HISTOCHEMISTRY
Theoretical and Applied

A. G. Everson Pearse

Volume 1
Preparative and Optical Technology

FOURTH EDITION

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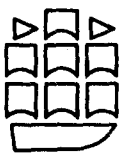

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Preface

I have no excuse for writing a fourth edition of this book, and little excuse for dividing it into three volumes.

It is 30 years since I wrote the first chapters of the first edition, and it is now obvious that histochemistry and cytochemistry have together developed to the point at which new criteria must be applied to their joint technology.

It is no longer appropriate, for instance, to treat the electron microscopical branch of the art as a separate entity; rather it must take its place as an applicable, if not yet too frequently applied, extension of the techniques of light microscopical histochemistry.

At this point in time, while undertaking a revision of the whole work, I have chosen to redesign the book in terms of content and format, taking advantage of the change in style to put electron microscopical histochemistry into its proper context throughout. This first volume is sub-titled *Preparative and Optical Technology*; the titles of the second and third volumes are, respectively, *Analytical Technology* and *Enzyme Technology*.

Once again I have been obliged to rely heavily on the testimony of friends and associates since it is, today more than ever, impossible for a single individual to compass the whole range of the technology which is involved.

The techniques of histochemistry and cytochemistry continue to be widely applied through the whole field of the biomedical sciences. 'Good wine needs no bush' but, as may be learnt from perusal of Chapter 1, statistics provided in the Science Citation Index® for the years 1961 to 1972 and with respect to non-journal items, show that more than 22 per cent of all citations in biomedicine for this ten year period were directed to histochemical texts. Notwithstanding, as with Volume 1 of the third edition, I remind myself that 'it is not the beginning but the end that crowneth the work'. I trust that this volume and, hopefully, the two which follow it, will further the cause of histochemistry and supply both a means and a stimulus for its further expansion.

Hertfordshire, 1980 A.G.E.P.

Acknowledgements

I cannot begin to acknowledge in person all my colleagues and associates who have been concerned with this book in its first, second, third and now, fourth editions. Their number is altogether too large. At the outset, however, I must acknowledge my great indebtedness to my former lecturer, Professor F. W. D. Rost, now Professor of Anatomy in the University of New South Wales, not only for the two chapters which he has contributed but also for suggesting to me, in a sufficiently compelling manner, the need for the whole book to be redesigned as well as revised. My present lecturer, Dr Julia M. Polak, and my secretary for over 11 years, Miss Shirley Green, have both played an indispensable role in allowing me a reasonable portion of that solitude which is a prerequisite for the composition of extensive texts. Miss Green has again, as for the former edition, compiled the author index.

I have received valuable assistance, in the preparation of individual chapters, through personal exchanges of information, the loan of manuscripts and the provision of unpublished data. I refer particularly to Mr K. V. Slee and Mr Robert Slee (Chapter 2); Mr T. G. Rowe and Mr Geoffrey Smith of Edwards High Vacuum Ltd (Chapter 3); Dr Denis Jones (Chapter 5); and Professor L. J. Roth (Chapter 9). I am especially grateful to Dr D. H. Marrian who undertook the task of correcting the formulae and equations, both new and old. All were drawn or redrawn for me by Mrs Louise Perks and Mr Doig Simmonds is responsible for the new colour diagrams.

I wish to thank the following authors, editors and others for permission to use the undermentioned illustrations. Acknowledgements carried over from the third edition of the book are given first. Figure 1, Professor N. Shimizu; Figure 19, L. J. Wright; Figures 24 and 25, Professor O. Eränkö; Figure 26, Professor W. A. Jensen; Figure 48, Dr J. Persijn and the Editor and Publishers, *Histochemistry*; Figure 73, Dr H. A. Nielsen; Figure 78, Dr Roger Daoust; Figure 84, Dr Robert Love; Figures 85, 91 and 92, Dr W. E. Stumpf and the Editors, Oak Ridge Symposium 'Radioactive Isotopes in Medicine'; Figure 87, Dr L. E. Ericson and the Editor and Publishers, *J. Ultrastructural Research*; Figure 88, Dr R. Speirs; Figure 89, Dr H. Flodh; Plate 6L, Professor J. H. Humphrey; Plate 10A, Professor Dr Robert Hess and Ciba-Geigy; Figures 4, 5, 6, 10 and 11, Slee Medical Equipment Ltd; Figure 7, L.K.B. Instruments Ltd; Figures 12, 13, 14 and 15, Dr T. C. Appleton and the Administrator, Royal Microscopical Society (*J. Microscopy*, **100**, 49, 1974); Figure 16, Union Carbide U.K. Ltd; Figures 30 and 31, British Oxygen Company Ltd, Edwards High Vacuum; Figure 39, Polaron Equipment Ltd; Figure 40, Balzers High Vacuum Ltd; Figures 42 and 43, Dr W. Umrath and Leyblond-Heraeus GMBH; Figure 44, Dr E. Remy; and BTG Biotechnik GMBH, Munich; Figures 45A and 45B, Professor Dr W. Meier-Ruge and the Editor, *Histochemical Journal* (**8**, 387, 1976); Figures 50 and 51, Dr J. Alqvist; Figures 74 and 75, Dr L. Pelliniemi, Miss Susan Van Noorden and the Editor and Publishers, *Histochemistry* (**53**, 271–272, 1977); Figures 76 and 77, Dr E. Weber and the Editor and Publishers, *Histochemistry* (**54**, 51, 1977); Figure 80, Dr. H.-W. Denker; Figure 81, Dr H.-W. Denker and the Editor and Publishers, *Acta Endocrinologica Kbh.* (**70**, 591–602, 1972); Figure 82, Dr H.-W. Denker and the Editor and

