral cortex and subcortical white matter. Heavily myelinated areas are black. Myelinated fibers can be seen radiating into all but the most superficial layers of the cortex.

masses whose margins are indistinct. The sharp boundary between gray and white matter seen in the cerebral hemispheres is also deceptive since, upon closer examination with a hand lens or with special stains, myelinated fibers can be seen to penetrate into almost every part of the gray matter (Fig. 3).

The microscopic anatomy of the nervous system is far more complex. The normal gray matter of brain contains at least five different cell types in close proximity to each other. These can be seen with Nissl stains for chromatin material (Fig. 4), and include the cell bodies of large and small neurons; the nuclei of the smaller, but more numerous glial cells: astrocytes and oligodendrocytes, which have supportive and metabolic functions, and microglia, which serve as scavenger cells; and endothelial cells which line the walls of small blood vessels. Nissl stains do not outline the entire cell, and the greater part of the tissue is left unstained. These pale areas are actually filled with an interlacing, fibrillary network made up of the axonal and dendritic processes of neurons and the fibrous extensions of glial cells. The white matter contains all of these elements, with the exception of the cell body and dendrites of the neuron.

Since all but the most minute samples of nervous tissue contain a variety of structures, it is not possible to attribute the results of biochemical investigations to any of them except by inference. Nevertheless, the reproducibility of a series of samples would not be affected were it not that the proportions of these elements vary from area to area, and vary in any single location throughout life. As early as 1907, twenty-eight subdivisions were described within the cerebral cortex (1,2). This work was done with unstained sections and a hand lens. Since that time, a number of investigators have approached the problem using the light microscope and simple cell stains (3–8). As a result over two hundred morphologically distinct areas have now been identified. The dorsal thalamus, a deep gray nucleus, contains at least 25 subnuclei which differ in structure as well as function (9). The same problem arises when examining almost every other nuclear mass within the central nervous system. Relatively little attention has been paid to the microanatomical organization of the white matter. However, variations occur in both fiber

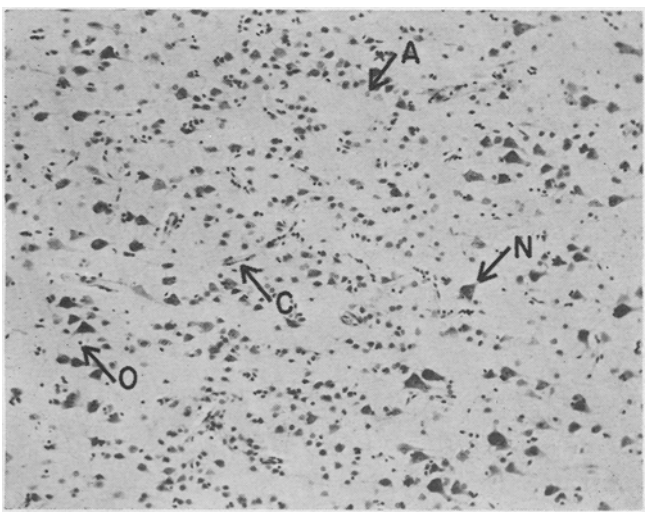

FIG. 4. A photomicrograph of eulaminate cortex. The triangular cell bodies of neurons (*N*) of many sizes can be seen interspersed among the nuclei of oligodendroglia (*O*) and of astrocytes (*A*). Numerous small blood vessels (*C*) are also present. (Nissl × 100).

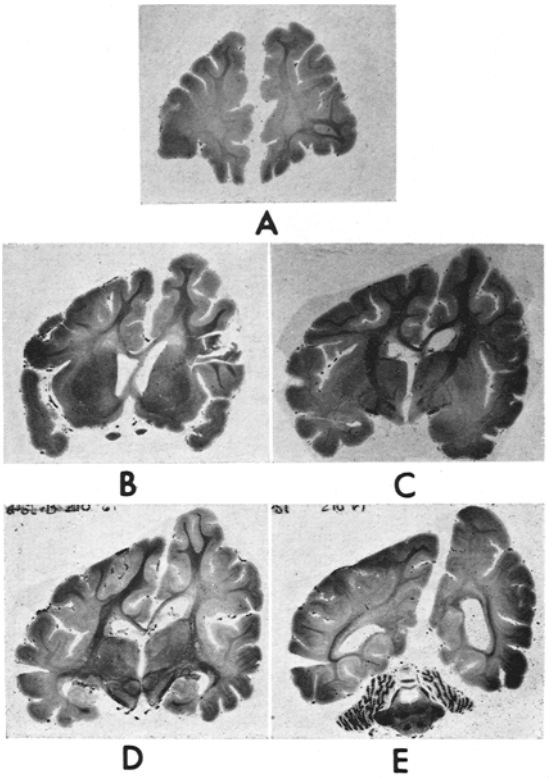

FIG. 5. Warren Museum Case #RPSL-B-210-61. Coronal sections of the cerebral hemispheres of an 11-week full-term infant. Heavily myelinated areas are black. The uneven distribution of myelin is apparent. In the anterior part of the frontal lobe (*A*) more myelin is seen near the base of the brain than elsewhere. The primary motor (*C*) and sensory (*D*) projections are relatively heavily myelinated. Tracts in the brain stem (*E*) are heavily myelinated when compared to those in the cortex.

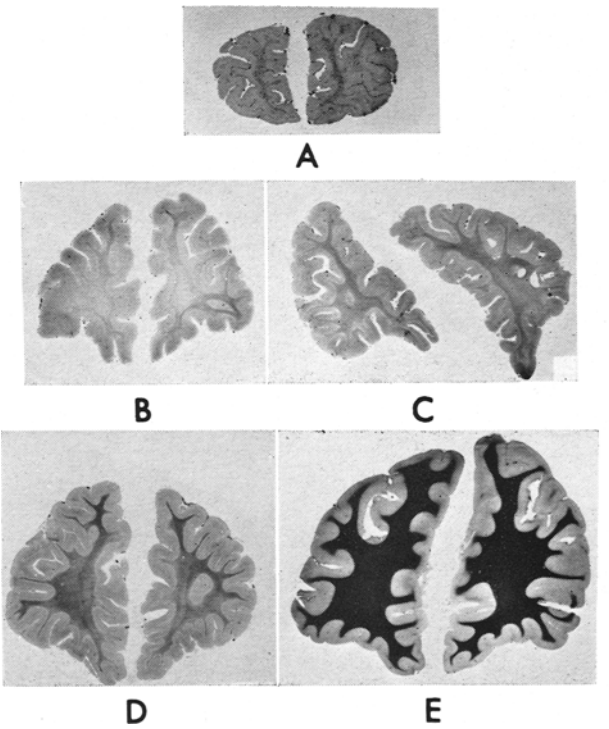

FIG. 6. Warren Museum Cases: Coronal sections through the frontal lobe anterior to the caudate nucleus and stained for myelin: (*A*) 42 week stillborn, (*B*) 11 days, (*C*) 4 months, (*D*) 7 months, (*E*) 8 years. The amount of myelin in this area accumulates throughout infancy and childhood.

