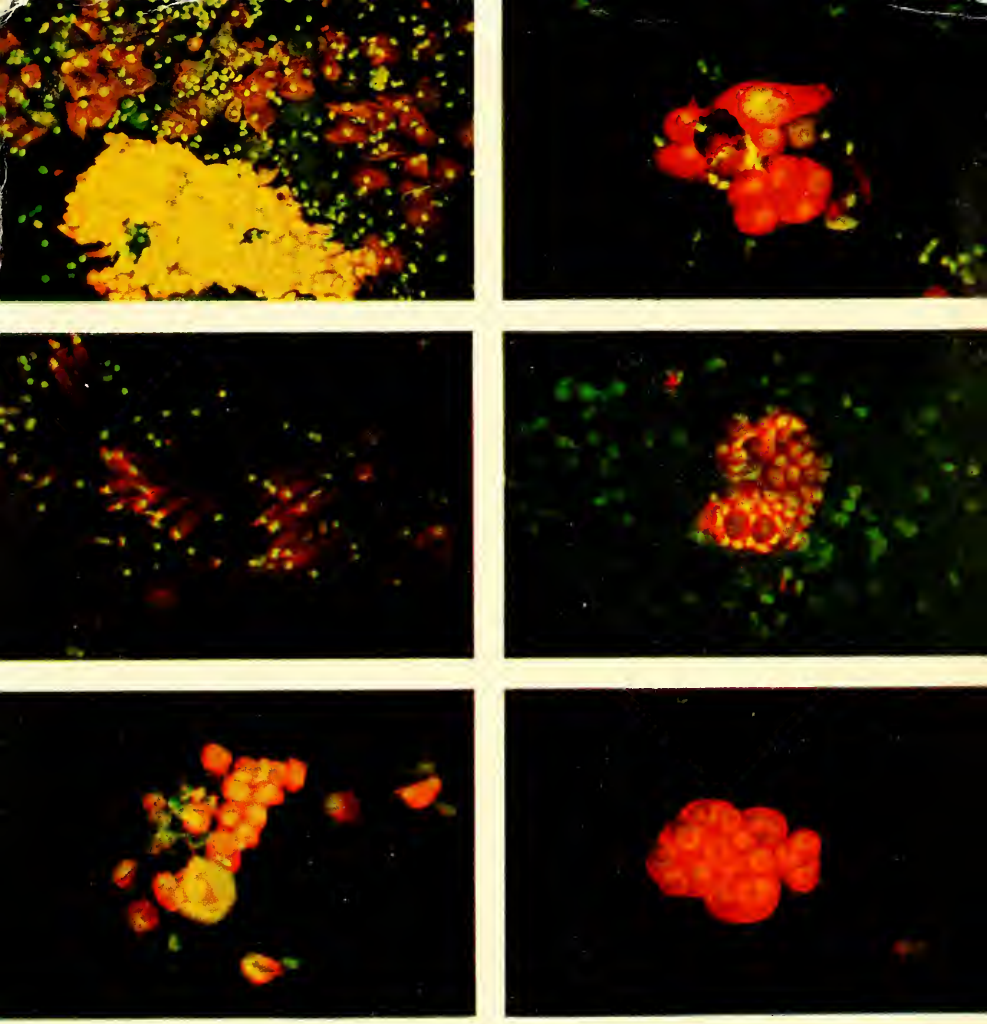
STAINING

Practical and Theoretical



(*Photograph by courtesy of Sandoz Ltd., Basle, Switzerland*)

Normal and Malignant Cell Elements as Observed with the Cytodiagnostic Acridine Orange Fluorescence Microscope Method (*by Bertalanffy*)

Top left.—Normal vaginal aspirate. In the upper portion of the picture are various types of squamous cells from the vaginal epithelium. The superficial cells have green cytoplasm and small green, pyknotic nuclei. The cytoplasm of the intermediate, parabasal and basal cells fluoresces brown to reddish brown; the nuclei are larger and greenish yellow. In the lower portion is a group of endocervical cells with yellow fluorescent nuclei that partially obscure the scanty reddish brown cytoplasm. Scattered between the squamous cells are green fluorescent, lobulated nuclei of polymorph granulocytes. ♀ 38, × 150. *Top right.*—Cervical aspirate from a case with squamous cell carcinoma of the cervix. The malignant cells show flaming red cytoplasmic fluorescence and yellowish nuclei. ♀ 71, × 300. *Centre left.*—Normal bronchial secretions showing several respiratory epithelial cells with reddish brown cytoplasm and greenish yellow nuclei. These cells are surrounded by green nuclei of neutrophil granulocytes. ♂ 56, × 250. *Centre right.*—Bronchial secretions from a case with adenocarcinoma of the lung. The malignant cells have bright red cytoplasmic fluorescence and yellow hyperchromatic nuclei. ♀ 57, × 360. *Bottom left.*—Pleural effusion showing a group of malignant cells of pulmonary origin. The malignant cells have bright orange fluorescent cytoplasm. ♂ 60, × 360. *Bottom right.*—Ascitic effusion with a group of adenocarcinoma cells of ovarian origin. The cytoplasm of the malignant cells is flaming red. Note the large red fluorescent, RNA-containing nucleoli. ♀ 78, × 450.

STAINING

Practical and Theoretical

by

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FOREWORD BY

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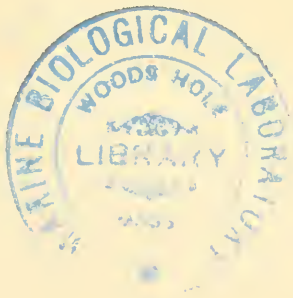
Professor of Pathology, University of Oxford



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BY THE SAME AUTHOR

Encyclopaedia of Microscopic Stains
Methods of Analytical Histology and Histochemistry
A Practical Manual of Medical and Biological Staining Techniques
Microscopic Staining Techniques

PRINTED IN GREAT BRITAIN BY
J. W. ARROWSMITH LTD., BRISTOL

FOREWORD

By Professor Sir HOWARD W. FLOREY,
F.R.S., M.D., PH.D., M.A., F.R.C.P.

President of The Royal Society, London
Professor of Pathology, University of Oxford

Mr. EDWARD GURR is known to many histologists and cytologists for the excellent reagents he produces and for his unfailing courtesy and helpfulness in dealing with the questions put to him about stains and staining methods. He has for many years made a study of the chemistry of dyes, and at the same time he has extensively studied their histological applications on which he has written a number of successful books.

In this new book he embodies his long experience on both the theoretical and practical aspects of staining. It should prove of the greatest value to all concerned with the art and mechanism of biological staining, and I wish it every success.

H. W. FLOREY.

PREFACE

The object of this book is twofold, namely:

(1) To present new ideas regarding the mechanism and theory of staining reactions, etc.

(2) To bring together, in concise form, a large number of staining procedures previously scattered throughout many books and journals.

This book, then, may be regarded as a laboratory guide, a book on theory, and a work of reference.

Up to comparatively recent times staining methods appear to have been based more on myth and lore than on established scientific facts. Empirical staining by the histologist, based on experience of a relatively small number of dyes, is giving way to a conscious selection proceeding from a knowledge of the chemical structure and affinities of what he uses. However, the use of stains as simple differentiators of anatomical components will continue to be an important part of histological art, and this is one of the reasons why so many different staining methods have been included in this book. Many of the methods given here are new; some are not so new, while others are very old. By studying the older methods and comparing them with the newer ones, in the light of the information given in the theoretical section of this book and the notes and observations given at the end of most staining procedures described here, some new ideas, which could be applied to other problems, might emerge.

As in my previous books, I have attempted to cater for a great variety of workers in the medical and biological spheres, hence the large number of recipes (over 700) and staining procedures (about 300) given here. Some of these methods, which may appear redundant to some technicians, will be required by others. Contact with some thousands of medical and biological laboratory workers scattered throughout the world has taught me this.

Some of the methods could perhaps be simplified: there is ample scope for experiment here. In this connection I would call attention to the Falg and Faviol methods. These are examples

of simple staining procedures, based on a deliberate and logical selection of dyes after consideration of their known chemical and physical properties, to supplant, and with better results, more complicated and time-consuming methods.

It should be made clear that no attempt is made in this book to teach the histologist or the pathologist his work. Indeed, I do not presume to be qualified to do so. I regard myself not as a practising histologist, but as an organic chemist with a specialized knowledge of biological stains, and with an awareness of some of the problems and requirements of the biologist and medical laboratory worker.

It is hoped that this book, particularly if used in conjunction with my last one, *Encyclopaedia of Microscopic Stains*, may provide fresh ideas and suggest new ways of approach to some of the problems facing medical and biological workers in the laboratory, whether they happen to be engaged in research or routine projects.

I wish to pay tribute to the excellent library facilities of the Royal Microscopical Society, London, which has been of considerable help to me. I also wish to place on record my grateful thanks to my wife, Mrs. F. P. Gurr, B.Sc., for her patience and forbearance during the writing of this book, which like all its predecessors was to have been the last from my typewriter! To her also are due my thanks for helpful criticism of the script and for the encouragement she has given me. I am also indebted to another very good friend, Mr. W. Leonard Hill, the chairman of Leonard Hill Ltd. and Leonard Hill (Books) Ltd., the publishers. The production staff of Leonard Hill (Books) Ltd., and the printers, J. W. Arrowsmith Ltd., of Bristol, are, I feel, to be congratulated on the meticulous care they have exercised in the handling of the typescript.

My special thanks are due to my friend and counsellor, Professor Michael MacConaill, M.R.I.A., of the Department of Anatomy, University College, Cork, on whose wisdom I have freely drawn. I count it a privilege to serve as a member of his staff. He has been a source of inspiration to me, with his unselfishness and sympathetic understanding.

My acknowledgements are also due to Miss Finula O'Donovan, B.A., Secretary, Department of Anatomy, University College, Cork, for the vast amount of typing performed by her in recording

the work carried out both jointly and separately by Professor MacConaill and myself in his department in Cork and in my laboratories in London on the fuchsinic acid dyes. Only a small part of this work is reproduced here. Acknowledgements are also due to Mr. J. Rafferty, Senior Technician, Department of Anatomy, University College, Cork for technical assistance in connection with histological trials of the fuchsinic acids, their component dyes and many others used in the studies carried out by Professor MacConaill and myself. Mr. Rafferty showed a great deal of initiative in the performance of this work. My thanks are also due to Mr. J. R. Thomas for relieving me of other work thereby making it possible for me to devote time to the writing of this book.

Apart from the publishers no one other than myself has seen every page of this book prior to publication. Therefore no one except myself can be held responsible for any errors that may have crept in.

In conclusion I wish to express my special gratitude to Sir Howard Florey, F.R.S., for his great personal kindness and the interest he has shown.

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STAINING PROCEDURES

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SECTION I

SOME OBSERVATIONS ON THE THEORY OF STAINING

When two dyes, one basic and the other acid, are brought together in aqueous solutions under suitable conditions, a precipitated compound dye is formed. If this precipitate is collected and thoroughly washed with water to remove any excess of one or the other dye and the colourless, water-soluble, by-product which is an inorganic salt, it will then be found impossible to separate the two component dyes by means of ordinary solvents such as water, alcohol, cellosolve, xylol, chloroform, etc. The reason for this is, of course, that the negatively charged (acid) dye-ion is united with the positively charged (basic) dye-ion to form a chemical compound. When such a dye is dissolved in alcohol and applied to tissues the union of the acid and basic dye-ions no longer holds in face of competition from various tissue elements of the opposite charge. There is no doubt about the chemical nature of the union between the two dyes *in vitro*, but there is a doubt concerning the nature of the union of the liberated dye-ions with the tissue elements. We know that the components of a chemical compound cannot be separated from each other by the action of ordinary solvents.

Then it follows that any dye that can be extracted from stained tissues, with ordinary solvents such as water or alcohol without prolonged soaking, could not have been in chemical combination with the tissue elements that were coloured with it. On the other hand, if the dye cannot be extracted at all, or if it cannot be completely removed from the tissues, this does not prove that the dye remaining in the tissues has entered into chemical union with the latter. Many basic dyes, used without mordants, can be completely extracted from tissues by ordinary solvents, as can a number of carboxylated (acid) dyes which are devoid of sulphonic groups. Eosin is an example of the latter class of dyes. Many but not all, sulphonated acid dyes hold on to the tissue elements stained by them; that is, after the normal histological process of washing away the excess dye, dehydration, etc. When any dye,

acid or basic, applied to tissues without the use of a mordant, remains in the tissues in sufficient strength to colour them, and cannot be extracted by ordinary solvents, as already indicated, it could be bound to the tissue elements through chemical union or by physical factors, such as adsorption.

Proteins which are found everywhere in tissues and which are the most important biological substances, constitute an enormous group composed of carbon, hydrogen, oxygen, nitrogen, and usually sulphur. Many proteins contain phosphorus in addition to the elements named above, and some contain small amounts of iron, iodine, copper, zinc, etc. Proteins are intimately associated, in biological cells, with nucleic acids, lipids, carbohydrates and their derivatives; hormones, enzymes, vitamins, etc. Hormones and enzymes themselves have been found to be protein in character. The chemistry of proteins is exceedingly complex. There is a greater degree of diversity in their molecular structure and chemical composition than in any other group of biological substances. The natural proteins consist of large numbers of molecules, of the same or different amino acids which are united through condensation with the loss of the elements of water. Proteins are characterized in biochemistry by their amino acid components. Amino acids are organic acids in which one or more of the hydrogen atoms has been replaced by an amino group. Amino acids are amphoteric because their molecules contain both acidic (carboxyl) and basic (amino) groups. Proteins also are amphoteric because they are composed of amino acids. Because proteins are amphoteric the possibilities of building up and otherwise modifying their molecules by processes of nature is enormous. In fact, the number of types of protein molecules varying in structure and chemical composition is almost infinite. Their molecular weights are in many cases enormous. As mentioned above, proteins found in tissues are intimately associated with nucleic acids, lipids, carbohydrates, etc., to form highly complex substances. It seems reasonable to imagine the molecules of such substances as consisting of chains of protein-complexes from which the tissue elements are woven by nature. Some tissue elements such as the envelope of the red blood corpuscle and neurokeratin are closely woven or "close-knit" (see page 77), while others such as collagen appear to be loosely woven or " loose-knit ". The outer surfaces of tissue elements might be imagined to

consist of a meshwork formed by molecular chains of proteins or protein complexes. This outer meshwork would be amphoteric. There would be different degrees of amphotericy, so that some tissue elements would be relatively more basic, and others relatively more acidic in reaction. According to Baker (1958), the envelope of the red blood corpuscle is strongly alkaline and one of the least pervious of all tissue elements.

It is not difficult to imagine this outer meshwork acting as a kind of filter, permitting certain molecules and dye-ions and other ions to pass through its lattices into the inner threshold of the tissue element. Further progress of the molecules and dye-ions, which have passed through this first barrier, would depend upon further obstacles to be met with. Although molecules of slender shape or of low molecular weight may be able to gain entry and perhaps pass along the inner labyrinth of lattices and corridors formed by tissue groups, they may not be able to get out again. They may be trapped by becoming wedged in narrow crevices, or they may be seized by tissue elements of opposite charge and thus held in firm chemical bondage.

On the acid side of their isoelectric points, proteins exist as cations, and on the basic side they exist as anions. The isoelectric point of a protein (or of a dye) is that pH at which the protein (or the dye) does not migrate in an electric field.

Acid and basic dyes combine with proteins to form salts and proteinates. The acid dye, eosin, for instance, combines with proteins that are on the acid side of their isoelectric points (protein^{+} eosin^{-}), whereas the basic dye, methylene blue, combines with proteins that are in solution alkaline to their isoelectric points (protein^{-} methylene blue^{+}). Basic dyes, in general, rely upon amino or imino groups or basic nitrogen atoms attached to their aromatic nuclei for their basicity: that is, their power to unite with negatively charged ions of tissues or with other substances such as acid dyes. Carboxylated (acid) dyes rely upon their carboxyl groups for their acidity: that is, their power to unite with positively charged ions of tissues, or with other basic substances.

Sulphonated dyes rely upon their sulphonic groups for their acidity. When two substances such as a tissue element and a dye unite chemically there is an exchange of ions between the two substances. Proteins already possess basic groups identical

with those offered by basic dyes. Proteins also possess carboxyl groups identical with those offered by carboxylated (acid) dyes. In other words, most basic dyes, and carboxylated dyes devoid of sulphonic groups, have nothing to offer relatively simple proteins which are not already possessed by the latter. This may be the reason why most basic dyes, used without mordants, and carboxylated acid dyes (devoid of sulphonic groups) can be readily extracted from most tissue elements, coloured by them, with ordinary solvents. In short, as the proteins already possess acidic and basic groups identical with those offered by basic dyes and carboxylated acid dyes (devoid of sulphonic groups), no impulse, therefore, exists on the part of the protein for an exchange of ions to be made, and any coloration that occurs would be due to physical and not chemical factors. Not all basic dyes, however, can be completely removed from tissues by ordinary solvents. In such cases presumably the basic dye is bound to the strongly acidic groups of tissues by chemical affinity. On the other hand it might be that the molecules of the dye have penetrated deep into the tissues and have been trapped in narrow crevices from which they cannot be dislodged by ordinary solvents. Here it should be mentioned that all basic dyes are of relatively low molecular weight (E. Gurr, 1960, pages 423–432). They are presumably, but not necessarily, of small molecular dimensions or volume which would enable their molecules to penetrate deeper into " close-knit " tissue elements than might the majority of acid dyes, most of which are of higher molecular weight than any of the basic dyes. If the basic dye is in chemical union with acidic groups of tissues, then it could not have united with carboxyl groups, if the hypothesis put forward above is valid.

As previously stated, proteins are found in intimate association with other substances in tissues to form protein complexes. When ordinary solvents will not remove an unmordanted basic dye, for example, from tissue elements that have been stained by it, then the dye may have entered into chemical union with tissue-elements. Such tissue elements might contain chemical groups that are more strongly acid than the carboxyl radical. Examples of such tissue groups might be the phosphate and the sulphate radicals.

When the two basic dyes, methyl green (molecular weight 458)

and pyronin Y or G (molecular weight 303), are in competition with each other, their differential staining of the two types of nucleic acids (see Kurnick's method, page 297) might conceivably be influenced by the differences between the molecular weight of these two dyes. Pyronin can be replaced by toluidine blue Korson (1951) for staining RNA. Toluidine blue is a basic dye whose molecular weight (306) is very close to that of pyronin Y. It might prove worth while to try other basic dyes of around the same molecular weight as pyronin Y, in place of the latter for RNA.

Acid fuchsin, as stated elsewhere in this book, is an amphoteric dye. In practice, however, it behaves as an acid dye when applied to tissues. It unites with tissue elements that are basic in reaction. It does, however, behave as a base towards other acid dyes. It also behaves as an acid towards basic dyes.

Basic dyes are usually employed for staining mitochondria. Examples of such dyes are janus green, amethyst violet, janus black, janus blue, methylene blue, pinacynaol, toluidine blue.

There appears to be a possibility, therefore, that when acid fuchsin stains mitochondria it is acting as a basic dye through its amino (basic) groups uniting with acidic groups (probably phosphate) of mitochondria. On the other hand, perhaps the acid fuchsin is acting as an acid dye and is not reacting with the same chemical groups, of mitochondria, as are the basic dyes mentioned above. Osmic acid, of course, stains mitochondria; in this case presumably through the lipid moiety of the latter, so that the staining reaction would not be the same.

In general, sulphonated (acid) dyes appear to hold on to tissues stained by them and are not completely removed by ordinary solvents. Mention is made elsewhere in this book of competition between pairs and trios of sulphonated acid dyes for certain tissue elements. One of the dyes in such staining procedures will replace the other in certain tissue elements. With few exceptions it is the sulphonated dye with the lower molecular weight that replaces the one with the higher molecular weight. Acid fuchsin (molecular weight 586), for instance, captures certain tissue elements previously stained by light green (molecular weight 793). Orange G (molecular weight 452) will replace acid fuchsin as well as light green. Many more examples could be quoted but space does not permit. But removal of one sulphonated dye by another

in tissues is, of course, something very different from the removal of a dye by an ordinary solvent. Probably the reason many sulphonated acid dyes usually hold on to tissue elements despite the application of solvents is that the dyes are either trapped within the tissues in the manner suggested (page 5), or because they are in firm chemical union with basic tissue elements. As already stated, sulphonated dyes rely upon their strongly acidic sulphonic groups for their acidity. Presumably such groups are not found in tissues under normal circumstances. Therefore when these strongly acid dyestuffs are in contact with tissue-elements there is apparently a strong attraction between the sulphonic groups of the dye and the basic groups (e.g. amino, imino) of the tissue elements. Hence, it seems reasonable to suppose that chemical union takes place between the sulphonated dye and basic tissue-elements. Since proteins are ubiquitous in tissues, so must be their basic groups (e.g. amino, imino). This may explain in part why many sulphonated acid dyes stain tissues with such intensity.

Here, another question might be asked. That is, why do some sulphonated dyes replace others with which the tissues have previously been stained. The answer may be found in the relative degrees of acidity of the competing ions. The one replaced may not be so strongly acid as the dye which replaces it in tissues. Orange G, sun yellow G, chlorantine fast green BLL, for instance, replace a number of less strongly acid dyes, including acid fuchsin and light green SF.

Presumably the strongly basic tissue elements, with which the less strongly acid sulphonated dyes were first united, cast off the latter for the more strongly acid sulphonated dyes for which the tissue elements have a greater affinity.

Another instance of competition between dyes and tissue-elements might be mentioned here. That is, the use of the acid dye light green for differentiating the basic dye, safranin. This is a case of competition between a strongly acid dye and a weakly acid tissue-element for a basic dye. Light green is a sulphonated dye. It appears unlikely that a carboxylated (acid) dye, devoid of sulphonic groups, such as eosin could be used in simple aqueous solution for differentiating safranin or any other strongly basic dye, because eosin is too weak an acid to capture basic dyes from the acidic tissue elements which depend upon their

carboxyl groups for their acidity just as does eosin itself. Eosin will, however, unite with basic dyes (methylene blue, for instance) in the absence of competition as mentioned on page 32.

A striking example of staining due to both physical and chemical factors is to be found in the strongly acid dye, solochrome cyanin R. The molecule of this dye, as can be seen from the structure on page 72, contains two carboxyl groups and one sulphonic. There are no basic groups, on the dye-ion, which might impart amphoteric properties. The dye-ion is wholly acid. Therefore it is rather surprising that this dye acts towards tissues as though it were an amphoteric dye. Gurr & MacConaill (1959) found that this dye, like the closely related dye used by Pearse (1957), acts as if it were acid towards basic tissue-elements, and as if it were basic towards acid tissue elements, notably DNA and RNA. As a test of an earlier hypothesis (MacConaill, 1949, 1951) regarding the relative basicity of the erythrophile elements that we included solochrome cyanin in the group of dyes used in our investigations. Given a suitable pH, solochrome cyanin R stains acidic tissue elements blue, and basic elements pink or red. Pearse first used a 2% solution of orthophosphoric acid to secure a suitable pH. In our work we found that Pearse's results appear to be reproduced equally well by using acetic acid as the dye-solvent. We also found that the best results were obtained with a 0·5% solution of the dye. The only difference between the dye used by Pearse (1957) and that used by us was that his was a monosodium salt and ours a trisodium salt.

Staining by solochrome cyanin R fully confirmed the inference that led us to the trial. The special elements (MacConaill, 1949, 1951) of the spinal cord, including the neurone nucleolus, showed a basic reaction, while those tissue elements that had been deemed to be acidic reacted correspondingly to this dye.

The molecular structure of solochrome cyanin R is such that there is no reason to think that it breaks up into an acidophil and a basophil portion when in solution. Solochrome cyanin is a hydroxytriphenyl methane dye, the colour changes of which are probably due to dissociation of its hydroxyl group as occurs in the case of the phthalein class of indicators. The latter may also be regarded as hydroxytriphenyl methane derivatives. Solochrome cyanin, therefore, may be looked upon as an indicator, rather than as a dye. For this reason we consider it

especially useful in determining the acidity or basicity of tissue elements stained by it: the method we used is described on page 422.

DYES AS DIFFERENTIATORS AND DECOLORIZERS

It has long been known to histologists that a few dyes when applied to tissue-elements previously stained with some other dye, will remove the first stain and leave the tissue-elements colourless, if the process is stopped at this stage. If the process is allowed to continue, however, the second dye will then stain the tissues; that is, by replacement of the first dye. We can see an example of this, in the standard Faviol method, which is particularly vigorous when the protective acid bath is omitted. In this case an aqueous solution of the acid dye, sun yellow G. acts both as a differentiator and as a decolorizer. It acts on the tissues by removing the dye which has already stained them. It also acts on the displaced dye by decolorizing it.

