ENZYME HISTOCHEMISTRY

and Its Application in the Study of Neoplasms

M. S. BURSTONE

ENZYME HISTOCHEMISTRY

and Its Application in the Study of Neoplasms

M. S. Burstone

National Cancer Institute
National Institutes of Health
Bethesda, Maryland

ACADEMIC PRESS • 1962
NEW YORK and LONDON

ACADEMIC PRESS INC.
111 FIFTH AVENUE
NEW YORK 3, N. Y.

United Kingdom Edition
Published by
ACADEMIC PRESS INC. (LONDON) LTD.
BERKELEY SQUARE HOUSE, LONDON W. 1

Library of Congress Catalog Card Number 62-13046

PRINTED IN THE UNITED STATES OF AMERICA

FOREWORD

Histochemical techniques may be considered as extensions of the routine histopathologic diagnostic staining procedures. The latter methods, although providing valuable morphologic information to the pathologist and histologist, lack chemical significance. Aware of the greatly expanded knowledge of biochemistry in recent years, the microscopist inevitably has been confronted with questions concerning the cytologic localization of newly discovered biochemical entities including enzyme systems in tumors. Attention has again focused on morphologic problems with those related to the structural organization of the metabolic machine being of central importance in the new field of molecular biology. The pathologist too has sought to interpret mechanisms based on altered biochemical systems. Of considerable practical importance is the expectation that evidence obtained by histochemical methods would aid in solving problems of diagnostic pathology. Thus modern chemical orientation in the morphologic sciences has demanded precise and highly specific cytochemical methods, particularly for enzyme systems.

The present text covers the subject of enzyme histochemistry in unprecedented depth clearly presenting the basic chemistry involved and evaluating critically the various aspects essential to the successful application of the methods. This work brings together from many sources information of value to both the developmental histochemist and biologist interested in applying new techniques, and furthermore provides insight into the conception and development of the relatively new field of enzyme histochemistry. The thorough compilation of the impressive number of available methods covers the historical development of the field in addition to the advances made, a number of which have come from the author's laboratory. As an interdisciplinary science, histochemistry utilized knowledge in several fields and the author has performed a unique and difficult service in bringing together the essentials of organic chemistry, biochemistry, and morphology upon which the methods are based.

The novel organization of the work with appended compilations of chemical data concerning organic dyes and substrates as well as the detailed outlines of the methods should prove useful for easily accessible reference. The extensive information on the synthesis of substrates and the detailed outlines of the methods including critical appraisal and evaluation will provide a ready aid to those applying the techniques to biological problems. Of particular interest is the chapter dealing with the relationship between histochemical substrates and carcinostatic agents, an area of potential clinical value which as yet has been little explored.

Director, National Cancer Institute
National Institutes of Health
Bethesda, Maryland
October, 1962

KENNETH M. ENDICOTT

ACKNOWLEDGMENT

I wish to acknowledge the support of those who encouraged this work, especially Dr. Michael B. Shimkin, Associate Director for Field Studies, National Cancer Institute, and Dr. Eli M. Nadel, Chief, Diagnostic Research Branch, National Cancer Institute. Particular thanks are due Dr. S. S. Spicer, National Institute of Arthritis and Metabolic Diseases, and Dr. Frank B. Johnson, Armed Forces Institute of Pathology, who have read the text in its entirety and who have offered helpful advice and suggestions. Also I should like to express my appreciation to my staff members, including Mrs. N. Joan Archard, Miss Sally Rockwell, and Miss Paula Weaver, who have done the major portion of the typing of the manuscript and related tasks. Much of the photographic data was done by Mr. John W. McGuire and associates. Other photographic data have been generously furnished by my friends and colleagues whose names are appropriately noted in the text. Proofreading of the galleys as well as assistance in the preparation of the subject index have been done by Mrs. Doris Chaney and Miss Linda M. Whiting.

