THE

RATIONAL USE OF DYES IN BIOLOGY

AND

GENERAL STAINING METHODS

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FOREWORD

This book is a completion of the author's *Staining Animal Tissues*, issued in 1962. It provides for the botanist, the microbiologist and the cytologist what the earlier work did for the animal histologist. For this alone it is to be welcomed, but there is another cogent reason also. Dr. Gurr has really now completed a comparative anatomy of staining. When I wrote a foreword for his *Encyclopaedia of Microscopic Stains* (published in 1960) I ventured to prophesy that it would be not only a well of reference but also a fount of inspiration. Events have justified this opinion, which was founded on the fact that he had, in effect, given us a comparative anatomy of biological dyestuffs which would make it easier for us to pick and choose not only for standard uses but also for experiment. Coupling the two volumes as an effective unity, we can say that they bring together the ways of two worlds in the examination of Life's many manifestations. In these specialist days contact between the botanist and the zoologist is all too rare. Each conducts the mysteries of his private altar which, though not intentionally, has become an *arcana disciplini* known only to the initiates of his own community. This book will enable the animal histologist to see the kind of thing that is done by the phytohistologist and probably suggest methods to him that he might not have thought of otherwise. It would be interesting to know how far conclusions about plant histo-chemistry on the results of many of the staining methods I have read here would be valid in the animal field.

In his preface (where, incidentally, he has been over-generous in his reference to me), Dr. Gurr specifically points out that few of the staining methods described can be rated as rational. However, no small part of the information collected by him deals with cytology, the vigorous and masterful child of the zoologist and the botanist. It is in this field particularly that we find a justification for so many seemingly empirical techniques being included in what is declared to be the *rational* use of stains in histology. In the scientific world I venture to say, the term 'rational' has two connotations. First, it may mean that a staining technique (to keep to our present universe of discourse) has been employed because the chemical effect of the stains used upon the tissues and the interaction of stains with each other is known, so that the method chosen leads to an interpretation of results in terms of known causes. This undoubtedly is what we histologists should aim at. But there is another and wider connotation of the word 'rational', a connotation that this word has when we apply it in the spheres of politics, of war and of the more daily affairs of men. In these instances we choose a particular policy from one of a group, a group whose members we select for consideration because experience rather than ratiocination has shown their worth in this case or in that. It is a matter of consideration of things that have shown their value empirically, followed by a decision that this rather than that should be applied here and now. An outstanding example of what I mean is furnished by the stains for chromosomes given in these pages. They have been found to work by skilled workers and used repeatedly in circumstances that are clearly stated. Some of them depend upon dyestuffs, notably orcein, whose exact mode of working is at best imperfectly understood. But the choice of methods offered the reader is so numerous and the notes in conection with each so

thorough that a rational decision, in the sense I have indicated, can be made by the investigator.

In the first section of this book, which is concerned with the rationalization of staining in the stricter sense, Dr. Gurr proposes a new classification of stains based on a better appreciation of their chemistry as reagents. This was in itself desirable and may have a further good effect. The chemistry of staining, not least its physical chemistry, has suffered of late years from the fact that biological editors consider it chemistry and chemistry editors consider it biology, so that it is difficult to have that thorough public discussion of the many problems raised in this connection. We have, for example, the often unexpected pH of a staining solution in which structurally acid dyes give a solution on the basic side of the neutral point, a converse phenomenon being observed with many structurally basic dyes. Our author has himself contributed lately much to the understanding of these things, for example, by his published observations on the pH of distilled water. This is another of the reasons why I think that this completion of the work begun by his *Encyclopaedia* should have a good effect in stimulating further work on this general topic by others.

I should like to end on a more personal note. Dr. Gurr is manifestly enthusiastic about his subject, a noteworthy character of Kepler, Harvey and Newton before him to cite some of those who did great things and saw great visions. Only the disinterested can be enthusiastic because, like the poet, they want others to see not only the truth of what they have seen but also its goodness and beauty. In these days, when technology is doing so much for the betterment of the sick and the poor, it is good to think that there are some who also look upon their work as does the artist. Histological staining is a form of painting, in which the canvas of the tissues is as active as is the human hand that deals with it. A picture is valueless unless we can look upon it with understanding. It is this understanding that Edward Gurr will help us to achieve more largely as the years go on.