Lee (1913) mentions the use of aurantia and of picric acid in alcoholic solutions as differentiators of certain other dyes, but this fact was well known before that time. Both aurantia and picric acid are acid dyes, but unlike sun yellow G, which is a sulphonated stilbene dye, these two are non-sulphonated and belong to the nitro group. It appears also that they are used in alcoholic solution and for the differentiation of basic dyes. They decolorize the tissue-elements, if the differentiation is allowed to proceed long enough, then stopped at that stage. If their action is allowed to continue they stain the tissues after displacing the basic dye. This applies to synthetic basic dyes used without a mordant. It also applies to certain acid dyes such as eosin and phloxine possessing carboxyl (acid) groups, but devoid of sulphonic groups. Tartrazine in cellosolve has been used (Lendrum, 1947) as a contrast stain to, and for differentiating phloxine, as described on page 368. Tartrazine contains two sulphonic groups attached directly to the aromatic nucleus and one carboxyl group attached to a side chain; that is, not directly attached to an aromatic nucleus. The structure of this dye is given by Gurr (1960). In this case the dye removed (phloxine) is not decolorized by the dye (tartrazine) used to displace it. The reason for this is that neither dye possesses groups which would

permit chemical interaction to take place between them. Phloxine is much more soluble (about 9%) in cellosolve than is tartrazine (about 2·5%) which is used as the differentiating dye. Also the strength of phloxine used in the aqueous staining solution is only 0·5%, as against the 2·5% of tartrazine (in cellosolve), but this matter will be discussed later in this chapter.

A differentiating dye can be regarded as a decolorizer of tissues, not of dyes. A decolorizing dye can be regarded as a decolorizer both of tissues and of other dyes. Both differentiating and decolorizing dyes if allowed to act long enough will not only cast out the synthetic dye with which the tissues have been previously stained, but will also take its place and colour the tissues.

The synthetic basic dyes used without a mordant, and carboxylated dyes devoid of sulphonic groups, such as the eosin group, which includes phloxine, are generally readily removed by immersing tissues stained with them in alcohol. Therefore the differentiating action of aurantia or picric acid must be due, to a large extent, to the alcohol in which these two differentiators are dissolved. Cellosolve may have the same action as alcohol in this respect. Like alcohol, it is an aliphatic solvent. Most dyes are more soluble in cellosolve than they are in alcohol. Alcohol, of course, has the power to remove picric acid itself from tissues, as is well known. It may be that dyes such as aurantia, picric acid and tartrazine reinforce the action of alcohol or cellosolve in detaching certain basic and acid dyes from tissues. Possibly the action of the molecules of these dyes is to bombard the other dyes out of the tissue elements.

Differentiation by dyes might, then, be due to:

(*a*) *Physical phenomena*, e.g. molecular bombardment, as mentioned above, by which the dye to be differentiated is dislodged from the tissues and is then carried away by the solvent of the differentiating dye.

(*b*) *Chemical affinities*. The tissue elements may have a greater affinity for the differentiating dye than for the dye which is cast off by them in favour of the differentiating dye. This appears to be the situation in the case of Orange G in the Falgog method, sun yellow in the standard Faviol method, and also probably in the case of the tartrazine method (page 368). However, it is hardly likely to apply to unmordanted basic synthetic dyes differentiated by

aurantia or picric acid; that is, if the basic dyes are united chemically with acidic tissue elements. In such a case a dye like aurantia or picric acid could not unite chemically with acidic tissue elements previously stained by the basic dye, because these two dyes are themselves acid and they do not possess any chemical groups which would confer amphoteric properties on their molecules. Moreover, these two differentiating dyes can themselves be removed from the tissues by alcohol. It would appear, then, that the coloration of tissues by picric acid or aurantia is purely a physical process.

(*c*) *A combination of both physical and chemical factors.* In the case of differentiation of safranin by picric acid it can easily be shown *in vitro* that the latter combines with safranin to form a compound, safranin picrate. If a test tube of aqueous picric acid is taken and a few millilitres of 1% aqueous safranin added a precipitate of safranin picrate will form: this is insoluble in aqueous picric acid. If another test tube is taken, but this time of alcoholic picric acid, and the same amount of safranin added as was added to the aqueous picric acid, a precipitate of safranin picrate will again be formed: this is insoluble in alcoholic picric acid. It appears reasonable, therefore, to suppose that the differentiating action of picric acid, as far as safranin and other synthetic basic dyes are concerned, is to attract the basic dye away from the tissues, then combine with it to form an insoluble neutral compound which is unable to attach itself to tissues and is washed out in the histological processes that follow. It would appear that this is a case of competition between the acidic tissue elements and the acidic dye (picric acid or aurantia) for possession of the basic dye which is more strongly attracted to the acid dye than to the acidic tissue-elements.

DECOLORIZING DYES

As stated elsewhere in this book, certain strongly acid dyes are capable of decolorizing certain tissue-elements that have been stained with certain quinonoid (e.g. triarylmethane) acid dyes. Not only are certain strongly acid dyes capable of decolorizing the tissue-elements, but they have another action. That is, on the quinonoid dye, which may also be decolorized by the strongly acid dye.

Light green units chemically with acid fuchsin to produce a blue dye, trifalgic acid. This colour change is brought about through the incorporation of resonance system of the acid fuchsin with that of the light green. This gives the possibility of longer resonance sequence of single and double bonds with consequent deepening of the colour of both component dyes. When this blue dye (trifalgic acid), is applied to tissues, partial ionization takes place, and a proportion of the molecules of the trifalgic acid are split into monofalgic and difalgic acids. These three acids are then taken up by various types of tissue-elements to give a polychrome picture.

Acid fuchsin and light green belong to the same chromophoric group. One might say that here the chromophore of each of the two dyes is compatible with the other.

When certain strongly acid dyes such as sun yellow, or orange G, which do not belong to the triarylmethane group, are brought into contact with acid fuchsin, under suitable conditions and in the absence of tissues or other stainable material, they unite with the acid fuchsin and bring about its decolorization. The reason appears to be that the chromophore of the acid fuchsin is incompatible with that of the strongly acid dye whose chromophore is of a different type.

When the two dyes unite, the resonance sequence of double and single bonds to which acid fuchsin owes its colour, is disrupted. On the other hand, the decolorizing dye retains its colour because its chromophore remains undisturbed by the union with acid fuchsin. This type of decolorization is of the Schiff type. Schiff's reagent (leuco basic fuchsin) is, of course, prepared by the action of sulphurous acid on basic fuchsin. The sulphurous acid is generally produced by the action of hydrochloric acid on potassium metabisulphate (page 400), although in some laboratories SO_2 is used instead. This gas is bubbled into the fuchsin solution to form sulphurous acid which unites with the dye in such a way as to disrupt the resonance system of the latter, thereby bringing about its decolorization. Similarly sulphurous acid acts on acid fuchsin and other acid and basic amino-triarylmethane dyes as well as many other quinonoid dyes. We can see at a glance that the basic fuchsin (or acid fuchsin) has been decolorized by the sulphurous acid, because the solution is colourless, if it has been properly prepared. It is not so obvious in

the case of acid fuchsin decolorized by an acid dye, because the decolorizing agent, unlike that used for preparing Schiff's reagent, is itself coloured; and it imparts its colour to the final solution which contains leuco acid fuchsin united with the decolorizing dye. So long as this compound of leuco acid fuchsin and the other acid dye sun yellow, for instance, remains out of contact with tissues or other stainable material, it holds together, retaining the original colour of the decolorizing dye. If, however, the solution is spotted on filter paper, at first the spot is the same colour as the solution. After a few seconds a colourless fluid exudes from the coloured spot and encircles the spot, spreading outwards. The outer periphery, that is the edge of the zone farthest away from the coloured spot, suddenly changes from colourless to red; the same colour as that of acid fuchsin. This red coloration then advances inward towards the central spot (which is blue in the case of dianil blue or violet-blue in the case of violamine, or deep yellow in the case of sun yellow G) until the whole of the zone previously occupied by a colourless liquid is coloured red. This may be taken to indicate that the colourless liquid which came out of the central coloured zone was in fact leuco acid fuchsin. This has been confirmed by chemical tests. The behaviour of the compound on filter paper would indicate that the union between the decolorizing dye and the acid fuchsin while strong enough to hold in the absence of competition from tissues or other stainable material (e.g. cellulose) is not strong enough when the acid fuchsin is given an opportunity to escape from the decolorizing dye, as it does on filter paper. It appears that in breaking away from the sun yellow as leuco acid fuchsin, and once out of reach of the decolorizing dye, its colour is restored by atmospheric oxidation. The restoration of the red colour of the leuco fuchsin band on filter paper is hastened and intensified by the application of heat. A leuco acid fuchsin of this type might possibly find application in histochemistry or in biochemistry. Wherever the decolorizing dye can reach the acid fuchsin in tissues, the latter dye will be decolorized.

Dutton (1928) states that nigrosin in aqueous solution acts on eosin (of Wright's stain) as a very powerful decolorizer. The dye said to be decolorized in this case (eosin Y) is a carboxylated (acid) dye, devoid of sulphonic groups. It contains no basic groups whatsoever and it does not, therefore, possess any

amphoteric properties, as does acid fuchsin and certain other acid dyes. It can only combine with basic dyes or acid dyes which possess basic groups attached directly to the aromatic nuclei. The structure of eosin Y is such that union with a basic dye or an amphoteric dye would not result in the decolorization of the eosin. With a suitable basic dye it would form an eosinate, an alcoholic solution of which, applied to microscopic tissue preparations, would produce a polychrome picture. If the eosin were to combine with an acid aminotriarylmethane dye containing one or more basic groups, or certain other acid dyes of the quinonoid group, it might be expected to decolorize or partially decolorize such dyes by disrupting their resonance sequence of double and single bonds. Water soluble nigrosin is a highly sulphonated (acid) dye of the azine series, the exact structure of which is not known. Azine dyes also belong to the quinonoid group.

The dye-ion of nigrosin possesses a basic nitrogen atom as well as sulphonic acid groups. In view of statements made above, one might reasonably expect the eosin to decolorize the nigrosin either wholly or partially, or at least to modify its colour. In fact *in vitro* tests which I have recently carried out show that this actually happens. Eosin Y unites chemically with water-soluble nigrosin to produce a water soluble compound dye which is much deeper in colour than eosin itself but lighter in colour than nigrosin. The compound is in fact bluish violet. It might be asked how then does nigrosin "decolorize" the eosin in Dutton's (1928) technique? It should be remembered that the concentration of the solid methylene blue eosinate of Wright's stain solution is only 0·3%. This means that the actual eosin content is only about 1·5%. Only a fraction of the Wright's stain, and consequently the eosin component, applied to the specimen, is taken up by the tissues. The bulk of the stain is washed away. The nigrosin is applied in a concentration which is many times greater than that of the eosin of the Wright's stain solution, and still greater than the concentration of the eosin left in the cytoplasm. Therefore the number of molecules of nigrosin present would be infinitely greater than the number of molecules of eosin. If it takes one, two, three or even as many as twenty molecules of nigrosin to unite with each of the eosin molecules present in the tissues and these nigrosin molecules are reduced in colour as a result of the union, then there is still an

overwhelming number of free nigrosin molecules whose colour has not been affected. It would seem that in uniting with nigrosin the eosin becomes detached and carried away from the cytoplasm. But in this case, the eosin is not replaced by the other acid dye (nigrosin) for some reason, not understood, but probably due to physical phenomena. The number of molecules of nigrosin reduced in colour by union with the eosin would, as already stated, be infinitesimal compared with the large excess of undecolorized nigrosin molecules. Therefore no reduction in the colour of the nigrosin as a whole would be observable.

Rosenbaum (1949) states that while it is generally agreed that any staining process involves both physical and chemical factors, the physical phenomena which occur during negative staining with nigrosin are of particular interest. As Rosenbaum remarks in his paper which deals with the negative staining of protozoa with water soluble nigrosin, negative staining consists essentially of staining the background of the field of observation, leaving the actual specimen unstained. He also states that all workers have reported water-soluble nigrosin as a strong decolorizing agent, and that in his opinion the dye depends upon colloidal reactions in its staining effects. Along these lines, Burri (1909) mentions Indian ink as ideally suited for negative-staining. Indian ink, Rosenbaum states, produces an opaque background film of suitable thickness for the process. Nigrosin, on the other hand, has the ability to disperse more freely in a liquid phase, and hence the resulting suspension is more fluid than Indian ink. The same author (Rosenbaum, 1949) states that the results of earlier work carried out by him on the effects of certain dye suspensions on *Pelomyxa* suggest that homogeneous particles of water-soluble nigrosin, form, in their dispersed phase, close contact with each other on body surfaces and in solution so as to produce a high surface tension of great capillarity.

This also, I would suggest, is probably a contributory factor in decolorizing action of water-soluble nigrosin.

Notes on
THE pH OF DYES IN AQUEOUS SOLUTION

It should be mentioned here that a true acid dye, as used in histology, is a metallic salt (but sometimes an ammonium salt)

of an aromatic organic acid. A basic dye, on the other hand, is an inorganic or aliphatic organic salt of an aromatic organic base. In both cases it is the aromatic organic (the dye-ion) part of the dye molecule which imparts colour to the tissue elements that have been treated with a solution of the particular dye.

In solution a dye undergoes electrolytic dissociation into its component ions. In the case of an acid dye, the dye-ion is negatively charged and is acid in reaction. The dye-ion of a basic dye, on the other hand, is positively charged and it is basic in reaction. This does not mean that an aqueous solution of an acid dye is necessarily acid in reaction, nor that an aqueous solution of a basic dye is necessarily basic in reaction. When we speak of a dye as being acid or basic we are really referring to the dye-ion itself, not a solution of the dye which is a solution of a salt, as previously mentioned.

It has generally been accepted that aqueous solutions of acid dyes are either neutral or slightly alkaline. Also, that aqueous solutions of basic dyes are either neutral or slightly acid. While this is substantially true in many cases, particularly with regard to acid dyes, further work carried out more recently in the author's own laboratories appears to show that there are more exceptions than previously suspected as the following Tables show:

TABLE I

SOME WATER SOLUBLE DYES

arranged in ascending order of pH

In all cases the pH given is that of a 1% solution of the particular dye in distilled water previously adjusted to pH 7·0 with alkali; the pH measurements were made at 20° C.

Key to symbols used in the Table (*pages* 19–23)*:*

a/ = acid dye
am/ = amophoteric dye
b/ = basic dye.

Dye groups are indicated as follows:

Symbols *Dye groups*

A = Anthraquinone (e.g. a/A = acid dye of the anthraquinone group).
AC = Acridine (*Note:* acridine dyes also belong to the xanthene group).
AHP = Anthrahydroxyphthalein.
AN = Azine.
AZ = AZO (*Note:* the small letters m, d or t, after AZ, indicate mono- di- and tri- respectively: e.g. a/AZm = acid dye of the mono-azo group).
DPM = Diphenylmethane
DPNM = Diphenylnapthylmethane (triarylmethane).
F = Fuchsinic acid.
HP = Hydroxypyrazol
HTPM = Hydroxytriarylmethane (see also P).
I = Indigoid
ID = Indamine.
N = Nitro.
NC = Natural colouring matter.
NS = Nitroso.
O = Oxazine.
P = Phthalein.
PDNM = Phenyldinapthylmethane (triarylmethane).
SP = Sulphonphthalein (these may also be classed as HTPM).
ST = Stilbene.
SU = Succein
TL = Thiazole.
TN = Thiazine.
TPM = Triphenylmethane (triarylmethane).
X = Xanthene.

Note: Michrome numbers given against the names of the dyes in the following Tables are taken from *Encyclopaedia of Microscopic Stains* (E. Gurr, 1960). Dyes marked with an asterisk (*) have molecules consisting of two univalent dye-ions in association with one divalent cation, or in the case of Nile Blue, with one univalent anion. Napththol green, however, has three univalent dye-ions.

Dye	*pH*	*Molecular weight*	*Group*	*Michrome No.*
Picric acid	1·35	229	a/N	707
Acriflavin	1·4	260	b/AC	430
Alizarin red S	2·15	342	a/A	23
Brilliant cresyl blue ..	2·2	318	b/O	404
Thionin	2·25	264	b/TN	215
Toluidine blue	2·25	306	b/TN	641
Solochrome cyanin R ..	2·3	536	a/HTPM	750
Azur C	2·35	278	b/TN	353
Malachite green	2·4	365	b/TPM	315
Methylene blue chloride ..	2·5	320	b/TN	416
Victoria blue B	2·6	506	b/DPNM	148
Celestin blue	2·6	364	b/O	66
Bismarck brown Y	2·65	384	b/AZd	334
Gallamine blue bisulphite	2·8	403	am/O	288
Pyronin B	2·8	359	b/X	44
Azo violet 4BS	3·0	483	a/AZm	1
Pyronin Y (Pyronin G) ..	3·0	303	b/X	339
Setoglaucine	3·1	399	b/TPM	241
Light green SF yellowish	3·1	793	a/TPM	240
Wool green S	3·2	577	a/DPNM	162
Rhodamine S	3·325	375	b/SU	38
Bismarck brown R ..	3·375	461	b/AZd	62
Guinea green B	3·4	663	a/TPM	141
Acid green L	3·4	691	a/TPM	647
Patent phosphine	3·5	315	b/AC	680
Turquoise blue	3·65	424	b/TPM	281
Chrysoidin Y	3·65	249	b/AZm	96
Luxol fast blue	3·7	1519§	a/PCY	331
Rhodamine 6G	3·9	451	b/X	68
Darrow red	4·0	—	b/O	—
Janus black	4·4	454	b/AZm	159
Azo acid blue	4·45	409	a/AZm	41
Meldola blue	4·8	339	b/O	222
Fuchsin acid	5·0	586	a/TPM	5
Neutral red chloride ..	5·1	289	b/AN	226
Nile blue sulphate.. ..	5·15	733†	b/O	212
Brilliant delphine blue ..	5·2	444	a/O	58
Alcian blue..	5·3	1341§	am/PCY	24
Acridine yellow	5·4	274	b/AC	8
Victoria blue 4R	5·425	520	b/TPM	307

Dye	*pH*	*Molecular weight*	*Group*	*Michrome No.*
Thionin blue	5·5	334	b/TN	150
Janus yellow	5·5	457	b/AZd	408
Methyl green	5·6	458	b/TPM	177
Tropaeolin O	5·65	316	a/AZm	193
Naphthochrome green G	5·7	506	a/PDNM	938
Disulphine green B ..	5·9	725	a/TPM	265
Coerulein (bisulphite) ..	5·95	554	am/AHP	80
Haematein	6·05	300	a/NC	360
Safranin O	6·1	351	b/AN	405
Gentian violet	6·15	380	b/TPM	417
Eriocyanin A	6·2	762	a/TPM	358
Janus green	6·25	483	b/AZm	183
Eriochrome verdone ..	6·25	484	a/AZd	292
Janus blue	6·25	506	b/AZm	160
Methyl violet 6B	6·3	484	b/TPM	180
Pararosaniline chloride ..	6·35	329	b/TPM	722
Aniline blue	6·4	738	a/TPM	167
Brilliant glacier blue ..	6·4	434	b/TPM	61
Thioflavine T	6·5	319	b/TL	164
Azocarmine G	6·5	580	a/AN	43
Patent blue A	6·5	1408*	a/TPM	201
Acridine orange N ..	6·5	302	b/AC	87
Toluylene blue	6·55	291	b/ID	367
Trypan red	6·55	1003	a/AZd	217
Crystal violet	6·6	408	b/TPM	103
Neutral red iodide ..	6·6	380	b/AN	399
Janus red	6·6	460	b/AZd	218
Phloroglucinol	6·65	162	¶	238
Methylene green	6·65	365	b/TN	184
Haematoxylin	6·7	302	a/NC	304
Fuchsin basic	6·7	—	b/TN	412
Eosin, yellowish	6·7	692	a/X	93
Erio green	6·7	561	a/DPNM	579
Indigo carmine	6·7	466	a/I	149
Eriochrome black T ..	6·75	461	a/AZm	261
Azocarmine B	6·75	682	a/AN	319
Fast green FCF	6·8	809	a/TPM	135
Carminic acid	6·85	492	a/A	214
Naphthol green B ..	6·85	878‡	a/NS	192
Azur B	7·0	306	b/TN	357

Dye	*pH*	*Molecular weight*	*Group*	*Michrome No.*
Orcein	7·0	500	am/NC	375
Auramine O	7·0	304	b/DPM	42
Cresyl fast violet CNS ..	7·0	340	b/O	520
Methylene blue thiocyanate	7·1	328	b/TN	268
Alizarin cyanin green ..	7·15	623	a/A	15
Patent blue V	7·2	1159*	a/TPM	712
Brilliant yellow S	7·2	465	a/AZm	636
Martius yellow	7·2	251	a/N	170
Ponceau 3R	7·2	494	a/AZm	210
Tropaeolin OOO No. 2 ..	7·25	350	a/AZm	181
Sun yellow G	7·3	837	a/ST	720
Durazol yellow	7·3	709	a/TL	109
Erythrosin	7·3	880	a/X	124
Anthraquinone violet ..	7·35	663	a/AZd	284
Dianil blue 2R	7·35	845	a/AZd	302
Chlorantine fast red ..	7·35	992	a/AZd	237
Chrysamine G	7·45	526	a/AZd	390
Chlorantine fast green BLL	7·5	1324	a/AZt	541
Phloxine B	7·5	830	a/X	206
Setopaline (Setocyanin) ..	7·6	427	b/TPM	76
Isamine blue	7·6	768	a/TPM	152
Fast yellow G	7·675	401	a/AZm	17
Tropaeolin OO	7·7	375	a/AZm	199
Azur A	7·7	292	b/AN	718
Chromosome red (M. A. MacConaill and E. Gurr)	7·7	6017	a/F	1091
Vital red	7·7	827	a/AZd	3
Congo violet	7·725	699	a/AZd	274
Vital new red	7·75	987	a/AZd	83
Carmoisine L	7·75	502	a/AZm	102
Chromotrope 2R	7·8	468	a/AZm	301
Fast light yellow	7·8	380	a/HP	646
Lacmoid	7·85	235	a/O	462
Carmoisine A	7·86	502	a/AZm	67
Tropaeolin OOO No. 1 ..	7·95	350	a/AZm	154
Azoeosin	7·975	380	a/AZm	28
Ponceau S	8·0	761	a/AZd	725
Lissamine fast red B ..	8·0	468	a/AZm	230
Ponceau de xylidine ..	8·0	556	a/AZd	565
Azofuchsin 3B	8·0	509	a/AZm	47

Dye	*pH*	*Molecular weight*	*Group*	*Michrome No.*
Biebrich scarlet	8·1	556	a/AZd	53
Naphthol yellow S ..	8·15	358	a/N	191
Wool violet 4BN	8·175	762	a/TPM	701
Cloth red 3B	8·2	510	a/AZd	543
Acid alizarin black ..	8·2	411	a/AZm	299
Amethyst violet	8·4	435	b/AN	317
Chlorazol sky blue FF ..	8·4	993	a/AZd	91
Violamine 3B	8·5	764	a/X	161
Chlorazol fast pink BK ..	8·5	991	a/AZd	674
Benzopurpurin 4B ..	8·5	725	a/AZd	82
Rosolic acid sodium salt ..	8·5	392	a/HTPM	157
Mercurochrome	8·5	751	a/X	530
Naphthol black B	8·5	861	a/AZm	190
Brilliant yellow I	8·65	510	a/AZm	30
Metanil yellow	8·65	375	a/AZm	178
Geranine G	8·7	498	a/AZm	75
Orange G	8·75	452	a/AZm	411
Naphthol yellow S ..	8·75	358	a/N	191
Erio fast fuchsin	8·75	663	a/X	699
Pontamine sky blue 5BX	8·8	993	a/AZd	106
Bordeaux B	8·8	502	a/AZm	54
Owen's blue	8·85	980	a/AZd	19
Chlorazol violet N ..	8·85	729	a/AZd	456
Naphthol blue black ..	8·875	617	a/AZd	60
Azo blue	8·9	727	a/AZd	300
Chlorazol paper brown ..	8·95	704	a/AZd	94
Nigrosin	9·0	—	a/AN	316
Chlorazol black E ..	9·05	782	a/AZt	92
Lignin pink	9·1	509	a/AZm	35
Trypan blue	9·1	960	a/AZd	186
Crystal ponceau 6R ..	9·1	502	a/AZm	664
Victoria green G	9·15	849	a/AZt	630
Congo rubin	9·2	698	a/AZd	95
Chrome azurol red ..	9·25	669	a/HTPM	74
Methylene violet (Bernthsen)	9·25	292	b/TN	339
Crocein scarlet	9·3	556	a/AZd	690
Congo red	9·35	697	a/AZd	400
Thioflavine S	9·35	490	a/TL	133
Chlorazol azurin	9·4	759	a/AZd	729
Acid alizarin blue SWR ..	9·45	304	a/A	98

Dye	*pH*	*Molecular weight*	*Group*	*Michrome No.*
Erie garnet	9·5	698	a/AZd	90
Metachrome brown ..	9·5	352	a/AZm	337
Chlorazol pink YK ..	9·5	600	a/AZm	255
Fluorescein (Uranin) ..	9·5	376	a/X	136
Titan yellow	9·5	696	a/TL	163
Indulin	9·625	566	a/AN	151
Alizarin brilliant blue BS..	9·65	372	a/A	262
Alizarin cyanin 2R ..	9·7	390	a/A	22
Benzoazurine G	9·725	759	a/AZd	50
Dianil brown	9·8	683	a/AZt	71
Benzopurpurin 6B ..	9·9	725	a/AZd	48
Manchester blue	10·0	845	a/AZm	336
Benzopurpurin 10B ..	10·1	757	a/AZd	81
Chlorazol green	10·8	879	a/AZt	675
Amaranth	10·8	604	a/AZm	37
Alizarin brilliant blue B ..	11·7	474	a/A	263

* The molecule consists of two dye-ions in association with a univalent cation.