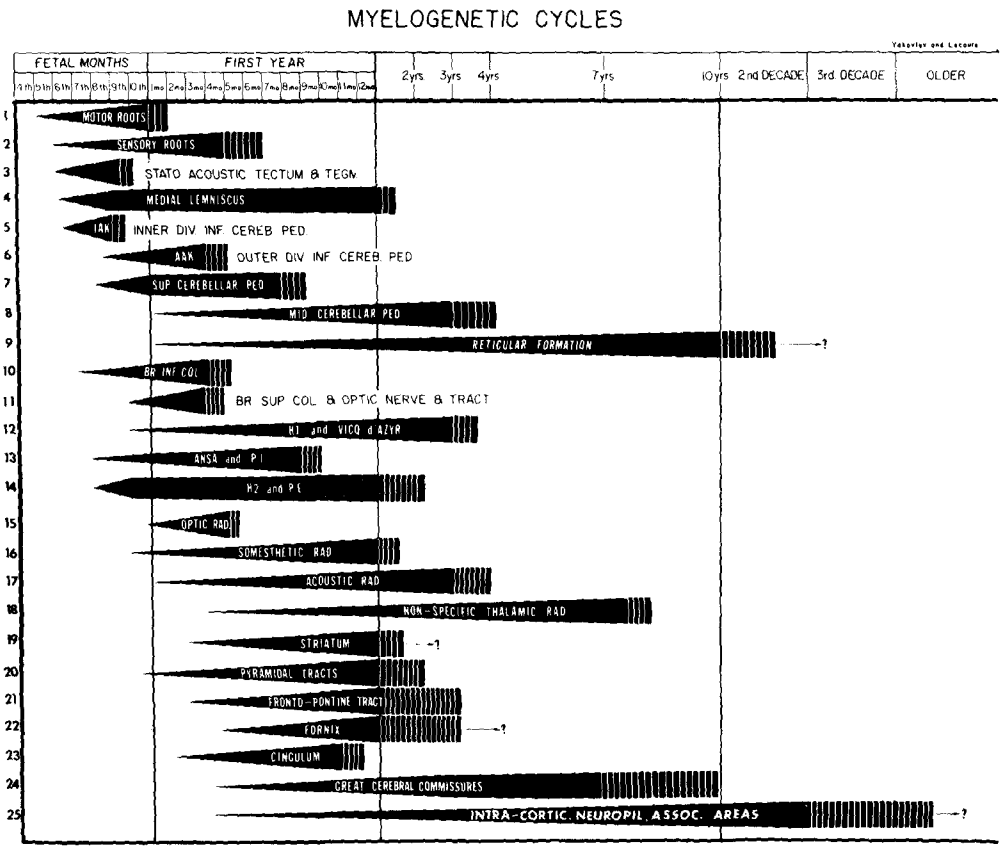

FIG. 7. Myelogenetic Cycles: The tracts and cell masses in the central nervous system myelinate at predictable rates. However, the schedule of myelination differs markedly in different tracts and nuclei. (Reproduced by permission of Drs. P. A. Yakovlev and A. R. Lecours in "Proceedings of the International Congress on Regional Maturation of the Brain," Paris, December 1964, A. Minkowski (ed.) Blackwell, London.)

density and cell composition throughout the neuraxis, and it is well established that the axons of different tracts vary considerably in the thickness of their myelin sheaths (10). The limits placed upon this type of morphological diversity are, in fact, arbitrary since in any one brain minor variations can be found when comparing sections separated by only a few micra.

The Effect of Maturation on Brain Structure

The morphological changes that occur throughout life take place most rapidly in the infant. Very few anatomical studies have been performed in humans in an attempt to demonstrate these variations. The work of Flechsig (11), Kaes (12) and Langworthy (13) on the pattern of myelination in infancy and childhood are early landmarks as is the work of Conel (14) on the cellular composition of the infant cortex. While their work is fundamental to our knowledge about the postnatal development of the human brain, their findings have been criticized because of the small number of cases studied (15). As a result, normal histological variations permissible in a single area of the brain at any one age have never been determined. These results are now being extended by the efforts of Paul Yakovlev and his collaborators at the Warren Museum of Harvard University (16,17). While the normal morphological variations that occur in infancy are being better defined, those associated with old age are largely unknown.

During the first two years of postnatal life, the weight of the brain increases almost threefold. This is accompanied by increasingly complicated patterns of secondary fissuration and the development of tertiary fissures. Changes occur in the width of the cerebral cortex, its cell density, the average cell size and the complexity of the axonal and dendritic network. However, it is the pattern of myelination that is presently of greatest interest to the lipid chemist. Myelin is first seen in the human central nervous system in the last half of fetal life. At birth the myelin is still, for the most part, confined to the tracts of the spinal cord, brain stem and diencephalon, though a small amount of myelin is found in the subcortical white matter of the limbic lobe and of the primary projection areas (i.e., pre- and post-Rolandic, calcarine and transverse temporal cortices) (Fig. 5). In the months immediately after birth, myelination spreads to adjacent areas of the subcortical white matter and along the tracts that project to and from these zones. This is a gradual process, and myelination of the central white matter is not completed for many years (Fig. 6). Within the cortex, particularly in association areas, the formation of myelin continues into the sixth or seventh decades involving first the deeper and then the more superficial parts of the gray matter (12).

The changes that occur during normal development are orderly but they are not uniform throughout the brain. Functional subunits, such as specific receptor areas or tracts, mature at rates that are independent of each other (Fig. 7) and independent of those of adjacent association areas. If the young brain is sectioned, as it usually is, in frontal, sagittal or horizontal planes that are 0.5 to 1.0 cm apart, these areas overlap, and the degree of maturation found in any single plane of section is a mosaic. This also is true in the anterior portions of the frontal lobes, which is an area commonly used for biochemical analysis because of the supposed homogeneity of its white

FIG. 8. Photomicrograph of the hippocompal formation showing selective destruction of a small segment of the neuronal population in a patient who had a severe anoxic episode. (Nissl stain.)

matter. However, in infants there is considerably more myelin at the base of the anterior part of the frontal lobe than there is in its lateral or more superior aspects. Therefore, unless meticulous attention is paid to matching the anatomical locus of a sample and its controls, and to minimizing age differences, seemingly significant biochemical results may only reflect variations in the normal anatomy and the degree of maturation. While this would not appear to be as serious a problem in older children and adults where the level of development is more uniform, several studies indicate that variations in the range of 3% to 10% do occur in brain lipids (18-22) and proteolipids (23) when samples are taken from different areas of either the gray or white matter.

The Effect of Disease and Injury

The pathological features of the diseased brain are superimposed on this shifting anatomical baseline. Since they too lack uniformity, the problem of making biochemical and morphological correlations is compounded. Some of the reasons for this are outlined below:

1) One cannot predict the extent, the severity or the location and distribution of a pathological change in the nervous system by the nature of the insult. General insults such as anoxia (Fig. 8), carbon monoxide poisoning, nutritional deficiencies (Fig. 9) or toxins need not produce diffuse disease. In fact, the neurological lesions they cause may be remarkably restricted. We are not certain of the mechanisms responsible for this selectivity, though it is assumed that they have to do with modifications of specific metabolic pathways in these areas making them especially vulnerable to a particular insult (24).

In contrast, very restricted insults such as a penetrating wound, a metastatic tumor nodule or a small infarct may produce widespread structural and biochemical derangements that are not directly related to the nature of the insult. There are several causes for this. Focal lesions may be associated with the accumulation of fluid in cerebral white matter. This may extend for some distance from the site of injury and, if left unchecked, may result in destruction of tissue and cell death.