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The dedication of this fourth edition remains the same as in the previous three editions. Nevertheless the completion of this volume, after three years' gestation, is due in great measure to the support given to me as always by my wife, Dr Elizabeth Pearse. She has sustained me through many periods of doubt, and some of exhaustion, and my indebtedness to her I can only signify, never repay.

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1. Historical Introduction

DEFINITIONS AND *OBITER DICTA*

Had its use not been pre-empted by the publication, in 1952, of George Gomori's classical text, the title of the first and succeeding editions of this book would have been *Microscopic Histochemistry*. The term has descended directly from the older term and older discipline, 'microchemistry', or, as it was in former times, 'chemistry observed with the microscope'. Microchemistry subsequently became a branch of biochemistry, concerned with chemical analyses on the smallest possible scale and as such it was extended, in modern times, to ultramicrochemistry. All three, as will be made clear in the historical section which follows, derive from the genius of the French botanist François-Vincent Raspail who was the first (1826) to perceive, and to practise, the art of chemistry in combination with the magnified vision of the microscope.

The hybrid discipline of histochemistry, 'a borderline field between histology and analytical chemistry or biochemistry' (Gomori, 1952), can further be defined as: *the identification, localisation and quantification, in cells and tissues and by chemical or physical tests, of specific substances, reactive groups, and enzyme-catalysed activities*. It is thus more a technology than a science and it stands or falls, as I have many times written and always believed, by its applicability to other disciplines.

The situation was not always so clear and very many and varied definitions have preceded the one given above. Doubtless many will succeed it but, at this moment in time, we can profitably survey the succession as part of the process of becoming acquainted with the complicated semantic struggles which have left the term histochemistry, at least, firmly in the hands of microscopists, whatever the wavelength of their illumination.

Histochemistry, according to Gérard in his preface to Lison's first edition (1936) of his *Histochimie Animale*, was a recent creation expressing a profound aspiration, the recognition in the cell, by means of appropriate chemical reactions, of different products formed during life. Earlier than this Voss (1933, 1952) had invented the term histotopochemistry specifically to signify the prime concern of histochemistry with localisation. Although this was, and still is, an excellent term, it was criticised as cumbersome and lacking euphony. Thus it never passed into general use. The distinction between histo- and cytochemistry, at one time assiduously preached by followers of the latter discipline as suggesting the division between an amateur technology and a professional science (cf. Danielli, 1953), has now virtually ceased to exist. The term histochemistry now contains cytochemistry as its premier division, despite the view of Gomori (1952) that the name of the lesser portion should be reserved for studies on the chemical organisation of the cell in general. This juncture is in accordance with the opinion of Vialli (1966) that the one is merely an extension of the other.

Early criticisms of histochemical techniques, either in general or in particular, were fully warranted. In his great treatise *Biochemistry and Morphogenesis* Needham (1950) expressed the view that 'histochemistry has a long way to go before it can attain the certainty of direct chemical methods' and for Danielli (1953) cytochemistry was 'an almost

undeveloped branch of biology' a field calling for a team of workers and demanding 'a high standard of knowledge in each of the fields of biology, chemistry and physics'. Fortunately for histochemistry this attempt to dissuade individual scientists, the majority then and now, from employing its techniques was a total failure.

It may be that even in its modern form histochemistry still has some way to go before it receives proper recognition from those workers in the basic sciences whom it serves so well. If verbal acknowledgement is still lacking, histochemists can take heart from the statistics provided by the records of the Science Citation Index® (Current Contents®) for the years 1961–72. During this period, in the total field of 'biochemistry and biomedicine', some 29 texts were cited more than 200 times. Five of the 29 were histochemical and these 5 amassed between them no less than 22.269 per cent of the total number of citations (13 543). The inference to be drawn from these statistics is obvious. It is that the techniques which comprise the discipline of histochemistry have been found useful, and that they are used, by workers in all the biological sciences. The results of their labours, and even the applications themselves, can still be classified as good, bad or indifferent and histochemistry must still bear some of the responsibility for the last two categories because it retains in its orbit methods known, and shown, to lack the requisite specificity and the proper capacity for accurate localisation. It is probable that the critical faculties of histochemists, which have always and rightly been criticised by workers in other fields, are still in need of some measure of sharpening.

THE FUTURE OF HISTOCHEMISTRY

'There is every indication that histochemistry is emerging now as an independent discipline with its own theoretical background, methods, and special problems, just as was the case with biochemistry shortly after the turn of the century'. With these words Gomori (1952) concluded the introduction to his book *Microscopic Histochemistry* and, a quarter of a century after their publication, we can ask ourselves whether they have been justified by events.