1965 M. A. MacConaill

PREFACE

This book is a companion volume to my previous work *Staining Animal Tissues, Practical and Theoretical*. The latter might have been called 'Rational Use of Dyes in Biology', Volume 1 (Animal and Human Tissues); Volume 2 (i.e. the present work) being devoted to general cytology, botany, haematology and that varied complex of organisms treated of in protobiology. It was felt, however, that each of the two works should be complete in itself, even though the present book contains much that will, I believe, interest those devoted to human and animal histology.

The object of this book is fourfold, namely:

(1) To present new ideas, following those already put forward in my last book, regarding the mechanism and theory of staining reactions.

(2) To emphasize the fact that synthetic dyes are not merely agents for imparting colour but are also highly reactive chemical substances. Many of them are in fact artificial amino-acids, capable of interacting with one another to produce polychromatic compounds which may be regarded as synthetic proteins.

(3) To encourage the rational, as opposed to the empirical, use of dyes. Dyes can only be used rationally if the biologist has some knowledge of their main chemical and physical characteristics. Not all biologists have the time to familiarize themselves with this branch of organic chemistry. It is believed that the first part of this new book will furnish this need in a concise form, in a simplified way which has not been presented elsewhere. It should be emphasized, however, that this is not a book on the chemistry of dyes.

The first part, among other things, deals with the morphological and functional anatomy of dye molecules, their interactions with one another and with tissue elements. It is hoped that a study of this part of the book will be of help to biologists in interpreting results of staining. It is also hoped that it will be of help in the selection of suitable dyes for particular purposes in research or in teaching. It seems to the author that much valuable time and material is wasted on random and empirical trials of dyes in microtechnique, etc. Much of this waste could be avoided by a brief preliminary study of the main chemical and physical characteristics of the dyes it is proposed to use for a particular piece of research.

(4) To bring together, in concise form, a variety of staining procedures previously scattered throughout the literature of many countries.

This book, then, may be regarded as a book on the morphological and functional anatomy (both practical and theoretical) of dye molecules; a work of reference; a laboratory guide to the rationalization of staining procedures; and a collection of staining methods, some of them very old, some very new, and others not so new. The greater proportion of the staining methods given here are empirical. I have not attempted to separate these from the rational methods, as the reader himself will be able to draw this distinction. It is not for me to sit in judgement on other workers' techniques, distinguishing those which are merely empirical from those which are not; many empirical methods are still useful as simple differentiators of anatomical components. I think it is proper, however, to emphasize the recently devised staining procedures of Professor MacConaill

and myself. Because of the manner of the planning of these methods I feel that they can be rightly cited as examples of planned and fully rational staining methods. Accordingly, these have been placed together at the end of the section dealing with staining methods. I wish to say that Professor MacConaill carried out by far the greater share of work in the development of these new and rational staining procedures. My special contribution was to be his adviser on the chemistry, interactions and choice of dyes, to carry out *in vitro* experiments in confirmation of his histological and *in vitro* results and to test our hypotheses regarding the chemical interactions involved.

As in my previous books, I have attempted to cater for a great variety of workers in the medical and biological spheres, hence the wide variety of staining methods given here. It is not claimed that this book is by any means a comprehensive work on methodology. Doubtlessly many other methods might have been included, but these had to be left out for reasons of space. On the other hand, some of the older methods given here may seem to be redundant to some workers and might not have been included. But contact with some thousands of medical and biological workers scattered throughout the world has taught me that many old and seemingly redundant methods are still in demand. This might be because of their historic interest or simply because they are still an important part of histological art.

In fact, one of the reasons for writing this book has been an attempt to save time. More and more biologists of almost every nationality write to me for details of staining methods; for advice on the choice of dyes for a multitude of purposes; for information on their chemical structure, reactions, pH values, solubilities, molecular weights, literature references, and so on. This book attempts to provide the answers in advance to at least a few of the kind of questions that are so frequently put to me. It is also an attempt to pass on some hitherto unpublished ideas and information regarding the character of dye molecules. There is needed even more co-operation between biologists, physicists and chemists. The subject of morphological and functional anatomy of dye molecules in relation to biology offers a rich field for research, the surface of which has as yet been scarcely scratched.

