Tracing Neural Connections with Horseradish Peroxidase

Edited by
M-Marsel Mesulam

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Preface for IBRO Handbook Series

During the last fifty years there have been two changes in the way in which scientists have studied the nervous system. First of all, the traditional and largely independent major scientific disciplines of physics, chemistry, physiology, pharmacology, and pathology gave rise to the more specialized sub-disciplines of neurophysiology, neurochemistry, neuropharmacology, etc., and the science of experimental psychology was born. Then, after about another generation, it became clear that a deeper understanding of the brain could not be achieved by seperate and unrelated studies in each of these sub-disciplines. Rather, a unified approach was needed in which the specialized methods were applied in a co-ordinated way to solve a particular problem. Indeed, combinations of methods could often yield results not obtainable by the application of any individual technique. Thus, scientists studying the nervous system began to call themselves neurobiologists or neuroscientists because they did not wish to be identified with any particular experimental discipline. Very soon meetings took place (e.g. in 1955 the First International Meeting of Neurobiologists) and organizations (Neuroscience Research Program, MIT) were founded to give formal recognition of this new approach to the study of the brain. In 1958 the decision was taken in Moscow to establish the International Brain Research Organization (IBRO), which became incorporated as an independent organization through a bill in the Parliament of Canada at Ottawa in 1961.

IBRO now has 2000 members, most of whom hold senior positions in research or teaching, in 52 countries of all political complexions. Through its National Corporate members, many of which are Academies of Sciences or national societies for neuroscience, the body of neuroscientists reflected in IBRO must be of the order of 15,000. One of the programmes of IBRO, all of which aim to serve the international community of neuroscientists, is the publication programme. IBRO publications include *IBRO News*, *Neuroscience* and the *IBRO Symposia Series*. With the present *Handbook Series*, IBRO aims to fill a major gap in the world literature. The neuroscientist needs to be able to turn to whichever specialized method that is most suited for the problem he is currently studying. The series on *Methods in the Neurosciences* will help to provide expert advice on exactly how to carry out the experiments, on what difficulties can occur and on the limitations of the method.

It is planned as a continuing series, so that new volumes can be published as and when new methods are developed, tested, and found useful. It is my hope that books in this series will have a significant impact on neuroscience throughout the world, by helping to provide the tools with which the scientist can tackle his problems.

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Preface

The tracing of neural pathways continues to be a central concern for neuroanatomy. During the second half of the nineteenth century, at a time when the foundations of neuroanatomy were being established, tracing pathways often required laborious dissections by hand. Subsequently, the availability of methods for staining Nissl substance, myelin, and axons offered alternative strategies. However, the modern era in neuroanatomy did not start until the introduction of the Nauta method and its variants. These techniques captivated the interest of many investigators and catalysed extensive progress in the clarification of neural pathways.

The Nauta procedures dominated research for almost a quarter of a century. Then, neuroanatomy experienced a methodological revolution in the beginning of the 1970s with the development of powerful techniques based on the axonal transport of tracers such as horseradish peroxidase and radiolabeled amino acids. The revolution continues. New methods, one more ingenious and powerful than the other, are rapidly being added to the armamentarium of the neuroanatomist. Limitations on the type of anatomical questions that can be asked have all but disappeared. The problem is no longer how to formulate the question according to the limitations of available methodology but to choose the most applicable method from among numerous alternatives. Whereas tracing pathways used to be the exclusive domain of neuroanatomists, it is now within the reach of investigators in other areas of neuroscience. Neuroanatomical problems that required infinite patience in the past now yield definitive answers rapidly and reliably. It is not surprising, therefore, that advances in methodology are currently attracting considerable interest.