'Of the many fields of cytology, cytochemistry has probably been that which has grown most rapidly and been the scene of the most intense activity in the past ten years. The next ten or twenty years must inevitably see the consolidation of this field, the refinement of many methods, and the invention of many new methods'. Thus Danielli (1953) began the final chapter of his critical monograph *Cytochemistry* reversing, with these words, the somewhat severe strictures of his earlier chapters. Again we may pertinently enquire whether the prophecy is justified or not. But first we can go back further still to add to the list some of those who have realised that morphology alone, even with the help of several extra orders of magnitude of resolution afforded by the electron microscope, is a self-limited exercise.

Foremost among these was Gheorghe Marinesco (1863–1938) who must be considered as one of the pioneers of neurohistochemistry, a field which within the past ten years has risen from comparative obscurity to a position of the highest importance and significance. For perfect comprehension of the phenomena of life, said Marinesco (1920), we shall shortly be obliged to have recourse to a physicochemical language to replace the descriptive and impressionistic one which presently dominates neuro-biopathology as well as normal histology and pathology in general. For the last word in this section we can return to Raspail (1845) who wrote, in his yearly handbook *Manuel-annuaire de la santé*, 'The microscope'—by this he meant without doubt microscopic histochemistry—'will unmask all causes of disease and replace traditional jargon . . . with the positive language of scientific observation'.

The future of histochemistry is a future of increasing, and increasingly successful, applications. As with its contribution to the prob-

lem of the distribution and dynamics of the nucleic acids which, in the hands of Brachet (1940a and b, 1942, 1944, 1946) was the direct fore-runner of work leading ultimately to the establishment of the genetic code, histochemistry must often be content to see its findings embraced by other disciplines whose techniques, such as those of molecular biology, can carry them into new dimensions of discovery.

The history of histochemistry, a survey

The historical survey given here first appeared in shorter form in a review of modern methods in histochemistry (Pearse, 1951). It was designed to give a true impression of the continuity of what is at the same time the youngest and one of the oldest of the biological disciplines. Below, as an introduction, appears a brief tabulated history. This is by no means complete, but it forms a skeleton on which to hang the otherwise unconnected individual references which follow.

1800–29 Isolated reports of the investigation of chemical as opposed to morphological structure in tissue preparations. Histochemistry unknown as a separate discipline.

1830–55 These years saw the beginnings of histochemistry as a discipline. In its origins it was primarily botanical; for some decades the whole practice of histochemistry in its true sense was in the hands of the botanists. Various works on the subject appeared: Raspail's *Essai de Chimie Microscopique Appliquée à la Physiologie*, 1830; his *Nouveau Système de Chimie Organique*, 1833; Lehmann's *Lehrbuch der physiologischen Chemie*, 1842; Raspail's *New System of Organic Chemistry*, 1834 (translation).

1856–71 Histochemistry, in the case of animal as opposed to plant tissues, was mainly biological chemistry, and most of its methods involved tissue destruction. More textbooks on this aspect of histochemistry began to appear, some of them being translations of earlier works: *Physiological Chemistry* of Lehmann, 1951 (translation); *Traité de Chimie Anatomique* by Robin and Verdeil, 1853; *Chemie der Gewebe des gesammten Thierreichs* of Schlossberger, 1856; *Handbuch der Experimental Physiologie der Pflanzen* of Sachs, 1865; and the *Handbuch der Histologie und Histochemie des Messchen* of Frey, 1867.

1872–98 At this stage histochemistry became divided, part left histology and became attached to physiology and part remained as biological chemistry. Articles and books published during this period included *Ein Beitrag zur Histochemie*, by Miescher, 1874; *Histology and Histochemistry of Man*, by Frey, 1874 (translation); *Traité de Chimie Physiologique* of Lehmann, 1883 (translation); Bunge's *Lehrbuch der physiologischen und pathologisches Chemie*, 1887; and Sachs' *Lectures on the Physiology of Plants*, 1887.

1899–1929 During this period the use of aniline dyes in histology, first described by Bencke in 1862, became widespread. The first quarter of the twentieth century saw the rapid expansion of descriptive histopathology. Histologists became more interested in new dyes and staining techniques and showed less interest in the chemistry of tissue structures. Although diagnostic significance was attached to many of the new colour reactions, no attempt was made to put them on a physical or chemical basis. Morphological studies overwhelmed histochemistry and Mann (1902) was rash enough to say that the study of mammalian micro-anatomy was 'almost complete' by 1900. Three contributions of this period described microchemistry and microphysiology; they were Mann's *Physiological Histology*, 1902, Ehrlich's *Encyclopädie der Mikroskopischen Technik*, 1903, and Macallum's *Methoden und Ergebnisse der Mikrochemie*, 1908, all classical works. Prenant (1910) reviewed the general state of histochemistry in a valuable paper, and Molisch's *Mikrochemie der Pflanzen*, 1913, contained much of histochemical and cytochemical interest. Other works published towards the close of this period, which show that histochemistry still flourished, were the *Review of Recent Developments in Histochemistry*, by Parat, 1927; *Animale Histochemie*, by Patzelt, 1928; *Prakticum der Histochemie*,

by Klein, 1929; and *Histochemische Methoden*, by Hertwig, 1929.

