



**HISTO-
CHEMICAL
TECHNIQUE**

W. G. Bruce Casselman

**METHUEN'S MONOGRAPHS ON
BIOLOGICAL SUBJECTS**

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Preface

Through its integration of studies on structure and chemical composition, histochemistry can contribute to many biological investigations and provide further insight into the nature of various physiological and pathological processes. The effective use of histochemical methods requires an appreciation of their potentialities and limitations as well as an adequate knowledge of their chemical bases. Mindful of these requirements, this monograph attempts to provide an introduction to the principles and practice of microscopical histochemistry, especially the chemical aspects, and to present a beginning selection of reliable techniques applicable to a variety of biological problems.

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Toronto, Canada
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Microscopical Histochemistry

Histochemistry is concerned with the chemistry of cells and tissues. It deals not only with the chemical nature of their structural components but also with the nature of the chemical processes going on in them. Sometimes, a distinction is made between 'histochemistry' and 'cytochemistry' on the basis of the level of organization being studied. However, many histochemical observations must be made at a cytological level so that 'histochemistry' properly includes 'cytochemistry' (Baker, 1952).

Histochemistry can be regarded as an extension of morphological investigations to include chemical composition. While histological preparations demonstrate the structural organization of cells and tissues, histochemical preparations enable the identification and localization of certain classes of chemical substances. At the same time, histochemistry resembles biochemistry in its concern with chemical constitution but it differs from biochemistry in being also concerned with morphological localization. The name 'physiological histochemistry' (Dempsey and Wislocki, 1946) emphasizes an aspect of histochemistry that is often overlooked. The greatest value is obtained from histochemical investigations when the observations on structure and composition are correlated with the functional state of the cells and tissues.

The ultimate aim of histochemistry is to describe the dynamic organization of cells and tissues in terms of their structure, composition, and function. The results achieved up to the present fall far short of this goal whose attainment depends upon at least three lines of investigation (Danielli, 1947):

- (a) determination of the nature, distribution and concentration of the chemical constituents of living cells and tissues.
- (b) determination of the nature of the chemical reactions

occurring in cells and tissues and the means whereby materials and energy are stored in them, and

(c) determination of the mechanisms for the functional utilization of energy provided by the chemical reactions, that is, the fundamental relationships between structure and function in cells and tissues.

1.1 Microscopical histochemistry

A wide variety of physical, chemical and biological techniques are available for studying chemical constituents in their natural locations in cells and tissues. Those methods in which the final observations are made microscopically on tissue sections or similar preparations constitute *microscopical histochemistry*, the subject of this monograph. This branch of histochemistry provides almost the only approach to certain biological problems: because the structural integrity of the cells and tissues is preserved and because the cells or their components can be taken as the units of investigation, information that would be completely lost in preparations such as homogenates can be obtained concerning individual cells or different types of cells in heterogeneous populations.

The early development of microscopical histochemistry has been outlined by Baker (1943) and by Pearse (1953). Raspail (1825, 1830) first recognized its possibilities. He devised methods for various organic and inorganic constituents as well as the techniques of frozen sectioning and microincineration. By the middle of the nineteenth century many microanatomists were studying both the structure and the chemical composition of tissues. With the introduction of stains, histochemistry was almost completely abandoned in favour of a paracheimistry based on staining properties. Mann (1902), however, published his important *Physiological Histology* during this period. Renewed interest in microscopical histochemistry was heralded by Lison's *Histochimie Animale* in 1936. Since then, histochemistry has developed steadily and rapidly. During the past two decades, innumerable investigations have been published and there are now several textbooks and journals devoted to histochemistry (see Bibliography on page 12).

Microscopical histochemistry is still in a relatively early stage of development. Some of the methods in use and some of the

conclusions reached in studies with them cannot withstand critical analysis (Gomori, 1950). Other methods, even though relatively non-specific, can serve useful purposes provided that the factors influencing the results are known and taken into consideration.

1.11 Limitations. All the methods of microscopical histochemistry have their limitations and it is important that these be recognized. Some of the limitations and sources of error result from the very conditions that characterize microscopical histochemistry. Only static or fixed constituents can be studied when chemical tests are applied to fixed tissues. In cells and tissues, especially in thin sections of them, the amounts and concentrations of some substances are often near, if not below, the limits of detection of the test. Sometimes the size of the particles of the constituents is near or below the limit of microscopical resolution. During the preparation of the tissues and during the performance of the tests, there are dangers of errors being introduced by adsorption, diffusion, solution, denaturation or inactivation with resultant alterations in location, chemical reactivity, or both. Because of technical difficulties such as those associated with fixing, dehydrating, embedding and sectioning the majority of histochemical investigations tend to be descriptive and qualitative. Further difficulties are introduced by the complex chemical nature of cells and tissues; for example, the demonstration of one constituent or one class of constituents must often be attempted in the presence of other substances that might interfere with the test or its interpretation.