This volume discusses the uses of horseradish peroxidase, one of the two tracers which initiated the revolution in neuroanatomical methods. Horseradish peroxidase now offers an unusually convenient method for tracing connections of central neurons, peripheral ganglia, nerves, muscles, skin, viscera, and related structures. The first chapter in this volume deals with the underlying theory of axonal transport, enzyme histochemistry, and lectin conjugation. The practical implications of these principles are then outlined with respect to tracing neural connections with the light microscope. The second chapter addresses the electronmicroscopic applications of horseradish

peroxidase histochemistry for determining the synaptic arrangements of neural pathways. The third chapter extends this analysis to intracellular injections of tracer when the experimental goal is to correlate the physiological properties of individual neurons with their morphological specializations and local connectivity.

Each chapter aims to provide a comprehensive analysis of the subject. Redundancy has been discouraged by frequent cross references among the three chapters. The intended audience includes the neophyte as well as the expert. For those with no prior experience, there are extensive practical guidelines, caveats, and appendices. For the seasoned investigator, there are detailed sections on background and on the potential for new directions. Each chapter contains an extensive table of contents so that the reader can rapidly identify the relevant section. The sequence of sections within each chapter attempts to reproduce the sequence of practical considerations, from the choice of tracer and injection device to the final interpretation of the results. I hope that this detailed account will facilitate the applications of horseradish peroxidase histochemistry, consolidate the interpretation of observations and perhaps also stimulate further developments.

During the preparation of this volume, it has been a pleasure to work with Dr. A. D. Smith, Director of Publications for IBRO, and Dr. S. D. Thornton, Publishing Editor for Life Sciences at John Wiley and Sons.

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CHAPTER 1

Principles of Horseradish Peroxidase Neurohistochemistry and their applications for Tracing Neural Pathways—Axonal Transport, Enzyme Histochemistry and Light Microscopic Analysis

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PART I INTRODUCTION

I.1 Foundations

The enzyme horseradish peroxidase (HRP) has a wide range of applications for tracing neural connections. Although the plasma membrane constitutes an effective barrier to its free diffusion into intact cells, extracellular HRP gains entry into neurons through a process of endocytosis. The membrane-delimited endocytotic vesicles which contain the enzyme are then transported along neural processes that emanate from the site of administration. While the HRP molecule is not itself visible, readily detectable reaction-product is obtained by enzymatic action at the site of administration as well as at sites of transport. In this fashion, the connectivity of the region injected with HRP can be determined quite conveniently.

More than a century has elapsed between the discovery of peroxidases and their application for tracing neural connectivity. Peroxidase activity appears to have been described first in 1855 by Schoenbein who noted that extracts of mushrooms and other tissues, in the presence either of air or hydrogen peroxide, turned guaiac solutions blue (Paul, 1963). Schoenbein's extracts were undoubtedly mixtures of oxygenases and peroxidases. A distinction between these two types of enzymes was made in 1897 when Bourquelot divided the oxidizing substances of living matter into four groups. Substances

in the first three groups could utilize the oxygen in the air for oxidation, whereas the fourth group contained substances which displayed oxidizing properties only in the presence of peroxides such as hydrogen peroxide. During the March 25, 1898 session at the Biological Society of Paris, Linossier (1898) suggested the term 'peroxidase' for substances in this fourth group, a term which has been retained to the present. Bourquelot (1898), on the other hand, preferred the term 'ferments oxydants indirects' and lamented Linossier's choice by stating that: 'Il me semble, avec plusieurs de mes collègues de la société qui l'ont déjà fait remarquer, que cette dénomination n'est pas heureuse.' Clearly, his preference did not prevail.