† The molecule consists of two dye-ions in association with one univalent anion.

‡ The molecule has three dye-ions.

§ Approx.

¶ This is not a dye but a colourless phenol used for producing colour in certain plant tissues, as a test for lignin.

An analysis of the above tables might bring some interesting points to light. Thus, in the following Tables (pages 25–26) which have been extracted from Table I, it will be seen that:

(1) There is very little difference in the pH ranges between acid and basic azine dyes commonly used in histology.

(2) Of the acid oxazine dyes in common use, two are slightly acid d; one is neutral, and the others show pH values which are very acid; two of the basic dyes are very acid in solution, as is the amphoteric member of this group.

(3) Four of the basic thiazine dyes are very acid in solution, while one is quite alkaline. The latter dye (methylene violet, Bernthsen) is an oxidation product of methylene blue. Gurr (1960) quotes French (1926) as stating that in using Holmes and

French (1926) azur C tissue stain, some samples of azur C did not work satisfactorily when the solutions were first made, but they improved with age. This fault was overcome by adding a very small proportion of methylene violet (Bernthsen) to the azur C, which is a lower oxidation product of methylene blue.

The azurs, A, B and C, normally contain a small proportion of methylene violet. It would appear from French's statement that solutions of azur C on standing undergo further oxidation with the production of an increased amount of methylene violet. Possibly the pH of the latter dye, which is considerably higher than that of any of the other thiazine dyes or of any other basic dye of any group, may have some bearing on its staining effects on the nucleus of the malarial parasite, etc.

(4) There is very little difference in the pH ranges between the acid and basic triphenylmethane dyes commonly used in histology.

(5) It has already been suggested (page 7) that basic dyes of around the same molecular weight as pyronin Y and methyl green might be used in place of these two for differentiating between RNA and DNA. As will be seen from the tables here, toluidine blue, used by Korson (1951) in place of pyronin Y for staining RNA, not only has a molecular weight very near to that of pyronin Y, but apparently its pH is very close to that of pyronin Y also. Pyronin B has a pH even closer to pyronin Y but there is an appreciable difference between the molecular weights of these two dyes. The former dye is unsatisfactory as a substitute for pyronin Y as a stain for RNA.

Methyl green has a molecular weight of 458 and a pH of 5·6. In selecting dyes on their molecular weights, or more precisely on the mass of their individual dye-ions for research, as suggested by E. Gurr (1960), it might be as well, in certain cases, to take the pH into consideration also.

(6) Tables II to VI given below may possibly serve to show whether further study and division of Table I, into mon-, di-, tris-azo and other dyes listed therein are likely to be of interest to individual readers. It is not, however, considered necessary to make further extensions here, as this is something which can be done quite readily by the reader himself should he be interested.

TABLE II

AZINE DYES

Basic	*pH*	*Molecular weight*	*Acid*	*pH*	*Molecular weight*
Neutral red chloride	5·1	289	Azocarmine G ..	6·5	580
Safranin O ..	6·1	351	Azocarmine B ..	6·75	682
Neutral red iodide	6·6	380	Indulin	9·625	566
Amethyst violet ..	8·4	435			

TABLE III

OXAZINE DYES

Basic	*pH*	*Molecular weight*	*Acid*	*pH*	*Molecular weight*
Brilliant cresyl blue	2·2	318	Brilliant delphine blue	5·2	444
Celestin blue ..	2·6	364	Lacmoid ..	7·85	235
Meldola blue ..	4·8	339			
Nile blue sulphate	5·15	733*			
Cresyl fast violet	7·0	340			

Amphoteric

Gallamine blue bisulphite	2·8	403

TABLE IV

THIAZINE DYES

Basic	*pH*	*Molecular weight*
Thionin	2·25	264
Toluidine blue	2·25	306
Azur C	2·35	278
Methylene blue chloride	2·5	320
Thionin blue	5·5	334
Methylene green	6·65	365
Azur B	7·0	306
Methylene blue thiocyanate	7·1	328
Azur A	7·7	292
Methylene violet (Bernthsen)	9·25	292

TABLE V

TRIPHENYLMETHANE DYES

Basic	*pH*	*Molecular weight*	*Acid*	*pH*	*Molecular weight*
Malachite green ..	2·4	365	Light green SF yellowish ..	3·1	793
Setoglaucine ..	3·1	399	Guinea green B	3·4	663
Turquoise blue ..	3·65	424	Acid green L ..	3·4	691
Victoria blue 4R ..	5·425	520	Fuchsin acid ..	5·0	586
Methyl green ..	5·6	458	Disulphine green	5·9	725
Gentian violet ..	6·15	380	Eriocyanin A ..	6·2	762
Methyl violet 6B	6·3	484	Aniline blue ..	6·4	738
Pararosaniline chloride ..	6·37	329	Patent blue A ..	6·5	1408*
Brilliant glacier blue	6·4	434	Fast green FCF	6·8	809
Crystal violet ..	6·6	408	Patent blue V ..	7·2	1159*
Fuchsin basic ..	6·7	—	Isamine blue ..	7·6	768
Setopaline ..	7·6	427	Wool violet 4BN	8·175	762

TABLE VI

XANTHENE DYES

Basic	*pH*	*Molecular weight*	*Acid*	*pH*	*Molecular weight*
Pyronin B.. ..	2·8	359	Eosin, yellowish	6·7	692
Pyronin Y ..	3·0	303	Phloxin B ..	7·5	828
Rhodamine 6G ..	3·9	451	Violamine 3B ..	8·5	764
			Mercurochrome	8·5	751
			Erio fast fuchsin	8·75	663
			Uranin	9·5	376

In selecting dyes, based on their molecular weights, in accordance with the earlier hypothesis (E. Gurr, 1960, pages 423–432, and E. Gurr and M. A. MacConaill, 1959) regarding the influence of the molecular weights of dyes on the kinds of tissue-elements stained by them, consideration should be given not to weight of the dye molecule as a whole but to the mass of the single dye-ion. Dyes marked *†‡ (*see* page 23) dissociate, in solution into two or three separate univalent dye-ions, and it is the mass of these single dye-ions, not the molecular weight of the dye-molecule as a whole which has to be taken into consideration.

The fuchsinic acid dyes are exceptional in that their molecules contain multiple dye-ions of different kinds. In the solid state these unions of different dye-ions are stable, but in solution a degree of ionization takes place with competition between the different kinds of dye-ions for different kinds of tissue-elements.

In the case of the more complex fuchsinic acids in solution, even in the absence of tissue elements or other stainable material, competition exists between the different component dye-ions of the same molecule. An example of this is to be found in trifavioxanthic acid where the solution changes colour due to conflict between the different component dye-ions signifying a change in the structure of the molecule.

Work on pH measurements of all dyes used in biology is now proceeding in the author's laboratories and the results will be published when the task has been completed. Meanwhile, it is thought that the above Tables, although incomplete, may be of interest to medical and biological research workers.

Some observations
on
THE EFFECTS OF HEAT ON THE pH OF AQUEOUS DYE SOLUTIONS

Following the syntheses of the Falgic acids *in vitro* (Gurr and MacConaill, 1959, 1960; MacConaill and Gurr, 1960*a*), the present author decided to make further investigations into the mechanism of the chemical reactions involved. In this later work it became necessary to make accurate pH measurements, over a wide range of temperatures, of aqueous solutions of certain acid dyes. Basic dyes were not investigated at the time, but work on these is being carried out in the author's laboratories at the present time and the results will be published elsewhere when the investigations have been completed.

The acid dyes investigated registered pH values over a range of approximately 2·5–4·0 for 1% aqueous solutions in distilled water at 20° C. In these experiments it was observed that as the temperature of the dye solution was gradually increased up to about 60° C so the pH shifted over to the more acid side As the temperature was gradually increased still further, the pH became less acid, until at 100° C the dye solutions registered the same pH values as those observed at 20° C. The solutions were then allowed to cool gradually to room temperature (20° C), and it was noted that during the cooling process the same pH changes took place but in reverse, so that at 20° C the pH readings were identical with those originally observed before the solutions had been heated.

It should be mentioned here that the necessary adjustments were made to the pH-meter to allow for readings over a temperature range of 0°–100° C.

Some observations
on
pH CHANGES INDUCED BY HEATING DISTILLED WATER AND LONDON TAP WATER

London tap water (pH 8·2) was found to behave in the same way as the solutions of acid dyes mentioned above. On heating gradually to 60° C, the pH fell gradually to 6·8. At this point, as the heating was continued the pH rose steadily until at 100° C the pH registered was the same as that at 20° C. On cooling the same changes took place only in reverse.

The same experiments were carried out with distilled water. Here it should be mentioned that the pH of distilled water, even freshly distilled, is often between 5·0 and 6·0, and not 7·0, as appears to have been generally believed in many biological laboratories. Therefore, when neutral distilled water is essential in a particular staining technique it is advisable, in cases of doubt, to buffer the water to pH 7·0.

In the experiments referred to above, a large number of samples of freshly distilled water were tested. It was found that whatever the pH of the sample at room temperature (20° C), when the water was boiled and then allowed to cool again the pH reverted to that originally registered at room temperature. It was found that as the samples were heated over the range 20°–100° C, the pH of the samples gradually rose by about 2·0. That is to say, if the pH at 20° C was 5·4, then at 100° C it was found to be pH 7·4. The experiments were checked afterwards by the use of indicators. The colour changes of indicators of the sulphonphthalein group, at least, are due to dissociation of the hydroxyl group. In some cases, bromocresol purple for instance, it was found that an irreversible change in the molecular structure of the indicator appeared to take place between 90° C and 100° C. On cooling samples of the boiled water containing this indicator, no further colour change took place, even after addition of acids and alkalis.

CLASSIFICATION OF DYES USED IN MICROSCOPY

With few exceptions, notably haematoxylin and carmine, the stains used for colouring microscopic tissue preparations are synthetic dyes. These dyes constitute a broad class of aromatic organic chemicals which can be divided and sub-divided into various groups according to their chemical and physical properties. Brief descriptions of some of these divisions are presented below:

ACID DYES

Strictly speaking, dyes of this class are those in which the balance of the charge on the dye-ion is negative. Acid dyes, with some exceptions, stain tissue elements that are basic in reaction.

BASIC DYES

A dye of this class is one in which the charge on the dye-ion is positive, but not all so-called basic dyes are ionic. Basic dyes stain tissue-elements that are acidic in reaction, but here also there are exceptions.

AMPHOTERIC DYES

Under certain conditions a number of dyes are amphoteric. This occurs among both acid and basic dyes. Many acid dyes possess both reactive acidic groups and reactive basic groups, in addition to their cations. These groups together confer amphoteric properties on the particular dye. Such dyes are normally regarded as being acid dyes. With one exception, there are no basic dyes whose molecules possess both reactive basic and reactive acidic groups, in addition to their anions, the exception being nono-fuchsinic acid, recently discovered (*see* page 85). This somewhat bizarre compound might be regarded as a strongly acid basic dye!

NEUTRAL DYES

Most of these are not really dyes at all, but are non-ionic organic colouring matters. Some of them contain acidic or basic groups, however, and for this reason such neutral " dyes " are sometimes classified as " acid " or " basic ". Among the neutral " dyes " are the Sudan colours (1, 2, 3, 4, etc.). This type of

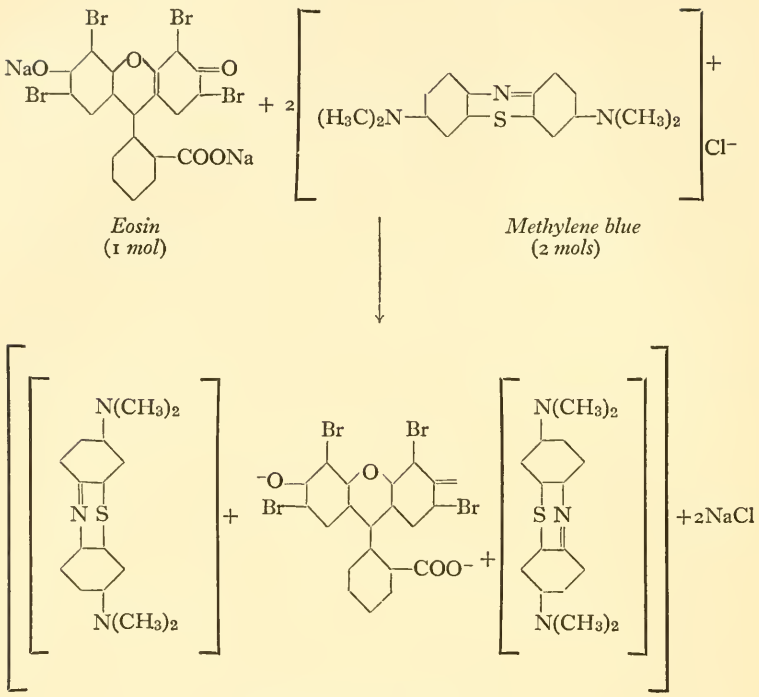
" dye " is probably absorbed or dissolved by lipids to which colour is imparted. A few non-ionic " dyes " such as Sudan black, which has two basic groups (imino), might conceivably unite chemically with acidic elements of certain tissues. All " neutral dyes " can be converted into true dyes by the process of sulphonation. They then become water-soluble dyes. In the strict sense a dye is a coloured aromatic, organic substance capable of electrolytic dissociation which enables it to unite by chemical and/or physical means with tissue-elements or other substances of opposite charge.

Neutral " dyes " should not be confused with " neutral " or compound stains. The latter are formed by chemical union between a basic and an acid dye. Such compounds of basic and acid dyes have long been known to histologists. The histologists were the first to invent them. It is doubtful, however, if the average textile dyestuff manufacturer is aware of such preparations. It is still more doubtful if they would be of any interest to the textile dyer.

COMPOUND DYES

As stated above these are neutral dyes of the kind that appear to be known only to the histologist. They are produced by neutralizing suitable acid dyes with suitable basic dyes. That is to say, the negatively charged dye-ion of the acid dye unites with the positively charged dye-ion of the basic dye when aqueous solutions of the two dyes are brought together, to form a precipitate which is a two-colour dye. If, however, one of the dyes is a polychrome stain, then the resultant compound stain will also possess the same polychrome properties plus the colour of the other dye. Such a compound dye is a true chemical compound and not a mixture. Among such dyes are Jenner stain (Jenner, 1899), Leishman stain (Leishman, 1901), methyl green-orange G (Kardos, 1911), neutral red-light green (Twort, 1924), and neutral red-fast green FCF (Ollet, 1947, 1951).

As will be seen from the following example which shows the reaction involved in the union of an acid dye (eosin) with a basic dye (methylene blue), an exchange of ions takes place. The sodium from the eosin unites with chloride from the methylene blue, and the organic part of one dye which is positively charged unites



Eosin-methylene blue (Jenner)
(1 *mol*)

with the organic portion of the other dye, which is negatively charged:

The precipitated Jenner stain is insoluble in water. It is dried and dissolved in alcohol. Out of contact with tissues or other stainable material the dye-ions remain united as one molecule, but as soon as the solution is applied to tissues dissociation takes place, but not completely, and we have a proportion of undissociated compound-dye molecules which impart shades of purple and violet to certain tissue elements. We also have a proportion of the component dye-ions which stain different elements present in the tissues. Accordingly, the positively charged methylene blue ion is attracted to the acidic tissue elements. The negatively charged eosin ions are attracted to the basic tissue elements. It would appear that this is a case of competition between an acid dye-ion and acidic tissue elements

for a basic dye-ion. Also competition between a basic dye-ion and basic tissue elements for acid dye-ions.

Another series of compound dyes known to histologists consists of mixtures of acid dyes of the Masson type. But these are not chemical compounds. Then there are mixtures of basic dyes such as methyl green-Pyronin (Pappenheim, 1901; Brachet, 1942, 1953; Kurnick, 1952, 1955): these also are mixtures, not chemical compounds.

Although it has long been known to histologists that a pair of suitable dyes, one acid and the other basic, will unite to form a chemical compound dye, it does not appear to have been known until quite recently that certain acid dyes will unite chemically with other acid dyes to produce water-soluble compound dyes which produce polychromatic effects when applied to tissues. Some of these dyes have been isolated in the dry form. However, compound dyes of this type are dealt with elsewhere in this book (pages 76–90) and readers requiring more detailed information should consult the original papers cited above.

Although it is possible to form chemical compound dyes by the union of many basic dyes with many acid dyes, as well as by the interaction of certain acid dyes with certain other acid dyes, it is not possible to produce chemical compound dyes by the interaction of any basic dye with any other basic dye. The reason for this is that there are no basic dyes, as there are acid dyes, whose molecules contain both reactive basic and acidic groups on the dye-ion itself.

CLASSIFICATION OF DYES BY MOLECULAR WEIGHT

It has been suggested (Gurr, 1960) that, with certain exceptions, the molecular weights of acid dyes influence their staining effects on tissues. In the work referred to above, tables are given in which some 430 dyes used in microtechnique are arranged in order of molecular weight, irrespective of the dyestuffs groups to which they belong. With the aid of these tables it was observed that those dyes that had been recommended by various authors over the years as satisfactory stains for collagen were acid dyes having molecular weights over 700. Such dyes, it appeared, had been found to be either poor or useless as cytoplasmic stains. It was also noted that the best cytoplasmic stains were acid dyes with molecular weights between about 350 and 590. During the

course of work carried out between the years 1947 and 1957 I had occasion to formulate and prepare a large number of trichrome mixtures of acid dyes for demonstrating keratins, etc., and had observed that under suitable conditions, acid dyes having molecular weights between 350 and 590 appeared to give satisfactory results with keratins. The lower molecular weights between these two limits gave excellent results. It was stated at the time that these were only general observations, and exceptions would no doubt be found. Exceptions, in fact, have since been found (MacConaill & Gurr, 1959; Gurr & MacConaill, 1959). For instance, patent blue A (molecular weight 1405) did not behave as was expected. The reason for this might be put down to the fact that the molecule of this dye is made up of two univalent dye-ions attached to one divalent cation. In solution ionization takes place with the release of these two dye-ions which are capable of association separately with two univalent cations to form two dye-molecules of approximately half the molecular weight of the original dye. A few other exceptions were also found. Although it would appear that other factors besides molecular weight—e.g. molecular volume, molecular dimensions, side-chains, diffusion phenomena—influence the penetrative powers of dyes, a study of literature over the past eighty years or so does appear to show that most acid dyes, when in competition with each other generally behave in accordance with the original hypothesis.

In many cases the molecular weight of an acid dye does give a reasonably clear indication whether the dye under consideration is likely to be a suitable alternative for another acid dye of around the same molecular weight. A few dyes may not behave in accordance with the original hypothesis, but this method of selection has been found very considerably quicker, and more economical in materials than the process of trying out a large number of acid dyes irrespective of their molecular weights. It appears that the molecular weight in the case of many acid dyes has far more influence on the type of tissue elements stained than has the chromophoric configurations of the dyes. In fact the side chains (e.g. sulphonic, carboxylic, hydroxyl, amino, imino groups, etc.) appear to have more influence on the staining effects than do the chromophores of most acid dyes.

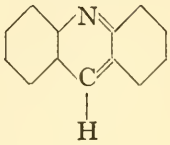
All dyes possess a configuration known as the chromogen.

The chromogen is responsible for colour. Associated with the chromogen is an arrangement of atoms and/or groups known as the chromophore. Dyes are classified into groups according to their chromophores. Some of these groups are described very briefly in the following pages where the structural formulae of representative dyes are set out with their molecular weights and solubilities in water and in absolute alcohol. Only about 80 examples are given here, although about 500 organic dyes are used in microtechnique, as described in more detail in my earlier work (Gurr, 1960), to which readers are referred for further information. However, the structural formulae, etc., of a number of dyes, most of them new ones (e.g. trifalgic acid), and pH values, given in the following pages were not available in time for inclusion in the above-mentioned work.

For reasons stated in the book mentioned above and an earlier one (Gurr, 1958*a*), colour index numbers were not used in either of these works. Instead a system of numbering used in the author's laboratories for many years was adopted. These numbers will not change over the years, and the author has, therefore, continued with their use in the following pages.

ACRIDINE GROUP

Chromophore:



This is a subdivision of the xanthene group. Only about ten acridine dyes are used in microscopy. They are all basic dyes of low molecular weight, around 300. All of them are fluorochromes. Some of them (e.g. acriflavine) are used as antiseptics. Atebrin, which is an acridine dye, is also an antimalarial agent.

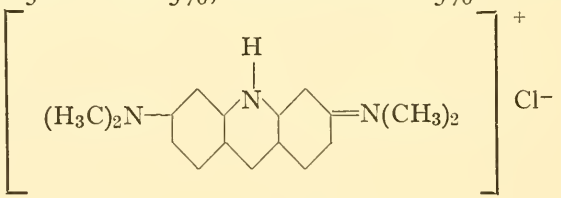
Acridine orange is used for differentiating nucleic acids. It is also used in a simple and rapid method (*see* pages 93–101) for the cytodiagnosis of cancer.

All the acridine dyes used in microscopy are yellow or orange in ordinary light. In fluorescence microscopy, however, they give polychrome pictures.

The following are examples of the acridine dyes:

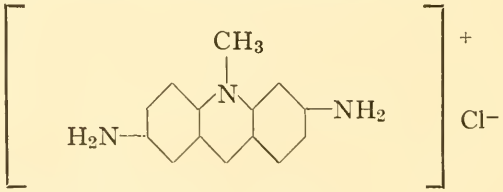
ACRIDINE ORANGE N

Michrome No. 87. *Molecular weight* 302. *A basic dye, pH* 6·5*. *Solubility at* 15° *C: water* 5%, *absolute alcohol* 0·5%.



ACRIFLAVINE

Michrome No. 430. *Molecular weight* 260. *A basic dye, pH* 1·4*. *Solubility at* 15° *C: water* 15%, *absolute alcohol* 1%.

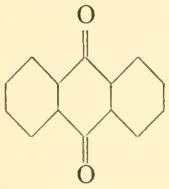


* 1% in neutral distilled water see pp. 17 and 29.

ANTHRAQUINONE GROUP

About twenty-five dyes of this group are used as microscopical stains. Some of these (e.g. alizarin red) are used as hydrogen ion indicators also. All anthraquinone dyes may be regarded as derivatives of anthraquinone, whose structure represents their chromogen:

ANTHRAQUINONE



In two or three cases one of the oxygen atoms is replaced by a nitrogen atom which links the chromogen to other aromatic organic groups.

To biologists perhaps the best known anthraquinone dye is alizarin and its water-soluble derivative, alizarin red S (alizarin sulphonate sodium). Alizarin was originally obtained from natural sources; i.e. from madder root.

Sudan violet, which is really a neutral " dye ", of the anthraquinone group, has two basic groups (amino). This dye is used for staining lipids. It is completely insoluble in water, although soluble to the extent of about 1% in 30% ethyl alcohol. This dye has the lowest molecular weight (210) of all organic dyes.

Of the anthraquinone dyes used in biology, anthraquinone violet has the highest molecular weight (633).

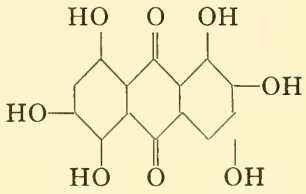
Among the anthraquinone colours is carminic acid. It is the active principle of carmine. Carminic acid is extracted from the dried bodies of the female species, *Coccus cacti*, a tropical insect which lives on certain species of cactus plants. At one time it was in frequent use as an acid-base indicator, but it is now little used for that purpose. Carmine, which is not a definite chemical compound, is a crimson pigment of great historical interest. It was used in micro-technique as far back as the eighteenth century;

some seventy years or so before the discovery of the first synthetic organic dye (mauvein or mauve) by a young British chemist, Perkin, in 1856.

Some examples of anthraquinone dyes are given below:

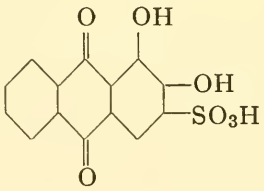
ACID ALIZARIN BLUE SWR

Michrome No. 98. *Molecular weight* 304. *An acid dye, pH* 9·45*. *Solubility at* 15° *C: water* 1%, *absolute alcohol* 0·75%.



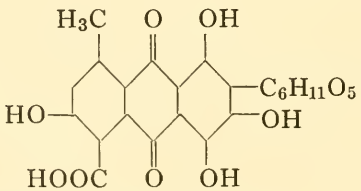
ALIZARIN RED S

Michrome No. 23. *Molecular weight* 342. *An acid dye, pH* 2·15*. *Solubility at* 15° *C: water* 6·5%, *absolute alcohol* 01·1%.



CARMINIC ACID

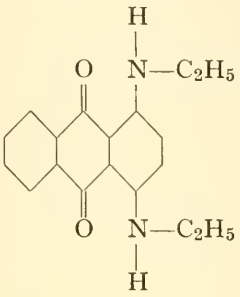
Michrome No. 214. *Molecular weight* 492. *pH* 6·85*. *A natural colouring matter. Solubility at* 15° *C: water in all proportions, alcohol* 0·2%.



* 1% in neutral distilled water; see pages 17 and 29.

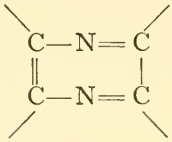
SUDAN BLUE

Michrome No. 158. *Molecular weight* 294. *A neutral colouring matter. Solubility at* 15° *C: water nil, absolute alcohol* 1·4%.