The process of Wallerian degeneration is also responsible for distant effects resulting from a focal lesion, and is of particular interest to the lipid chemist. When a neuron is destroyed or its axon sectioned, that part of the axon which is distal to the site of the lesion degenerates. This is accompanied by disintegration and resorption of the myelin sheath. Wallerian degeneration occurs in the central as well as the peripheral nervous system, and as axons in the central white matter may run for many centimeters, there may be considerable destruction some distance from the site of the primary lesion (Fig. 10). Severing the axon may also result in swelling of the body of the neuron and eventually in cell death (central chromatolysis). This is a less predictable response than Wallerian degeneration and is likely to occur in nutritional deficiencies, particularly that involving nicotinic acid.

2) While diffuse degenerative or storage diseases involve all parts of the cerebral cortex or the white matter, the morphological changes they induce are often unevenly distributed. This is especially true in the earlier stages of these disorders when some areas may be devastated while others are minimally involved.

3) Microscopic examination of a diseased area generally reveals many processes occurring simultaneously. Neuronal lipid storage may be accompanied by death of neurons, degeneration of axons with secondary destruction of their myelin sheaths, and proliferation of glial cells. The processes of destruction and repair often overlap in lesions that advance slowly. The biochemical information obtained from

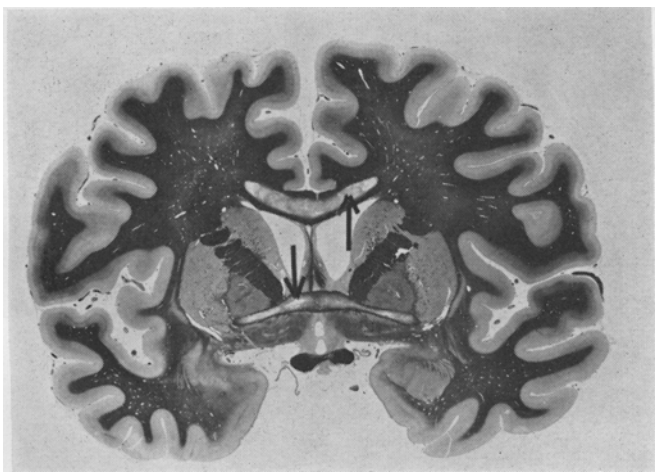

FIG. 9. A coronal section of the cerebral hemispheres at the level of the anterior commissure stained for myelin, in a patient with Marchiafava-Bignami disease. There is selective destruction of the medial part of the corpus callosum and of the commissure while the white matter elsewhere is normal.

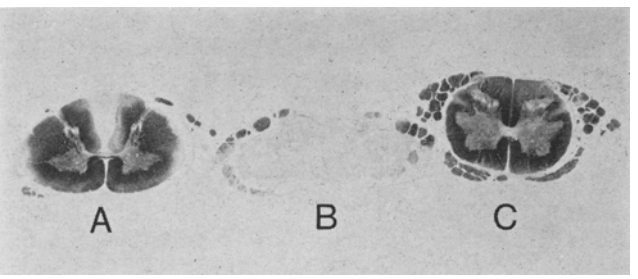

FIG. 10. A photomicrograph of three levels of the spinal cord, (*A*) lower cervical, (*B*) mid-thoracic and (*C*) lumbar, stained for myelin. An old traumatic injury has almost completely destroyed the mid-thoracic cord. Areas of pallor are seen in the dorsal and anterior-lateral regions of the cervical cord and the lateral columns of the lumbar cord. They are the result of Wallerian degeneration of nerve fibers that were damaged when passing through the thoracic region.

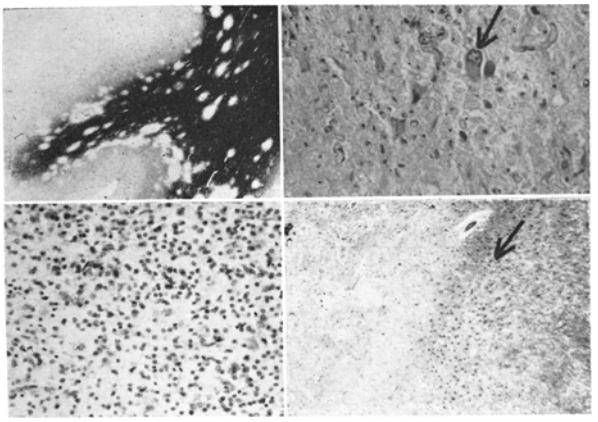

FIG. 11. Upper left: A case of progressive multifocal leukoencephalopathy. Cerebral cortex and white matter are stained for myelin, and numerous patchy areas of demyelination are seen. Upper right: A photomicrograph of a demyelinated area in this disease (stain: hematoxylin and eosin). Large, bizarre glial cells are seen. Lower left: A photomicrograph of a recently demyelinated area (Nissl stain) in a patient with acute Multiple Sclerosis. There is an outpouring of inflammatory cells. Lower right: A photomicrograph of an old area of demyelination in a patient with Schilder's Disease. A wall of astrocytes is seen. (Cajal gold stain for astrocytes.)

a tissue sample is a summation of all the processes occurring at that time and, as a result, such an analysis may be of very little use in focusing on the defect initially responsible. In those diseases in which a single process clearly predominates, as in Tay-Sachs disease where virtually every neuron is distended by stored ganglioside, random tissue blocks can usually be expected to yield relevant biochemical information. To date, lipid analyses of the central nervous system tissue have been most successful when applied to the examination of the storage diseases. However, biochemical studies of the storage diseases of later life, such as juvenile amaurotic idiocy, have not helped to clarify the cause of the disorder (25,26) though there is an increase in neuronal pigment identified histochemically as lipofuscin (27). Neuronal death is a prominent feature of these latter diseases and distended cells are few, if present at all. It is possible that pertinent biochemical changes are obscured when analyzing random samples of tissue of this type, where only a small fraction of the cells present in the tissue are involved in the active process at any one time and where relatively large numbers have been destroyed.

4) The cellular composition of a lesion is not just a function of the nature of the initial insult. The morphological picture also depends upon the age of the lesion and how acutely it began (Fig. 11). In acute lesions, destruction of normal tissue often is accompanied by an intense cellular response which involves polymorphonuclear leukocytes, lymphocytes and plasma cells. Microglial cells then migrate to the area and metamorphose into macrophages which ingest cellular debris. As this process continues, astrocytes begin to increase in number as do small blood vessels of capillary size. This is particularly striking at the margin of a lesion. If the necrosis of normal tissue is complete and the lesion is sufficiently large, a cavity may result. This will be surrounded by a capsule of fibrous glial tissue with a few strands of glia usually bridging the walls of the cavity. In processes that advance more slowly, gliosis occurs but its histological appearance is more subtle. There is only a moderate increase in astrocytic cell bodies, but when the tissue is suitably stained there is evidence of a dense fibrillary gliosis throughout the involved area (Fig. 12). In both cases, the processes of destruction and repair are superimposed.