1930–44 This stage saw the rebirth of histochemistry and its partial return to the domain of histology. The most important work of the period was Lison's *Histochemie Animale* (1936) in which the author proclaimed the new science of histochemistry without tissue destruction. It is impossible to overestimate the effect of this book upon the progress and practice of histochemistry. It remained, until superseded in 1953 and 1960 by second and third editions of broader scope, the acknowledged bible of histochemists in all fields of the basic sciences. Other excellent, though less important, publications were the *Handbook of Chemical Microscopy*, by Chamont and Mason, 1930; *Histochemische Methoden* of Romeis, 1932; *Die Mikroveraschung als histochemische Hilfsmethode*, by Policard and Okkels, 1932; and Linderstrøm-Lang's *Problems in Histochemistry*, 1936.

1945–58 The first published work of this period was Glick's *Techniques of Histo- and Cytochemistry*, 1949. This dealt somewhat briefly with the theory and practice of histochemistry, as it concerns the histologist. In it was reviewed the entire compass of histochemistry, physiological, physical and histological, and much of the technical information was beyond the scope of any but specialists in small individual fields. After a brief interval there appeared four works largely or wholly devoted to histochemistry. These, in order of appearance, were Gomori's *Microscopic Histochemistry*, 1952; Danielli's *Cytochemistry*, 1953; the first edition of my book, 1953; and Lison's second edition *Histochimie et Cytochimie Animales*, 1953. Bourne's *Functional Histology*, 1953, though not intended as a histochemical treatise, was based largely on the application of histochemical techniques, and Lillie's second edition with its new title *Histopathologic Technic and Practical Histochemistry*, 1954, represented an important addition to histochemical literature though only partly devoted to this subject. A stimulating and critical review of modern histochemistry was given by Vialli in his *Introduzione alla Ricerca in Istochimica*, 1955, in which the merits and demerits of most aspects of the science were discussed. Evidence of the expanding outlook of modern histochemistry was offered by Mellors' *Analytical Cytology*, 1955, a work of many authors concerned with all types of microscopy and their application, and by Eränkö's *Quantitative Methods in Histology and Microscopic Histochemistry*, 1955. These two works stressed particularly the quantitative side of histochemistry, whose future development was clearly closely bound up with progress in this direction. In 1958 appeared the first volume of a very comprehensive series of individual volumes, the *Handbuch der Histochemie*, dealing with the general methodology of the subject, as well as its applications. The rapidly developing discipline of histochemistry, which until 1950 had no journals or periodicals devoted to it, acquired in this period no less than seven. If one counted those publications in which histochemical or cytochemical papers formed a substantial part or even a majority of the contributions the number was clearly even larger. The main histochemical and cytochemical journals of this period were, with dates of first publication, *Experimental Cell Research* (1950), *Journal of Histochemistry and Cytochemistry* (1953), *Acta Histochemica* (1954), *Journal of Biophysical and Biochemical Cytology* (1955), *Rivista di Istochimica* (1955), *Annales d'Histochimie* (1956), *Histochemie* (1958).

1959–65 During this period there was again a considerable increase in the number of new books, and new editions, devoted to histochemistry. A small but effective volume entitled *Histochemical Technique* was produced by Casselman in 1959 and in the following year appeared three new texts. These were McManus and Mowry's *Staining Methods, Histologic and Histochemical*, the second edition of Mellors' *Analytical Cytology*, and the second edition of my own book. In 1962 Burstone's *Enzyme Histochemistry and its Application to the Study of Neoplasms* was published and in the same year Jensen's *Botanical*

Histochemistry, Principles and Practice. In the following year appeared Barka and Anderson's *Histochemistry, Theory, Practice and Bibliography*. Further volumes and parts of volumes of the *Handbuch der Histochemie* appeared, spasmodically and in no particular order; these included Vol. II 'Polysaccharide' (1962 and 1964), Vol. III 'Nucleoproteide' (1959), Vol. V 'Lipide' (1964), Vol. VII 'Enzyme' (1960, 1962, 1963, 1964). Two further monographs, in the German language, were published in 1964. The first, written by two Hungarian authors, Kiszely and Pósalaky, was entitled *Mikrotechnische und Histochemische Untersuchungsmethoden.* This work was intended largely as an illustrated laboratory handbook as also was the second work, Spannhof's *Einführung in die Praxis der Histochemie.* A third, much augmented, edition of Lillie's classical text appeared in 1965. This was a veritable mine of information, much of which was unobtainable elsewhere. New journals of the period were fewer than in the preceding epoch. The Japanese Histochemical Association commenced, in 1960, the publication of an annual volume of its proceedings in the English tongue. In 1962 the title of the *Journal of Biophysical and Biochemical Cytology* was changed to the *Journal of Cell Biology* without change in the editorial policy giving priority to electron microscopy and electron cytochemistry. The Polish Histochemical Society, founded in 1961, produced the first volume of its journal *Folia Histochemica et Cytochemica* in 1963. This journal was published substantially but not exclusively in English. With the formation of sections for histochemistry and cytochemistry (1964) and for electron microscopy (1965), the Royal Microscopical Society expanded its journal in order to accept an increased number of papers in these fields. To mark the occasion of the 2nd International Congress of Histochemistry, held in Frankfurt in 1964, there was published a small volume edited by Sandritter entitled *Hundert Jahre Histochemie in Deutschland.* The English version, edited by Sandritter and Kasten, followed shortly after as a significant contribution to the history of histochemistry.