AZIN GROUP

Chromophore:

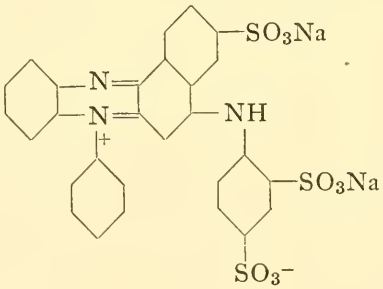


These dyes also contain a quinonoid ring structure to complete the chromophore. Because of this configuration, the azins belong to the broader class of quinonoid dyes. The latter group also includes the indamines, the indophenols, the indulins, the oxazins, the thiazins, the triarylmethanes, etc.

Only about twelve azin dyes are used as microscopical stains. Of these, two are acid dyes; the rest are basic. Some examples of azin dyes are given below:

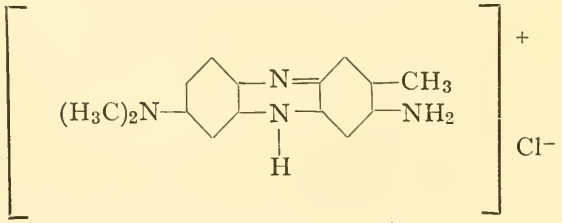
AZOCARMINE B

Michrome No. 319. *Molecular weight* 682. *An acid dye, pH* 6·75*. *Solubility at* 15° *C: water* 2%, *absolute alcohol* 0·05%.



NEUTRAL RED

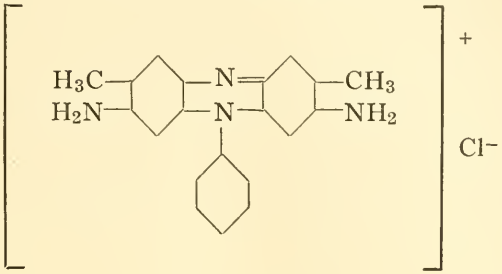
Michrome No. 226. *Molecular weight* 289. *A basic dye, pH* 5·1*. *Solubility at* 15° *C: water* 4%, *absolute alcohol* 1·8%.



* 1% in neutral distilled water; see pages 17 and 29.

SAFRANIN O

Michrome No. 405. *Molecular weight* 351. *A basic dye, pH* 6·1*. *Solubility at* 15° *C: water* 4·5%, *absolute alcohol* 3·5%.



AZO GROUP

Chromophore: —N=N—

The azo dyes form by far the largest group of dyestuffs. There are at the present time over one thousand manufactured. About one hundred of these are used in biology. The vast majority of them are acid dyes. There are a number of different classes of azo dyes. Space does not permit more than a very brief outline of two or three broad divisions of this vast group.

The azo dyes can be subdivided into mono-, dis-, tris-azo, etc., according to whether they contain one, two, or three or more azo groups to the molecule.

About eighty mono-azo dyes are used as microscopical stains. All but a few of them are acid dyes. Most of the acid mono-azo dyes in biology are used as cytoplasmic stains. The molecular weights of these range from about 350 to about 696. The basic ones fall into a lower molecular weight range. Some of the acid dyes, with molecular weights around 690 will stain collagen as well as cytoplasm. Those between about 350 and 500 will stain keratin selectively, under suitable conditions.

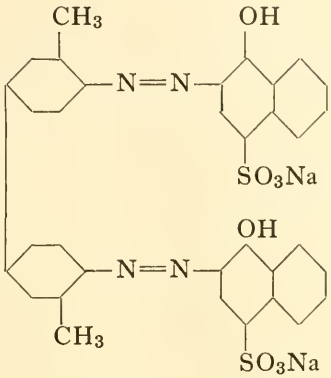
There are over seventy dis-azo dyes used as microscopical stains. All of these are acid except two.

Only eight tris-azo dyes have been used in biology up to the present time. These are all acid dyes. One of them, chlorantine fast green BLL, possesses a very high molecular weight (1324). This dye stains collagen as well as cytoplasm.

Some examples of azo dyes are given:

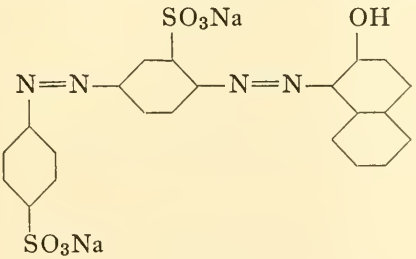
AZO BLUE

Michrome No. 300. *Molecular weight* 727. *An acid dis-azo dye, pH* 8·9*. *Solubility at* 15° *C: water* 2·5%, *absolute alcohol* 0·02%.



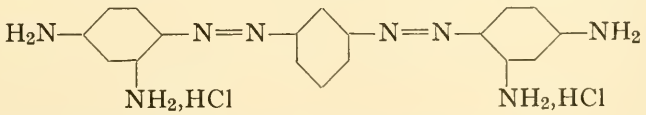
BIEBRICH SCARLET, AQUEOUS

Michrome No. 53. *Molecular weight* 556. *An acid dis-azo dye, pH* 8·1*. *Solubility at* 15° *C: water* 5%, *absolute alcohol* 0·25%.



BISMARCK BROWN Y

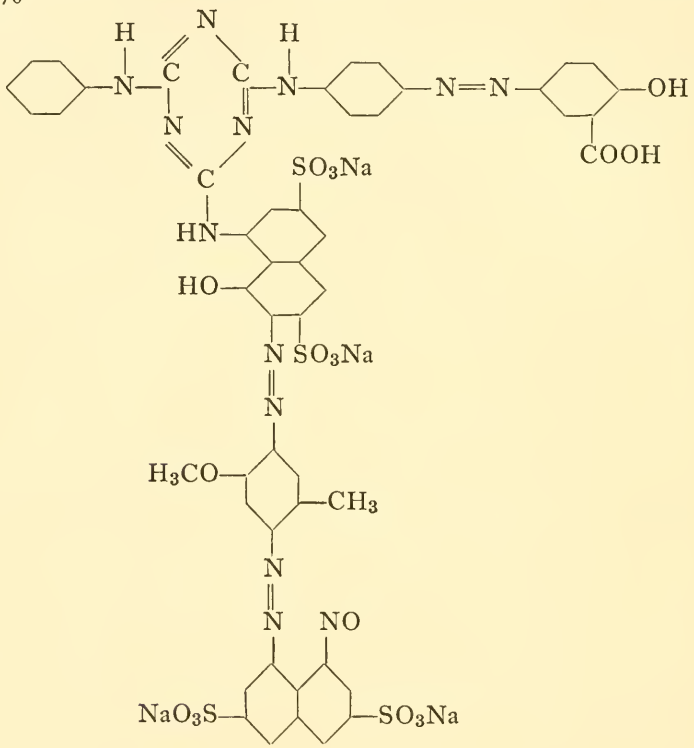
Michrome No. 334. *Molecular weight* 384. *A basic dis-azo dye, pH* 2·65*. *Solubility at* 15° *C: water* 1·5%, *absolute alcohol* 3%



* 1% in neutral distilled water; see pages 17 and 29.

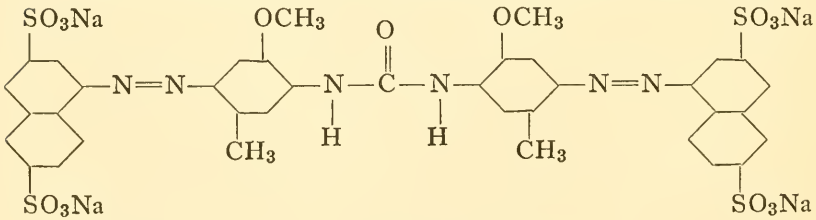
CHLORANTINE FAST GREEN BLL

Michrome No. 541. *Molecular weight* 1324. *An acid tris-azo dye, pH* 7·5*. *Solubility at* 15° *C: water* 5·25%, *absolute alcohol* 0·5%.



CHLORANTINE FAST RED

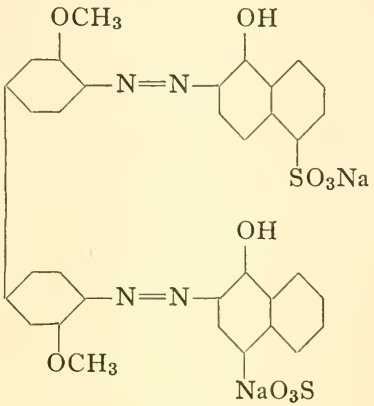
Michrome No. 237. *Molecular weight* 992. *An acid dis-azo dye, pH* 7·35*. *Solubility at* 15° *C: water* 1%, *absolute alcohol* 0·45%.



* 1% in neutral distilled water; see pages 17 and 29.

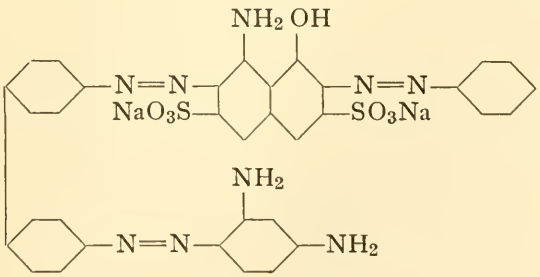
CHLORAZOL AZURINE

Michrome No. 729. *Molecular weight* 759. *An acid dis-azo dye, pH* 9·4*. *Solubility at* 15° *C: water* 1·5%, *absolute alcohol* 0·1%.



CHLORAZOL BLACK E

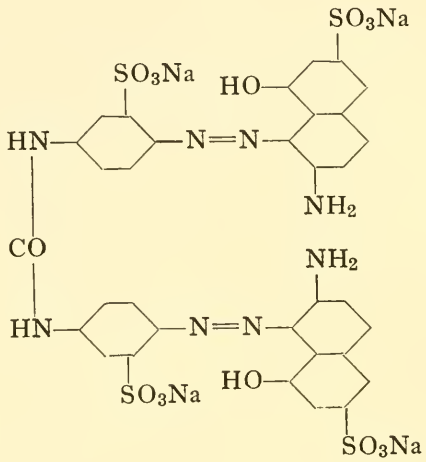
Michrome No. 92. *Molecular weight* 782. *An acid tris-azo dye, pH* 9·05*. *Solubility at* 15° *C: water* 6%, *absolute alcohol* 0·1%.



* 1% in neutral distilled water; see pages 17 and 29.

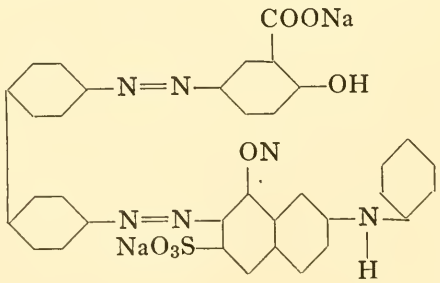
CHLORAZOL FAST PINK BK

Michrome No. 674. *Molecular weight* 991. *An acid dis-azo dye, pH* 8·5*. *Solubility at* 15° *C: water* 1·3%, *absolute alcohol nil.*



CHLORAZOL PAPER BROWN

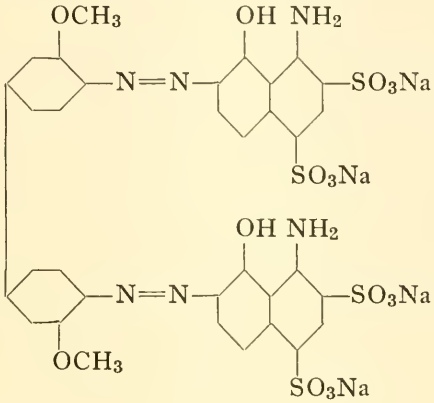
Michrome No. 94. *Molecular weight* 704. *An acid dis-azo dye, pH* 8·95*. *Solubility at* 15° *C: water* 4·5%, *absolute alcohol* 1·25%.



* 1% in neutral distilled water; see pages 17 and 29.

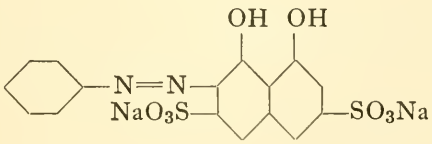
CHLORAZOL SKY BLUE FF

Michrome No. 91. *Molecular weight* 993. *An acid dis-azo dye, pH* 8·4*. *Solubility at* 15° *C: water* 4·75%, *absolute alcohol, nil.*



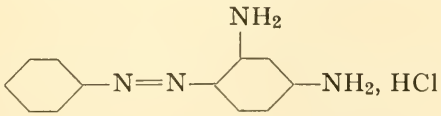
CHROMOTROPE 2R

Michrome No. 301. *Molecular weight* 468. *An acid mono-azo dye, pH* 7·8*. *Solubility at* 15° *C: water* 19%, *absolute alcohol* 0·15%.



CHRYSOIDIN Y

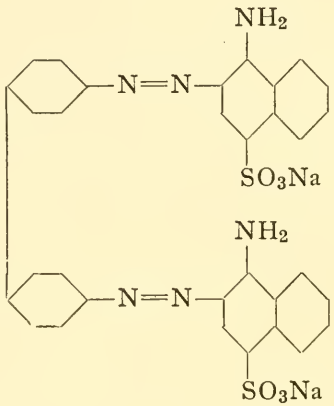
Michrome No. 96. *Molecular weight* 249. *A basic mono-azo dye, pH* 3·65*. *Solubility at* 15° *C: water* 5·5%, *absolute alcohol* 4·75%.



* 1% in neutral distilled water; see pages 17 and 29.

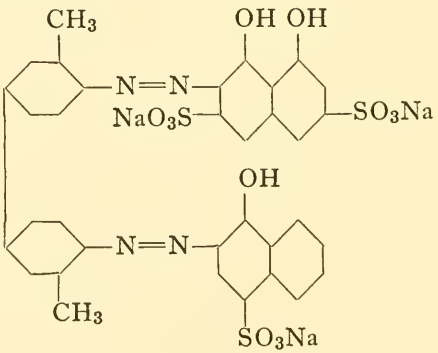
CONGO RED

Michrome No. 400. *Molecular weight* 697. *An acid dis-azo dye, pH* 9·35*. *Solubility at* 15° *C: water* 5%, *absolute alcohol* 0·75%.



DIANIL BLUE 2R

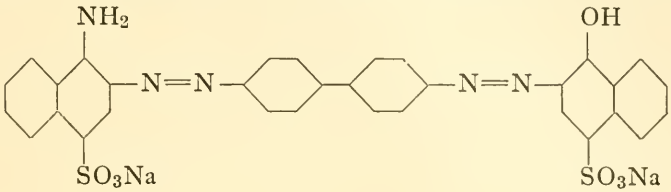
Michrome No. 302. *Molecular weight* 845. *An acid dis-azo dye, pH* 7·35*. *Solubility at* 15° *C: water* 5%, *absolute alcohol* 0·01%.



* 1% in neutral distilled water; see pages 17 and 29.

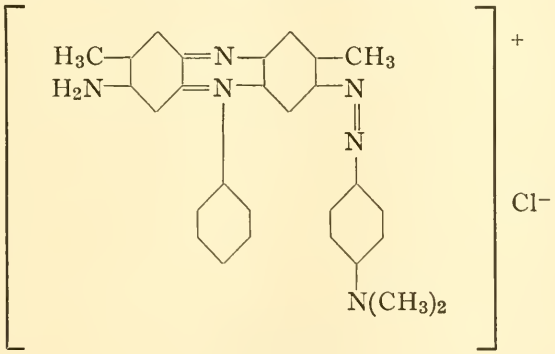
ERIE GARNET

Michrome No. 90. *Molecular weight* 698. *An acid dis-azo dye, pH* 9·5*. *Solubility at* 15° *C: water* 5%, *absolute alcohol* 0·1%.



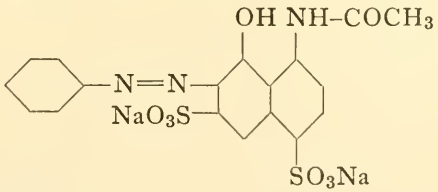
JANUS GREEN B

Michrome No. 183. *Molecular weight* 483. *A basic mono-azo dye, pH* 6·75*. (*Note—This dye also contains the azine group.*) *Solubility at* 15° *C: water* 5%, *absolute alcohol* 1%.



LIGNIN PINK

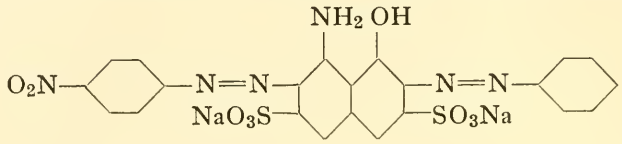
Michrome No. 35. *Molecular weight* 509. *An acid mono-azo dye, pH* 9·1*. *Solubility at* 15° *C: water* 12%, *absolute alcohol nil.*



* 1% in neutral distilled water; see pages 17 and 29.

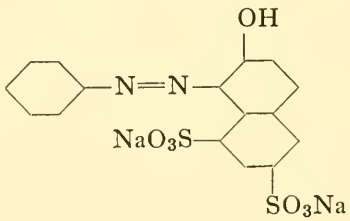
NAPHTHOL BLUE BLACK

Michrome No. 60. *Molecular weight* 617. *An acid dis-azo dye, pH* 8·875*. *Solubility at* 15° *C: water* 2·5%, *absolute alcohol* 3%.



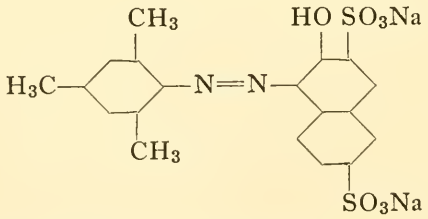
ORANGE G

Michrome No. 411. *Molecular weight* 452. *An acid mono-azo dye, pH* 8·75*. *Solubility at* 15° *C: water* 8%, *absolute alcohol* 0·22%.



PONCEAU 3R

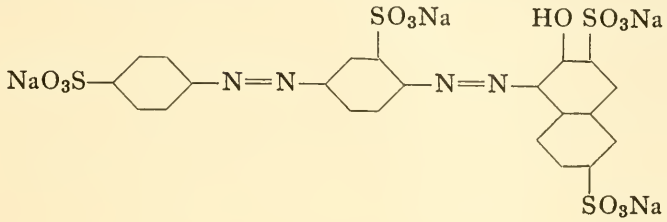
Michrome No. 210. *Molecular weight* 494. *An acid mono-azo dye, pH* 7·2*. *Solubility at* 15° *C: water* 3%, *absolute alcohol* 0·3%.



* 1% in neutral distilled water; see pages 17 and 29.

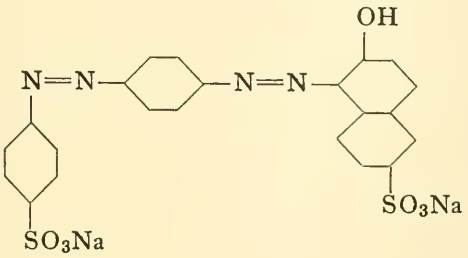
PONCEAU S

Michrome No. 725. *Molecular weight* 761. *An acid dis-azo dye, pH* 8·0*. *Solubility at* 15° *C: water* 1·35%, *absolute alcohol* 1·2%.



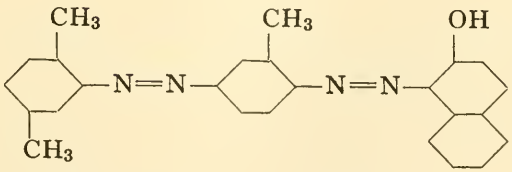
PONCEAU DE XYLIDINE

Michrome No. 565. *Molecular weight* 556. *An acid dis-azo dye. Solubility at* 15° *C: water* 5%, *absolute alcohol* 0·1%.



SCARLET R

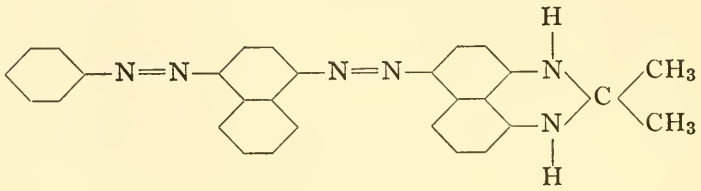
Michrome No. 576. *Molecular weight* 394. *A neutral dis-azo colouring matter. Solubility at* 15° *C: water nil, absolute alcohol* 0·6%.



* 1% in neutral distilled water; see pages 17 and 29.

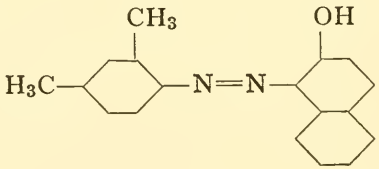
SUDAN BLACK B

Michrome No. 165. *Molecular weight* 457. *A neutral dis-azo colouring matter. Solubility at* 15° *C: water nil, absolute alcohol* 0·25%.



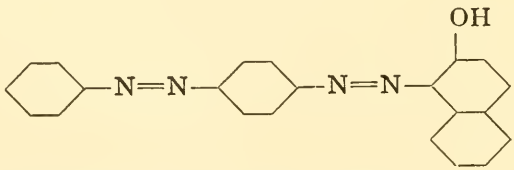
SUDAN 2

Michrome No. 153. *Molecular weight* 276. *A neutral mono-azo colouring matter. Solubility at* 15° *C: water nil, absolute alcohol* 0·28%.



SUDAN 3

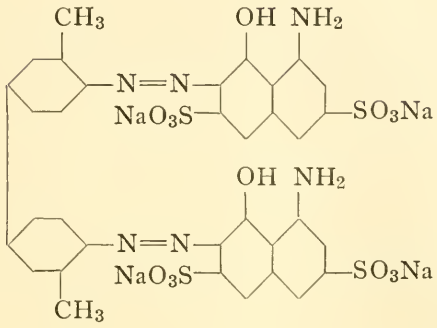
Michrome No. 312. *Molecular weight* 352. *A neutral dis-azo colouring matter. Solubility at* 15° *C: water nil, absolute alcohol* 0·25%.



* 1% in neutral distilled water; see pages 17 and 29.

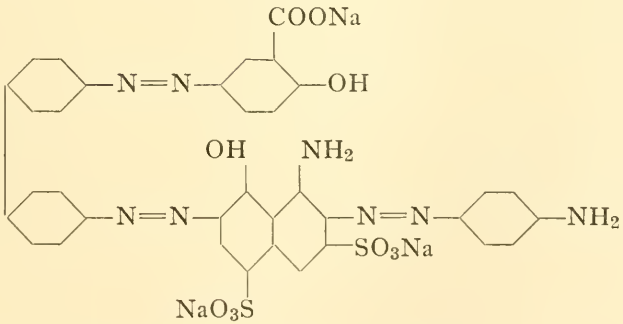
TRYPAN BLUE

Michrome No. 186. *Molecular weight* 961. *An acid dis-azo dye, pH* 9·1*. *Solubility at* 15° *C: water* 1·0%, *absolute alcohol* 0·02%.



VICTORIA GREEN G

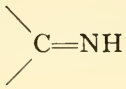
Michrome No. 630. *Molecular weight* 849. *An acid tris-azo dye, pH* 9·15*. *Solubility at* 15° *C: water* 1·85%, *absolute alcohol* 1·85%.



* 1% in neutral distilled water; see pages 17 and 29.

DIPHENYLMETHANE GROUP

Chromophore:

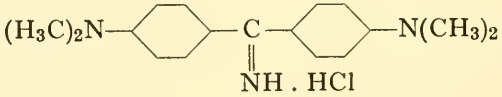


This is a very small group. Only one dye, auramine O, is used as a microscopical stain. This is used as a fluorchrome for tubercle bacilli. It is a basic dye.

AURAMINE O

Michrome No. 42. *Molecular weight* 304. *A basic dye, pH* 7·0*. *Solubility at* 15° *C: water* 1·0%, *absolute alcohol* 4·0%.

Note: Solutions of auramine should be prepared by shaking the required quantity of the dye with the solvent at room temperature: if the solutions are heated above 38–40° C decomposition takes place.



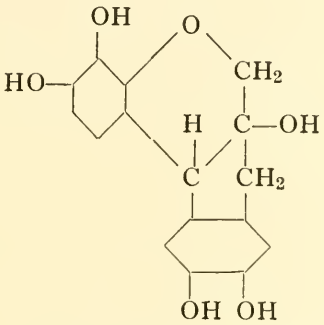
* 1% in neutral distilled water; see pages 17 and 29.

NATURAL COLOURING MATTERS

The most important of these, as far as the histologist is concerned, is *haematoxylin*.

HAEMATOXYLIN

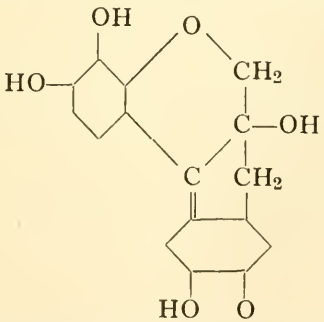
Michrome No. 304. *Molecular weight* 302. *An acid colouring matter, pH* 6·7*. *Solubility at* 15° *C: water* 10%, *absolute alcohol* 10%.



This is obtained from the wood of a species of tree, the *Haematoxylin campechianum*, Linn., which is indigenous to Mexico, but is cultivated in the West Indies. Haematoxylin is not itself a dye. It owes its tinctorial properties to the formation of one of its oxidation products.

HAEMATEIN

Michrome No. 360. *Molecular weight* 300. *An acid colour, pH* 6·05*. *Solubility at* 15° *C: water* 1·5%, *absolute alcohol*, 1·5%.