October, 1962 M. S. BURSTONE

CONTENTS

INTRODUCTION

According to the review edited by Sumner and Myrbäck (1950, p. 2), the term enzyme was introduced by Kühne in 1878. Preliminary studies on enzymes, however, were carried out between 1830 and 1840 by Liebig, Wohler, and Berzelius, who viewed the role of enzymes as similar to that of inorganic catalysts whose function was not necessarily associated with a living organism.

Sumner and Myrbäck (1950, p. 1) state that in one sense life may be defined as an orderly function of enzymes. Disease may manifest itself as a disorder, inhibition, or hyperfunction of enzymes. Greenberg (1960, p. 3) describes enzymes as an invention of nature designed to accelerate and control in a specific manner the numerous chemical reactions that determine the metabolism and vital activity of cells, and thus of the organism. By their unique specificities, by their interlocking assembly line functioning, by changes in the physical environment such as pH and temperature, by their changing content, by the rise and ebb of their substrates, and by the influence of activators and inhibitors, including hormones, enzymes maintain an orderly balance of physiological processes necessary for the preservation of the organism. Thus, enzyme control and regulation is initiated with conception and continues throughout all phases and all functions of the life cycle of the organism in health and disease.

Many aspects dealing with the vast literature on classical enzymology are considered in the reviews by Sumner and Myrbäck (1950, 1951a,b, 1952), Hoffmann-Ostenhof (1953, p. 219), Sumner and Somers (1953), Lindley (1954), Alberty (1956), Mehler 1957, Boyer *et al.* (1959), Best (1960), Boyer (1960), and Clifton (1960, p. 212). Dixon and Webb (1958) emphasize the ramifications of enzymology and its close relation with many sciences including biochemistry, physical chemistry, bacteriology and microbiology, genetics, botany and agriculture, pharmacology and toxicology, pathology, physiology, medicine, and chemical engineering. The application of enzymes in clinical medicines has been the subject of a recent review by Innerfield (1960). Enzyme histochemistry has also become significantly associated with a number of research and clinical areas, including diagnostic and research pathology, oral histology and pathology, and veterinary medicine.

In general, biochemical analysis of enzymatic activity has involved homogenization techniques in which the cells are ruptured. Holter (1952)

indicates that this technique is susceptible to various sources of error, the primary ones being the effects of autolysis and elution or resorption during the process of separation. The total enzymatic activity of the homogenate may be assayed, or else the suspension may be separated into various fractions by centrifugation at varying speeds. Of interest is the fact that Bensley and Hoerr (1934), who were the first to isolate mitochondria by differential centrifugation of disrupted guinea pig liver cells, stained and examined microscopically their preparations during various stages of the procedure. Danielli (1953) has termed the technique used to isolate cellular organelles including mitochondria as maceration procedures, and comments that changes in the submicroscopic organization and composition of both nuclear and cytoplasmic bodies may be the result of both the homogenization procedure as well as the solutions which are used in fractionation since the nucleus and cytoplasm contain complex colloidal systems.

By contrast, definitive microscopic histochemical techniques utilize tissue sections the architecture of which is not grossly disturbed, and employ known chemical reactions to produce insoluble chromogenic precipitates precisely at the cytological sites associated with specific enzyme activities. Thus, localization of a variety of enzymes may be accomplished at the cytological level; and even of greater importance, sites of enzyme activity may be delineated to one or two specific cells in a relatively large heterogeneous sample. Although such techniques are not quantitative from a strictly numerical standpoint, the relative activity of the reactive cells may be approximated by the rapidity at which the dye formation or precipitation occurs. The microscopic techniques, however, may be quantitated by the ingenious approach developed by Linderstrøm-Lang and associates (Glick, 1949) in which different but essentially comparable tissue samples are employed for microscopic examination and for quantitative microanalysis, respectively. Another approach to the quantitation of certain types of histochemically produced dyes involves the pooling of tissue sections and extraction of the dye which is then measured colorimetrically.