1966–76 The expansion of histochemistry, as an applied science or technology, continued into the last quarter of the century with no slackening in the pace of its advance. Several large, and some smaller texts appeared in print. The first, and for several years the largest of these (1966) was Thompson's compilation *Selected Histochemical and Histopathological Methods.* Almost encyclopaedic, it contained a most useful and comprehensive bibliography and can be contrasted with Bancroft's (1967) much more selective text *An Introduction to Histochemical Technique,* the success of which can be judged from the appearance of a second edition in 1976. A third edition of Culling's *Handbook of Histopathological and Histochemical Techniques* appeared in 1974.

Next to appear, the largest of all the books appearing in this period, were the two volumes *Histochimie normale et pathologique* by Ganter and Jolles (1969). Written by an excellent combination, pathologist and chemist, this French text was produced, presumably as a replacement for the missing 4th edition of Lison, by his publishers Gauthier-Villars. It contains a substantially greater proportion of chemical or biochemical data than most other works in the field. Three volumes of a multiple author work *Techniques of Biochemical and Biophysical Morphology,* edited by Glick and Rosenbaum, appeared successively in 1972, 1975 and 1977. Sadly, this potentially extremely useful series was terminated with the appearance of the third volume.

During this decade occurred the expected increase in the number of new techniques for electron microscopic localisation. These were collected together by Geyer (1973) in an excellent and timely monograph *Ultrahistochemie,* published by the Gustav Fischer Verlag in Jena. A series of monographs on the *Electron Microscopy of Enzymes,* in four volumes and by a large series of individual authors, appeared at the same time (1973, 1974), edited by Hayat and published by Van Nostrand Reinhold. Also dedicated to the technology of enzymes was the first edition of Lojda's (Lojda, Gossrau and Schiebler)

German text *Enzym-histochemische Methoden*. This book fully reflects the first author's complete mastery of the field and he has been very ably assisted by his two collaborators in the production of what should become the definitive authority for the technology of light microscopic enzyme histochemistry. Two paperback volumes by Luppa, entitled *Grundlagen der Histochemie* appeared in 1977. Dealing with both practical and theoretical aspects of the discipline these constitute, for those who read German, one of the least expensive ways of achieving familiarity with its technology.

Two more new editions appearing during the period under review were the second edition of Barka and Anderson's *Histochemistry, Theory Practice and Bibliography* (1963) and Lillie's (Lillie and Fullmer) 4th edition (1976), the latter very completely revised and brought up to date. The *Handbook of Histochemistry* by the end of the decade, had reached the 4th part of its 8th volume under the editorship of Graumann and Neumann. This work, in its later volumes, was largely devoted to considerations of the application of histochemistry to selected regions of the mammalian organism, or to diseases and disorders thereof.

New journals appearing in the decade 1966–76 were, not unnaturally, rather fewer in number than in the previous decade. There were, however, a substantial number of reorganisations and some withdrawals, as well as a few additions. The first volume of the official journal of the Japan Society of Histochemistry and Cytochemistry, published in English as *Acta Histochemica et Cytochemica*, appeared in 1968 and in 1970 began the publication of a series of monographs, mostly by single authors, under the comprehensive title *Progress in Histochemistry and Cytochemistry*. This series, published mainly in English by the Gustav Fischer Verlag, fills a very real need in supplying a medium where selected specialist topics in the technology or application of histochemistry, of greater length and substance than could be accommodated in other journals, can appear without prejudice or abbreviation. First published in 1968, the *Histochemical Journal* in 1973 became associated with the Royal Microscopical Society, by agreement with its publishers, Chapman and Hall. This association coincided with an alteration of editorial policy affecting the *Journal of Microscopy* which, up to this time had carried a proportion of papers in the field of applied histochemistry. The change of title (1974) from *Histochemie* to *Histochemistry* set the seal on this journal's position as one of the leading English language journals, without other alteration in its format or general content.

The history of histochemistry, a continuum

In a series of papers entitled *Contributions to the History of Microchemistry* Harms (1931–32) gave a detailed account of the histochemical works of the French pharmacist, botanist and microscopist, François-Vincent Raspail (1794–1878), and he concluded, with the support of other competent observers, that Raspail should be regarded as the founder of the science of microchemistry. These views were carried a stage further by Baker (1943, 1945) in a monograph of the Quekett Microscopical Club and in the first edition of his book on cytological technique. He too considered that Raspail was the real founder of histochemistry. His claims to that title are clearly unassailable. An excellent account of Raspail's life and works was given by Weiner (1959) in a monograph which concentrated largely on his extensive philanthropies. This was followed (1968) by the same author's *Raspail, Scientist and Reformer*, containing a chapter by his great-granddaughter Simone Raspail giving the fullest details of his early years as a young researcher, when (1822–30) virtually all his histochemical work was carried out. As in previous editions, the frontispiece shows Raspail in his younger days before he deserted the infant discipline of histochemistry for polemics and politics.