5) Multiple lesions, though part of the same disease process, need not be identical. Multiple sclerosis is an excellent example. Since the lesions occur in white matter and involve the focal destruction of myelin sheaths with relative preservation of their axons, this disease is classed as demyelinating. However, if the onset of the lesion is acute, axons and, in fact, all tissue elements in the area may be destroyed. In most sclerotic plaques there are few inflammatory cells and these, for the most part, are limited to lymphocytes in the perivascular spaces. However, in acute lesions there may be an intense proliferation of inflammatory cells. Not all the lesions in multiple sclerosis are of the same age. Some that are more recent still contain numerous lipid-filled macrophages. In older lesions, these cells may have disappeared entirely or only a few may be seen near the blood vessels. In many sections, the pathology that is apparent is not due to initial insult but is the result of Wallerian degeneration.

Even parts of a single lesion need not be of the same age. Schilder's disease, another demyelinating disorder, illustrates this principle. The advancing margin of the lesion shows evidence of active disease, while the more central areas represent the results of an older injury. Under these circumstances, analysis of the entire lesion may obscure important biochemical changes at the active margin.

Sampling Methods

Variables such as these suggest that the biochemist interested in diseases of the nervous system must design his sampling procedures to ensure a) that he is dealing with the primary lesion and not a secondary change, b) that the area he examines is representative of the disease, c) that he is dealing with tissue that represents the active stage of the disease, whenever possible, d) that his findings are not the result of a florid but nonspecific cellular reaction, e) that pertinent biochemical findings will not be obscured by others resulting from the death of neurons, the destruction of myelin and reactive gliosis. Pathological criteria exist that permit such distinctions; however, similar biochemical criteria are not yet available. Therefore, biochemical information, in order to be of significance, must be associated with a careful pathological study of the entire nervous system, and the

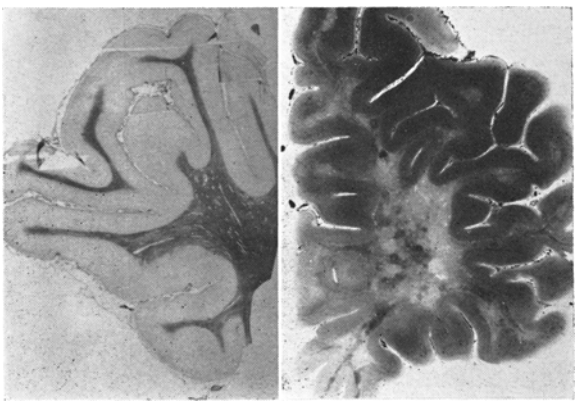

FIG. 12. Coronal sections in a case of Pellizaeus-Merzbacher's disease. The section on the right is stained for myelin and severe demyelination in the white matter is apparent. The section on the left is stained for glial fibrils (Holzer stain) which are markedly increased throughout the white matter.

samples selected for analysis must also be subjected to a simultaneous pathological investigation in order to determine their place in the overall disease process. When the pathology of the disease is well known, it may be possible to use this information to choose a type of sample that will yield the most meaningful biochemical results.

In the past thirty years, several new methods of sampling tissue have been developed by those interested in establishing more precise biochemical and morphological relationships. Three approaches have been valuable: the isolation of subcellular structures, the use of single cells or small tissue samples whose cell composition is nearly homogenous, or the use of very large samples whose boundaries are easily defined. While each of these approaches can be criticized, they can be extremely useful in relating alterations in structural lipids with morphological changes when applied selectively to special problems.

Isolation of Subcellular Structures

The isolation of subcellular particles is now done routinely in many laboratories. Several excellent reviews of the general technique and its application to nervous tissue have been published recently (28,29), and there are a number of papers concerned with special techniques designed to isolate myelin (30–36). All utilize the same method: the centrifugation of homogenized tissue against a continuous or discontinuous density gradient usually made up of water solutions containing different molar concentrations of sucrose. With this technique, it has been possible to analyze the lipid composition of relatively pure nuclear, mitochondrial, microsomal and myelin membranes.

There are several drawbacks inherent to the fractionation-centrifugation technique. The initial sample must be large since material is lost during the process of purification. Because of this loss, only indirect measurements of the total lipid contributed by a class of particles to a tissue sample can be made. Furthermore, when there is more than one type of cell in a tissue, it is not possible to isolate the organelles of each cell type independently. In cerebral tissue, isolated mitochondria, microsomes or nuclei are derived from nerve cells, glial cells and epithelial cells. Therefore, changes in the lipid content of any one of these subcellular fractions cannot be ascribed to the organelles of a single cell type. In diseased tissue, where destruction of one class of cells may be accompanied by a reparative process involving proliferation of other cell types, it is difficult to relate analytical biochemical information obtained from subcellular fractions to the pathology. Attempts to isolate individual classes of cells (i.e., neurons, astrocytes, oligodendroglia) by centrifugation techniques prior to fractionating their subcellular particles have thus far failed (37).

TABLE I

Disorders Which Chiefly Involve the Myelin Sheath

Type of disorder	Distinctive biochemical abnormalities
I. Disorders in which myelin is destroyed:	
A. Leukodystrophies	
1. Sudanophilic	Increased cholesterol ester and other neutral lipids
2. Metachromatic	Increase in sulfatide
3. Krabbe	A relative increase in cerebroside compared to sulfatide
4. Pelizaeus-Merzbacher	Unknown
5. Canavan (spongy degeneration)	Unknown
6. Hyaline body	Unknown
7. Late life	Unknown
B. Demyelinating	
1. Multiple sclerosis	Unknown
2. Postinfectious and postvaccinal encephalomyelitis	Unknown
3. Schilder's disease	Unknown
4. Acute hemorrhagic leukoencephalitis	Unknown
II. Disorders in which the formation of myelin is delayed:	
A. Amino acidurias (phenylketonuria, maple syrup urine disease)	Proportionately less proteolipid than other myelin elements
B. Nutritional deprivation (experimental animals)	Unknown
C. High doses of steroids (experimental animals)	Unknown
D. Irradiation (experimental animals)	Unknown

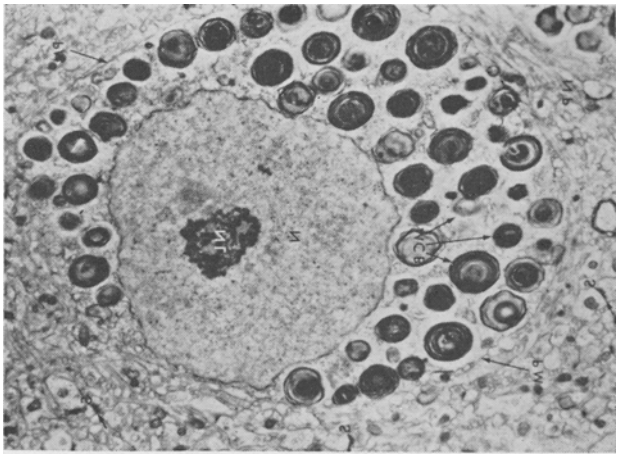

FIG. 13. An electronphotomicrograph (X9800) of cytoplasmic inclusions (membranous cytoplasmic bodies) in the neuron of a patient with Tay-Sachs disease. (Reproduced by permission of R. D. Terry and M. Weiss in J. Neuropath. Exp. Neurol. *22*, 18 [1963].)