In applied histochemistry the quality of microscopic localization or in other words the accurate reflection of enzymatic activity as revealed by a dye precipitate is, of necessity, the determining factor as to the value of any technique. Histochemists trained as histologists or histopathologists should expect to see clear-cut microscopic pictures provided that proper histochemical techniques are employed. Available evidence at our present state of knowledge indicates that sharply delineated staining patterns are significant even when there is an overall cytoplasmic activity (Chapter 4). The all too facile description of blurred diffuse dye deposits including small dye crystals (unrelated to any known cellular organelles) as accurate

enzymatic localizations must be avoided, since such interpretations merely represent attempts to rationalize poor results obtained with outmoded methods or improper histochemical techniques.

From the standpoint of the experimental and practicing pathologist, however, microscopic enzyme techniques are of potential value in detecting histological and cytological alterations which are not discernible with routine non-specific stains. The development of new and sophisticated procedures makes it reasonable to assume that enzyme histochemical techniques of the future will be employed as diagnostic tools because of their capability of revealing early metabolic alterations which occur concomitantly with the neoplastic process. With reference to present-day procedures, of interest is the suggestion that the aminopeptidase azo-dye technique is of use in detecting lymph node metastases of gastric carcinoma (Chapter 10, p. 415). The well-known application of the alkaline phosphate procedure in the differentiation of "leukemoid" reactions and leukemias (Chapter 5, Section II, D) also warrants further evaluation. The use of the Mylar film or tape technique (Chapter 5, p. 276) may lend itself to the automation of histochemical diagnostic procedures, once appropriate diagnostic histochemical methods have been developed.

An important but relatively unexplored area lies in the application of enzyme techniques in the study of a variety of non-neoplastic pathological conditions. Included are diseases of the musculo-skeletal and nervous systems, to which a number of presently available oxidative enzyme procedures may be applied. In addition to some of the more prosaic applications in experimental histopathology and embryology, enzyme techniques may be of considerable significance in the study of drug metabolism. For example, many hydroxyl-containing aromatic compounds are conjugated *in vivo* as glucuronides which in turn are hydrolyzed by β-glucuronidase in certain sites (Fishman, 1961, p. 31). It is conceivable that increased activity of the aforementioned sites associated with metabolic transformations may be revealed by histochemical techniques. An analogous situation involves the hydrolysis of bis(2-amino-1-naphthol)phosphate by phosphatase to produce the carcinogenic 1-hydroxy-2-naphthylamine (Troll *et al.*, 1959).

The application of enzyme histochemistry in the study of lesions including tumors which are related to virus or virus-like agents has not received even cursory consideration. Of interest are the findings by Riley (1961) that certain mouse tumors exhibit a virus-tumor synergism which is shown by an accelerated growth of the tumor and by elevation of lactic dehydrogenase in the host plasma. The possible tissue sites of increased dehydrogenase activity could be explored by means of histochemical procedures.

An important application of enzyme histochemistry lies in the study of

various enzyme systems which are associated with the calcification process, especially as it relates to bones and teeth of mammalian species. Although at the present time such studies have primarily tended to reveal specific sites of enzyme activity from a morphological standpoint, a considerable amount of evidence has also been gleaned which tends to shed light upon the mechanisms by which the processes of bone apposition and resorption occur. Among the enzyme systems of calcifying tissues which have been delineated by histochemical procedures are phosphatases, esterases, dehydrogenases, and oxidases (Burstone, 1960a,b).

The enzymatic changes which are associated with aging tissue have received considerable interest from a biochemical standpoint (Sinex, 1961). For example, Barrows *et al.* (1960) have reported a decreased succinoxidase activity in rat kidney associated with aging, and correlated it with a decrease in the number of mitochondria per cell. Application of histochemical procedures and their correlation with biochemical methods may augment the evaluation of enzymatic alterations which occur with aging.