It should be emphasized that no attempt is made in this book to teach the medical or biological laboratory worker his job. I do not regard myself as a practising histologist, but as an organic chemist with a specialized knowledge of dyes, and with some experience of microtechnique which has made me aware of some of the requirements and a few of the very difficult problems of the biologist and the medical research worker.

A book of this kind neither could nor should be written without recourse to as many learned periodicals as possible. For access to no small number of these I have to thank the Librarians of the Royal Microscopical Society, the Linnean Society, the Chemical Society and the British Medical Association. Their help was both unfailing and most kind.

I wish to place on record my gratitude to my wife, Mrs. F. P. Gurr, B.Sc., for inspiration, constructive criticism and moral support. Like all my other books, this one, I felt, was needed; the idea presented itself to me as a challenge. It began as a pleasure. Like all its predecessors, it was believed at a later stage (when it was not a pleasure!) to be the last book from my typewriter! I am grateful to my wife for her patience and forbearance.

My thanks are due to Mr. J. R. Thomas, Mrs. C. Pegler, Mr. B. F. White, Mrs. N. Samuel, Mr. J. Jones, Mrs. A. Austin and my wife for relieving me of other work, thereby making it possible for me to make time to write this book.

I also wish to thank the Publishers and the Printers for their painstaking and meticulous care in transforming my typescript into print.

Acknowledgements are due also to Miss Finula O'Donovan, B.A., Secretary, Department of Anatomy, University College, Cork, for undertaking the vast amount of typing involved in recording the work carried out jointly and separately by Professor Michael MacConaill, M.R.I.A., and myself in his department in Cork and in my laboratories in London on our polyanionic dyes.

Mr. J. Rafferty, Senior Technician, Department of Anatomy, University College, Cork, has shown great energy and initiative in assisting us with histological and cytological trials of a large number of the dyes used in this special work, and I wish to record my grateful appreciation of his help.

Last, but by no means least, I wish to place on record my grateful thanks to Professor Michael MacConaill, my friend, counsellor, and academic chief, on whose wisdom and experience I have freely drawn. He has been a source of inspiration to me. I count myself fortunate to have been a research student of his and regard it an honour to be a member of his staff as Research Associate. By far the larger portion of the credit for the success of our joint work is due to him. Without him the set of new discoveries regarding the interactions of anionic dyes with one another and with tissue elements might not yet have been made.

Finally it should be added that apart from the Publishers, no one but myself has seen the whole of this book prior to publication. Therefore, no one but myself can be blamed for any errors that may have crept in.

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CONTENTS

PART 1 - THEORETICAL

A GUIDE TO THE RATIONAL USE OF DYES IN BIOLOGY

ON THE THEORY OF COLOUR IN RELATION TO ORGANIC MOLECULES

Ordinary white light is composed of light of the total spectral range. If an object appears coloured in ordinary white light it is because that object absorbs some of the spectral radiation but not all. The unabsorbed colour is reflected or transmitted and is the colour of the object as it appears to the eye. Objects which appear black absorb the whole of the spectral range without transmitting or reflecting any component of the white light. If a coloured object, illuminated by a source of white light, is examined with a spectroscope it will be seen that the light reflected or transmitted by the object does not show the complete visible spectral range. Some of the colours of the spectrum will be missing. If the object examined is red, for example, then the blue-green will be missing from the spectrum. If the object is blue-green on the other hand, then red will be missing from the spectrum. Red and blue-green are, therefore, regarded in this respect as complementary colours. The wave-lengths of the more important colours of the visible spectrum are given in the following table.

Wave-lengths in mμ	*Colour*	*Complementary Colour*
400–430	violet	greenish-yellow
430–490	blue	yellowish-orange
490–510	blue-green	red
510–530	green	purple
530–560	yellowish-green	violet
560–590	yellow	blue
590–610	orange	greenish-blue
610–750	red	blue-green

Absorption often takes place in more than one region of the visible spectrum and the colour observed also depends upon the *intensity* of absorption. Since light is made up of radiations of different wave-lengths and the velocity of the whole is constant, then the shorter the wave-length of any one component radiation the higher the frequency of its wave. Conversely, the longer the wave-length the lesser the frequency.