The greatest usefulness of the fractionation-centrifugation techniques thus far has been in the isolation of myelin. Since this lipid-rich membrane is unique, we do not have to be concerned with the possibility that it is derived from more than one type of cell. It is now possible to obtain the composition of pure myelin by direct analysis rather than by inference based on the evaluation of myelin-rich and myelin-poor tissues. Since the myelin sheath is severely involved in a number of neurological diseases (Table I), a more exact knowledge of its normal composition is of great importance. Recently, several attempts have been made to study diseased myelin isolated by fractionation-centrifugation techniques. O'Brien found that the myelin in fragments isolated from patients with metachromatic leukodystrophy, a disease in which there is an abnormal accumulation of cerebroside sulfate, contained extremely large amounts of cerebroside sulfate per gram of lipid weight suggesting that this compound accumulates to excess within the myelin sheath (38). Gerstl et al. (39) isolated myelin from the central nervous system of patients with multiple sclerosis and found that its composition differed from normals, being particularly deficient in plasmalogens. These investigators compared their results to control material that was matched for age and the area sampled. The precautions noted for the analysis of small blocks of tissue also apply to the analysis of subcellular fractions, since it has yet to be proved that the lipid composition of the myelin sheath or of the membranes of any organelle is constant throughout the central nervous system.

A second fruitful application of centrifugation techniques has been the isolation of unusual cells or

TABLE II

Diseases in Which Unusual Cells or Inclusions are Present in Brain

Disease	Most frequent site	Name	Biochemical composition
I. *Inclusions*			
A. Extracellular			
Alzheimer's disease	Cerebral cortex	Plaques	Unknown
B. Intracellular			
1. Viral or probably viral:			
a. Herpes simplex	Neurons and glia of cerebral cortex		Unknown
b. Subacute inclusion body encephalitis	Neurons and glia of cerebral cortex		Unknown
c. Encepholitis lethargica	Neurons of substantia nigra	Neurofibrillary tangles	Unknown
d. Rabies	Cerebellum-Purkinje cells Neurons of Ammons Horn	Negri bodies	Unknown
2. Metabolic:			
a. Familial myoclonic epilepsy	Dentate nucleus of cerebellum	Lafora bodies	Glycogen
b. Tay-Sachs disease[a]	Neurons of cerebral cortex	Membranous cytoplasmic bodies	Large amounts of ganglioside, cholesterol and phospholipid
c. Late infantile lipidosis[a]	Neurons of cerebral cortex		Unknown
d. Hurler's syndrome (gargoylism)[a]	Neurons of cerebral cortex		Unknown
e. Metachromatic leukodystrophy[a]	Neurons of cerebral cortex		Possibly sulfatide
3. Degenerative:			
a. Alzheimer's disease	Neurons of cerebral cortex	Neurofibrillary tangles	Unknown
b. Pick's disease	Neurons of cerebral cortex	Argentophilic bodies	Unknown
c. Paralysis agitans	Neurons of the substantia nigra	Lewy bodies	Unknown
II. *Unusual cells*			
A. Krabbe's leukodystrophy	Cerebral white matter	Giant cells	Lorge amount of galactocerebroside
B. Progressive multifocal leukoencephalopathy	Cerebral white matter	Giant astrocytes. Enlarged oligodendrogial cells	Unknown
C. Gaucher's disease	Perivascular in cerebral cortex and white matter (Rare)	Gaucher cells	Large amount of glucocerebroside

[a] These inclusions are identified with aid of the electronmicroscope.

inclusions in diseased nervous tissue (Table II). The most notable of these achievements has been the biochemical analysis of lipids of membranous cytoplasmic bodies (Fig. 13). These were first described in diseased neurons by Terry and Weiss (40) using the electron microscope, and then isolated and analyzed by Samuels et al. (41). This technique should have further applications to a number of neurological diseases since intracytoplasmic membranous inclusions have been seen under the electron microscope in other storage diseases including metachromatic leukodystrophy (42), gargoylism (43), and late infantile lipidosis (44). Terry has recently reviewed the subject in its entirety (45). Austin was able to document the increased cerebroside content of giant cells found in Krabbe's leukodystrophy (Fig. 14) by homogenizing cerebral tissue in sucrose and then using density gradient centrifugation to separate these cells (46). Recently, he has analyzed the structures of Lafora bodies found in familial myoclonic epilepsy, separating them by a similar method (47).

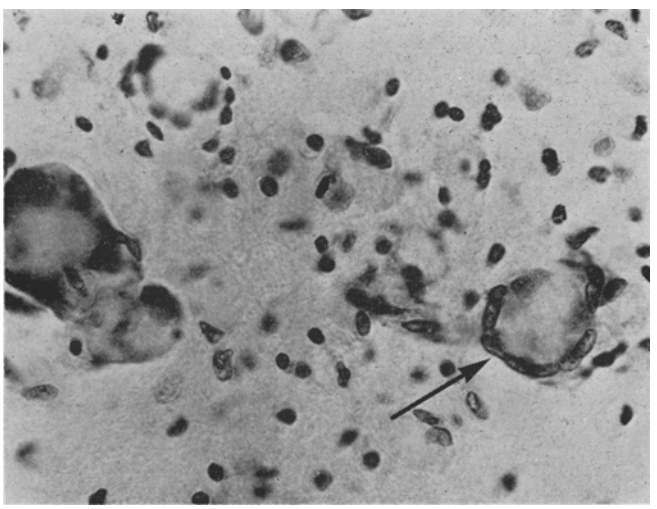

FIG. 14. A photomicrograph of cerebral white matter showing giant cells in a case of Krabbe's leukodystrophy.

Isolation of Small Groups of Cells

Quantitative histochemistry was the name given by Linderstrøm-Lang and Holter (48,49) to biochemical analysis of small samples of tissues. Punch biopsies of a tissue are taken and frozen rapidly. They are then sectioned serially in a cryostat at −10C to −20C; each section is approximately 40 μ thick. Biochemical analyses are performed on every second or third section while the remainder are saved for histological studies. Changes in a single biochemical parameter may then be related to changes in the cell population of adjacent areas. This method was modified by Pope and his associates (50,51) to be of use in dealing with small samples of cerebral cortex about 2 mm in diameter. He first analyzed the distribution of several enzymes, ribose nucleic acid (RNA) and deoxyribose nucleic acid (DNA) in human frontal cortex (52,53). However, in recent years Pope, Hess and their co-workers have developed biochemical techniques that permit the analysis of total solids, total lipids, proteolipid protein, residual protein (that remaining after extraction of the tissue in 2:1 chloroform-methanol (v:v)), gangliosides and cerebrosides on samples with dry weights in the range of 10 to 100 μg (54–58). Similar sampling procedures have been used by Robins and his group, combined with a somewhat different method of biochemical analysis, to determine the lipid and enzyme composition of the different layers of the motor and occipital cortices of the monkey (59–61).

The purpose of this method, as defined by Pope (62) is to establish "correlations between biochemical composition and metabolic behavior on the one hand, and anatomical fine structure on the other. . . ." In the absence of concomitant quantitative histological methods, the authors have tried to formulate biochemical indices which would reflect the structural integrity of the tissue. They consider cerebroside and

proteolipid an index of the amount of myelin, DNA of cell population (both neurons and glial cells), and acetyl-cholinesterase and gangliosides of the structural integrity of the neuron. They have tried to develop indices to measure the potential for turnover of tissue elements as well, by suggesting that RNA be used as an index of synthetic potential and peptidase and lipase as indices for the potential of a tissue to degrade proteins and lipids respectively (54).