Peroxidase activity is virtually ubiquitous in living tissue, especially in plants. The root of horseradish (*Cochlearia armoracia*) and fig sap are two of the richest sources but abundant activity is also present in Japanese radish, broad bean leaves, sweet potato, wheat germ, petunia, yeast, and even tea leaves (Paul and Stigbrand, 1970; Pruidze *et al.*, 1976). The isolation and eventual purification of a peroxidase was successfully undertaken by Wilstätter and his colleagues between 1917 and 1924 (Keilin and Hartree, 1951). The crystallization of peroxidase from horseradish root, one of its richest natural sources, was reported by Theorell in 1942 and also by Keilin and Hartree in 1951. The availability of crystalline horseradish peroxidase (HRP) resulted in the determination of a molecular weight around 40,000 (44,100 by Theorell in 1942 and 39,800 by Cecil and Ogston in 1951). Furthermore, direct microspectroscopic examination of slices cut from horseradish root led Keilin and Mann (1937) to the conclusion that HRP is a hematin-containing protein. Subsequent investigations revealed that HRP also contained carbohydrate groups in addition to amino acids (Welinder, 1976). Native HRP was thus found to consist of a glycoprotein apoenzyme containing a covalently bound hematin group. Horseradish root contains several peroxidase isoenzymes which differ greatly in isoelectric point, amino acid composition and enzymatic activity (Kay *et al.*, 1967; Paul and Stigbrand, 1970; Shannon *et al.*, 1966). Isoenzyme C of Shannon *et al.* (1966) and IIIb of Paul and Stigbrand (1970) constitute the most abundant variety. The amino acid sequence and the sites of the 8 carbohydrate attachments of this HRP isoenzyme were described by Welinder (1976) who reported the presence of 308 amino acid residues containing four disulfide bridges. The molecular diameter of the complete HRP molecule was calculated to be 4.74 nm with a 0.3 nm hydration shell (Steiner and Dunford, 1978).

Research on the enzymology of peroxidases has largely relied on chromogenic substances which change color when oxidized by the peroxidase–peroxide complex. Thus, the darkening of guaiac solutions upon oxidation was used as the marker for peroxidatic activity in the initial observations by Schoenbein, Bourquelot, and Linossier. A great many substances other than guaiac can act as chromogenic markers for peroxidases. Even as early as 1904,

Adler and Adler listed more than two dozen substances which could be employed for detecting the weak peroxidatic activity of blood. Although each of these substances was suitable for the detection of peroxidatic activity *in solution*, only a few proved adequate for *histochemical* applications which require the oxidized chromogen to precipitate as an insoluble polymer and to remain as a visible marker at the tissue sites containing enzyme activity.

One of the chromogens used by Adler and Adler (1904) was benzidine, a substance which subsequently proved useful for histochemical applications. Benzidine had been discovered by Zinin in 1853 (Scott, 1962). Although Fischel (1910) claims priority for the description of a histochemical procedure based on benzidine, he was apparently unable to obtain uniform results so that the brownish reaction-product in his preparations tended to fade in about a week. The consistency and stability of the benzidine procedure was improved by Kreibich (1910) who acidified the incubation medium (Graham 1918–19). The suitability of benzidine as a sensitive histochemical marker was further demonstrated by Graham (1918–19) who obtained selective staining of peroxidatic activity in the granules of myelogeneous leukocytes so that they could be differentiated from other types of white blood cells. Graham had previously found alpha-naphthol and pyronin quite suitable for this purpose but was forced to look into an alternative method when the first world war made the reagents unavailable in the United States. However, stability of the benzidine reaction-product appeared difficult to secure and the initial green stain in Graham's preparations tended to fade into a brown color. This phenomenon led to the introduction of sodium nitroferricyanide as an effective stabilizing agent for the blue reaction-product (Goodpasture, 1919). Gomori (1952) subsequently evaluated the cumulative experience with benzidine and described a reliable procedure based on sodium nitroferricyanide as the stabilizer.