It is appropriate to define here three terms which will be referred to later in this book. The first of these is *hypochromy*. The other two are *bathochromy* and *hypsochromy*.

Hypochromy or hypochromic effect means an increase in the *intensity* of absorption.

Bathochromy or bathochromic effect means a shift of the absorption band towards lower frequencies, that is to say, towards longer wave-lengths, and corresponds, therefore, to a deepening of the colour seen from yellow, through orange, purple, violet, red, blue and green, to black.

Hypsochromy or hypsochromic effect means a shift of the absorption band

towards higher frequencies, that is to say, towards shorter wave-lengths, with consequent lightening of colour.

Bathochromy and hypsochromy are opposite effects; hypsochromy and hypochromy are also opposites. On the other hand bathochromy and hypochromy are not. Care should be taken to avoid confusing the two terms *hypsochromy* and *hypochromy*.

Saturated organic molecules do not absorb light except in the far ultra-violet region. They are, therefore, colourless in ordinary white light. All coloured organic compounds are unsaturated in character. But all unsaturated organic compounds are not coloured in ordinary light. For instance, benzene (benzol) belongs to the latter class. It is colourless in ordinary light but coloured in the ultra-violet range, as are many other colourless unsaturated aromatic organic compounds.

In order to become coloured in ordinary light, unsaturated aromatic organic compounds need to have at least one *chromophore*. The latter may be defined as a configuration which has a group containing one or more multiple bonds. The action of a chromophore is to shift the absorption of the unsaturated organic molecule into lower frequencies, that is to say, into longer wave-lengths.

Colour may be deepened and intensified progressively by modifications to the dye molecules whereby the number of chromophores is increased. Or the colour of a dye may be deepened and intensified by the replacement of weaker chromophores by stronger ones.

Venkataraman (1952) states that auxochromes are more difficult to define than chromophores and that there has been some confusion regarding the nature and function of auxochromes. An attempt has been made (Gurr, 1962c) to obviate some of this confusion by the introduction of a new term to distinguish certain kinds of auxochromes from others. This new term, *colligator*, is a functional one and will not be found elsewhere in published literature.

From the standpoint of the colour of organic compounds, Venkataraman defines an auxochrome as a substituent atom or group which increases the intensity of the absorption of light due to a chromophore. An auxochrome may also shift the main absorption band to lower frequencies, that is to say, to a longer wave-length, thereby deepening the colour of the organic molecule. The function of an auxochrome, in such a case, is analogous to that of a second chromophore when conjugated with a first. The second chromophore increases the intensity of absorption due to the first chromophore. The second chromophore also shifts the main absorption band to a longer wave-length, thereby deepening the colour of the organic molecule.

It would appear then that both auxochromes and additional chromophores may have a bathochromic effect. But according to Venkataraman there are adequate reasons for associating auxochromes with hypochromy (not hypsochromy!) and additional chromophores with bathochromy. These may or may not be accompanied by bathochromy in the case of an auxochrome, or by hypochromy (i.e. intensification of absorption) in the case of the additional chromophore.

A given auxochrome may have a hypochromic effect only on certain chromophores. The hypochromic effect may only come into operation when the auxochrome is in a certain position with reference to the chromophore in the aromatic nucleus. The same auxochrome may decrease the absorption for other

chromophores. In such cases the auxochrome would have a hypsochromic effect (i.e. lightening of the colour).

An atom or group functions as an auxochrome only under certain conditions. For this reason the effect of auxochromes and chromophores must be considered conjointly. An 'auxochromic effect' has thus, according to Venkataraman, more significance than an 'auxochrome' and he considers that Brooker's suggested composite term *auxochromophoric system* (Brooker, 1945) is an acceptable one to express the combination of auxochromes and chromophores in dye molecules. The distinction between auxochromes and chromophores in light absorption is one of degree rather than of kind. It is convenient, however, to distinguish between auxochromes and chromophores. The specific effect of auxochromes of increasing the intensity of absorption should also be taken into consideration.