The terms constitutive and adaptive enzymes describe those enzymes present in fixed and variable concentrations, respectively (Mehler, 1957, p. 392). A variety of adaptive enzymes has been described in both microorganism and animal tissues. Definitive localization of the distribution and changes in activities of adaptive enzymes may be elucidated by the histochemical approach. Of interest is the observation that liver cytochrome oxidase activity is greatest adjacent to the portal areas where the oxygen tension is highest (Burstone, 1959).

Of particular significance are the newer applications of histochemical techniques in the study of enzyme activity at the electron microscope level, as well as the relationship of histochemical substrates to cancer chemotherapeutic agents, topics which are discussed in Chapters 13 and 15, respectively.

A particularly intriguing aspect of enzyme histochemistry lies in its potential application to *exobiology*, the study of extraterrestrial life. Lederberg (1960) has suggested the application of cytochemical techniques which are adaptable to automation and telemetric recording procedures. Thus, a transparent tape ribbon could be employed to collect samples which would then be incubated in suitable substrate media, following which the cytochemical reaction sites could be detected and evaluated by a microscope-Videcon-transmitter system. A related area involves the application of enzyme histochemistry in the study of the effects of ionizing radiations including those of extraterrestrial origin as well as well controlled laboratory experiments employing known isotopes. In general, enzyme histochemical techniques should be valuable tools in the study of radiation effects and damage in tissue sections.

In large part, the recent advances in the field of enzyme histochemistry have been directly related to the synthesis and development of new substrates and techniques. At the termination of the second World War, only two or three histochemical enzyme procedures were available, while during the relatively short intervening period some forty to fifty new enzyme techniques and modifications have been developed. Although many completely new substrates have been synthesized, cognizance should be taken of the fact that many of those presently employed were first synthesized and reported around the turn of the century. Specific examples are α- and β-naphthyl phosphates which were reported by Kunz in 1894 and α- and β-naphthyl acetates which were synthesized by Miller (1881a,b). In fact, the synthesis of almost all simple esters of α- and β-naphthol including sulfates, glucuronides, and glucosides has been described in the earlier German chemical literature. Indoxyl acetate which was used as the basis of the first so-called indoxyl esterase procedure was first prepared by Vorländer and Drescher in 1901. Even the more complex naphthol AS acetate, whose histochemical application was originally described by Gomori (1952b), was first synthesized in 1909 by Graff. Thus it is apparent that the design and synthesis of really new and specific histochemical reagents has received serious consideration only during recent years. If the present rapid rate of progress of developmental histochemistry continues, a large series of new and specialized techniques for both basic research and diagnosis should be available in the foreseeable future.

The format of the present text includes an appendix at the end of each chapter. In the case of specific enzyme systems, the appendix is divided into two parts, A and B, respectively. Part A gives specific laboratory procedures for the synthesis of various substrates and related compounds, while part B enumerates methods and laboratory procedures for the microscopic demonstration of enzymes. Also included are the various types of fixation and embedding procedures which have proved of value in enzyme histochemistry. A brief review dealing with the application of specific enzyme techniques is incorporated in specific chapters. An integral part of many chapters is a table indicating a large number of potential substrates which either have been applied in enzyme histochemistry or biochemistry, or else may have potential application with reference to new substrate design. These tables also include pertinent data on molecular structure, melting points, and bibliographic references. Although the various appendices may serve as a comprehensive laboratory manual, it is hoped that the organization of the text and general approach to the problem stresses the significant relationship between organic chemistry, and in particular dyestuff chemistry, and microscopic enzyme histochemistry.