* 1% in neutral distilled water; see pages 17 and 29.

Solutions of haematoxylin must be "ripened"—that is, partially oxidized to produce the dye, haematein—before they are ready for use. This ripening occurs gradually, through spontaneous atmospheric oxidation over a period of weeks and months, after the solutions have been made up and left to stand. However, the solutions may be ripened artificially so that they are ready for immediate use, by adding minute quantities of oxidizing agents such as hydrogen peroxide, mercuric oxide, potassium permanganate or potassium iodate.

Haematoxylin will not attach itself to tissue-element unless a mordant is used. A mordant is a substance, such as alum, employed to make certain dyes "bite" and hold on to tissue-elements. A mordant acts as the link between the dye with which it unites to form a "lake", and the tissue-elements with which it also combines. It appears, however, that a conventional mordant is not required when haematoxylin is used with certain acid dyes; e.g. light green. The latter not only stains basic tissue-elements when used in conjunction with haematoxylin, but they also act as mordants between acidic tissue-elements and haematoxylin (see Rafferty's stain, page 389).

Haematein is a feeble reddish-yellow dye with a negative charge, which according to Baker (1956, 1958), quoting Seki (1933), has an isoelectric point of about 6·5. When used without a mordant, haematein has no affinity for tissue components and it is quite useless as a stain, although it is used in this form as a hydrogen ion indicator. Therefore, mordanting in one form or another is essential when haematoxylin (haematein) solutions are used for staining. The mordants most commonly used are ammonia alum, potash alum and iron alum (ferric ammonium sulphate). The lakes formed with these and other mordants carry a strong positive charge. Accordingly they behave as strongly basic dyes. In some cases the mordant is incorporated in the stock solution of the haematoxylin: e.g. Delafield, and Ehrlich. In other cases the mordant is kept as a separate stock solution, e.g. Heidenhain, and Weigert.

Other natural colouring matters of importance are *orcein* and *carminic acid*.

NITRO GROUP

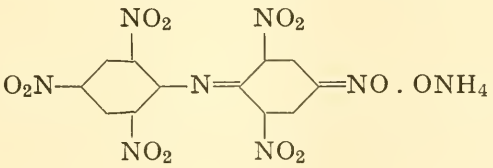
Chromophore: NO_2

The nitro dyes form a comparatively small group. Picric acid (2, 4, 6-trinitro-phenol) belongs to this group. All nitro dyes are yellow in colour. They are all acid dyes. Members of this group used as microscopical stains are: martius yellow, Manchester yellow, naphthol yellow S, aurantia, and naphthol yellow T.

Examples of nitro dyes are given below:

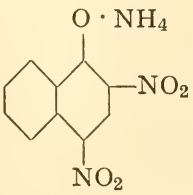
AURANTIA

Michrome No. 4. *Molecular weight* 456. *An acid dye. Solubility at* 15° *C: water* 0·1%, *absolute alcohol* 0·55%.



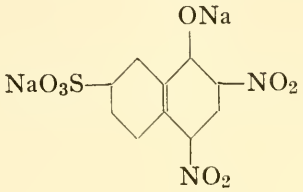
MARTIUS YELLOW

Michrome No. 170. *Molecular weight* 251. *An acid dye. Solubility at* 15° *C: water* 1·25%, *absolute alcohol nil.*



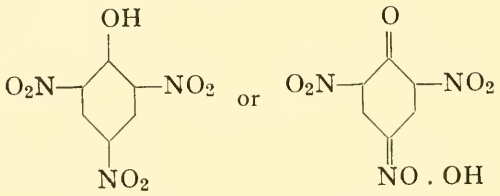
NAPHTHOL YELLOW S

Michrome No. 191. *Molecular weight* 358. *An acid dye, pH* 8·15*. *Solubility at* 15° *C: water* 12·5%, *absolute alcohol* 0·65%



PICRIC ACID

Michrome No. 707. *Molecular weight* 229, *pH* 1·35*. *Solubility at* 15° *C: water* 1·2%, *absolute alcohol* 9%.



* 1% in neutral distilled water; see pages 17 and 29.

NITROSO GROUP

Chromophore: —N=O

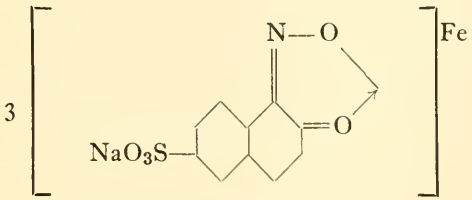
The nitroso dyes form a very small group. They are all acid dyes. Only two, namely, naphthol green B and naphthol green Y, have so far been used in biology.

Naphthol green B is used as a stain for collagen, etc.

Naphthol green Y has not, as far as I am aware, been used for staining microscopic tissue preparations; its tinctorial properties are poor. However, Ingraham & Visscher (1935) and Visscher (1942) employed the dye, macroscopically, in their studies of the secreting mechanism of gastric glands and pancreas.

NAPHTHOL GREEN B

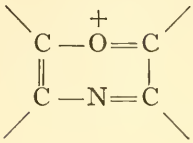
Michrome No. 192. *Molecular weight* 878. *An acid dye, pH* 6·85*. *Solubility at* 15° *C: water* 10%, *absolute alcohol* 3%.



* 1% in neutral distilled water; see pages 17 and 29.

OXAZINE GROUP

Chromophore:

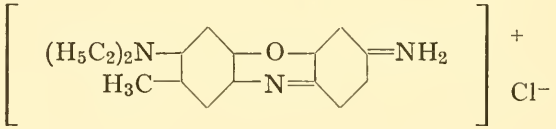


About twelve dyes of this group are used in microtechnique. One is an acid, two are amphoteric, and the rest are basic dyes.

Examples of oxazine dyes are given below:

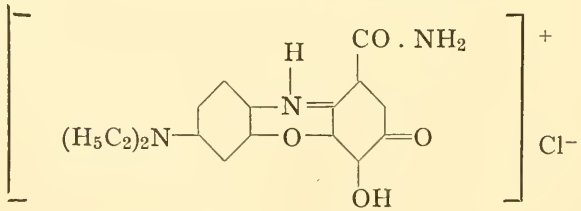
BRILLIANT CRESYL BLUE

Michrome No. 404. *Molecular weight* 318. *A basic dye, pH* 2·2*. *Solubility at* 15° *C: water* 3%, *absolute alcohol* 2%.



CELESTIN BLUE

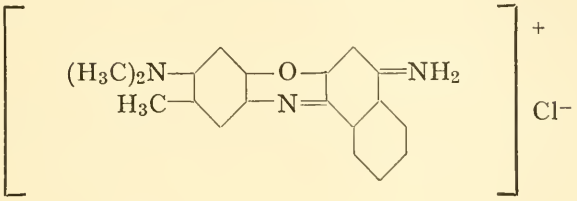
Michrome No. 66. *Molecular weight* 364. *A basic dye, pH* 2·6*. *Solubility at* 15° *C: water* 2%, *absolute alcohol* 1·5%.



* 1% in neutral distilled water; see pages 17 and 29.

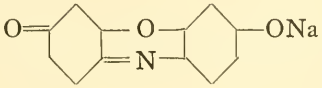
CRESYL FAST VIOLET CNS

Michrome No. 530. *Molecular weight* 340. *A basic dye, pH* 7·0*. *Solubility at* 15° *C: water* 9·5%, *absolute alcohol* 6%.



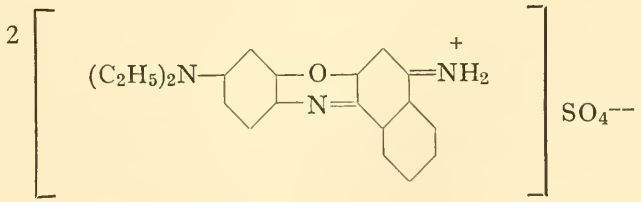
LACMOID

Michrome No. 462. *Molecular weight* 235. *An acid dye, pH* 7·85.* *Solubility at* 15° *C: water* 10%, *absolute alcohol* 10%.



NILE BLUE SULPHATE†

Michrome No. 212. *Molecular weight* 733. *A basic dye, pH* 5·15*. *Solubility at* 15° *C: water* 6%, *absolute alcohol* 5%.



* 1% in neutral distilled water; see pages 17 and 29.

† *Note:* Nile blue sulphate has the highest molecular weight of any basic dye used in microscopy. This is due to the fact that its molecule is composed of two univalent dye-ions attached to one divalent sulphate group, as their anion. Such a dye in solution would split into two separate dye-ions each capable separately of associating with univalent dye-ions to form a dye of approximately half the molecular weight of the original dye (Gurr, 1960, pp. 423–432).

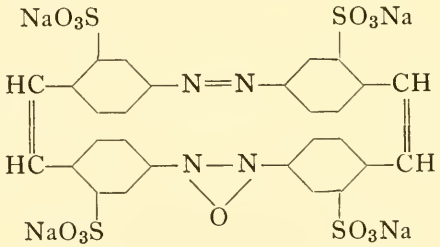
STILBENE GROUP

This is a subdivision of the azo group. Stilbene dyes contain one or more azo groups in their molecules. They are all condensation products of 5-nitro-toluene sulphonic acid. The chromophore of the stilbene dyes may be the azo group or the azoxy group, or both.

Up to the present time only two stilbene dyes appear to have been used in microscopy. They are both acid dyes of high molecular weight. One is Mikado orange G, and the other Sun yellow G. The structure of the latter dye is given below:

SUN YELLOW G

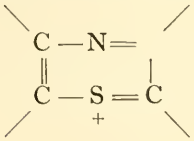
Michrome No. 720. *Molecular weight* 837. *An acid dye, pH* 7·3*. *Solubility at* 15° *C: water* 1%, *absolute alcohol* 0·008%.



* 1% in neutral distilled water; see pages 17 and 29.

THIAZINE GROUP

Chromophore:

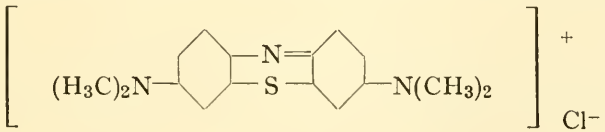


The chromogen consists of this configuration as the inner ring of a three-ring arrangement, the outer two being aromatic nuclei.

About eleven thiazine dyes are used in microtechnique (Gurr, 1960). Only one of these is acid; the others are basic dyes. Members of this group, best known to biologists, are methylene blue, toluidine blue, and thionin. The structures of these three dyes are given below:

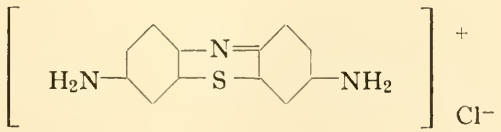
METHYLENE BLUE

Michrome No. 416. *Molecular weight* 320. *A basic dye, pH* 2·5*. *Solubility at* 15° *C: water* 9·5%, *absolute alcohol* 6%.



THIONIN (Ehrlich)

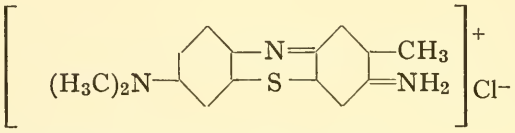
Michrome No. 215. *Molecular weight* 264. *A basic dye, pH* 2·25*. *Solubility at* 15° *C: water* 1%, *absolute alcohol* 1%.



* 1% in neutral distilled water; see pages 17 and 29.

TOLUIDINE BLUE

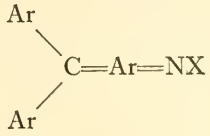
Michrome No. 641. *Molecular weight* 306. *A basic dye, pH* 2·35*. *Solubility at* 15° *C: water* 3·25%, *absolute alcohol* 1·75%.



* 1% in neutral distilled water; see pages 17 and 29.

TRIARYLMETHANE GROUP

Chromophore:



X = H or H_2 or O or an aryl or aliphatic group.
where Ar = an aromatic nucleus;

The triarylmethane dyes, all of which are quinonoid dyes, can be divided into three main sub-groups, namely:

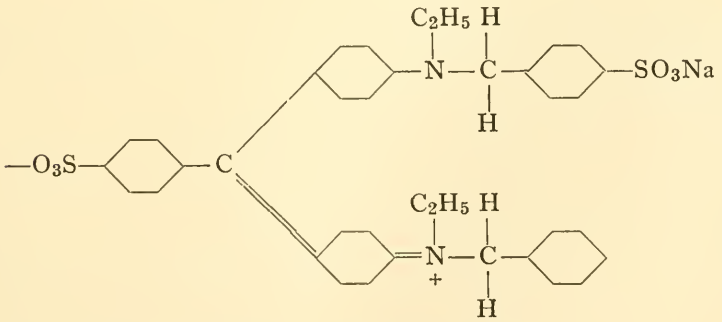
1. Triphenylmethane.
2. Diphenylnaphthylmethane.
3. Phenyldinaphthylmethane.

Each of these can be further divided, but space does not permit a discussion of these in this present work.

Examples of the triarylmethane dyes are given below:

ACID GREEN L EXTRA

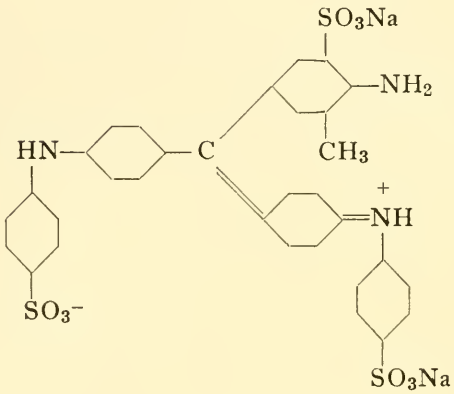
Michrome No. 647. *Molecular weight* 691. *An acid triphenylmethane dye, pH* 3·4*. *Solubility at* 15° *C: water* 5%, *absolute alcohol* 5%.



* 1% in neutral distilled water; see pages 17 and 29.

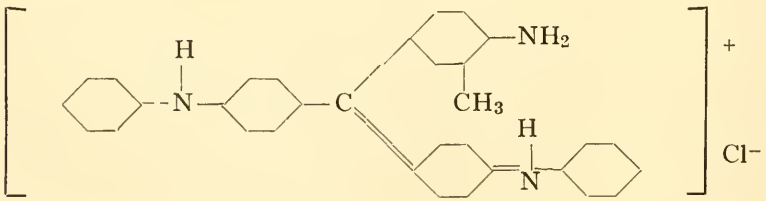
ANILINE BLUE, AQUEOUS

Michrome No. 167. *Molecular weight* 738. *An acid triphenylmethane dye, pH* 6·4*. *Solubility at* 15° *C: water* 50%, *absolute alcohol nil.*



ANILINE BLUE, ALCOHOLIC

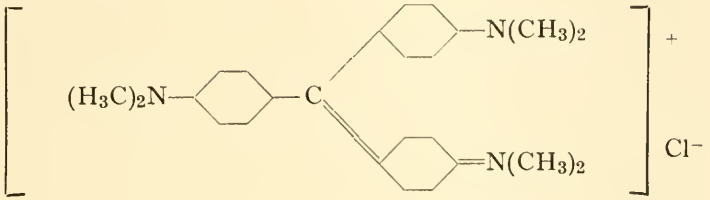
Michrome No. 221. *Molecular weight* 490. *A basic triphenylmethane dye. Solubility at* 15° *C: water nil, absolute alcohol* 1·5%.



* 1% in neutral distilled water; see pages 17 and 29.

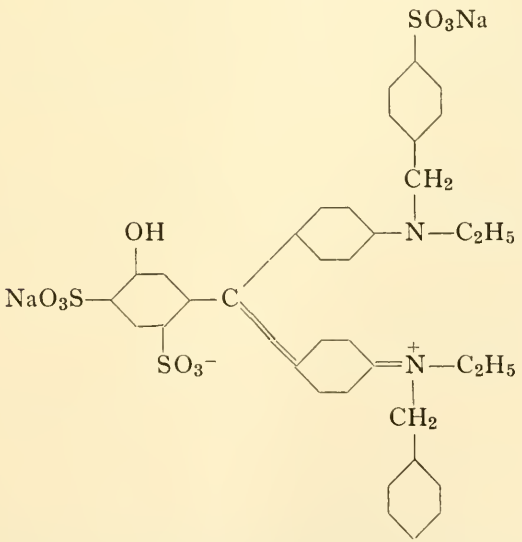
CRYSTAL VIOLET

Michrome No. 103. *Molecular weight* 408. *A basic triphenylmethane dye, pH* 6·6*. *Solubility at* 15° *C: water* 9%, *absolute alcohol* 8·75%.



FAST GREEN FCF

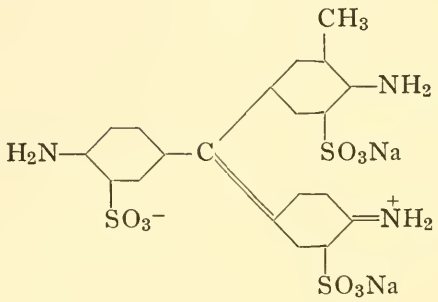
Michrome No. 135. *Molecular weight* 809. *An acid triphenylmethane dye, pH* 6·8*. *Solubility at* 15° *C: water* 4%, *absolute alcohol* 9%.



* 1% in neutral distilled water; see pages 17 and 29.

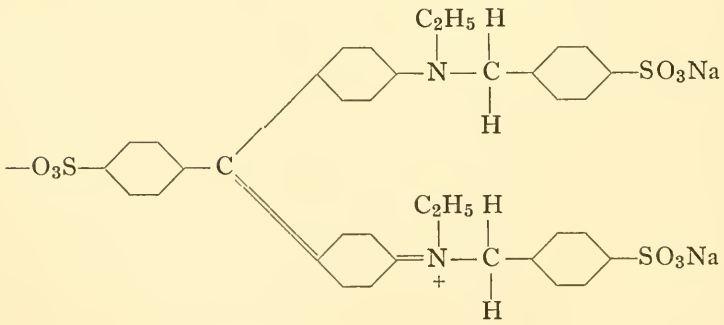
FUCHSIN ACID†

Michrome No. 5. *Molecular weight* 586. *An acid triphenylmethane dye, pH* 5·0*. *Solubility at* 15° *C: water in all proportions, absolute alcohol* 3%.



LIGHT GREEN SF YELLOWISH

Michrome No. 240. *Molecular weight* 793. *An acid triphenylmethane dye, pH* 3·1*. *Solubility at* 15° *C: water* 20%, *absolute alcohol* 4%.

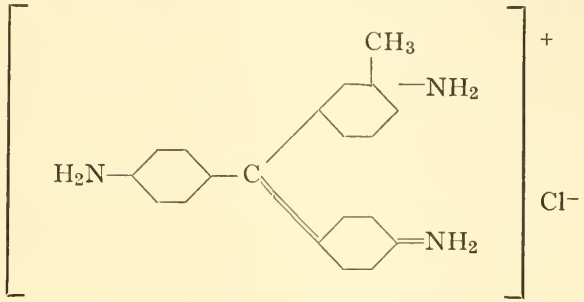


* 1% in neutral distilled water; see pages 17 and 29.

† *Note:* Fuchsin acid is the parent substance of a new group of dyes, the fuchsinic acids, recently discovered.

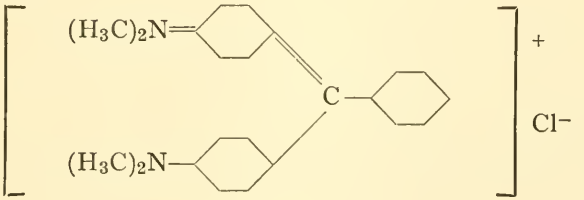
MAGENTA, BASIC†

Michrome No. 623. *Molecular weight* 338. *A basic triphenylmethane dye. Solubility at* 15° *C: water* 1%, *absolute alcohol* 10%



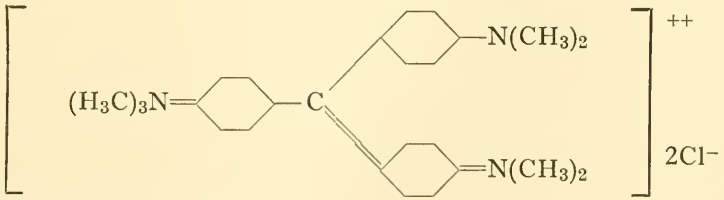
MALACHITE GREEN

Michrome No. 315. *Molecular weight* 365. *A basic triphenylmethane dye, pH* 2·4*. *Solubility at* 15° *C: water* 10%, *absolute alcohol* 8·5%.



METHYL GREEN OO

Michrome No. 177.‡ *Molecular weight* 458. *A basic triphenylmethane dye, pH* 5·6*. *Solubility at* 15° *C: water* 8%, *absolute alcohol* 3%.

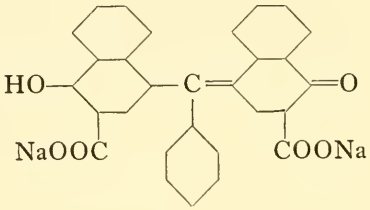


* 1% in neutral distilled water; see pages 17 and 29.
† *Note:* This is one of the component dyes of basic fuchsin.
‡ *Note:* In a previous work (Gurr, 1958*a*) this number was given in error as 77.

NAPHTHOCHROME GREEN

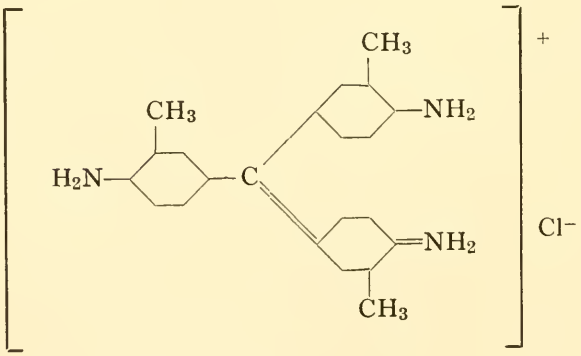
Michrome No. 938. *Molecular weight* 506. *An acid hydroxy-triarylmethane* (*hydroxy-dinaphthylphenylmethane*) *dye, pH* 5·7*. *Solubility at* 15° *C: water* 3%, *absolute alcohol* 1·5%.

This dye might possess hydrogen ion indicator properties. If used at a suitable pH it might also impart polychrome pictures to tissues in much the same way as solochrome cyanin R.



NEW FUCHSIN†

Michrome No. 624. *Molecular weight* 366. *A basic triphenylmethane dye. Solubility at* 15° *C: water* 1%, *absolute alcohol* 10%.

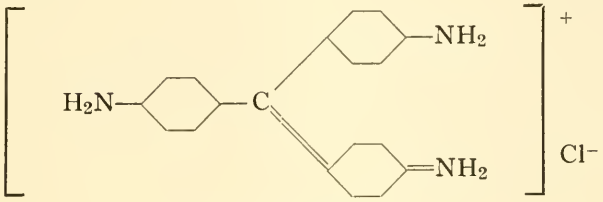


* 1% in neutral distilled water; see pages 17 and 29.

† *Note:* This is one of the component dyes of basic fuchsin.

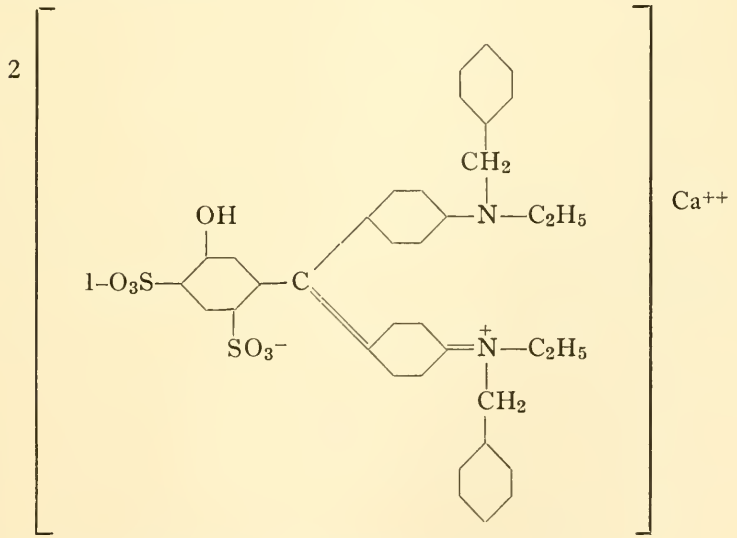
PARAROSANILINE CHLORIDE†

Michrome No. 722. *Molecular weight* 329. *A basic triphenylmethane dye, pH* 6·35.* *Solubility at* 15° *C: water* 1%, *absolute alcohol* 10%.



PATENT BLUE A

Michrome No. 201. *Molecular weight* 1408.‡ *An acid triphenylmethane dye, pH* 6·5*. *Solubility at* 15° *C: water* 5%, *absolute alcohol* 7·75%.



* 1% in neutral distilled water; see pages 17 and 29.

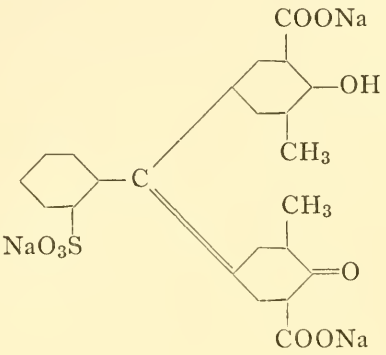
† *Note:* This is one of the three component dyes of basic fuchsin.

