METHODS OF NEUROCHEMISTRY

Volume 1

Edited by RAINER FRIED

Department of Biochemistry
Creighton University
Medical School
Omaha, Nebraska

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INTRODUCTION

Research and knowledge in the neurosciences have been progressing rapidly, and significant contributions are being made in many fields that will permit us to gain a better understanding of the metabolism and function of the brain, and the mode of operation of the mind. Due to the complexity of the nervous system and its function, a special methodology has developed; its mastery is essential for successful and meaningful research.

The objective of the present collection is to present a thorough, well-documented series of methods prepared by experts in their respective fields that should serve as a reliable manual for pertinent laboratory procedures. Rather than giving a compilation of untested methods reported in the literature, the authors stress the methodology used in their own laboratory with which they are well familiar and which they have been using successfully. Where different basic principles of experimentation are available, several methods will be reported in consecutive volumes of this series.

We hope to be able to present a collection of modern methods, covering all important areas of the neurosciences, mainly in the fields of biochemistry and pharmacology. The series is planned to be open-ended, and we intend to publish additional volumes as soon as sufficient material is available. Each volume will contain methods related to several distinct areas of research rather than obey a unifying concept. Suggestions of subjects to be covered and expert authors would be welcome. It is our hope that the present new series will fill a need and will deserve a place in the library of neurosciences. Suggestions, comments, and criticisms of readers and users would be greatly appreciated.

I should like to thank the Publishers for their interest and for making it a pleasure to work with them in this new venture. I should like to express my appreciation to the authors for their effort and valuable contributions and to Miss Louise Starman and Miss Margaret Howse for their efficient and dedicated aid in preparing this series.

RAINER FRIED

PREFACE

The emergence of neurochemistry as one of the fastest growing life sciences during the last decade has resulted in an outpouring of neurochemical publications. This makes the task of the editor of a book in the field of neurochemistry especially difficult because in addition to the general editorial problems of selection of subject matter and selection of form of treatment of the subject, the elusive and treacherous questions of timeliness and of relevance are now added.

In the present volume, Dr. Rainer Fried has met all these challenges with a fair degree of success. Instead of trying to cover the vast material encompassed by the title *Methods of Neurochemistry*, he has limited himself to essentially monographic coverage of circumscribed themes that appear to have been carefully chosen for their interest to the bench chemist, and to the medical scientist who wishes to initiate research in neurochemistry.

The five subjects chosen have been covered by authors of generally recognized competence in each particular field: They are: myelin, by L. C. Mokrasch; phospholipids, by G. B. Ansell and S. Spanner; catecholamines, by D. F. Sharman; microiontophoresis, by K. Krnjević; and biochemical screening and diagnostic procedures in mental retardation, by D. O'Brien. All five subjects are timely and highly relevant to the mainstream of neurochemical research. Each has been treated in a form to be of interest to the general neurochemist and with technical descriptions that will be found useful by all interested laboratory workers. Dr. Fried has himself contributed the final chapter in the form of a "Tabulation of compounds of importance in neurochemistry." This chapter is a pioneering task. He has wisely refrained from making his listing exhaustive and has concentrated on compounds of clear neurochemical interest. Each com-

pound is briefly discussed from the chemical and the physiological points of view. The usefulness of this list is that it constitutes a timesaving device that will permit rapid access to the main sources of information. It is possible that this chapter may win for Dr. Fried criticism as well as approval according to how helpful it turns out to be in each particular case. A clear use of this chapter is to permit the reader to find readily the source material for subjects other than the ones discussed in the preceding five chapters.

The Editor has studiously avoided making this text competitive with other publications; instead he has attempted to make it complementary, on the one hand, to the more extensive treatises, and, on the other hand, to the truly specialized monographs. As a consequence, the book may bridge some of the gaps left between these two other types of presentation of particular subjects. Indeed, the main use of this book may be as a benchside text that at the same time contains enough theoretical and background information to permit the exploration in depth of a given subject.