Among the previously published texts and reviews on histochemistry

including enzyme histochemistry which may be employed as references are those by Glick (1949, Techniques of Histo- and Cytochemistry); Gomori (1952a, Microscopic Histochemistry); Danielli (1953, Cytochemistry, A Critical Approach); Eränkö (1955, Quantitative Methods in Histology and Microscopic Histochemistry); Arvy (1957–1958, Les Techniques Actuelles D'Histoenzymologie); Deane *et al.* (1960, Histochemical Methods for the Demonstration of Enzymatic Activity); Pearse (1960, Histochemistry, Theoretical and Applied); McManus and Mowry (1960, Staining Methods, Histologic and Histochemical); Lison (1960, Histochemie et Cytochemie Animales, Vols. I and II); Lillie (1954, Histopathologic Technic and Practical Histochemistry); and Wachstein (1962, Histochemistry of Enzymes in Tumors).

References

Alberty, R. A. (1956) *Advances in Enzymol.* **17**, 1–6.

Arvy, L. (1957–1958) "Les Techniques Actuelles d'Histoenzymologie." Collection of reprints from *Biologie méd. (Paris)* 46–47.

Barrows, C. H., Jr., Falzone, J. A., Jr., and Shock, N. W. (1960) *J. Gerontol.* **15**, 130–133.

Bensley, R. R., and Hoerr, N. L. (1934) *Anat. Record* **60**, 449–455.

Best, J. B. (1960) *Intern. Rev. Cytol.* **9**, 129–186.

Boyer, P. D. (1960) *Ann. Rev. Biochem.* **29**, 15–44.

Boyer, P. D., Lardy, H., and Myrbäck, K. (1959) "The Enzymes," 2nd ed., Vol. 1. Academic Press, New York.

Burstone, M. S. (1959) *J. Histochem. and Cytochem.* **7**, 112–122.

Burstone, M. S. (1960a) *In* "Metabolism of Oral Tissues" (R. F. Sognnaes, ed.). *Ann. N. Y. Acad. Sci.* **85**, 431–444.

Burstone, M. S. (1960b) *In* "Calcification in Biological Systems," pp. 217–243. Am. Assoc. Adv. Sci., Washington, D. C.

Clifton, E. E. (1960) *In* "Enzymes in Health and Disease" (D. M. Greenberg and H. A. Harper, eds.), pp. 212–268. Charles C Thomas, Springfield, Illinois.

Danielli, J. F. (1953) "Cytochemistry, a Critical Approach." Wiley, New York.

Deane, H. W., Barrnett, R. J., and Seligman, A. M. (1960) *In* "Handbuch der Histochemie" (W. Graumann and K. Newmann, eds.) Vol. 7, Part 1. Fischer, Stuttgart, Germany.

Dixon, M., and Webb, E. C. (1958) "Enzymes." Academic Press, New York.

Eränkö, O. (1955) "Quantitative Methods in Histology and Microscopic Histochemistry." (In statistical collaboration with Jaakko Kihlberg.) S. Karger, Basel, New York.

Fishman, W. H. (1961) "Chemistry of Drug Metabolism." Charles C Thomas, Springfield, Illinois.

Glick, D. (1949) "Techniques of Histo- and Cytochemistry." Interscience, New York.

Gomori, G. (1952a) "Microscopic Histochemistry." Univ. Chicago Press, Chicago, Illinois.

Gomori, G. (1952b) *Intern. Rev. Cytol.* **1**, 323–335.

Graff, J. (1909) *Ann.* **367**, 253–265.

Greenberg, D. M. (1960) *In* "Enzymes in Health and Disease" (D. M. Greenberg and H. A. Harper, eds.), pp. 3–25. Charles C Thomas, Springfield, Illinois.