The first clear appreciations of the science of microscopic tissue chemistry undoubtedly

came from Raspail (1825a and b, 1829). This author, after formulating his four resolutions (quoted in full by Harms, 1931, and partly also by Baker, 1943), settled down to a study of the processes of fertilisation in flowers and fruits of the *Graminaceae*. The most important reaction which he used for this purpose was the iodine reaction for starch, first described by Colin and de Claubry in 1814, and by Stromeyer (1815), and employed in a microscopic study of starch grains by Caventou in 1826. It is not certain whether priority for the histochemical use of iodine solutions should go to Raspail or to Caventou; the point is not of great importance since the latter did not pursue his histochemical studies any further. Raspail, on the other hand, discovered and applied many other histochemical reactions which are still of importance today. In 1829 he used the xanthoproteic reaction for protein and the hydrochloric acid (furfural) test for carbohydrate which became more widely known as the reaction of Liebermann (1887); the latter is not now employed in animal histochemistry. In applying sulphuric acid to his plant tissues to demonstrate the presence of protein he was, in fact, using the aldehyde method for tryptophan which, modified by many workers since his time, is still applied in histochemistry as the Voisenet-Fürth reaction. According to Reichl (1889) this type of benzylidene condensation reaction was used by Mikosch to demonstrate protein in plant tissues with a mixture of benzaldehyde, sulphuric acid and ferric sulphate.

Raspail is now usually credited with the discovery of micro-incineration (in 1829 according to Baker). He was also the first person to study the pH of protoplasm, using an indicator dye, turnsole, obtained from a species of sun spurge found in the Mediterranean region. This dye, normally blue, turned pink in acid solution.

Almost contemporary with Raspail's discoveries were those of several other botanists who published accounts of true histochemical reactions. Amongst these the work of Mohl (1831) on the iodine reaction, and of Schleiden (1838) on the iodine-sulphuric acid reaction may be mentioned. Apart from botanical work, however, progress in histochemistry was very slow and little work which can be described as histochemical, even in in the broadest sense, was recorded until after 1860. Among the oldest published techniques are those for demonstrating iron, and some of these had their origin in the work of Vogel (1845, 1847), who detected iron in the tissues by its conversion to black ferrous sulphide with yellow sulphide of ammonia. According to Lillie (1972), however, the iron tannin reaction can be carried back at least to 1807 when it was used bv Link to demonstrate fluid circulation in oak leaves and twigs. In 1867 Perls introduced his Prussian-blue method for demonstrating iron which remains the method of choice up to the present day. Perls was followed in 1868 by Quinke, who used Vogel's sulphide method, and his technique also survives to the present day in practically unmodified form. In 1844 Millon described his reaction for proteins which Hoffmann, in 1853, showed was actually a test for tyrosine. This reaction was not used in histochemistry until 1888 (by Leitgeb), although Payen (1843) had already demonstrated nitrogenous substances in vegetable tissues with mercury proto-nitrate. The first recorded localisation of starch in the chloroplast was by Sachs (1887) in his monograph on Plant Physiology.

Pigment histochemistry properly begins with the extensive studies of Virchow (1847) on the products of haemoglobin breakdown in the tissues. He was the first to use the term *haematoidin* for the yellow crystalline pigment appearing in areas of extravasation of blood. Incidentally, the classic phrase 'Omnis cellula e cellula', which is usually attributed to Virchow, in his role as the founder of Cellular Pathology (1858), was used by Raspail in 1825 as the epigraph to his paper on the development of the starch grain, published in that year in *Annales des Sciences Naturelles*. The term *haemosiderin* was proposed by Neumann (1888) for an intracellular iron-containing pigment distinct from Virchow's haematoidin. Von Recklinghausen (1889) first described haemofuscin and the term *melanin* was intro-

duced by Langhans although Virchow (1859) had already described a black pigment in the cells of the central nervous system. The characteristics of melanin in tumours were investigated later by Berdez and Nencki (1886).

In 1850, though the result was not published until 1859, Claude Bernard performed his celebrated experiment by the injection into dogs of iron lactate and potassium ferrocyanide, locating the resulting Prussian blue, which developed in the presence of acid, not only in the gastric glands but on the surface of the gastric mucosa. Such *in vivo* techniques really belong to the domain of physiology but this particular example may justly be claimed for histochemistry. In 1850, also, Schulze first demonstrated his chlor-zinc-iodine method for cellulose. A description of this method, which is still in use today, was given by Fürnrohr (1850).

The use of enzymes for tissue digestion was first reported in 1861, by Beale, who used gastric juice in order to remove unwanted tissues from the nerve fibres which he was studying. As such, the technique was really microanatomical, but it developed by the end of the century into a well-recognised technique described as enzymal analysis (Kossel and Mathews, 1898).