J. FOLCH-PI

CONTRIBUTORS TO VOLUME I

G. B. ANSELL, Department of Experimental Neuropharmacology, The Medical School, Birmingham, England

RAINER FRIED, Department of Biochemistry, Creighton University Medical School, Omaha, Nebraska

K. KRNJEVIĆ, Department of Research in Anesthesia, McIntyre Medical Sciences Building, McGill University, Montreal, Quebec, Canada

WALTER MENZIES, Department of Biochemistry, Creighton University Medical School, Omaha, Nebraska

LEWIS C. MOKRASCH, McLean Hospital Research Laboratory, Belmont, Massachusetts

DONOUGH O'BRIEN, Department of Pediatrics, University of Colorado Medical Center, Denver, Colorado

D. F. SHARMAN, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge, England

SHEILA SPANNER, Department of Experimental Neuropharmacology, The Medical School, Birmingham, England

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CHAPTER I

PURIFICATION AND PROPERTIES OF ISOLATED MYELIN

LEWIS C. MOKRASCH

MC LEAN HOSPITAL RESEARCH LABORATORY
BELMONT, MASSACHUSSETTS

I. INTRODUCTION

A. Definition and Critique

Myelin may be defined as the lamellar investment of an axon by the membrane of a satellite cell. In the central nervous system, the satellite cell is an oligodendroglion, and in the peripheral nervous system, the satellite cell is the Schwann cell. This definition is most valid when applied to myelin in its normal state in the nervous tissue or in histological sections thereof and when applied to myelin as a purely structural entity. When one considers myelin as a functional entity or as an isolated product, some abiguities in the definition may be noted.

Satellite cell cytoplasm and organelles are frequently found intercalated in the spiral winding. Should these be considered to be an integral part of myelin or an accessory part? Is the myelin actually the satellite cell membrane or is it derived therefrom? Should the satellite cell body be considered to be an integral part of the myelin structure or an accessory part? Without the satellite cell body and without the axon, is the isolated product myelin or a myelin form?

These points are generally of little concern to the investigator who is interested primarily in removing nonmyelin contaminants from his preparation and then studying the chemical composition or the physical structure of the preparation. Sooner or later, however, the physiological significance of material studied *in vitro* must be related to the precursor *in vivo*.

Some authors use the relative absence of nucleic acid as an index of purity of their myelin preparations (*1*). Nucleic acid in a myelin preparation could represent contamination from axoplasm or satellite cell cytoplasm, if the latter is not regarded as being an integral part of the myelin sheath. Other investigators regard at least part of the nucleic acid found in the myelin to be a characteristic component of myelin (*2*).

There is evidence to suggest that the myelin membranes are different in composition from the cell membranes from which they are presumed to be formed (*3*). Furthermore, there are changes in the composition of myelin which are not paralleled in extra-myelin structures (*4*). There are also changes in metabolic activities of myelin which do not parallel those of other cellular constituents from which the myelin could be derived (*5*). Therefore, myelin must be regarded as a part different from the cell wall of the satellite cell.

From the fact that isolated myelin has essentially the same ultrastructural

features as myelin in histological preparations (*6,7*), one cannot infer that isolated myelin is unchanged from its native state. The osmotic responsiveness of myelin is well documented (*8–10*). It is probable that myelin passes through a state of considerable disorganization during the homogenization, if not also during its other periods of suspension, during which authentic myelin constituents could be lost, enzymes denatured, and subtle rearrangements in its constituents occur. When the myelin is recovered in a concentrated state, such as a centrifugal residue, it seems probable that even a completely disaggregated system of myelin membranes could reform the classical myelin structure, in the same manner as its hypotonic disaggregation is reversible. Indeed, other lipoprotein membrane systems are capable of re-forming to their native ultrastructural appearance after being completely disaggregated (*11,12*), and the formation of myelin figures appears to be a naturally favored mode of reaggregation even in synthetic systems (*13–15*).

Finally, another criterion of purity of isolated myelin is its solubility in chloroform–methanol mixtures (*16*). However, Lees (*17,18*) has shown that the solubility of nerve tissue proteins in chloroform–methanol mixtures depends upon the ionic environment of the tissue preparation at the moment of extraction. Indeed, if isolated myelin is suspended in an isotonic buffer system rather than an isotonic sucrose solution, it is never completely soluble in chloroform–methanol mixtures (*19*).