Since, as we have noted previously, the primate cortex contains over 200 histologically distinct areas, samples taken for this type of analysis must be very carefully controlled with respect to location. Robins et al. (59) clearly showed that a different biochemical pattern exists in the motor and visual cortex, as might be expected from histological differences. Furthermore, the cellular composition of the cortex, the number of neurons, glial cells, vessels and myelinated axons varies considerably with depth from the surface of the brain and with age. This is reflected in the changing biochemical composition of the cortex (Fig. 15). As a result, it would be virtually impossible to pair sections accurately in diseased and normal cortex. A large number of sections must be analyzed biochemically, comparisons made with the histological picture of adjacent sections, and a pattern developed relating the changes noted in a single chemical constituent with changes in cell composition or structure. This anatomical-biochemical pattern can then be compared with that seen in disease. This requires multiple analyses and, of course, is time-consuming and tedious. Furthermore, the relationships derived are still the result of inference, though more precise than those obtained from larger samples.

Within the central nervous system, even sections that are only 40 μ apart may show significant histological variation. Thus, the quest for homogeneous or, at least reproducible, cell samples has extended to even more micro levels, the ultimate end being the analysis of a single cell. The separation of single cells has been pioneered by Lowry and his co-workers (63–65) using freeze-dried tissue, and by Hydén and his associates using fresh tissue (66,67). Both methods involve freezing small tissue samples, making thin sections in a cryostat and eventually dissecting out single cells by hand or with a micromanipulator. Neurons can be distinguished from glial cells with the aid of a dissecting microscope. Recently, Roots and Johnston have tried to increase the number of cells that can be isolated in a short period of time by passing brain tissue through molecular sieves of graded sizes (68). This concentrates the cell bodies but does not completely separate neurons or individual species of glial cells from each other. However, by using a dissecting microscope and a small hand-manipulated loop it is possible to harvest 300 to 600 neurons from the resulting suspension in a 3-hr period.

Biochemical investigations of samples of this size have primarily involved studies of oxidative and glycolytic enzymes and nucleotides. While discussion of these elegant methods is beyond the scope of this paper, it is interesting to note that Hydén and his co-workers studying isolated neurons and glial cells have made several interesting observations on the reciprocal metabolic relationships that exist between these two elements normally (69,70) and during the course of certain extrapyramidal diseases in man, notably Parkinsonism (71).

Lowry and his associates have developed methods for determining cholesterol (72), lipid phosphorus

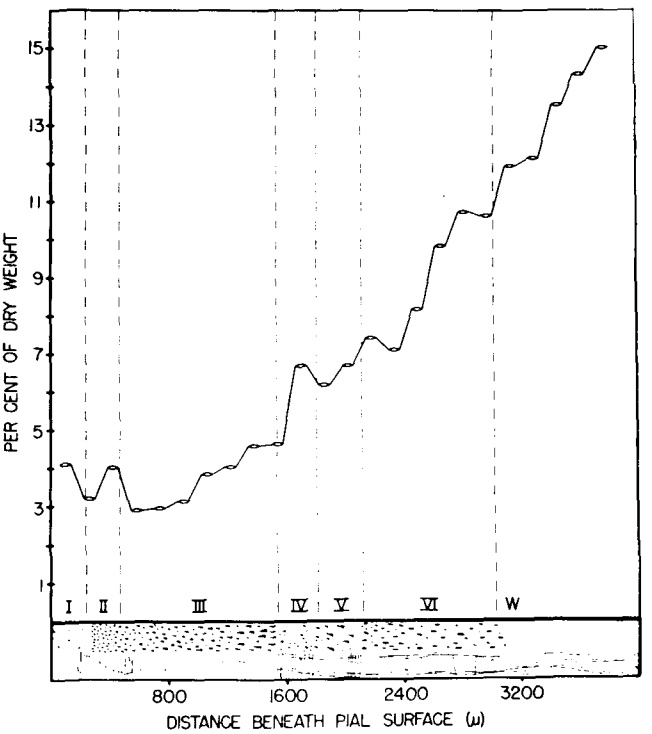

FIG. 15. A graph showing the intralaminar distribution of cerebrosides in the human eulaminate frontal cortex. The average thickness of the cortical layers is shown along the abscissa. (Reproduced by permission of E. Lewin and H. H. Hess in J. Neurochem. *12*, 213 [1965].)

(73), and sphingolipids (74) on dried samples weighing about 10 μg. They have attempted to use these procedures in the characterization of relatively homogeneous areas of normal brain such as Ammon's Horn of the hippocampal formation (75) and cerebellar cortex (76). Roots (77) has studied the lipids of isolated neurons.

The analysis of isolated cells of the same class solves the problem of relating lipid composition to cell type. However, we do not yet possess sufficient information to appraise whether or not cells of the same type in different areas of the brain have a similar lipid composition or whether the lipid composition of a cell changes during a normal life span. This information would be helpful in evaluating changes in isolated diseased cells. Furthermore, all the techniques

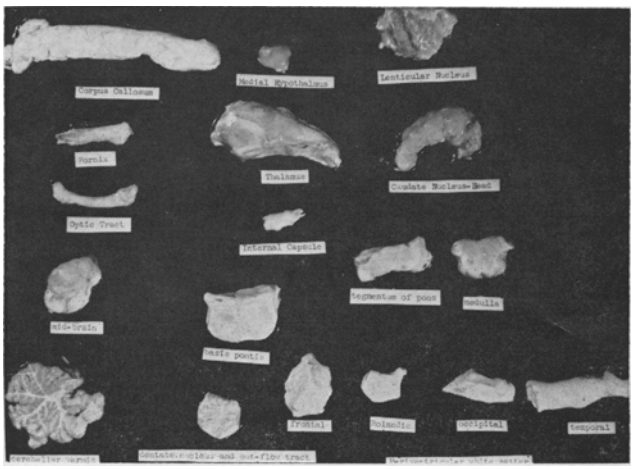

FIG. 16. Sixteen samples of human brain which may be isolated and stored for future biochemical analysis in cases in which the pathological nature of the disease is not known.

TABLE III

Diseases Which Involve the Neurons of the Cerebral Cortex

	Major biochemical abnormality
I. Storage disorders with excessive accumulations of one or more metabolites:	
A. Infantile amaurotic idiocy (Tay-Sachs)	Storage of ganglioside (G5)
B. Late infantile amaurotic idiocy	?Storage of ganglioside
C. Juvenile amaurotic idiocy (Batten-Spielmeyer-Vogt)	Unknown (? Lipofuscin)
D. Adult form of amaurotic idiocy (Kuf)	Unknown
E. Gargoylism	Storage of mucopolysaccharides and gangliosides (G5, G6)
F. Niemann-Pick disease	Storage of sphingomyelin
G. Farber's disease	Unknown
H. Glycogen storoge disease (Type II)	Storage of glycogen
I. Metachromatic leukodystrophy	Storage of sulfatide
J. Fabry's disease	?Storage of ceramide-trihexoside and ceramide-dihexoside
II. Neuronal degenerations:	
A. Alper's disease	Unknown
B. Ford-Christensen-Krabbe disease	Unknown
C. Jakob-Creuzfeldt disease	Unknown
D. Alzheimer's disease	Unknown
E. Pick's disease	Unknown
F. Degenerations associated with a systemic carcinoma	Unknown

of isolation described to date refer primarily to the cell body, and at best the most proximal portion of the cell processes. Dissection of a complete cell has thus far proved impossible. It is quite possible that disease could begin in a part of the cell not yet separated by these techniques. Finally, in order to obtain a complete picture of the biochemical and morphological changes occurring in a tissue, one would have to know the changes in the number of cells in an area affected by a disease process. Once again, this would involve the use of quantitative histological techniques that are not yet available.