Demonstration of the presence of enzymes in tissue began with the work of Klebs (1868) and Struve (1872), both of whom showed that tincture of guaiac gave a blue colour with pus, thus first recording the presence of peroxidases now well known to occur in the granules of the leucocytes. Brandenburg (1900) first demonstrated the peroxidase reaction in the latter site. Cytochrome oxidase was first demonstrated by Ehrlich (1885), though not, of course, under that name. He performed the 'Nadi' reaction (Vol. III) *in vivo* by injecting α-naphthol and *p*-phenylenediamine into animals, observing the formation of indophenol blue in situations where 'Nadi oxidase' was present.

Besides some of the methods mentioned above, during the period 1856–98 a number of other histochemical methods appeared. Heidenhain, in 1868, showed that ergastoplasm, the deeply basophil substance at the base of secreting gland cells, contained a material which could be precipitated with acetic acid. This is now recognised as ribonucleic acid. In 1870 he described the development of a brown colour in certain cells of the adrenal medulla when these were treated with chromic acid and this phenomenon is now called the chromaffin reaction. According to Lison, its discovery should be attributed to Henle in 1865. The use of enzymes for digestion is recorded during this period by Miescher (1871a), who employed pepsin to free nuclei from cytoplasmic material, and by Stirling (1875) who isolated elastic fibres by means of digestion with gastric juice. These two were hardly histochemical techniques in the modern sense, but they serve to illustrate the destructive nature of much of the research into the chemistry of the tissues which was taking place at that time. It was this destructive element which caused Lison to separate the older histochemistry from his new non-destructive science.

In 1873 Miescher isolated nuclear chromatin by making use of its selective affinity for methyl green, and Ehrlich, 1878–79, observed the effects of heat coagulation in increasing the affinity of haemoglobin for nitro dyes. This last work finds a modern echo in inquiry into the effects of denaturation on the combination of dyes or histochemical reagents with specific groups in the tissue proteins. In a letter to the Editor entitled 'A centenary of nuclear chemistry' James (1970) provides much additional information on Miescher's contribution to histochemistry. He gives, at the same time, some fascinating details of Miescher's choice of material (pus) for his earliest extractions and of the curious circumstances which delayed, until 1871, the publication by Hoppe-Seyler of Miescher's report on the chemistry of pus cells. When he became Professor of Physiology in Basel in 1872 Miescher transferred his affections from pus to Rhine salmon sperm as the source of his acid 'Nuclein' and its associated basic protein 'Protamin'. Greenstein (1943) quotes a letter from Miescher to his uncle, friend and teacher, the Basel histo-

logist Wilhelm His, which contains these imperishable words: 'I know better than anyone else that my work is only the preliminary study to a future histochemistry'. Without doubt Miescher must join Raspail, to be venerated as one of the true founders of histochemistry, despite his vituperations against the 'guild of dyers'. Many further details of Miescher's career are to be found in Mirsky's (1968) extensive historical survey entitled *The Discovery of DNA*. He describes, *inter alia*, how His advised Miescher to devote himself to histochemistry because, in his own histological investigations, he was 'constantly reminded that the ultimate problems of tissue development would be solved on the basis of chemistry'.

During this same period (1856–98) the aniline dyes came into general histological use, following their introduction into the field by Bencke (1862). This caused a revolution in the practice of histology and provided a check to the progress of histochemistry. The various dyes were largely used without any attempt to correlate their performance with the chemical nature of the tissue components that were stained, although every endeavour was made to record the correlation of colour with structure in the histological sense. Notwithstanding this criticism, a considerable amount of work was done by a few authors in order to find out how the various stains attached themselves to the tissues. The physical theory of staining was upheld by Witt (1890–91) and particularly strongly by Fischer (1899), who explained all staining on the basis of absorption. Miescher and Ehrlich, and also Knecht (1888), believed that staining was a chemical process. Mann's (1902) comment on all this is interesting. The object of staining, he says, is first to determine morphological facts and second, 'to recognise microchemically the existence and distribution of substances which we have been made aware of by macrochemical means.' 'It is not sufficient,' he goes on, 'to content ourselves with using acid and basic dyes and speculating on the basic or acid nature of the tissues, or to apply colour radicals with oxidising or reducing properties; but we must endeavour to find staining reactions which will indicate not only the presence of certain elements such as iron or phosphorus, but the presence of organic complexes such as the carbohydrate group, the nucleins, protamines, and others.' These remarks show that Mann, at least, was aware of the problem, but few practical attempts to meet it were made by histologists in general.

Some of the reactions involving the use of aniline dyes were in fact histochemical, although their significance was often unappreciated or wrongly appreciated. In most cases the significance is still not fully understood. Puchtler *et al.* (1975) surveyed the history of basic fuchsin, and of the aldehyde-Schiff reactions, from 1862–1935. They described, in some detail, the early studies of Hoffmann (1862) on the synthesis and chemistry of pararosanilines and his reluctance to assign to his dyes any precise chemical structure. Various theories concerning the combination of basic fuchsin with aldehydes, also described, include those of Schiff himself (1865a and b) on this subject, together with his views on the composition of his eponymous reagent (1866, 1867a and b).