A colligator has already been defined here as a special kind of auxochrome. It is by means of its colligator(s) that a dye is able to unite chemically with tissue elements or with certain other dyes and compounds. There are two main divisions into which colligators may be placed. These are (*a*) acidic colligators and (*b*) basic colligators.

The most important acidic colligators are SO_3^-, SO_3H, SO_3Na, COOH, COONa, OH and ONa (or OK). These groups are met with in acid dyes, although the last four occur in a few basic dyes. The acidic colligators enable the acid dye ion to attach itself to tissue elements that are basic in reaction, or to basic dye ions or other basic substances.

Basic colligators are NH_2, NH and N^+. These groups occur in basic dye ions and in many acid dye ions. They enable basic dye ions to attach themselves to acidic tissue elements and also to unite with acidic dye ions. In amphoteric anionic dyes, these groups enable the dyes to unite with more strongly acidic dyes.

Although we have distinguished between chemically reactive and unreactive auxochromes by calling the reactive or conjugative kind colligators, both kinds have a common function. Ordinary auxochromes, as we may call the non-colligator kind of auxochromes, have been defined above in accordance with Venkataraman. But the colligators have the same function as ordinary auxochromes as well as the ability to bring about conjugation of the dye ions, to which they are attached, with certain other dye ions.

The primary function of the sulphonic group is to render a dye water-soluble. Its influence on the colour of dyes is of minor importance. But if certain other auxochromes are present in the same ring and if they and the sulphonic group occupy appropriate positions on the same aromatic nucleus in relation to each other and to the chromophoric group, then the sulphonic group may have a hypsochromic or a bathochromic effect. Under similar conditions other colligators also affect the colour of the dye ion containing them, but the topic is too involved to be dealt with here and readers are referred to Venkataraman's scholarly work for detailed information on this subject.

ON THE EARLY HISTORY OF BIOLOGICAL STAINING

It is difficult to establish the priorities in the matter of the introduction of stains in biological research. The honour of being the first to use stains in microscopy has been ascribed in literature to a number of different workers. These include Leeuwenhoek (1719), Sarrabat (1733) and Hill (1770). Rooseboom (1956) and other writers have credited Leeuwenhoek, a Dutch microscopist who died in 1723 at the age of 90, as being the founder of microbiology. But investigations by the present author appear to show that the honour belongs to Grew (1682). He was the son of a Coventry dentist and was trained in medicine at Leiden University, Holland, where he took the degree of M.D., returning to England to practise medicine in London. In later life he turned to the study of botany, in which field he distinguished himself. For his contributions in the sphere of plant biology Grew was made a Fellow of the Royal Society and later became Secretary of that Society.

The stain used by Grew was cochineal extract. This substance is of animal origin since it comes from the dried bodies of the female of the species, *Coccus cacti*, a trqpical insect which lives on certain kinds of cactus plants. Thus the first stain used in microtechnique was used on plant tissues and was of animal origin.

Leeuwenhoek, a naturalist, was the first to use a stain on animal tissues. The stain he used was an extract of saffron which is of plant origin, being the dried stigmata of *Crocus sativus*.

Before the discovery of mauve or mauvein, in 1856, by a young English chemist, Perkin, the stains used by microscopists were natural colouring matters, such as cochineal, cudbear, gamboge, indigo, madder, seaweed extract and a few inorganic substances such as copper sulphate.

Although Perkin founded the synthetic dyestuff industry, picric acid was prepared long before his time. Woulfe was the first to make this dye when in 1771 he produced it by the action of nitric acid on the natural colouring matter, indigo. But neither he nor other chemists at the time recognized picric acid as a derivative of benzene. All synthetic dyes known today are derivatives of benzene, a hydrocarbon which was not discovered until 1825, by Faraday. In 1834 Runge produced the dye rosolic acid. He prepared this by oxidizing phenol, but a practical process was not worked out until 1861, by Kolbe and Schmitt. Hofmann was the first to isolate benzene from coal-tar in 1845 but its chemistry was not understood until some years later, after Perkin's discovery of mauvein.