HOFFMANN-OSTENHOF, O. (1953) *Advances in Enzymol.* **14,** 219–260.
HOLTER, H. (1952) *Advances in Enzymol.* **13,** 1–20.
INNERFIELD, I. (1960) "Enzymes in Clinical Medicine." McGraw-Hill, New York.
KUNZ, P. (1894) *Ber.* **27,** 2559–2565.
LEDERBERG, J. (1960) *Science* **132,** 393–400.
LILLIE, R. D. (1954) "Histopathologic Technique and Practical Histochemistry," McGraw-Hill, New York.
LINDLEY, H. (1954) *Advances in Enzymol.* **15,** 271–299.
LISON, L. (1960) "Histochimie et Cytochimie Animales," 3rd ed., Vols. I and II. Gauthier-Villars, Paris.
MCMANUS, J. F. A., AND MOWRY, R. W. (1960) "Staining Methods, Histologic and Histochemical." Hoeber, New York.
MEHLER, A. H. (1957) "Introduction to Enzymology." Academic Press, New York.
MILLER, O. (1881a) *Ber.* **14,** 1600–1602.
MILLER, O. (1881b) *Ann.* **208,** 223–248.
PEARSE, A. G. E. (1960) "Histochemistry, Theoretical and Applied," 2nd ed. Little, Brown, Boston, Massachusetts.
RILEY, V. (1961) *Science* **134,** 666–668.
SINEX, F. M. (1961) *Science* **134,** 1402–1405.
SUMNER, J. B., AND MYRBÄCK, K., eds. (1950) "The Enzymes," Vol. I, Part 1. Academic Press, New York.
SUMNER, J. B., AND MYRBÄCK, K., eds., (1951a) "The Enzymes," Vol. I, Part 2. Academic Press, New York.
SUMNER, J. B., AND MYRBÄCK, K., eds., (1951b) "The Enzymes," Vol. II, Part 1. Academic Press, New York.
SUMNER, J. B., AND MYRBÄCK, K., eds., (1952) "The Enzymes," Vol. II, Part 2. Academic Press, New York.
SUMNER, J. B., AND SOMERS, G. F. (1953) "Chemistry and Methods of Enzymes," 3rd ed. Academic Press, New York.
TROLL, W., BELMAN, S., AND NELSON, N. (1959) *Proc. Soc. Exptl. Biol. Med.* **100,** 121–122.
VORLÄNDER, D., AND DRESCHER, B. (1901) *Ber.* **34,** 1854–1860.
WACHSTEIN, M. (1962) *In* "Handbuch der Histochemie" (W. Graumann and K. Newmann, eds.), Vol. 7, Part 2, pp. 73–153. Fischer, Stuttgart.

CHAPTER I

FIXATION AND EMBEDDING TECHNIQUES

I. INTRODUCTION

Fixation techniques for enzyme histochemistry include the frozen-section procedure, utilization of aqueous fixatives, freeze-drying, and freeze-substitution. Unfixed frozen sections are included in this group since some degree of denaturation may occur during freezing and thawing or subsequent processing.

Although definitive histochemistry usually necessitates proper fixation, most of our knowledge of fixation has been empirically established. Each type of fixation procedure must be carefully selected for the specific problem at hand. Unfortunately, no one type of fixation offers a panacea for the histochemical laboratory.

For enzyme localizations, there exists a close relationship between initial fixation, substrate used, products of hydrolysis, and the final dyestuff pattern. In a general way, an enzyme technique is no better than the state of fixation of a given tissue. For example, a procedure designed to give good histochemical localizations may have limited use with acetone fixed paraffin embedded kidney, since such parenchymatous tissues may be considerably distorted by acetone fixation. The use of unfixed frozen sections in conjunction with techniques for the demonstration of hydrolases may involve considerable hazard, not simply because of loss of enzyme, but also because of solubility and instability of unfixed cytoplasmic constituents. Thus, it may be possible to lose relatively insoluble enzyme simply because of movement of cytoplasm.

Fortunately, many hydrolytic enzymes are well preserved following fixation as well as embedding in paraffin. The most desirable fixative would be one which renders proteins insoluble without resulting in loss of enzyme activity. It is known, for example, that urease treated with alcohol becomes irreversibly insoluble but remains enzymatically active (Sumner, 1948).