The metachromatic staining of amyloid with methyl violet was first described by Cornil (1875). Almost simultaneously Heschl (1875) and Jürgens (1875) were working on general problems of metachromasia. Ehrlich's reaction for mast-cells, using a saturated alcoholic solution of dahlia containing 8 per cent acetic acid, is essentially similar to modern methods using the thiazine dyes and may well be considered histochemical. Other reactions whose mechanism was poorly understood were the myelin methods of Weigert (1884) and Marchi (1892) and the anilin-violet method of Gram (1884). The chemical theory of staining was strongly supported by Mathews (1898) in his experimental work with albumins and albumoses and important researches into the nucleohistones were conducted by Saint-Hilaire (1898), who noticed, in his attempts to evolve a method for uric acid in the tissues, that nuclei were occasionally stained. He concluded that the presence of histone was responsible for the nuclear reaction. Saint-

Hilaire also showed that nucleohistones, precipitated in the tissues by acetic acid, were dissociable by means of dilute solutions of hydrochloric acid, leaving the histones *in situ*. Variations on this theme are widely employed at the present time.

Especially in the second half of the period we are considering, a great deal of work was done on the nature of protoplasm, particularly by Stöhr (1882), who, as the result of coagulation studies, concluded that a protein substance was present in gastric parietal cells, and by Flemming (1882), Kossel (1882, 1886), Altmann (1886, 1889), Schwarz (1887), and Mann (1890). Flemming (1876) had already described a cement substance holding together the fibrils which compose the loose connective tissue bundles. This he considered to be of mucinous nature. Altmann (in 1889) developed his method of fixation by freezing and drying, which has become an important modern tool of histochemistry in the hands of Gersh and his successors. Others continued to work on the chemical nature of staining and much of their work has modern applications. Among works of particular merit are those of Griesbach (1886), who postulated that tissue dye compounds should have properties differing from those of the free radical; of Unna (1887), who tried to confirm this; and of Lilienfeld (1893), who investigated the staining of mucins. Hoyer (1890) demonstrated metachromasia in the cells of the mucous salivary glands with thiazine dyes and, following his discovery, metachromatic methods for mucin became quite popular. Before Hoyer published his paper mucins were usually stained by techniques making use of their strong basophilia. List (1885) was the first to use Bismarck brown for this purpose and the same dye, in alcoholic solution, was used by Hardy and Wesbrook (1895) to stain water-soluble mucoproteins and mucopolysaccharides. In the same year, Heine (1895) made some important observations on the nature of chromatin. He observed that segments of this substance were intensely stained by Millon's reagent and tried, unsuccessfully, to distinguish between nucleoproteins and nucleic acids using mixtures of methyl green and rubin S. Also in 1895, Macallum demonstrated that, after treatment with sulphuric acid, the nuclei were stainable by the usual methods for iron in the tissues. Since this time the question of whether this iron is really present in the nuclei, or adsorbed from elsewhere, has been debated at length.

Enzyme methods of the closing years of the nineteenth century are represented by the contributions of Mall (1891), who investigated the swelling of collagen in various solutions and the action upon it of crude preparations of pepsin and trypsin. Nothing approaching the modern concept of enzymal analysis was achieved at this time. Daddi, in 1896, first used Sudan III for the *in vitro* staining of fat which, after being subsequently ingested by animals, was demonstrated in the tissues by its red colour. Sudan IV was proposed as a fat stain by Michaelis in 1901. This author showed that the staining of fats with Sudan dyes was purely physical, depending on solution of the inert dyes in the fats themselves.

Among methods for revealing inorganic salts in the tissues may be mentioned the techniques of Molisch (1893) who stained tissue-iron by converting it to the red thiocyanate, and of Lilienfeld and Monti (1892), who evolved an ammonium molybdate technique for demonstrating phosphate. This method was modified by Pollacci (1900) and it has been further modified by other workers in the twentieth century. The forerunner of a number of very similar techniques for demonstrating metal salts in the tissues was de Michele's (1891) method for mercury, which he converted to its sulphide by means of H_2S. The method which we still use to demonstrate the presence of calcium in the tissues (though in fact it demonstrates the phosphate radical) was described by von Kóssa in 1901; even then it was preceded by the more specific 'gypsum' method, described by Schujeninoff (1897), which also survives to the present day.

It is probable that this brief account of the history of histochemistry has failed to give the reader a true sense of the continuity of the discipline from decade to decade. This is at least partly due to the fact that real continuity

was lacking. Nevertheless, it is to be hoped that I have been able to show that histochemistry as formulated by Raspail, and the principles expressed by that great man, continued without serious interruption from the 1820s to the twentieth century. When the nineteenth century came to a close, the majority of histologists were occupied in reaping the rich harvest presented by new developments in the art of staining and few had time to spare for histochemistry. The subject therefore remained for the most part in abeyance, though kept alive by a few practitioners until its revival in the 1930s and its establishment on a modern footing as an independent branch of histology by Lison with his great work *Histochimie Animale*. Since his time expansion has been rapid and progress almost equally so, until now, in the second half of the twentieth century it is to be hoped that the majority of morphologists, using both light and electron microscopes, would wish to 'recognise microchemically' some at least of the substances in the tissues with which they deal.

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