‡ *Note:* A molecule of this dye consists of two univalent dye-ions in association with one divalent cation (Ca^{++}).

In solution such dyes ionize to produce two separate dye-ions, each capable of separately associating with one univalent cation to form a dye of approximately half the molecular weight of the original.

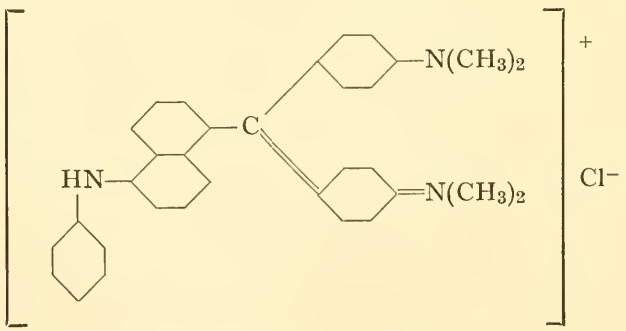
SOLOCHROME CYANIN R

Michrome No. 750. *Molecular weight* 536. *An acid dye of the hydroxy-triphenylmethane series, pH* 2·3*. *Solubility at* 15° *C: water* 7%, *absolute alcohol* 5%.



VICTORIA BLUE B

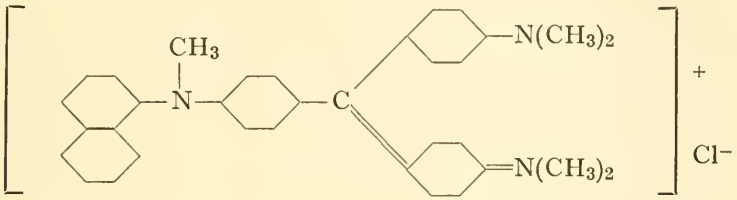
Michrome No. 148. *Molecular weight* 506. *A basic diphenyl-naphthylmethane dye, pH* 2·6*. *Solubility at* 15° *C: water* 4·3%, *absolute alcohol* 8·25%.



* 1% in neutral distilled water; see pages 17 and 29.

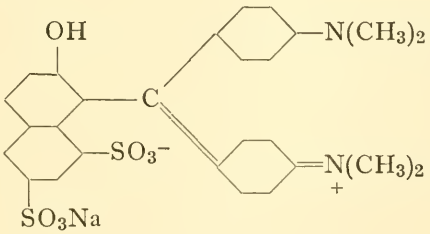
VICTORIA BLUE 4R

Michrome No. 307. *Molecular weight* 520. *A basic triphenylmethane dye, pH*5·425*. *Solubility at* 15° *C: water* 3%, *absolute alcohol* 20%.



WOOL GREEN S

Michrome No. 162. *Molecular weight* 577. *An acid diphenylnaphthylmethane dye, pH*3·2*. *Solubility at* 15° *C: water* 4%, *absolute alcohol nil.*



* 1% in neutral distilled water; see pages 17 and 29.

XANTHENE GROUP

This group of dyes contains a number of sub-groups. One of the most important of these, as far as the biologist is concerned, is the fluorene group, perhaps better known as the amino derivatives. Among the amino derivatives are:

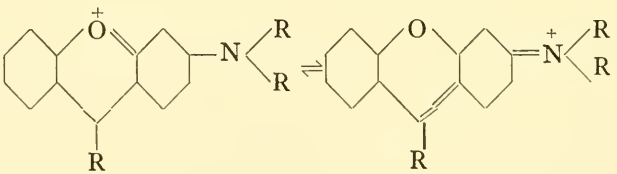
(*a*) The pyronines, examples of which are pyronin B and pyronin Y (or G).

(*b*) The rhodamines, which include rhodamine B and rhodamine 6G.

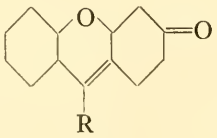
The hydroxy derivatives (fluorone dyes) is another division. Among these are the fluoresceins, eosins, phloxines, rose Bengal, and gallein.

The xanthene group also includes the anthrahydroxyphthaleins, which constitute a very small subdivision, consisting of only four or five members, only one of which (coeruleine) is used in microtechnique.

Aminoxanthene dyes contain the following resonance hybrid as their chromophore:

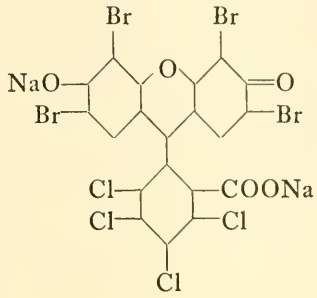


where R = hydrogen or a benzene or a naphthalene nucleus. The hydroxyxanthenes are stabilized, by the loss of a proton, to form an uncharged system having the quinoid structure, as follows, as the chromophore:



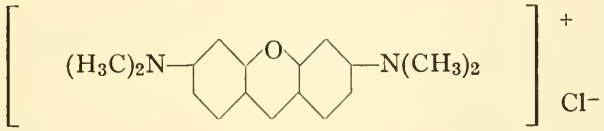
PHLOXIN B

Michrome No. 206. *Molecular weight* 830. *An acid dye, pH* 7·5*. *Solubility at* 15° *C: water* 10·5%, *absolute alcohol* 5%.



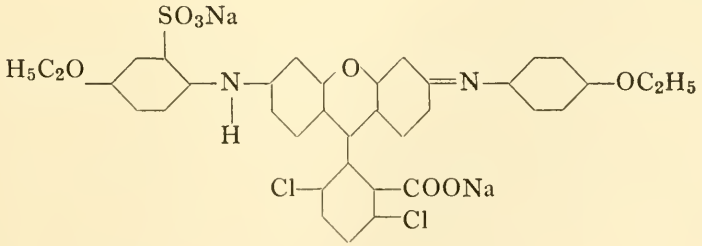
PYRONIN Y OR G

Michrome No. 339. *Molecular weight* 303. *A basic dye, pH* 3·0*. *Solubility at* 15° *C: water* 9%, *absolute alcohol* 0·5%.



VIOLAMINE 3B

Michrome No. 161. *Molecular weight* 764. *An acid dye, pH* 8·5*. *Solubility at* 15° *C: water* 5·25%, *absolute alcohol* 10·5%.



* 1% in neutral distilled water; see pages 17 and 29.

FUCHSINIC ACID DYES

It has recently been discovered (M. A. MacConaill & E. Gurr, 1959–62; E. Gurr & M. A. MacConaill, 1959–62) that certain acid dyes will unite chemically with certain other acid dyes to form compound stains which impart polychromatic pictures to tissue preparations viewed under the microscope. The first three compound dyes of the series were originally given the name of the MG or Falg dyes, for want of a better name at the time. Later the name was changed to the Falgic acids, for reasons explained below (page 78).

The discovery came about during the course of an investigation which was originally intended to serve a dual purpose, namely:

1. As a continuation of an earlier study (MacConaill, 1949,* 1951) upon the erythrophile elements found in the nervous system, including the so-called neurokeratin. The elements mentioned were called erythrophile because they had a special affinity for the red dye, acid fuchsin. The latter dye belongs to the triphenylmethane group, which is a division of the triarylmethane group of dyes. It seemed desirable to carry out a comparative study of the effect of other dyes of the same group, and of other groups, as a preliminary to more detailed work upon the erythrophile substances themselves.

2. As a test of another hypothesis (E. Gurr, 1960) regarding the influence of molecular weights of acid dyes on their staining effects. According to this hypothesis, acid dyes of high molecular weight do not usually stain close-knit tissues, while dyes of low and medium molecular weights usually stain close-knit tissues preferentially. Dyes of the former class usually stain close-knit tissues lightly, if at all.

During the early stages of our work (1959, 1960) on neurological tissues, our experiments took the form of using a series of acid triphenylmethane dyes having molecular weights ranging from 536 to 808, as well as a series of acid dyes from other groups, having molecular weights from 580 to 759. We were able to confirm the hypothesis regarding molecular weights of acid dyes on the material investigated. We were also able to confirm

* See page 258. MacConaill's *Lead Haematoxylin–Acid Fuchsin Technique.*

another hypothesis advanced earlier (MacConaill, 1949, 1951), that the erythrophile substance of the neuraxon is basic in reaction and that dyes which stain it also stain red blood corpuscles and the nucleolus of the nerve cell. We also discovered a new series of dyes, as mentioned above.

Although acid fuchsin is generally regarded as an acid dye, it is really amphoteric as its formula (page 68) indicates. As a dye, acid fuchsin stands out alone*. For many years it has been one of the most widely used plasma stains. It has also been used for a large number of special purposes in biology. But one very important point about this dye seems to have passed unnoticed until recently, and that is that it is the only dye which contains three unsubstituted amino (basic) groups and the same number of acidic (sulphonic) groups. In this respect alone, acid fuchsin is unique. No other acid triphenylmethane possesses even one unsubstituted amino group. Acid fuchsin has three. Some acid dyes of other groups possess unsubstituted amino groups, but the latter are in all cases outnumbered by acidic groups (e.g. sulphonic and/or carboxyl) also forming part of the dye-molecule.

Theoretically, acid fuchsin could attach itself to either basic tissue elements, by one or more of its sulphonic groups, or to acidic tissue elements by one or more of its amino groups. In fact it appears to combine with basic tissue elements only, whence its function as an acid dye. The three amino groups of this dye are, therefore, available for conjunction with acid groups of other chemical substances, both *in vitro* and in a stained specimen of tissue (*in tela*). This gives the possibility of combining acid fuchsin with other acid dyes to form *compound fuchsinic acids*.† It should be possible to form three such compounds corresponding to a conjunction of the acid fuchsin molecule, through its amino groups, with one, two, or three molecules of certain other acid dyes, through their sulphonic groups. If the molecule of acid fuchsin is represented as Fa, and the other acid dye as D, then the possible compounds can be represented by FaD, FaD_2, and FaD_3. Complete sets of such compounds have been prepared and isolated in the pure state; and they explain the colours found

* But see chlorazol black structure (page 45). This dye, like acid fuchsin, is really amphoteric and worthy of further investigations.

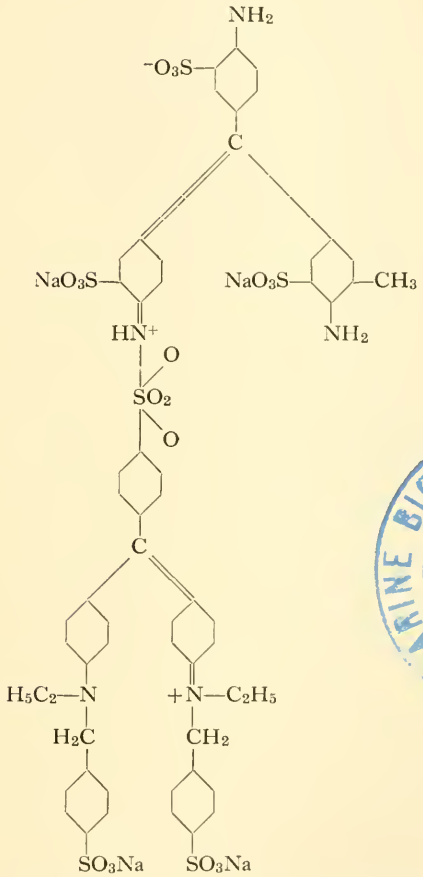
† *Note:* Fuchsinic acid is a synonym for acid fuchsin.

by staining a tissue with acid fuchsin as the primary stain, followed by the other acid dye as the secondary stain (see Falg, standard and basic Faviol techniques, pages 198–206).

This type of reaction was first observed in tissues (Gurr & MacConaill, 1959, 1960*b*; MacConaill & Gurr, 1960*a*) which had been stained first with acid fuchsin then with light green. It was expected that the picture to be seen under the microscope would be in red and green. Instead, a polychromatic effect was produced: various tissue-elements were stained in shades of red, mauve, violet, and blue. It was the last colour which excited our interest, as while the mauve shades could possibly be due to an admixture of the red primary stain (acid fuchsin) and the green secondary stain (light green), blue being a primary colour could not have been produced by mere admixture of red and green, therefore a chemical change appeared to have taken place, and investigations were then made into the phenomena. This resulted in the production of three new compound dyes *in vitro*. These, and other fuchsinic acid compound dyes were afterwards isolated in the solid form. The first three produced by the interaction of acid fuchsin (fuchsinic acid) and light green were called the Falg colours (synonyms: MG dyes), the first two letters of the name representing acid fuchsin (fuchsinic acid, FA) and the other two, LG, representing light green. Since FA behaves as a base towards LG, the Falg or MG compounds may not properly be described as fuchsinates, but they could be described as Falgic acids because each of the three compounds has two " free " sulphonic groups attached to each LG component. Thus, the following terms were used, in which Fa symbolizes the acid fuchsin radical and Lg the light green radical:

(1) FaLg = MONOFALGIC ACID

Formed when the acid fuchsin molecule accepts one molecule of light green.



Synonyms: MG purple, Falg purple

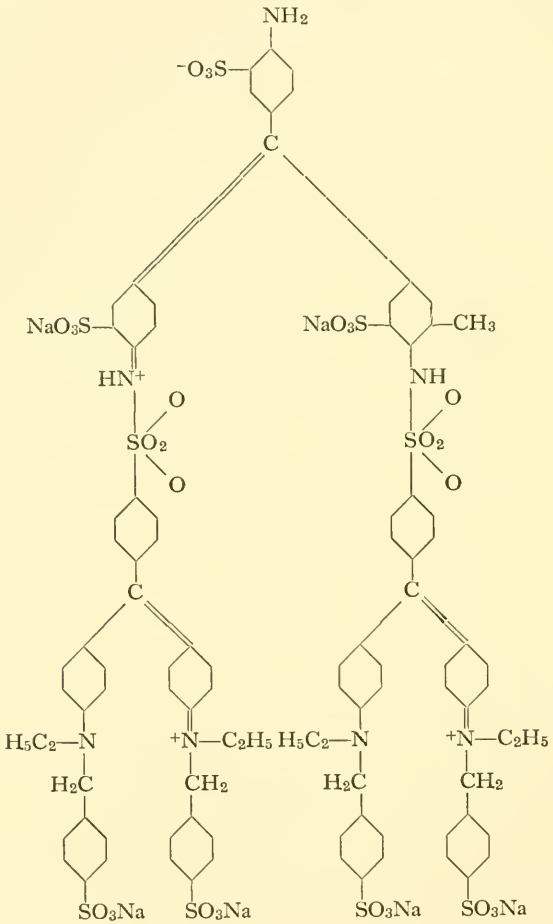
$(C_{57}H_{47}N_5O_{14}S_5Na_4)$

Michrome No. 820. *Molecular weight* 1278·304. *A purple, acid dye, pH* 4·55*. *Solubility at* 15° *C: water all proportions, absolute alcohol nil.*

* 1% in neutral distilled water; see pages 17 and 29.

(2) FaLg2 = DIFALGIC ACID

Formed when the acid fuchsin accepts two molecules of light green.



Synonyms: MG violet, Falg violet.

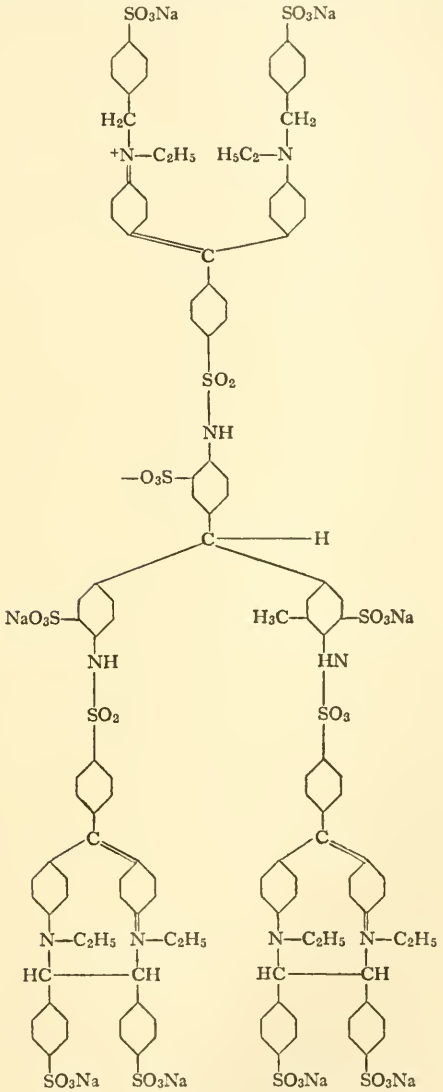
$(C_{94}H_{84}N_7O_{22}S_8Na_6)$

Michrome No. 819. *Molecular weight* 2058·178, *pH* 4·5*. *Solubility at* 15° *C: water all proportions, absolute alcohol nil.*

* 1% in neutral distilled water; see pages 17 and 29.

(3) FaLg = TRIFALGIC ACID

Formed when the acid fuchsin accepts three molecules of light green.



Synonyms: MG blue, Falg blue

($C_{131}H_{113}N_9O_{33}S_{12}Na_8$)

Michrome No. 818. *Molecular weight* 2,911, *pH* 4·2*.
Solubility at 15° *C: water all proportions, absolute alcohol nil.*

* 1% in neutral distilled water; see pages 17 and 29.

As already indicated, the chemical hypothesis was verified by the synthesis and isolation of each of these compounds in the pure form. The syntheses were confirmed by chromatography and electrophoresis. Although these and other fuchsinic acid compound dyes are now available commercially it appears that the two-stage techniques (e.g. Falg, page 198) are to be preferred to the one-step methods (e.g. Trifalgic acid, page 200) in histology and cytology. However, one of the compound dyes (trifalgic acid) has been found to be of value in protein-electrophoresis by Dr. John Bodman (Bodman, 1960), who kindly tested a sample of the dye for us electrophoretically. Among other compound fuchsinic acid dyes later discovered were the Faviolic acids. These are formed in the same manner as are the Falgic acids, the difference being that violamine 3B was used in place of light green.

The name of this series was derived from Fa for acid fuchsin and Violic for violamine 3B. Using the symbol Fa again to denote the acid fuchsin radical, and V to denote the violamine radical we have:

1. FaV = monofaviolic acid (red).
2. FaV_2 = difaviolic acid (violet).
3. FaV_3 = trifaviolic acid (blue).

Another series is the FADIANIC acids produced by the interaction of acid fuchsin and dianil blue 2R. Many other compound fuchsinic acids have since also been isolated. Some of these will be dealt with elsewhere in literature. Only the compounds of acid fuchsin-light green (Falg technique and modifications) acid fuchsin-violamine (basic Faviol technique) and acid fuchsin-sun yellow-violamine (standard Faviol technique), will be discussed here, in brief.

THE FALG TECHNIQUE

Practical details for carrying out this procedure are given on page 198. It should be noted that the light green attaches itself to those tissue elements that have already been stained by the acid fuchsin; and indeed to the stain (acid fuchsin) by which they have been stained. This is a special kind of counterstaining. We have to distinguish here between two chief kinds of staining, primary

and secondary. When, for example, we use the common haematoxylin-eosin stain, the haematoxylin is the primary stain and the eosin is the secondary stain. In the Falg and basic Faviol methods, the acid fuchsin is the primary stain and the light green or violamine is the secondary. But there is a difference between the haematoxylin-eosin method and the compound fuchsinic acid methods. In the haematoxylin-eosin method the secondary stain colours those tissue elements that have not been stained by the primary stain (haematoxylin). As stated by the authors (Gurr & MacConaill, 1960*b*), we can regard the eosin in the haematoxylin-eosin technique as a complementary secondary stain, while the light green in the Falg technique can be regarded as a supplementary secondary stain. Thus, it appears that there are two types of counterstaining: (1) *complementary* secondary staining, and (2) *supplementary* secondary staining.

THE FAVIOL TECHNIQUE

Practical details for carrying out this procedure are given on pages 203–6. What has been written in the above paragraph applies equally to the Faviol technique.

RESULTS OF FALG AND FAVIOL TECHNIQUES

The results of staining by the Falg or Faviol techniques are most easily expressed in the terms of concept of *erythrophilia* (MacConaill, 1949). A tissue element is said to be strongly erythrophile when it has a marked affinity for acid fuchsin. Tissue elements that have a moderate affinity for acid fuchsin are stated to be moderately erythrophile, while those which exhibit only a weak affinity for that dye are said to be weakly erythrophile. *Erythrophobe* tissue elements are those which have no affinity for acid fuchsin. As observed by Professor MacConaill, the Falg and Faviol methods demonstrate these degrees of erythrophilia by, in effect, transforming differences in monochrome intensity (amplitude) into differences in colour (wavelengths). Strongly erythrophile substances remain red, usually somewhat deeper than the colour of acid fuchsin. Moderately erythrophile elements become violet, and weakly erythrophile elements become blue. Erythrophobe are unstained and remain transparent. For an explanation of these phenomena, readers are referred to the

authors' original papers: the full references to these are given in the bibliography of this book.

The violet and blue obtained by using the basic Faviol technique are deeper than the corresponding colours obtained by the Falg method. This circumstance would perhaps be of help in determining a choice by workers after having had experience of both methods.

Among strongly erythrophile elements are the nucleoli of cells, probably on account of their histone component; red blood corpuscles, and neurokeratin. The oldest fibres formed by fibroproteins (e.g. keratin and collagen fibres) are strongly erythrophile, the youngest fibres being weakly erythrophile. Indeed there appears to be a tendency for erythrophilia to increase with age in all erythrophilic tissues. Much work remains to be done upon the relative erythrophilia of various tissue elements, both with regard to each of these two techniques separately, and by comparison between them in this respect. As a general rule, not yet entirely confirmed, the Falg method is preferable for adult gland tissues.

MODIFIED FALG AND FAVIOL TECHNIQUES

It has long been known that some dyes (e.g. aurantia, nigrosin, orange G, tartrazine, picric acid) decolorize certain tissues that have been stained with certain other dyes. This matter is dealt with in some detail on pages 10–16 of this book. However, it should be mentioned here that orange G is a notable example of this class of decolorizing or differentiating dyes. Its action can be seen in the Mallory triple stain for connective tissues. In that technique the red blood corpuscles, stained by the acid fuchsin, become coloured orange by the subsequent application of orange G. It was found accordingly that sections stained by the Falg and the basic Faviol methods, then treated with an aqueous solution of orange G of appropriate strength, were completely decolorized. If, however, the stained specimens are first immersed in a dilute aqueous solution of acetic acid of appropriate strength, then washed with water before the orange G is applied, the strongly erythrophile elements retain their red colour. Thus it is possible to obtain a picture of the strongly erythrophile elements alone. This is called the Falgog method (pages 201–2). It has been

found that the picture of the strongly erythrophile elements obtained by application of the Falgog method is more satisfactory than that produced by simple staining with acid fuchsin. Probably the orange G and glucose combine with and decolorize (reduce) the definite chemical compounds (Falg-tissue) present, thereby producing sharper pictures of the erythrophile elements. *In vitro* experiments have shown that acid fuchsin units through its amino groups with acetic acid to form a series of compounds to which have been given the name of *acetofuchsinic acids*, a number of which have been isolated in the solid form. The acetofuchsinic acids have been found to be of a much brighter red than ordinary acid fuchsin.

Still more recently it has been found (MacConaill & Gurr, 1961) that if acid fuchsin is kept in acetic acid solution for a few weeks it overstains so that the Falg and the Faviol reactions do not take place properly. The reason for this is that the acetic acid units with the acid fuchsin at room temperature over a prolonged period to form nono-acetofuchsinic acid (the highest homologue of the series) which is devoid of reactive basic groups. In other words the amino groups of the acid fuchsin have been blocked by the acetate radical, and the new dye, which is of a more intense red colour than the original acid fuchsin, has no basic groups available to permit union with other acid dyes.

In the Falgose technique, described on pages 202–3, the orange G is dissolved in 1% glucose solution instead of water alone. It was found that this technique gave rather better results than the Falgog method for sections up to 100 μ in thickness. The Falgog procedure is, however, better suited for sections over 100 μ in thickness.