The isolation of peroxidase from horseradish root and parallel advances in detecting tissue-bound peroxidase activity in blood smears offered the conditions necessary for the use of HRP as a histochemical tracer, a development which can be attributed to the work of Straus. Straus turned to HRP because he needed a more sensitive marker than egg white for tracing the endocytosis of intravenously administered protein into renal cells. In his initial experiments, Straus (1957) homogenized the kidneys and then calorimetrically determined the distribution of endocytosed HRP by reacting specific homogenate fractions with hydrogen peroxide and dimethyl-*p*-phenylenediamine, a chromogen that yields a red pigment upon oxidation. Although these experiments introduced HRP as a macromolecular *tracer*, they did not involve its *histochemical* demonstration at tissue sites containing enzyme activity. This subsequent development was also introduced by Straus (1959) who described a histochemical method for the microscopic demonstration of endocytosed HRP. For these histochemical applications, Straus (1959)

employed benzidine as the chromogen according to the recommendations that had been outlined by Gomori (1952) and was able to detect a specific precipitation of an intensely blue reaction-product within HRP-containing endocytotic vesicles in the liver and kidney of rats that had been injected intravenously with HRP. In two ensuing publications, Straus (1964a,b) consolidated the pertinent methodology by addressing the factors which determine the stability, sensitivity, and specificity of the benzidine-blue reaction for the histochemical detection of tissue-bound HRP.

Despite many successful applications, the Straus procedure was displaced by the Graham and Karnovsky (1966) method which introduced two major innovations: fixation with a glutaraldehyde–paraformaldehyde mixture and the use of diaminobenzidine as the chromogen. Diaminobenzidine yielded a brown reaction-product which could then interact with osmium tetroxide to provide electron-dense precipitates at sites of HRP activity. The procedure was simple, the specificity high and the ultrastructural detail exquisite. The Graham and Karnovsky (1966) procedure rapidly gained widespread acceptance and became established as the method of choice for the histochemical detection of HRP activity during the period when HRP histochemistry was being developed as a neuroanatomical method. The initial use of HRP as a neuroanatomical tracer was therefore profoundly influenced by two special properties of the diaminobenzidine procedure. First, the excellent ultrastructural detail enabled rapid developments in determining the subcellular compartments which participate in the uptake and transport of HRP (La Vail and La Vail, 1974; Turner and Harris, 1974). However, the low level of histochemical sensitivity of the initial diaminobenzidine procedures also resulted in frequent and often unsuspected underestimations of neural connectivity.

I.2 Neuroanatomical Applications

As had been the case for the kidney, HRP was introduced into the nervous system in order to monitor the pinocytosis of macromolecules from the extracellular space into neurons. Prior to the use of HRP, pinocytosis into neurons had been investigated with several electron-dense tracers, especially ferritin (Brightman, 1965; Rosenbluth and Wissig, 1964). Many of these tracers could not be identified by the light microscope. The additional advantage of obtaining a tracer that could produce a visible reaction-product at the light microscopic level provided some of the motivation for the investigation by Becker, Hirano, and Zimmerman (1968) who placed pellets of HRP directly into the brain of the rat. At the light microscopic level, they described the presence of the diaminobenzidine reaction-product within lysosome-like intracytoplasmic organelles of dendrites and perikarya. Their

ultrastructural analysis of this preparation demonstrated reaction-product in multivesicular bodies, simple vesicles, dense bodies, and within elements of the endoplasmic reticulum. Endocytotic uptake was suggested as the mode of entry for the HRP by the demonstration of reaction-product within coated pinocytotic vesicles. This pioneering investigation was followed by reports from Holtzman and Peterson (1969) that intact mammalian neurons can incorporate intravenously administered HRP by means of endocytosis. Furthermore, a simultaneous observation by Zachs and Saito (1969) showed that intramuscular injections of HRP in the mouse led to the rapid uptake of label into coated vesicles of nerve endings in the neuromuscular junction.

The next development necessary for utilizing HRP histochemistry in tracing neural connectivity was to show that the endocytosed enzyme was transported. There were many reasons for expecting the existence of such a phenomenon. Indeed, the occurrence of intra-axonal transport had already been described by Weiss and Hiscoe in 1948. Subsequently, direct cinémicroscopic examination by Hughes (1953) showed that pinocytotic vacuoles which formed at the nerve tip moved within the axon. He suggested the presence of a bidirectional intra-axonal traffic of such organelles. Thus, there were encouraging if not compelling reasons to expect that extracellular HRP would not only be incorporated into neurons by pinocytosis but that it would also be transported. Indeed, Becker *et al.* were perhaps the first to notice such transport in 1968 when they described particulate staining in the corpus callosum following hemispheric HRP implants. However, no special significance was attributed to this observation.