Macro-Sampling Techniques

In contrast to the effort devoted to micro-sampling procedures, there have been few innovations in macro-sampling techniques. This is unfortunate, since many of the diseases that involve the nervous system are either diffuse neuronal processes involving the storage of unusual materials or cell degeneration and death (Table III), or fall into the category of "system" degenerations in which a single tract or nucleus or a set of related tracts and cells deteriorate (Table IV). Many of these areas can be isolated in bulk and reproducibly, if one is familiar with the gross anatomy of the nervous system. Large samples are advantageous in that they average the effects of local histological and pathological variations. Rouser and his associates (21), in the process of performing biochemical analyses on a group of diffuse disorders of infancy and senescence, chose to separate the gray and white matter of an entire cerebral hemisphere since anatomically this is the most easily defined unit in the nervous system. Aliquots of each were then analyzed for their lipid content. This method yielded results that were more reproducible than those obtained from the analysis of small blocks of tissue. A second method is described in "Dissection of Brain." It can be used for storing samples of brain conveniently when the pathology of the disorder is not known as well as for isolating specific nuclei that are known to be selectively involved by a disease process. This technique involves the classical dissection of fresh brain after it is cooled rapidly to 4C. This facilitates the separation of gray and white matter as well as some white matter tracts along natural planes of cleavage. By making use of these planes as well as certain external landmarks, reproducible, easily identifiable areas of the cerebral hemisphere and brain stem can be separated. Unfortunately, the method cannot be applied to the brains of infants under about 6 months of age. It is of most value when combined with micro-sampling techniques appropriate to the area being studied.

The disadvantages of these macro-sampling procedures are by now obvious; essentially they are similar to those enumerated for 1- to 2-g blocks of tissue. It is not possible to correlate biochemical changes with cell morphology except by inference; the changes that occur with the processes of distintegration and repair are summated as are primary changes due to the initial insult and secondary changes that result from Wallerian degeneration. Minor changes occurring early in a disease may be obscured by the large amounts of normal tissue still present; the samples must still be meticulously controlled for age. Nevertheless, when the pathology is extensive and well-defined, these relatively simple methods yield useful information.

Storage of Tissue

Regardless of the sampling procedures used, further problems arise when preparing the same block of tissue for both microscopic and biochemical investigations. These difficulties result from the way in which the tissue is stored and the methods used to fix it prior to sectioning. Freezing the tissue is the most satisfactory way of storing it for biochemical analysis, since this preserves the integrity of the lipids, and, for brief periods at least, some of the metabolic reactions of the tissue. A specimen that is packaged in tightly sealed plastic bags shows no significant change in water content when stored at −50C for long periods. On the other hand, freezing the tissue can result in the formation of ice crystals and the disruption of membranes, which may distort the appearance of the cell, particularly the nucleus, and leave empty clefts and spaces in the standard preparation (78). Very rapid freezing and thawing minimizes this process as does dehydrating frozen tissue in absolute alcohol for several days prior to fixation.

TABLE IV

System Diseases (Degenerations Which Involve Related Nuclei and Tracts)

Disease	Areas involved	Major biochemical abnormality
I. *Pyramidal*		
Amyotrophic lateral sclerosis	Neurons of precentral gyrus Spinal motor neurons Corticospinal tract	Unknown
II. *Extrapyramidal*		
A. Basal ganglia:		
1. Paralysis agitans	Substantia nigra Lenticular nucleus	Unknown
2. Huntington's chorea	Caudate nucleus	Unknown
3. Dystonia musculorum deformans	Corpus striatum Globus pallidus Corpus Luysi	Unknown
B. Spinocerebellar degenerations:		
1. Olivo-ponto-cerebellar	Neurons of olives, pons and cerebellar cortex	Unknown
2. Friedreich's ataxia	Spinocerebellar tracts Dorsal and lateral columns of spinal cord	Unknown
3. Cerebellar-cortical atrophy	Neurons of the cerebellar cortex, especially Purkinje cells Neurons of the cerebral cortex	Unknown
4. Dentato-rubral atrophy (Ramsey-Hunt syndrome)	Neurons of the dentate nucleus of the cerebellum and the red nucleus	Unknown

Freezing the tissue also alters its metabolic activity. Many enzyme systems, particularly those involving oxidative phosphorylation, are totally or partially destroyed. In contrast to structural changes, the enzymatic activity of a tissue appears to be affected least when freezing and thawing is performed slowly (79). Chilson et al. (81) suggest that these phenomena are probably the result of denaturation of proteins secondary to changes in the pH and the ionic strength of cellular fluid as the eutectic point of many of these solutes is approached.

Tissue that is being saved for pathological studies is usually stored in a solution of 10% to 40% formalin. This method of preserving tissue has been known to lead to false biochemical analyses since 1929, when Weil (81) first showed that phospholipids were hydrolyzed with the release of phosphoric acid. The effect of formalin on the phospholipid composition of brain has been investigated several times since (19,82–84) and it is now established that storage in formalin for periods as brief as 24 hr results in a marked reduction in phosphatidyl ethanolamine and some loss of phosphatidyl serine (84). Longer storage transforms about 25% of the lecithin present to lysolecithin (82). Cerebrosides and cholesterol tolerate storage in formalin solutions, but cholesterol esters are gradually hydrolyzed. Polysialogangliosides are degraded to their monosialo forms slowly, and eventually even this last monosialic acid residue is split to yield ceramide hexosasides (85). Recently it has been observed that tissues fixed in solutions of gluteraldehyde retain normal structure and enzyme function for up to 24 hr (86). Cerebral tissue has been fixed by intravascular perfusion of this compound, and the content of each lipid class has been shown to be stable (87). However, this technique is only useful when working with laboratory animals.

The problems connected with the selection of samples and storage of tissue suggest that cooperation between the biochemist and the neuropathologist should begin with the fresh specimen. Anatomical landmarks are seen more easily in fresh tissue, wet weights are more accurate, and dissections are simpler to perform. The nature of the studies to be done can be decided upon, and the most suitable sampling procedures chosen. One or more samples of tissue can be fixed by methods that will best satisfy the biochemist, histochemist and pathologist.

The type of sampling procedure selected is based on what is known about the pathology of disease. Table V outlines the sampling techniques that seem to be most suited to different categories of pathological disease in the central nervous system. In most instances, accurate correlations between biochemical and morphological information require the use of more than one type of sample. When the nature of the disorder is unknown, the brain is divided by a midsagittal section; one-half is used for pathological studies and the other half frozen for possible chemical analyses at a later date.

Many technical problems remain still to be solved. However, rapid methods for separating lipid classes from small samples combined with improved microchemical techniques are already being developed, and it should soon be possible to analyze a number of small samples in a reasonable period of time. Histochemical methods designed to locate specific lipid classes in tissue slices are also progessing rapidly and have recently been reviewed by Adams (88,89). However, it is still possible to find significant biochemical abnormalities in tissues that appear histologically normal (90,91). The major obstacles to progress in this field at the moment are the limited techniques available to the quantitative histologist. Very few efforts have been made to estimate the number of cells in normal or diseased brain (92–94), and the current methods are so time-consuming as to make the process untenable on a large scale. Furthermore, there are no satisfactory methods for separating entire cells of a single type either from homogenates or tissue slices, or for estimating the weight or volume contributed to a tissue sample by the cells of any one type. Data of this type will have to be gathered before exact correlations between biochemical and morphological events can be made in areas as complex and variable as those of the nervous system.