Sun yellow G, an acid dye of the stilbene group, whose structure is given on page 62, has an effect similar to that of orange G on Falg- and Faviol-stained preparations. It is, however, an intensely yellow dye, so that when it is used in place of orange G as the decolorizer, the preparations are actually stained yellow everywhere. Here again the dilute acetic acid protects the acid fuchsin. It also permits the distinctive staining (violet) of moderately erythrophile elements by violamine 3B, whose structure is given on page 75. The blue colour of weakly erythrophile elements, however, is replaced by yellow, so that a three-colour picture is obtained in red, violet and yellow. Since most cellular

TRIFAVIOXANTHIC ACID

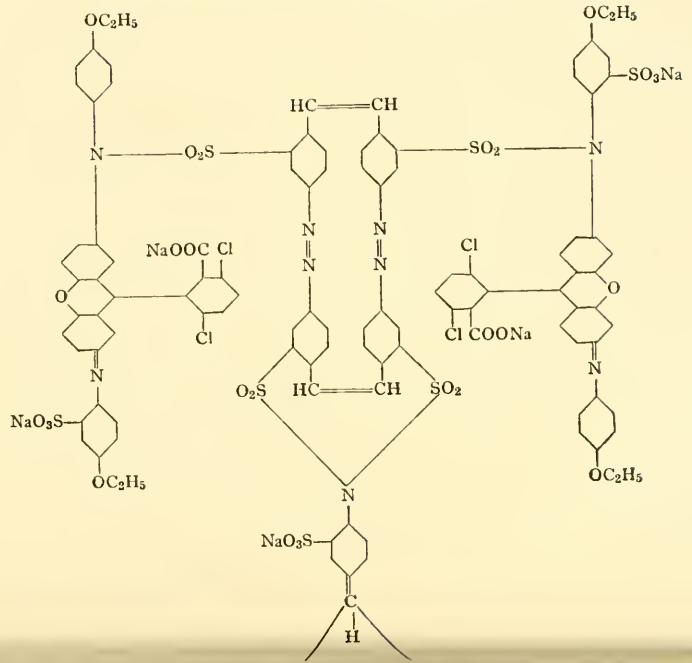
$(C_{320}H_{194}N_{27}O_{83}S_{21}Na_{16}Cl_{12})$

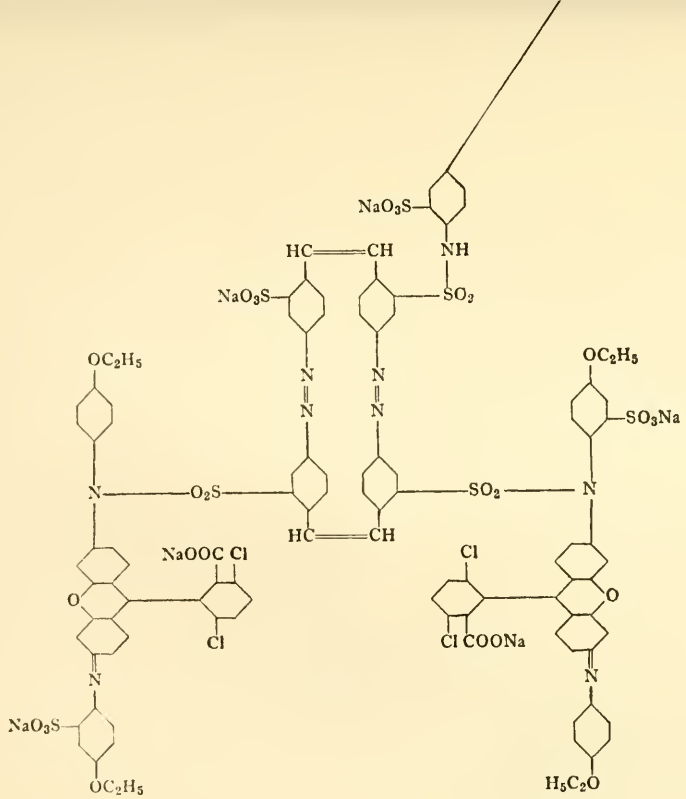
Michrome No. 1096. Molecular weight: 7,212.

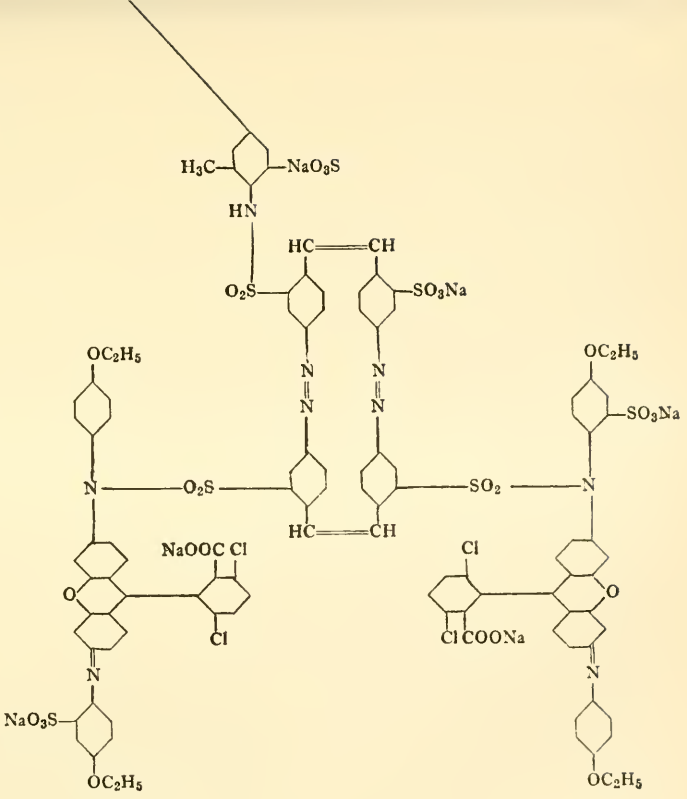
Formed when one molecule of acid fuchsin accepts three molecules of sun yellow and three units (two molecules each) of violamine 3B.

Note: While this is first and foremost a complex acid triphenylmethane dye, it will be noted from the following structure that it also is a sulphonamide, as are the falgic acids, pages 79–81.

Aqueous solutions of this dye, exposed to the atmosphere in an open dish, undergo a remarkable cycle of rapid colour changes, including shades of brown, khaki, red, blue, violet, etc.




NaO3S
HC=CH
NH
NaO3S
SO2
OC2H5
OC2H5
SO3Na
N
N
N
N
N
O2S
SO2
N
HC=CH
NaOOC Cl
Cl
Cl
Cl COONa
O
O
N
N
NaO3S
OC2H5
H5C2O


H3C
NaO3S
HN
HC=CH
O2S
SO3Na
OC2H5
OC2H5
SO3Na
N
N
N
N
N
O2S
SO2
N
HC=CH
NaOOC Cl
Cl
Cl
Cl COONa
O
O
N
N
NaO3S
OC2H5
OC2H5

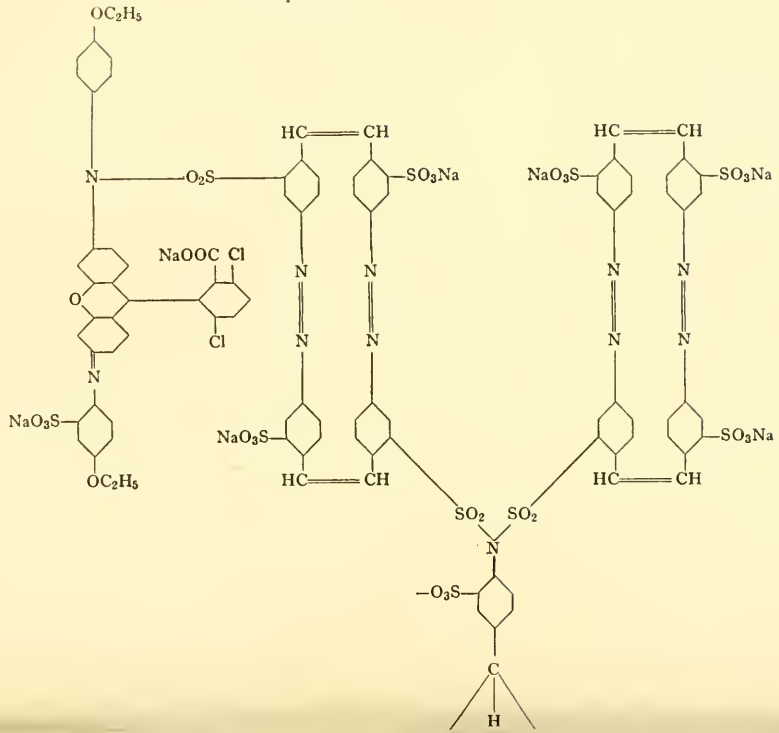
HAPLOFAVIOXANTHIC ACID

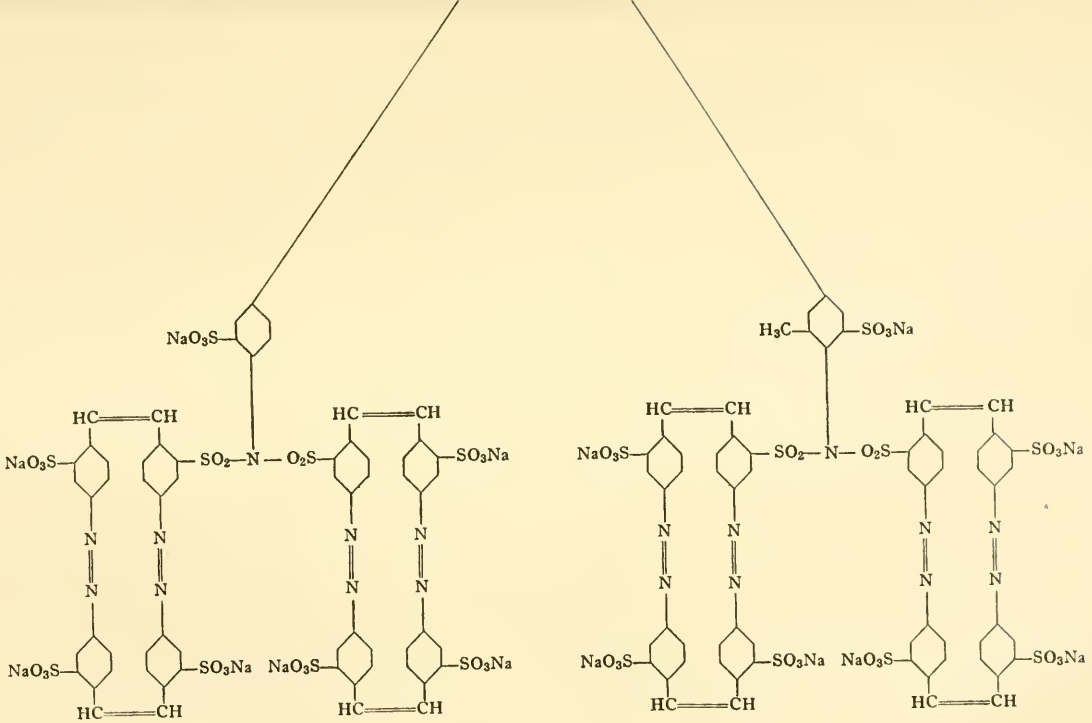
$(C_{224}H_{133}N_{39}O_{82}S_{28}Na_{21}Cl_2)$

Synonyms: Chromosome red (M. A. MacConaill and E. Gurr). Michrome No. 1091. Molecular weight: 6,134.

This dye is formed when one molecule of acid fuchsin accepts six molecules of sun yellow and one-half unit (one molecule) of violamine 3B.

Note: While this is first and foremost an acid triphenylmethane dye, it will be noted from the following structure that it is also a disulphonamide.




NaO3S
HC
CH
SO2
N
O2S
SO3Na
H3C

elements, except in certain glandular tissues, are either weakly erythrophile or erythrophobe, it follows that the red-stained strongly erythrophile tissue-elements are seen against a yellow background. The red colour of acid fuchsin is always made more distinct by the use of a yellow filter; consequently the red colour of the strongly erythrophile elements, notably of the chromosomes, is made more distinct by the use of sun yellow as an adjunct to the Faviol technique. This modified Faviol method is called the Standard Faviol technique. The procedure is described on pages 204–6. It differs from the Basic Faviol technique in two associated respects: (1) the violamine solution is replaced by a mixture of violamine and sun yellow; (2) a protective acid bath is given to the tissue preparations stained by acid fuchsin before they are exposed to the modified violamine solution. The Standard Faviol method is particularly advantageous when black and white photographs have to be taken of the stained preparations. In such photographs the red-stained elements appear as full black, violet as dark grey, and yellow as light grey. Moreover, better pictures are obtained without a colour filter than with one.

During the course of our work (MacConaill & Gurr, 1960*b*, 1961, 1962) it was decided to attempt to prepare *in vitro* the colours which had been produced *in tela*. As a result, not only were these compound dyes successfully isolated in the solid stain, but a number of others in addition. Among such dyes prepared and now available commercially are trifavioxanthic acid and haplofavioxanthic acid, the latter also being known as chromosome red. The structures of these two dyes are given above (pages 86–9). The haplofavioxanthic acid was not one of the compounds that we had produced previously in tissues and its preparation *in vitro* was performed by the present author to satisfy his own curiosity. Initial trials with this new dye carried out by Professor MacConaill appear to indicate that it may have useful application in cytology and medical research. Trifavioxanthic acid appears, at present, to be of little value as a biological stain, although due to its seemingly photosensitivity, oxidation-reduction and possibly hydrogen-ion indicator properties it may find application in biochemistry, etc. Other compound dyes of the series isolated but not yet tried out include mono- and di-favioxanthic acids.

STAINING PROCEDURES

ACRIDINE ORANGE

Fluorescence Microscopy Method for Cytodiagnosis of Cancer and Differential Staining of Nucleic Acids

Used as a fluorchrome at the appropriate pH, acridine orange presents highly polychrome pictures. DNA of the nucleus, for instance, is demonstrated in green or yellow fluorescence, while RNA of the nucleolus and of the cytoplasm fluoresces in reddish brown to orange with increasing concentrations to bright red. Proliferating malignant cells are readily characterized by an RNA content which greatly exceeds that of the non-malignant cells of origin (Brachet, 1950; Chargaff & Davidson, 1955; Pirozynski & L. Von Bertalanffy, 1955; L. Von Bertalanffy & F. D. Bertalanffy, 1960). These cytoplasmic changes are readily observable by the acridine orange fluorescence method.

The method, which was first developed for exfoliative cytology in gynaecology and later applied to the diagnosis of malignancies of the respiratory system, is based upon the metachromatic properties of acridine orange, giving differential staining of the two types of nucleic acids of the cell (L. Von Bertalanffy & I. Bickis, 1956). It is claimed that because of the striking differences between normal and malignant cells shown by this method, malignant cell elements can be recognized even by observers with little cytological training.

F. D. Bertalanffy (1960) states that cytodiagnosis is one of the most readily available and effective means of cancer diagnosis. All that is needed for the diagnosis of the presence or absence of malignant lesions in certain parts of the body, is a small sample of sputum, vaginal smears, or other material collected during routine examination.

The acridine orange method is widely used clinically in Canada and in the United States of America, and the information given here has been compiled almost entirely from reprints and personal communications from the original author, Professor Felix D.

Bertalanffy of the Department of Anatomy, University of Manitoba, Winnipeg, Canada.

Samples of sputum, bronchial excretions, gastric washings, urine, vaginal and curvical aspirates, etc., always contain normal epithelial cells. When malignant tumours arise in epithelial structures, tumour cells likewise desquamate and mingle with normal cells. Bertalanffy (1960) states that in recent years cancer diagnosis by exfoliative cytology has found increasing clinical application both in the form of large-scale screening in hospitals as well as, to a smaller extent, in private practice. However, the private medical practitioner has to rely upon the services of a cytological laboratory to which the exfoliative specimens are submitted, the reason for this being the lack of trained cytologists. Because of the high costs involved in routinely submitting to a cytological laboratory exfoliative material for evaluation, Bertalanffy states, many physicians may forego cytological cancer diagnosis, to the misfortune of the malignant cases that could have been cytologically diagnosed earlier. It is claimed that the acridine orange fluorescence method makes it possible for the physician or his technician, both of whom may be relatively untrained in cytology, to perform pre-screening of routinely collected exfoliative specimens. Only six minutes are required for the preparation of a specimen for examination with the fluorescence microscope, and if an average of three minutes is allowed for screening, a diagnosis may be obtained in less than ten minutes.

In this way, the author states, most normal samples can be discarded, leaving the number of samples to be submitted to a cytological laboratory greatly reduced, and in consequence a proportionate saving in costs of cytodiagnosis is effected.

The high mortality rate in cancer can be effectively lowered, the author states, only by early diagnosis, and this can be effected in two ways:

1. By routine cytodiagnosis by the physician.
2. By mass screening programmes for large proportions of the population.

Whereas conventional cytodiagnostic techniques are based primarily upon morphological criteria, the fluorescence method utilizes cytochemical changes in malignant cells. Because the

latter may precede morphological changes, which themselves may be a consequence of abnormal cytochemistry, the fluorescence method may allow an earlier detection of abnormal changes.

The Staining Technique

Equipment:

1. A high-pressure mercury vapour 200 watt burner (e.g. HBO 200) in a lamp housing.

2. Two blue filters, to be inserted between the light source and the microscope mirror.

3. One (or a combination of two) yellow filter(s) to be placed in the ocular(s) to prevent ultra-violet light from reaching the eyes.

Note: The items listed above are essentially the same as those specified by E. Gurr (1951, 1956), for general fluorescence microscopy.

It might also be mentioned here that an expensive microscope is not necessary. Good results are obtainable in fluorescence microscopy even with a cheap or antiquated microscope so long as it is fitted with objectives up to 1/6-inch focal length and has a suitable ocular. Moreover a completely darkened room is not necessary (E. Gurr, 1960).

Photography:

Photographs can be taken with a 35-mm. camera back, attached to the microscope and using a fast colour film, such as Daylight High Speed Ektochrome or Tungsten Super Anscochrome films. Exposure times range from a few seconds to several minutes, depending on magnification, fluorescence set-up, and length of time that the mercury burner has been in use.

Pre-screening:

Smears are carefully scrutinized field by field to ascertain whether or not they contain cells with orange or red fluorescence. Most normal smears show only cells with greenish, brown, or reddish brown fluorescence, and these samples should be discarded. Cells exhibiting orange or red cytoplasm are to be found

in some smears, and these should be regarded as suspicious and put aside for further evaluation, as " fluorescence suspicious ". Not all these smears will contain malignant, or even cytologically suspicious cells. Cells found to fluoresce in orange or red may prove to be the normal " active " type. Such cells, because of their increased proliferation, contain more RNA and thus fluoresce more intensely than " inactive " cells. During final evaluation, smears showing such cells will be eliminated on the basis of their normal morphology. Material from the female genital tract, body fluids, and urine, is particularly suitable for pre-screening by those who, while not having had extensive training and experience in cytology, have undergone a short course of instruction and have acquired also a reading knowledge of the subject. The pre-screening procedure thus lends itself especially well for the private medical practitioner, reducing the number of smears of some material to be submitted to a cytologist or cytopathological laboratory by 80% or 90%.

It is stressed, however, that pre-screening of respiratory material, oral smears, and gastro-intestinal material should be undertaken only by those who have acquired some considerable experience in pre-screening other material first.

Screening for final diagnosis:

This should be performed only by fully experienced cyto-pathologists or cytologists, or others fully trained and experienced in exfoliative cytology. The final evaluation should not be based upon cytoplasmic fluorescence only, and should not be attempted by individuals not fully competent in exfoliative cytology, as previously stated. The trained cytologist, scanning smears with low magnification (e.g. × 100) regards increased fluorescence of some cells as a warning signal. The final diagnosis is established by consideration of the morphological characteristics of these cells. Increased fluorescence of suspicious and malignant cells, serving as a warning signal, enables the cytologist to evaluate cytological specimens more rapidly. Thus after using the acridine orange fluorescence method, an increase of 100% in output has been reported in some laboratories, according to F. D. Bertalanffy (1960).

Staining Procedure

Solutions required:

A.	Ethyl alcohol (95% or absolute)	1 volume
	Ether	1 volume
B.	Acetic acid 1% aqueous	
C.	Acridine orange (Michrome No. 87)	2 gm.
	Distilled water	100 ml.
*D.	Disodium hydrogen phosphate (Na_2HPO_4)	9·465 gm.
	Distilled water	to 1 litre
*E.	Potassium dihydrogen phosphate (KH_2PO_4)	9·072 gm.
	Distilled water	to 1 litre
*F.	M/15 phosphate buffer pH 6·0 ..	
	Solution D	50 ml.
	Solution E	300 ml.

Note: **The original author (F. D. Bertalanffy, 1960) directs that solution F be checked with a pH meter to pH 6·0. I would suggest, however, that in some laboratories it might be preferable that the M/15 phosphate buffer should readily and simply be prepared as follows:**

G.	5 Michrome buffer tablets pH 6·0	
	Distilled water	500 ml.

Crush the tablets between the folds of a piece of clean paper with the thumb or with a coin, then dissolve the powder by shaking with the water. This not only obviates the preparation of solutions D, E, and F, but also the necessity for checking the pH with a pH meter.

H.	Solution C	0·5 ml.
	Solution F or G	99·5 ml.
I.	Calcium chloride ($CaCl_2$) ..	100 gm.
	Distilled water	to 1 litre
J.	Solution I	111 ml.
	Distilled water	889 ml.

Technique:

1. Fix smears immediately, while still wet, in solution A for at least five to fifteen minutes.

Note: **The smears may remain in the fixative indefinitely at room temperature.**

2. Rinse for about ten seconds in 80% alcohol.
3. Transfer to 70% alcohol for about ten seconds.
4. Transfer to 50% alcohol for about ten seconds.
5. Rinse in distilled water for about ten seconds.
6. Immerse in solution B (1% acetic acid) for ten seconds to one minute.

Note: **This is to prevent rapid fading of fluorescence.**

7. Rinse in distilled water for ten to fifteen seconds.
8. Stain in the buffered acridine orange (solution H) for three minutes or a little longer.
9. Immerse directly into phosphate buffer (solution F or G) for at least one minute to remove excess dye.

Note: **The smears may be left in the buffer solution for several hours in cases where it is desired to re-examine them later, or where preparations are processed in batches and screened successively.**

10. Differentiate for one to two minutes, or longer if found necessary, with solution J (i.e. M/10 calcium chloride), controlling by examination under the microscope while the smear is still wet, until the nuclei, especially of granulocytes, fluoresce in bright translucent green.

Note: **The calcium chloride solution promotes differentiation between RNA and DNA.**

11. Rinse with phosphate buffer (solution F or G).
12. Mount with a coverslip placed over a drop of the buffer solution, and examine with the fluorescence micrsocope.

Note: **Permanent preparations can be made by mounting in Uvak (Edward Gurr), which is an aqueous non-fluorescent mountant.**

Results:

Normal cells from the tracheal, bronchial and bronchiolar epithelium (celiated columnar cells, goblet cells, and basal cells), and pulmonary macrophages (alveolar cells) show brown to dull reddish-brown cytoplasm. Squamous cells from the oesophageal

and oral epithelia (present in sputum), exhibit brown or green cytoplasm, depending whether they originate from the basal or the superficial layers of the stratified epithelium.

In contrast, malignant cells fluoresce conspicuously in bright orange to red.*

On account of their distinct and very conspicuous fluorescence, malignant cells in sputum, bronchial secretions and pleural fluids are readily recognizable even with magnifications as low as × 100.

Malignant cells, whether they occur in the respiratory, digestive, urinary or female genital tract, body fluid, or other material are characterized by bright orange to flaming red fluorescence of the cytoplasm, and often by greenish-yellow hyperchromatic nuclei.

Readers should consult the original papers (F. D. Bertalanffy, 1960*a*, 1960*b*; L. von Bertalanffy & F. D. Bertalanffy, 1960) for further information and colour plates.

Notes:

(*a*) After screening, smears may be destained in 50% alcohol and reprocessed for any conventional procedure. They may then be returned to the fixative, which also removes the acridine orange, to be restained later with that dye if desired; or they may be dried and stored.

(*b*) It is stated in the original paper that the acridine orange fluorescence method has been tested in 25,000 cases in which there was a high rate of malignancy of the genital tract. Specimens consisted of screening material from asymptomatic women in the general population, routine samples from the gynaecologist, and exfoliated material of all kinds.

(*c*) It should be noted that *Trichomonas vaginalis* organisms have brownish-red cytoplasm and small, yellow nuclei. The mycelia and spores of *Monilia albicans* fluoresce bright red, while bacteria appear red to reddish brown.

(*d*) As already stated, when acridine orange is applied to exfoliated cells according to the procedure described above, DNA of the nucleus fluoresces in green, while RNA of the cytoplasm fluoresces in brown, reddish-brown, orange, or bright red, depending upon whether relatively small or relatively large

* But not all cells that fluoresce in these colours are necessarily malignant. Bright orange or red fluorescent cytoplasm is indicative of a high content of RNA, and this occurs in normal " active " cells (see page 96).

amounts of RNA are present. This specific histochemical reaction, the author states, is a manifestation of the fact that RNA is closely associated with protein synthesis of the cell. In this connection I would suggest that a more recent paper (I. Leslie, 1961), on the biochemistry of heredity, which treats the relationships between DNA, RNA and histones, may be of interest to readers.

(*e*) Instead of the alcohol–ether mixture (solution A) absolute alcohol (or 95% alcohol) may be used alone as the fixative. Formaldehyde should be avoided.

(*f*) The whole procedure from fixation (step 1 to step 11) is best carried out in Coplin jars.

(*g*) If the pH of the staining solution is shifted too far into the acid range, gradual loss of cytoplasmic fluorescence occurs. A shift into the alkaline range results in overstained cells. This, however, may be desirable, especially for pre-screening by those with little knowledge of cytology. In such cases the buffer solution used may have a pH of 7·0, but no higher, to accentuate cytoplasmic fluorescence of suspicious cells.

Note: Buffer tablets having a pH of 7·0 are also available for this purpose.

(*h*) The author (F. D. Bertalanffy, 1960) emphasizes that the acridine orange should be of good quality. A good indication for this and also for a proper working technique is when after differentiating the smears, the nuclei of the leucocytes, superficial squamous cells, etc., show clear, translucent green colour, and the cytoplasm, depending upon the cell type, appears in clear brown, reddish brown, or orange or red fluorescence. Some preparations of acridine orange available are considered unsuitable for the fluorescence method. These, due to contained-impurities, stain nuclei in ochre yellow and the cytoplasm in hazy brownish and reddish hues.