The definitive demonstration of a neural connection by means of transported HRP was not accomplished until the experiments of Kristensson and Olsson in 1971. Kristensson (1970) had already shown the retrograde transport of albumin (labeled with Evans Blue) from the gastrocnemius muscle of the suckling mouse to the corresponding motoneurons in the spinal cord. The motivation for using HRP instead of labeled albumin in these experiments was provided by the desire to obtain a more easily detectable tracer. Thus, in 1971, Kristensson and Olsson reported that the retrograde transport of intramuscularly administered HRP could be readily demonstrated in spinal cord tissue fixed and processed with diaminobenzidine according to Graham and Karnovsky (1966). Subsequently, LaVail and LaVail (1972) demonstrated an analogous retrograde transport in the central nervous system, from the retina to the isthmo-optic nucleus of the chick. The subcellular compartments that subserve this transport were then rapidly elucidated in elegant detail (LaVail and LaVail, 1974; Turner and Harris, 1974). These developments introduced HRP histochemistry as a major method for tracing neural connections.

The minute quantity of HRP that is transported by an individual neuron is by itself virtually invisible under light or electron microscopic examination.

At least four types of markers have therefore been introduced for detecting the distribution of the HRP in neural tissue. One approach is based on covalent binding of the HRP molecule to a fluorescent substance which can act as a specific marker when the tissue is examined by fluorescent microscopy (Hanker *et al.*, 1976; Norden *et al.*, 1976). Another approach includes the immunohistochemical detection of the enzyme through the formation of HRP–anti-HRP complexes and their subsequent fluorescent or histochemical detection (Vacca *et al.*, 1975; Sofroniew and Schrell, 1980). A third alternative is to attach a radioactive label to the HRP molecule so that the distribution of the tissue-bound enzyme can be traced by autoradiography (Geiseit, 1976). The fourth and most traditional strategy is to use the enzymatic activity of the HRP itself to produce a visible reaction-product (Straus, 1959). Although the uptake and transport of the HRP molecule remains unaltered, each method for detecting the resultant distribution of the tracer introduces an individual set of goals during tissue processing. The autofluorescent method depends on the preservation of the fluorescent label; the immunohistochemical method requires the preservation of immunological properties of the HRP molecule; and the direct histochemical method depends on the preservation of enzymatic activity. The autoradiographic method, on the other hand, appears most resilient since the radiolabel is extremely stable and since its activity and location are not influenced by the immunological or enzymatic integrity of the parent HRP molecule. However, the HRP molecule needs to undergo biochemical manipulation for autoradiographic and autofluorescent labeling and this could conceivably alter its ability to be incorporated and transported.

The threshold for detecting HRP, the ratio of sensitivity to specificity, and the resultant morphological detail are likely to vary from one method of detection to another. For example, the sensitivity and specificity of immunohistochemical detection is closely influenced by the individual preparation of antiserum. Furthermore, current methods that are available for fluorescent and radioactive labeling offer variable and often low levels of specific activity which may interfere with the sensitivity of detection. Although each of these four approaches for labeling the HRP molecule has been used successfully for tracing neural connections, the original method based on the direct histochemical generation of a reaction-product at sites of HRP activity remains as the most convenient, flexible, and popular approach and will therefore constitute the focus of attention in this chapter.

In experiments based on the direct histochemical detection of HRP, the distribution of reaction-product at the time of microscopic examination provides the only information for determining the site of HRP administration, the distribution of resultant transport, and consequently, the pattern of neural connectivity. The accuracy in determining neural pathways is therefore influenced by a number of factors related to the enzymatic activity of HRP.