Dissection of Brain

Using external landmarks and natural planes of cleavage, it is possible to subdivide the human brain into a number of well-defined areas corresponding to classical anatomical designations. Unfortunately, few of these areas are structurally or functionally homogeneous. Nor do pathological processes usually conform to their boundaries. Despite these drawbacks, this type of gross dissection has several advantages. These subunits are easily identifiable and can be removed in a reproducible manner providing the chemist with a series of normal baselines; smaller areas cannot be isolated in their entirety with this degree of consistency. When the pathological process is diffuse, removal of large units allows the investigator to minimize local variations. When the location of the pathology is uncertain, virtually every area of the brain can be sampled, proper histological controls made and then stored conveniently.

The dissection is performed shortly after the brain is removed at autopsy and is best done at 4C.

1) Remove the Circle of Willis. Free its major branches from the circle toward the periphery removing the accompanying meninges with them. The remaining meninges can then be stripped from the cortex by blunt dissection, exerting traction with a forceps and loosening adherent meninges with one's fingers.

2) Make a shallow midsagittal cut on the ventral surface of the brain separating mammillary bodies and bisecting the optic chiasm.

3) Turn the brain over and expose the dorsal surface of the corpus callosum by spreading apart the

TABLE V

Sampling Techniques Most Suited to Various Types of Cerebral Pathology

Site of the most distinctive pathology	Useful sampling techniques
1. Myelin sheath	Density-gradient centrifugation White matter of the entire hemisphere Small samples of white matter, with suitable controls matched for age and area
2. Neurons	
a. Storage diseases	Separation of single cell bodies by dissection or filtration Cortex of the entire hemisphere Small samples of cortex with suitable controls matched for age and area
b. Degenerative diseases	Quantitative histochemistry Dissection of single cells
3. Inclusions or unusual cells	Density-gradient centrifugation Dissection of single cells
4. Focal or multifocal lesions in white or gray matter	Excision of whole lesions Quantitative histochemistry Dissection of single cells
5. System diseases	Classical dissections of involved tracts or gray matter Dissection of single cells

medial surfaces of the cerebral hemispheres. Bisect the corpus callosum, the roof of the third ventricle and the massa intermedia. The posterior commissure is now exposed.

4) The brain stem and cerebellum can now be separated from the cerebral hemisphere in a reproducible plane formed by a line running from the posterior commissure to the posterior margin of the mammillary body and perpendicular to the fibers of the cerebral peduncle.

5) Free the cerebellum from the brain stem by dissecting away the roof of the fourth ventricle and then sectioning the cerebellar peduncles at the point at which they enter the substance of the brain stem.

6) Split the cerebellar hemispheres by a midline longitudinal incision through the vermis.

7) Divide the brain stem along the midline with a rostrocaudal incision using the aqueduct of Sylvius and the midline groove on the floor of the fourth ventricle as guides.

At this point, the brain has been split in half and each of the halves subdivided into three sections: cerebral and cerebellar hemispheres and brain stem. The right half is generally saved for histological study and further dissection is limited to the left.

A. The Cerebellar Hemisphere

1) A parasagittal incision 2 mm from the midline removes a block of the vermis. The V-shaped core of central white matter is excised. The folia of the vermis are now almost completely separated by their fissures and may be totally separated by a small incision in the plane of the fissure.
2) A central horizontal cut halves the remainder of the hemisphere and exposes the dentate nucleus. The cells of the dentate and their efferent fibers which exit through the hilum of the nucleus are removed *en bloc* with a scalpel or razor and stored. The sections that contain the dentate nucleus are not reproducible from brain to brain since the planes of cleavage between gray and white matter are artificial.

B. The Brain Stem

1) The brain stem is divided into three sections: mid-brain, pons and medulla by ventrodorsal cuts at the superior and inferior margins of the base of the pons.
2) The base of the pons is separated from the tegmentum.
3) Parts of individual nuclei can be removed as desired. Again planes of cleavage are artificial and one cannot be sure of removing identical areas of each nucleus in every brain dissected.

C. The Cerebral Hemisphere

1) The hemisphere is approached from its medial surface. The septum pellucidum is removed exposing the lateral ventricle. Gentle traction strips the choroid plexus from the ventricular wall.
2) The fimbria of the hippocampus is identified posterior to the thalamus and traced to the body of the fornix which is stripped from the inferior surface of the corpus callosum by blunt dissection using the handle of a scapel. The entire fornix is now free from the fimbria to the septal area at the level of the anterior commissure and is removed.
3) The gray matter of the cingulate gyrus is stripped back from the superior surface of the corpus callosum and the medial 5 mm of the callosum is removed by making a parasagittal cut extending from the genu to the splenium.
4) The optic tract is freed to the point at which it penetrates the brain.
5) The mammillary body and medial hypothalamus are removed by a cut extending from the superior surface of the mammillary body to the anterior commissure.
6) The caudate nucleus and the thalamus form two protrusions on the lateral wall of the ventricles. First the head and neck of the caudate nucleus are removed. Blunt dissection can be used to remove the adjacent white matter from the superior and anterior surfaces of the nucleus. Since the caudate and putamen are joined at their anterior inferior surfaces, an arbitrary cut is made separating them in the plane tangential to the lateral margin of the caudate. The white matter penetrating the lateral and inferior surfaces of the caudate are removed while the inferior margin is still attached to the ventricular ependyma and used as a fulcrum for traction. At the end of this phase of the dissection, this attachment is cut and the caudate removed.
7) The optic tract which had previously been isolated up to its entry into the brain is now used to place traction on the thalamus by pulling it gently in a lateral direction. Blunt dissection with one's fingers is usually sufficient to find a plane along the lateral margin of the pulvinar which can be followed anteriorly exposing the lateral surface of the thalamus and subthalamus. Since the superior and medial surfaces face the ventricle and the anterior surface was freed in removing the head of the caudate, the thalamus and subthalamus can now be removed. These two areas cannot be separated from each other in a reproducible fashion, however.
8) Gentle traction and blunt dissection easily separate the lateral wall of the lenticular nucleus from the external capsule. The dissection begins at the anterior margin of the putamen previously exposed when the caudate was removed. The medial white matter, which consists, for the most part, of fibers of the internal capsule, can also be stripped away by blunt dissection. However, a scalpel is needed to trim the base and superior surfaces and strip away the remnants of the internal capsule.
9) At this point, the internal nuclei have all been removed and the entire medial face of the brain consists of white matter of the centrum semiovale. Further horizontal cuts along the medial wall of the occipital or frontal lobes expose the central white matter of these areas. Samples may be taken at any level desired using the outline of the ventricle as a landmark.
10) An alternative approach suitable for subcortical white matter or for gray matter is to select any gyrus desired and free a segment of it by two cuts perpendicular to the plane of

the gyrus and a cut parallel to the surface at its base. White matter may be taken from the central core of the gyrus, leaving the gray mantle. This yields fairly reproducible samples if the same gyrus is chosen on each occasion and if the distance between the two perpendicular cuts is identical.

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