1.12 Criteria of accuracy. An additional problem impeding progress in microscopical histochemistry has been the lack of exact criteria of accuracy with which to assess a method and to determine its limitations. A somewhat useful, albeit arbitrary, basis for assessment has been that 'those methods are useful which yield results that are in essence in agreement with, or confirmed by, directly related data obtained by means of simpler, chemically valid or physiological (functional) techniques' (Gersh, 1941). Some of the general characteristics of reliable histochemical methods have been defined (Mann, 1902; Prenant, 1910; Parat, 1927) so that, now, there are established criteria of histochemical validity (Lison, 1936, 1953):

(a) Morphologically, there should be precise *localization* of

the substance sought and excellent preservation of the *structural integrity* of the cells and tissues being studied.

(b) Chemically, the *mechanism*, the *sensitivity*, and the *selectivity or specificity* of the test should be known.

The general significance of these requirements is discussed in the following chapters on the preparation of tissues for histochemical studies and on histochemical analysis.

REFERENCES

- BAKER, J. R. (1943). *J. Queckett Micr. Club* (ser. 4) **1**, 256
(1952). Chap. 1A in Bourne, G. H. (Ed.), *Cytology and Cell Physiology*, 2nd edn. (Oxford, Clarendon Press), p. 1
DANIELLI, J. F. (1947). *Symp. Soc. Exp. Biol.* **1**, 101
DEMPSEY, E. W., and WISLOCKI, G. B. (1946). *Physiol. Rev.* **26**, 1
GERSH, I. (1941). *Physiol. Rev.* **21**, 242
GOMORI, G. B. (1950). *Ann. N.Y. Acad. Sci.* **50**, 968
LISON, L. (1936.) *Histochemie Animale, Méthodes et Problèmes*. Paris, Gauthier-Villars
(1953) *Histochemie et Cytochimie Animale, Principes et Méthodes*, 2^{ème} edn. Paris, Gauthier-Villars
MANN, G. (1902). *Physiological Histology, Methods and Theory*. Oxford, Clarendon Press
PARAT, M. (1927). *Biol. Rev.* **2**, 285
PEARSE, A. G. E. (1953). *Histochemistry, Theoretical and Applied* (London, J. & A. Churchill), chap. I
PRENANT, A. (1910). *J. de l'Anat. et de la Physiol.* **46**, 343
RASPAIL, F. V. (1825). *Ann. Sci. Nat.* **6**, 224
(1830). *Essai de Chimie Microscopique Appliquée a la Physiologie*. Paris, Raspail

SELECTED BIBLIOGRAPHY

- BRACHET, J. (1957). *Biochemical Cytology*. New York, Academic Press
DANIELLI, J. F. (1953). *Cytochemistry, A Critical Approach*. London, Chapman & Hall
ERANKO, O. (1955). *Quantitative Methods in Histology and Microscopic Histochemistry*. Boston and Toronto, Little Brown and Co.

- GLICK, D. (1949). *Techniques of Histo- and Cytochemistry*. London and New York, Interscience Publishers
- GOMORI, G. (1952). *Microscopic Histochemistry, Principles and Practice*. Chicago, University of Chicago Press
- LILLIE, R. D. (1954). *Histopathologic Technic and Practical Histochemistry*. New York and Toronto, Blakiston Co.
- LIPP, W. (1954). *Histochemische Methoden*. München, R. Oldenbourg. (4 sections issued yearly)
- LISON, L. (1953). *Histochimie et Cytochimie Animales, Principes et Méthodes*, 2^{ème} edn. Paris, Gauthier-Villars
- PEARSE, A. G. E. (1953). *Histochemistry, Theoretical and Applied*. London, J. & A. Churchill
- RAWLINS, T. E. and TAKAHASHI, W. N. (1952). *Technics of Plant Histochemistry and Virology*. Millbrae, Calif., National Press
- VIALLI, M. (1956). *Introduzione alla Ricerca in Istochimica*. Milan, Italy, Industria Poligrafica Lombarda
- CECCALDI, P. F. (1953). 'L'histochimie, méthodes, possibilités', *Rev. Gen. des Sci.* **60**, 24
- DEMPSEY, E. W. and WISLOCKI, G. B. (1946). 'Histochemical contributions to physiology', *Physiol. Rev.* **26**, 1
- GERSH, I. (1941). 'Recent developments in histochemistry', *Physiol. Rev.* **21**, 242
- GLICK, D. (1944). 'Histochemistry', *Ann. Rev. Biochem.* **13**, 705
- GOMORI, G. (1951). 'Histochemical staining methods', Chap. 1 in Visscher, M. B. (Ed.), *Methods in Medical Research* (Chicago, Year Book Publishers), vol. 4, p. 1
- (1953). 'Cytochemistry and histochemistry', in Homburger, F. and Fishman, W. H. (Eds.), *The Physiopathology of Cancer*. New York, P. B. Hoeber
- HOGEBOM, G. H. (1951). 'Separation and properties of cell components', *Fed. Proc.* **10**, 640
- McMANUS, J. F. A. (Ed.) (1952). 'A survey of techniques for the histochemical approach to pathology', chap. 6 in *Recent Progress in Fundamental Medicine*. Philadelphia, Lea & Febiger
- MINER, R. W. (Ed.) (1950). 'Structure in relation to cellular function', *Ann. N.Y. Acad. Sci.* **50**, 815
- MOOG, F. (1952). 'Histochemistry', in Avery, G. S., Jr. (Ed.),