B. Historical Review

The term 'myelin' was assigned by Virchow (*20*) to a structure which he thought had more of the characteristics of a core than of a sheath or cortical structure. By X-ray and optical polarization techniques, it was discovered that myelin had a laminar structure with the laminae paralleling the axon (*21*). Although the histologists of the early part of this century were aware of the investment of the axon by the Schwann cell, it remained for Geren (*22*) to propose that myelin was a part of the Schwann cell membrane and that it had a spiral configuration. Later, it was correctly suggested that a single satellite cell could invest more than one axon (*23*).

While the morphology of membranes was being clarified, the chemical structure of membranes began to receive attention. The first widely accepted hypothesis concerning the chemical structure of membranes was that of Davson and Danielli (*24*). A modification of this classical bimolecular model was that of Robertson (*25*) which he termed the 'unit membrane.'

In this hypothesis, all membranes had an essentially similar chemical structure: a double molecular sheet of lipids sandwiched between layers of protein in a highly ordered configuration. Largely on the basis of the osmiophilic properties of membranes, particularly myelin, it was proposed that the lipids were oriented with their polar or ionic moieties toward the outer protein layer. The protein layer was ordered to present a highly polar surface toward the lipid layer and a hydrophobic or nonpolar surface to the exterior of the membrane. The work of Finean (*26*) provided support for the protein–lipid–protein 'sandwich' structure of myelin; from this and from the known chemical composition of myelin, models were described whose dimensions agreed well with the data obtained by electron microscopy and X-ray diffraction (*27,28*).

More recently, however, the 'unit membrane' hypothesis and the postulated chemical structure consistent with it have been re-examined (*29*). Briefly, more accurate information on the structural specificity of OsO_4 (*30*) led to the proposal that membranes are more likely to have a chemical arrangement opposite in sense to that previously proposed, viz., the lipid core has its molecules oriented with their nonpolar structures toward the protein layers and that the protein layers present a hydrophobic inner surface and a hydrophilic outer surface. This proposed configuration has received support from a number of sources (*31–33*).

II. PREPARATIVE PROCEDURES

A. Preparation of Tissue

1. Smaller Animals

For animals in the size range mouse to guinea pig, a light ether anesthesia before decapitation is recommended. The severed head is placed on a paper towel with its major axis at about 45° with respect to the edge of the work bench and the wound toward the worker. The pointed tip of a pair of scissors with a blunt and a pointed tip is inserted through the foramen magnum and guided against the interior surface of the skull while cutting along a coronal section just over the ears and toward the eyes on both sides. The cut portion of the skull is folded up and forward along the coronal suture with the aid of blunt forceps or the tip of a small spatula. The flat tip of a small spatula is inserted between the severed end of the spinal cord and the skull and moved laterally in a circular rotation so as

to sever the cranial nerves. Then the tip of the spatula is slid forward along the brain pan as far as it will go. A beaker containing a copious volume of an isotonic solution at 0° should be ready to receive the excised brains. If the skull is inverted over the beaker and a slight scooping retraction of the spatula is made, the brain should fall free and be in a fairly undamaged condition. With a little practice, the investigator can have the brain in the cold saline within 15 sec after decapitation.

2. Larger Animals; Separation of White and Gray Matter

For larger laboratory animals in the size range rabbit to dog, ether or pentothal anesthesia is recommended. Since some force will need to be used to open the skull, decapitation is not recommended: The rest of the carcass helps in handling the skull by anchoring it, as it were. Using bone shears, the skull is opened and brain removed, essentially in the same manner as with the smaller animals.

Normally one would receive brains from larger animals by arrangement with a slaughterhouse or autopsy facility, the precaution of chilling the brains in ice or cold saline being observed. After washing the brains in cold saline to remove blood and other foreign matter, gray and white matter may be separated by the following procedures.

a. Central Gray Matter

This is the most easily separated portion of larger brains. First, the meningeal membranes must be removed. This can be accomplished by positioning the brain with its inferior surface on a cold porcelain plate. With a pair of blunt forceps, the membrane can be stripped away and discarded. The exposed gray matter can be obtained by carefully scraping at the surface with a spatula, scalpel, or knife until the underlying white matter is barely visible. The yield of myelin from gray matter will be quite small.

b. White Matter—Method A

The brain is placed on a porcelain plate and meninges removed. Then the brain on the plate is placed in a freezer and frozen solid. When the tissue is completely frozen, it is then removed to the laboratory at normal room temperature. After about 15 min, the gray matter will have thawed and can be wiped or scraped away from the still-frozen white matter. After an additional 30 min or so, the brain is cut into slabs a centimeter or so thick and any gray matter nuclei appearing in cross section are excised. What remains is essentially pure white matter.