Beneke (1862) was the first to use a synthetic dye in microtechnique. The dye was called 'lilac aniline' at the time. It may well have been the same dye as that discovered by Perkin in 1856 and called mauve, mauvein or aniline purple. From 1869 onwards the use of synthetic dyes in microtechnique increased by leaps and bounds until today the number of such dyes used for one purpose or another is at least a thousand.

As the number of readily available dyes increased so did the manner of their use become more elaborate. In the beginning such dyes as carmine, haematoxylin and methylene blue were used singly. Later it became common practice to use two or more stains either together or in succession. Schwartz (1867) appears

to have been the first to have done this. He stained tissues with picric acid after ammonia carmine primary staining. In the following year Ranvier (1868) combined these two stains into one solution thereby initiating the picro-carmine technique still used today.

Up to the time of Ehrlich (1879a, b) all staining procedures were empirical. Investigators used dyes on the 'try it and see' principle. They could not have done otherwise because of the lack of co-ordination between biology and chemistry in this field at the time.

Long after the use of synthetic organic dyes had become firmly established in microtechnique, however, various distinguished biologists were still searching elsewhere for new stains. For example, Lee (1890, 1893) refers to the use of red cabbage, bilberry juice, blackcurrant juice, walnut juice and litmus. These natural substances had recently been tried and recommended as biological stains by Lavdowsky, Fol, Leon and Lawson Tait respectively.

In the first edition of his book, Lee (1885) mentions a bilberry juice-eosin double stain which he ascribes to Lavdowsky (1884). The primary bilberry juice staining solution, which contained a small proportion of alcohol, was described as giving 'a red colour with fresh neutral objects, or lilac when the acid of the fluid is neutralized by an alkali or a neutral salt. The latter is more durable. It stains well the nuclei of all cells, and shows karyokinetic figures very plainly'. He then went on to write 'The reporter of the *Journ. Roy. Mic. Soc.** criticizes the publication of this stain (which he considers to be probably useless) as an instance of the "modern fashion of recommending every conceivable substance which by any chance will furnish a stain". I consider the criticism unfortunate. A stain that is capable of showing karyokinetic figures plainly in fresh objects is certainly not useless, and it appears improbable that so accomplished an histologist as Lavdowsky should take trouble to recommend a useless process'.

Incidentally, in his preface Lee (1885) made special acknowledgement of the great assistance rendered him by the Journal of the Royal Microscopical Society, adding '. . . in many respects the best-edited periodical known to me'.

It is perhaps amusing now to note that in the preface to his third edition (1893), this famous author, Bolles Lee, whose valuable book had already become widely recognized as a classic in the sphere of microtechnique, suggested, apparently not without justification, that '. . . the reckless publication of crude and needless histological methods should be stopped'.

He then went on to complain that 'The really useful matter is smothered in a sea of rubbish. Some idea of the magnitude of the evil may perhaps be gleaned from the fact that this book has now been out of print for several months, the mass of literature that it has been necessary to digest for the purpose of a new edition being so great that the appearance of the work has been delayed far beyond the contemplated time. It is useless to remonstrate with the persons of whom I am complaining; they cannot grasp the fact of their ignorance, and cannot be brought to see that nobody heeds them. I would appeal earnestly, therefore, to those with whom the power lies, to put an end to this nuisance'.

The developmental pattern of staining procedures emerging from a study of the history of staining will show that the progress has been on the one hand from single to multiple staining and on the other hand from empirical to rational methods. Empirical methods have by no means disappeared. Some of them are

Journ. Roy. Mic. Soc.* (1884), **IV, 652.

being transformed into rational methods as more and more becomes known of the chemical reactions of synthetic dyes with one another and with tissues, and of the physico-chemical conditions that produce the best results. Others are unlikely ever to become rationalized because of the conglomeration of alchemic ingredients of the staining solutions. There is little scope for alchemy today, and as Baker (1958) states, dabbling with dyes by persons ignorant of the chemistry of what they are doing has no counterpart in the rest of science and cannot be regarded as scientific activity.