- Survey of Biological Progress* (New York, Academic Press), vol. 2, p. 197
- NOVIKOFF, A. B. (1955). 'Histochemical and cytochemical staining methods', chap. 2 in Mellors, R. (Ed.), *Analytical Cytology*. New York, Academic Press
- PEARSE, A. G. E. (1951). 'A review of modern methods in histochemistry', *J. Clin. Path.* **4**, 1
- 'Fine Structure of Cells', a symposium held at the VIIIth Congress of Cell Biology, Leiden, 1954. International Union of Biological Sciences (1956), Ser. B, No. 21. New York, Interscience; Groningen, P. Noordhoff
- 'Tissue fine structure', *J. Biophys. and Biochem. Cytol.* **2**, suppl. 1956

Histochemical Analysis

Most, if not all, of the applications of microscopical histochemistry* in biological research are analytical, being concerned with the detection or determination of substances in their natural locations in cells and tissues. The many problems that can arise in such studies require that the methods employed have sound scientific bases. These problems originate partly in the variety of substances that occur in tissues and partly in the varied conditions under which they are present. As a result, a method that is reliable under one set of circumstances could fail completely under other conditions. Such variations must be taken into consideration when choosing a suitable method.

2.1 Some general considerations of chemical analysis

The success of chemical analyses, whether histochemical or other, depends upon practical experience and precisely specified methods. Moreover, the theoretical basis for each method should be known so that the method can be used intelligently and so that new or improved versions can be developed. Progress in histochemical analysis as in other forms of qualitative analysis requires regular review of all those aspects of chemistry that might lead eventually to developing more sensitive and more reliable procedures.

Chemical analysis ultimately depends upon being able to convert the substance to be determined or detected into another characteristic compound. While there are innumerable reactions resulting in such changes, only certain ones are suitable for analytical applications and the requirements to be met depend upon whether the analysis is to be quantitative or qualitative.

* In this and subsequent chapters, 'histochemistry' is used synonymously with 'microscopical histochemistry'.

A quantitative determination requires that there be practically complete and rapid conversion of the material to be estimated, that the reaction be stoichiometric, and that the amount of either the reaction product or the reagent used be measurable. Most histochemical investigations, however, are qualitative. The requirements for qualitative detection are quite different from those for quantitative determination. Neither completeness of reaction nor measurability is essential for a qualitative test. Instead, the results of the reaction must be observable readily and without doubt. A qualitative method must have adequate sensitivity and selectivity or specificity. The infallibility of the method becomes especially important in analysing mixtures such as tissues.

2.11 Sensitivity. The characterization of chemical tests in terms of their *sensitivity* has been discussed by Feigl (1940, 1943). The sensitivity of a test can best be expressed in terms of its identification and concentration limits. The *identification limit* is the least quantity of material that can be just detected by a given reaction. The *concentration limit* is the lowest concentration in which the material can be present and still be detected by the reaction. Both characteristics of a reaction must be considered in judging its sensitivity. A truly sensitive test must be both quantity- and concentration-sensitive. Unfortunately, so far, very little attention has been paid to these characteristics of histochemical methods even though often an important requirement is high sensitivity. Lison (1953) does mention identification limits occasionally.

2.12 Specificity or selectivity. Regardless of the importance of sensitivity in evaluating a method for qualitative analysis, this should not be the sole basis for its appraisal. Seldom if ever will a histochemical test be applied to a single pure substance. For this reason, the selectivity or specificity of a method is also of great importance. A method demonstrating only one material is said to be *specific* while a method leaving a narrow choice between several substances is said to be *selective*. Accordingly, methods are either specific or not, but the latter can be more or less selective depending upon the number of choices. Most, if not all, histochemical methods are selective rather than specific. Sometimes, it is possible to increase their selectivity by inactivating or removing certain materials that are present and give similar