This method is not recommended when the isolation of relatively undamaged subcellular organelles other than myelin is also desired. In addition, the thawed tissue has a semiliquid consistency which makes it difficult to handle; otherwise, this method is comparatively easy in terms of manipulation except for the larger brains with numerous convolutions.

c. White Matter—Method B

The brain is placed on a porcelain plate and the meninges removed. With a flat blade, sagittal sections, 0.5–1 cm thick, are cut and laid flat on another cold porcelain plate. The cortical gray matter and transsected nuclei are trimmed away with a blade. What remains is practically pure white matter.

This method is the fastest of the three, and is one most suitable for the largest preparations. By adapting the method to thinner sections, one could select milligram samples of white matter from discrete myelinated tracts.

d. White Matter—Method C

The brain is placed on a cold porcelain plate and a midsagittal section is made. The easily recognized corpus callosum is excised and dropped into the cold saline. Next, each hemisphere is sagittally sectioned into approximately equal portions which are laid with their cortical surfaces on a cold porcelain plate. With a scalpel, the exposed white matter is stripped away from the underlying gray matter and deposited in the cold saline.

This method is the most tedious of the three, but it assures the purest white matter.

3. Dispersion of Tissue

There are several points at which the preparative technic determines the value of the preparation according to the objective of the experiment. The first of these is the method by which the tissue is dispersed into a uniform suspension or 'homogenized.' The second point is the choice of medium which will be used as the suspending solution.

a. Dispersing Devices

Commonly, prior to dispersion, the tissue is minced with scissors or razors or is macerated in a mortar. This author prefers the use of a tissue press (*34*) which simultaneously macerates the tissue and removes fibrous tissue. After weighing the minced or macerated tissue, it is gently suspended in the suspending solution.

The breaking of the cells is best accomplished by the use of the Potter–Elvehjem homogenizer having a precision bore glass tube and a Teflon or Kel-F pestle (*35*). Glass (*36*) or metal pestles are not suitable because the friction of the glass surface against the pestle quickly produces a sol of fine glass or metal particles; clearly neither is desirable. Commercial Teflon–glass homogenizers (*37*) with a smooth glass surface and a nominal clearance of 0.2 mm give a good preparation with negligible attrition of the plastic. For large preparations, a larger version of this basic instrument has been devised (*38*). A detailed discussion about the clearance tolerance without specifying temperature is not warranted because the plastics mentioned have large thermal coefficients of expansion. Ordinarily, the pestle is machined at one temperature (20–25°) and used at another (0–4°).

Some investigators use the Dounce homogenizer (*39*) for preparing the homogenate. This author prefers to use this tool for resuspending centrifugal residues.

Because of its toughness, a preparation of peripheral nerve must begin with the use of a Waring blender or similar device. Ordinarily, this would be avoided because of an inadequated degree of control between insufficient cell breakage and excessive destruction of subcellular particles (*40,41*). The same reservation applies to the use of other devices using the same shearing principle (*42,43*).

The use of an annular orifice homogenizer (*44*) is very effective in dispersing tissue, but it has been reported to be excessively destructive to subcellular organelles (*45*) when used according to the original specifications. Accordingly, its use for myelin preparations is not recommended.

b. Suspending Medium

Since the finding of Schneider and Hogeboom that isotonic sucrose was an ideal medium for the isolation of subcellular particulates from liver (*35*), this medium has been adopted for the study of brain subcellular particulates as well. Since most mammalian tissues lack invertase, sucrose is regarded as an inert solute. It has been shown, however, that sucrose is not entirely inert (*36,46,47*).

Isotonic mannitol has been reported to allow the isolation of brain particles with superior metabolic properties (*48,49*) and to interfere less in the solubility properties of brain proteins (*17*) than sucrose solutions. For these reasons, this author prefers the use of isotonic mannitol for the isolation of brain particles when their enzymic integrity is critical.