(*i*) If a very rapid diagnosis is necessary smears may be passed immediately into 80% alcohol and followed by distilled water, then processed in accordance with steps 6–12, described on page 98.

(*j*) Smears should not be blotted as this may result in the removal of cells from the specimen.

(*k*) Erythrocytes do not appear to fluoresce. This may be due to the iron content of the haemoglobin: heavy metals exert a quenching effect on fluorescence (E. Gurr, 1951*b*, 1953, 1956).

Haemorrhagic smears, therefore, present no obstacle in this technique because erythrocytes appear transparent and one is able, therefore, to " see through " them as if they were not present.

(*l*) The screening time for the experienced technician is about three minutes per slide. Relatively inexperienced workers may require five minutes or even longer. The screening time also depends upon the size of the coverslip and the magnification used. Thus, using the normal procedure including fixation, a diagnosis can be made within about twenty minutes after a sample has been collected. Only ten minutes or less is required for the rapid procedure, however.

References:
Bertalanffy, F. D. (1960*a*, 1960*b*).
Bertalanffy, L. von & Bertalanffy, F. D. (1960).

(*m*) The following general notes on fluorescence microscopy are given by E. Gurr (1951*b*, 1953, 1956):

Fluorescence is the property possessed by many substances of converting short wavelengths of light into longer wavelengths. In microscopy the substances and structures of most interest are those which convert ultraviolet light into light of the visible spectrum, as only substances of this character can be observed directly. It is, of course, well known that most living organisms are profoundly affected by short light waves, and a great deal of information as to their structure has been obtained by the study of the appearance of these organisms under the influence of invisible light rays. If individual cells or structural units are examined before, during, and after ultraviolet treatment, enough of this effect should be discovered to impart some understanding as to the changes which occur in the animal, or plant, as a whole.

In fluorescence microscopy structural details are rendered visible by: (*a*) *innate auto-fluorescence*, a property possessed by most tissues which when excited by short light waves become clearly visible since they become luminous and glow or " fluoresce " with a radiance of their own, or by (*b*) *secondary fluorescence* which is known as " fluorochromy " and is brought about by the process of treatment of the tissues with fluorescent dyes and certain alkaloids (e.g. Berberine sulphate) and other substances.

It is proposed to deal only with fluorochromy in the short space of this chapter.

Fluorescent dyes and other substances used for this purpose are known collectively as "fluorochromes"; these materials are selectively absorbed by certain parts of the cell. When tissues, bacteria or protozoa, which have undergone treatment with fluorochromes are examined under the microscope, using ultraviolet light instead of transmitted light of the visible spectrum, they become visible as bright luminous objects against a dark background. Cells stained with fluorochromes absorb the ultraviolet rays of short wavelength, and emit this energy in the form of fluorescent light in the visible spectrum. Basic and acidic fluorochromes act specifically to stain certain cellular structures as do the more common microscopic stains such as, for instance, methylene blue and eosin. The colour and the intensity of fluorescence depends on the relative basophilia and acidophilia of the individual cells, and upon the nature of the particular fluorochrome.

Fluorochromy may be employed with advantage in the study of living organisms: for instance, uranin, a non-toxic stain, may be injected into mice and frogs and the living organs can be studied without interfering with their functioning. Fluorochromy is also of practical importance for the demonstration of diphtheria bacilli, tubercle and leprobacilli, malaria, etc., as well as for virus research.

Contrary to general belief, the apparatus required for fluorescence microscopy is fairly simple and inexpensive. (A special microscope is not required.) The following are sufficient.

1. B.T.H. Mazda Mercury Vapour Lamp (box type) ME 250 w/50/5 (or a similar lamp as stipulated by Bertalanffy, above).

2. A simple convex lens to project the image of the lightsource through a suitable blue filter to the microscope mirror. The lens and light source should be encased with a black hood to prevent scattering of the rays.

3. A yellow filter which is placed in the oculars of the microscope to prevent any harmful effect of ultraviolet light to the microscopist's eyes. For this purpose Ilford's minus blue Micro 4 is recommended.

It is, of course, essential that fluorescence microscopical examinations must be carried out in a darkened room to be successful. It has been stated that microscope slides of special glass are

necessary for fluorescence microscopy, but provided they are not more than 1·3 mm. in thickness ordinary microscopic slides have been found quite satisfactory.

Notes on fluorochromic staining technique:

(*a*) Fixatives containing salts of heavy metals (with the exception of zinc); chlorine, bromine, iodine, and nitro compounds should be avoided as these exert a quenching effect on fluorescence. The most suitable fixatives are 5 to 10% formalin, or Kahle's fluid.

(*b*) If tissues are embedded in paraffin wax, all traces of the wax, which is auto-fluorescent, must be removed before sections are stained and examined.

(*c*) A special grade of immersion oil known as fluoroil (E. Gurr, 1951), which is non-fluorescent, should be used for high-power examination, since cedarwood oil and most of the immersion oils available for ordinary microscopy are unsuitable for fluorescence work.

(*d*) The usual mounting media, as used for ordinary microscopy, contain highly fluorescent materials which render them unsuitable for fluorescence work, and should, therefore, be avoided. For temporary mounts, glycerine may be used, and for permanent mounts there is a satisfactory xylol miscible medium on the market under the name of *Fluormount* (E. Gurr, 1951*b*). However, if the fluorochrome used is removed from the tissues by alcohols, dehydration has to be avoided, in which case permanent preparations can be made by mounting direct from water in Uvak which is a moderately quick-drying, aqueous, non-fluorescent mountant. The coverslips can be secured firmly immediately after mounting by the application of Laktoseal.

Fluorochromes, of which auramine O, coriphosphine, acridine yellow H107, aesculine, acridine orange, primuline, thiazole yellow are examples, are frequently used in very dilute solutions to produce characteristic fluorescent colours, and when preparations which have been treated with these solutions are examined in transmitted light of the visible spectrum, they appear to be unstained or only very faintly tinted.

Some explanation as to the colour differentiation obtained by the use of general-purpose fluorochrome of which acridine yellow H107 is an example, is offered by the fact that fluorescence

colour is effected by hydrogen-ion concentration, and as fluorochromes also exhibit differential absorption by various tissues, the production of a great variety of fluorescent colours is brought about by the influence of these two factors. Nuclei can be differentiated from cytoplasm by the use of any one of the following general fluorochromes: acridine yellow H107, coriphosphine, phosphine 3R, acridine orange or acriflavine.

Titan yellow, rhodamine B, phosphine 3R, methylene blue are all excellent fluorochromes for fat, while berberine sulphate is much used for protozoal parasites, particularly for malaria. Thioflavine has been found satisfactory for virus and for bacteria, as well as for general fluorochromic staining, while acriflavine has been used for trypanosomes and as a general stain, and uranin is one of the most suitable fluorochromes for intravital staining. All these fluorochromes are used in very dilute aqueous solutions, that is to say, something to the order of 0·1 to 0·01 per cent.

Intravital Staining with Fluorochromes

Dyes used for this purpose must be water soluble, non-diffusible in the living body, non-toxic in the workable dilutions required, and highly fluorescent even in greatly diluted solutions. Uranin possesses all these qualifications and is one of the most useful fluorescent dyes for intravital work, as stated earlier in this chapter. Acriflavine is another useful dye for this purpose, although it is not so intensely fluorescent as uranin. The fluorescence of uranin is impaired in basic solution so that it appears most readily in organs of an acid reaction. It is used in 0·1% solution in physiological saline, in which form it should be injected into the animal's blood stream or into the organ to be studied. The colour of its fluorescence varies with hydrogen-ion changes and consequently it is of great value as an intravital hydrogen-ion indicator. The colour changes are easily visible in dilutions to the order of one part in ten millions and in dilute solutions the intensity of the fluorescence has a definite relation to the concentration of the dye and consequently the intensity of the fluorescence serves as an indicator of the amount of uranin present. Primulin, thioflavine, rhodamine B and berberine sulphate have also been found useful for intravital work.

ACRIDINE ORANGE–MASSON TRICHROME STAIN

A selective fluorescence stain for mucin

Solutions required:

A.	Iron alum	5% aqueous
B.	Regaud's haematoxylin	
C.	Picric acid, saturated in 95% alcohol	20 ml.
	Alcohol 95%	10 ml.
D.	Ponceau fuchsin	
E.	Phosphomolybdic acid	1% aqueous
F.	Aniline blue, water soluble ..	2·5 gm.
	Glacial acetic acid	1 ml.
	Distilled water	49 ml.
G.	Acridine orange, 0·1% in distilled water	

Technique:

1. Fix pieces of tissue in Bouin's fluid for three days, or in Regaud's fluid for one day.
2. Embed in paraffin wax as usual and cut sections at 5 μ.
3. Attach sections to slides and take down to distilled water in the usual manner.
4. Mordant in the iron alum (solution A) for five minutes at 45° C to 50° C.
5. Wash well in several changes of distilled water.
6. Stain for five minutes in Regaud's haematoxylin (solution B), for five minutes at 45° C to 50° C.
7. Rinse in distilled water.
8. Differentiate in picric alcohol (solution C), controlling by examination under the microscope, while the preparation is still wet.
9. Wash in running tap water for a minute or so.
10. Stain for five minutes in ponceau fuchsin (solution D).
11. Rinse in distilled water.
12. Differentiate in the phosphomolybdic acid (solution E) for five minutes.

13. Add 0·5 ml. of acetic aniline blue (solution F) to the phosphomolybdic acid on the slide and mix by gently rocking the slide. Allow this mixture to act for five minutes.
14. Pour off excess liquid.
15. Rinse in distilled water.
16. Immerse in acridine orange (solution G) for two minutes.
17. Wash briefly in water.
18. Mount in Uvak.
19. Examine in a blue light, with a yellow eyepiece filter.

Results:

A yellow fluorescence of remarkable brilliance is observable in the intracellular mucus, while other structures of the mucosa and of the bowel wall are barely distinguishable, in dull greenish colour.

Notes:

(*a*) The authors applied acridine orange solution to an old section, after removing the coverslip and mountant, of bowel already stained by one of the Masson trichrome methods. The result, described above, was totally unexpected, since Masson trichrome stains do not attach themselves to mucus.

(*b*) In their previous work (Hicks & Matthaei, 1955), tissues had been treated with acridine orange (without Masson trichrome stain) and the authors had found that connective tissue, muscles, nuclei and other structures fluoresced strongly, but mucin showed an almost insignificant brown. A series of experiments then undertaken pointed to the fact that the presence of iron inhibits or quenches the normal production of fluorescence on staining with acridine orange in practically all tissue components except mucus, and this substance is permitted to develop its normal fluorescence in the more concentrated solution of this dye used in the present technique.

Note: In their previous work the authors used a considerably weaker (0·01%) solution of the dye.

(*c*) The original paper, which carries six colour photomicrographs, should be consulted for further information.

Reference: Hicks, J. D. & Matthaei, E. (1958).

ALCIAN BLUE

For acid mucopolysaccharides

Solutions required:

A. Alcian blue 1% aqueous
B. Haemalum

Technique:

1. Fix material in Bouin and embed in paraffin wax.
2. Fix sections to slides and carry through to distilled water as usual.
3. Stain for fifteen to forty-five minutes in the alcian blue solution.
4. Rinse in distilled water.
5. Stain in the haemalum solution for five to ten minutes.
6. Counterstain if desired.
7. Dehydrate through the usual graded alcohols.
8. Clear in xylol and mount in D.P.X. or Clearmount.

Results:

Acid mucopolysaccharides are stained blue-green, while nuclei are dark blue.

Notes:

(*a*) Alcian blue stains mucin clearly and conspicuously, combining with it in such a way that additional stains produce little, if any, alteration in colour.

(*b*) All common histological reagents will not remove alcian blue from mucin: only prolonged treatment with acid alcohol will reduce the colour of an overstained section. Once applied, the dye will resist indefinitely water, alcohols, alkalis, and hydrocarbons.

(*c*) Alcian blue will not distinguish chondroitin sulphuric acid complexes from mucoitin sulphuric acid: the dye, therefore, stains cartilage and mucin equally.

(*d*) A crystal or thymol or a few drops of chloroform should be kept in the alcian blue solution to inhibit the growth of air-borne micro-organisms: it is best to prepare the solution in small quantities as required for a day's use, however.

Reference: Steedman, H. E. (1950).

ALCIAN BLUE - CHLORANTINE FAST RED

For selective staining of mucopolysaccharides and for morphological studies of connective tissue, cartilage and bone

Solutions required:

A. Ehrlich haematoxylin

B. Alcian blue 1% aqueous .. .. 50 ml.
Acetic acid 1%, aqueous .. .. 50 ml.

C. Phosphomolybdic acid 1% aqueous

D. Chlorantine fast red 0·5%, aqueous

Technique:

1. Fix tissues in Bouin or in 10% formalin and embed in the usual way.

2. Stain sections in Ehrlich haematoxylin for ten to fifteen minutes.

3. Blue in tap water or in lithium carbonate solution.

4. Wash in distilled water.

5. Stain for ten minutes in the Alcian blue.

6. Wash in distilled water.

7. Immerse in the phosphomolybdic acid solution for ten minutes.

8. Wash in distilled water.

9. Stain for ten minutes in the chlorantine fast red solution.

10. Wash in distilled water.

11. Dehydrate; clear in xylol and mount.

Results:

Nuclei are stained purplish blue. Mucin, granules of mast cells, ground substance of cartilage and some types of connective tissue fibres: bluish green. Collagen fibres and ossein: cherry red. Cytoplasm and muscle: pale yellow.

Reference: Lison, L. (1954).

ALDEHYDE FUCHSIN

(after G. Gomori, 1950)

For elastic tissue, mast cells, beta cells of the pancreatic islets, etc.

Solutions required:

A.	Alcohol 70%	100 ml.
	Iodine crystals	0·5 gm.
B.	Sodium thiosulphate	0·75 gm.
	Alcohol 96%	10 ml.
	Distilled water	90 ml.
C.	Basic fuchsin	0·5 gm.
	Alcohol 70%	100 ml.
	Hydrochloric acid, conc.	1 ml.
	Paraldehyde	1 ml.

Dissolve the basic fuchsin in the alcohol; to the cold solution add the acid and the paraldehyde; shake well; then leave to stand for about twenty-four hours until the colour of the solution has changed to violet (almost indistinguishable in appearance from gentian violet). As soon as this change has taken place the stain is ready for use.

Note: The solution will keep for about four weeks at room temperature, but as the stain ages during the four weeks, longer staining times are necessary.

Technique:

1. Tissues may be fixed in almost any fixative, but those containing dichromate are not recommended as they adversely effect the clearness of the final picture.

2. Embed in paraffin wax for preference: if celloidin is used, the celloidin must be removed completely from the sections before staining as it is impervious to the stain.

3. Fix sections to slides and remove paraffin wax in the usual way.

4. Wash with absolute alcohol followed by 90% alcohol.

5. Immerse in solution A in a stoppered jar for ten minutes to one hour.

Note: This treatment with iodine is recommended for all tissues, whether they have been fixed in mercurial fixatives or not, as it often shortens the staining time necessary and makes the shade deeper.

6. Wash well with water.

7. Immerse in solution B for about one half to two minutes until the natural colour of the section has been restored.

8. Wash well with water.

9. Stain for five minutes to two hours in a coplin jar filled with solution C.

Note: Elastic fibres, five to ten minutes. Beta cells, fifteen to thirty minutes, or longer. Pituitary, thirty minutes to two hours.

10. At intervals examine the slide, after rinsing with 90% alcohol, under the microscope to ascertain the depth of staining, but taking care that the preparation is not allowed to dry. If the desired depth of staining has not been attained, the slide may be returned to the stain and rinsed with alcohol again before further examination: this process may be repeated any number of times until the desired degree of staining has been reached.

11. If desired a counterstain may now be applied: haematoxylin - Orange G is best for most purposes, but for pancreas and pituitary, a trichrome stain of the Masson type or the Mallory-Heidenhain technique can be used to bring out all types of cells. In either case, Light Green or Fast Green, FCF should be used in place of the aniline blue as their shades contrast better with the purple of the aldehyde fuchsin.

12. Dehydrate with absolute alcohol; clear in xylol and mount.

Results:

The following are stained deep purple:

(I) Elastic fibres of all tissues, whatever fixative has been used.

(II) Mast cells, after any fixative.

(III) The chief cells of the gastric mucosa, particularly well stained after fixation in formalin or Bouin.

(IV) Beta cells of the pancreatic islets of all species, after formalin, mercuric chloride-formalin or Bouin. Particularly beautiful results are obtained in the islets of man and the sheep (the beta cells of the latter are particularly difficult to stain otherwise).

(V) Functioning tumours of the islets are also stained selectively.

(VI) Certain basophils of the anterior pituitary, after the same fixatives as in (V). In the pituitary of the rat and of the pig the two kinds of basophils are usually quite conspicuous.

Notes:

(*a*) After fixatives containing mercury the background is pale mauve. After formalin or Bouin the background is colourless.

(*b*) If the stain is prepared by adding paraldehyde to Feulgen's fuchsin the resultant solution will stain the beta cells very distinctly but leave the elastic fibres unstained. Old solutions of the aldehyde fuchsin will stain elastic fibres very selectively, but leave the beta cells unstained.

Reference: Gomori, G. (1950*b*).

ALDEHYDE - FUCHSIN

A simplified method for neurosecretory cells

Solutions required:

A. Gomori's fluid.

B. Sodium metabisulphite .. 2·5 gm.
Distilled water .. .. .. 100 ml.

C. *Gabe's aldehyde fuchsin, modified*
Basic fuchsin .. .. .. 1 gm.
Distilled water .. .. .. 200 ml.

Heat the water to about 50° C in a flask then add the dye. Heat to boiling point, then allow to boil for one minute. Allow to cool to room temperature; then add:

HCl, conc. .. .. .. 2 ml.
Paraldehyde .. .. .. 2 ml.

Plug the neck of the flask with non-absorbent cotton wool and leave it to stand for about four days, until the red colour of the fuchsin has disappeared and no further precipitate has been thrown down.

Filter off the precipitate and discard the liquid. Wash the precipitate on the filter; drain; then dry the filter paper with the precipitate still on it in the oven, at a temperature not exceeding 80° C. Remove the dried precipitated aldehyde-fuchsin from the filter paper and store it in a reagent bottle.

To prepare the staining solution:

Aldehyde-fuchsin, prepared as above	0·25 gm.
Absolute alcohol	35 ml.
Distilled water	15 ml.

Note: This solution will keep for at least six months.

D. Halmi's mixture (as page 349, solution G)	
E. Alcohol, 96%	25 ml.
Acetic acid, glacial	1 drop

Technique:

1. Attach paraffin sections to slides, and dewax with xylol.
2. Hydrate through the usual graded alcohols.
3. Oxidize in solution A (Gomori's fluid), for one minute.
4. Rinse in solution B until all permanganate stain has been removed.
5. Wash in distilled water.
6. Wash with 30% alcohol.
7. Wash with 70% alcohol.
8. Immerse in aldehyde-fuchsin stain (solution C), for two to ten minutes, in a closed dish or tube.
9. Immediately wipe the back of the slide and rinse in 95% alcohol.
10. Immerse the slide in a second lot of 95% alcohol for two to five minutes, until no more aldehyde-fuchsin comes out of the section.
11. Wash in 70% alcohol.
12. Rinse in 30% alcohol.
13. Rinse in water.
14. Counterstain in Halmi's mixture for twenty to thirty seconds.
15. Wipe the back of the slide, then differentiate in solution E until no more colour comes out of the section.
16. Rinse in 95% alcohol.

17. Dehydrate with absolute alcohol.

18. Mount in Michrome mountant or:

19. Treat with a second lot of absolute alcohol.

20. Clear in xylol.

21. Mount in D.P.X., Clearmount, Emexel or Michrome mountant.

Notes:

(*a*) The method incorporates some of Gabe's (Gabe, 1953) modifications of Gomori's aldehyde-fuchsin (Gomori, 1950) together with the counterstain of Halmi (1952), and the authors state that it has been used in their laboratory for two years with complete success.

(*b*) The authors state that it was found unnecessary to acidify the aldehyde-fuchsin staining solution with acetic as in Gabe's modification of Gomori, since in the staining of neurosecretory material no difference could be seen between sections stained with and without the acid.

(*c*) For more detailed information, reference should be made to the original paper.

Reference: Cameron, M.D. & Steele, J. E. (1959).

ALDEHYDE FUCHSIN-LUXOL FAST YELLOW-PONTACYL BLUE BLACK

For the differential staining of elastic fibres, collagen fibres and mucin

Solutions required:

A. Gomori's aldehyde fuchsin

Basic fuchsin	0·5 gm.
Alcohol, 70%	100 ml.

Dissolve; then add:

Hydrochloric acid, conc.	1 ml.
Paraldehyde	1 ml.

Shake well; then plug the flash with cotton wool and allow the preparation to stand overnight at

room temperature. Next morning shake with about 1 gm. of decolorizing charcoal before filtering. Store in a refrigerator.

B.	Pontacyl blue black SX	1 gm.
	Water	100 ml.
C.	Potassium dichromate	2 gm.
	Distilled water	100 ml.
D.	Solution B	30 ml.
	Solution C	10 ml.
E.	Fast yellow TN	2 gm.
	Alcohol, 95%	100 ml.

Technique:

1. Fix pieces of tissue in Bouin or Zenker or formalin.
2. Embed in paraffin wax as usual.
3. Fix sections to slides and remove paraffin wax with xylol.
4. Wash in absolute alcohol.
5. Wash in 90% alcohol.
6. Wash in 70% alcohol.
7. Treat for the removal of mercurial deposits if a fixative containing mercury has been used.
8. Rinse in 70% alcohol.
9. Immerse in Gomori's aldehyde fuchsin (solution A) for thirty minutes.
10. Rinse well in 70% alcohol.
11. Rinse in distilled water.
12. Immerse in the pontacyl blue black SX-bichromate (solution D) for fifteen minutes.
13. Rinse in tap water.
14. Differentiate by dipping the slide into 70% alcohol until no more blue colour runs out of the section.
15. Immerse in the fast yellow TN (solution E) for five minutes.
16. Rinse in 95% alcohol.
17. Dehydrate in absolute alcohol.
18. Clear in xylol.
19. Mount in Clearmount or D.P.X. or Emexel.

Results:

Elastic fibres: deep red. Collagen: brilliant yellow. Mucin of goblet cells: reddish purple. Cytoplasmic granules in cells of

mucus-secreting glands of the respiratory tract: reddish purple. The nuclei of all cells are stained blue green, while the cytoplasm is yellow to green depending upon the cell. Cartilage: deep purple. Decalcified bone: green. Striated muscle: green and the banding very distinct. Smooth muscle cells: yellow with a greenish tinge. Nerve trunks: yellow.

Notes:

(*a*) The cells of lymph nodes are particularly well stained, and Sharpey's fibres are shown up quite satisfactorily and can usually be traced from the bone through the periosteum. The components of the walls of blood vessels are distinctly delineated.

(*b*) The durability of the stain, according to the authors, appears to be of a high degree, since there was no fading of any of the dyes in even the oldest slide which had been on hand for seven months.

(*c*) The authors state that both Ehrlich and Harris haematoxylin were used in place of pontacyl blue black and both gave good results. Celestin blue was also substituted for the nuclear stain with quite good results.

(*d*) The authors observe that a great deal of effort has been devoted, since staining with synthetic dyes was introduced in the last century, to devising multiple staining techniques which will differentially colour a variety of tissues. The most successful and the best known of the many excellent procedures, the authors state, are those of Mallory or Masson and of Kornhauser. In all of these and in their many variants, either water soluble aniline blue or fast green FCF was used as the stain for collagen.

The authors had previously* tested a group of dyes not previously used in biology, and one of these (luxol fast yellow TN) in alcoholic solution stained both collagen and cytoplasm a brilliant yellow. An attempt was then made to devise a new trichrome staining procedure by combining this yellow dye with a series of other dyes of different colours, as a result of which the technique described above was evolved.

(*e*) For further information and photomicrographs the original paper should be consulted.

Reference: Green J. A. & Wood, Mary L. (1959).

* *See* pages 381–4.

ALDEHYDE FUCHSIN - HAEMATOXYLIN LIGHT GREEN - ORANGE G - CHROMOTROPE 2R

For the differentiation of two types of Basophils in the Adenohypophysis of the rat and the mouse

Solutions required:

A. Bouin's fixative with the acetic acid replaced by 0·5% trichloracetic acid.

B. Lugol's Iodine

C. Sodium thiosulphate 5% aqueous

D. *Aldehyde-fuchsin*

Basic fuchsin	0·5 gm.
Absolute alcohol	60 ml.
Distilled water	40 ml.
Paraldehyde	1 ml.
Hydrochloric acid, conc.	1·5 ml.

Note: The solution turns purple in 24 hours, and is ripe and ready for use after being kept at 20° C. for three days or in two days if kept at about 37° C. The stain deteriorates after four or five days.

E. Ehrlich Haematoxylin

F.

Ethyl alcohol 70%	99·5 ml.
Hydrochloric acid, conc.	0·5 ml.

G. Lithium carbonate, saturated aqueous

H.

Orange G 2% aqueous	50 ml.
Light Green SF 1% aqueous ..	20 ml.
Distilled water	30 ml.
Chromotrope 2R	0·5 gm.
Phosphotungstic acid	0·5 gm.
Glacial acetic acid	1 ml.

Dissolve the phosphotungstic acid in the distilled water, then add and dissolve the chromotrope 2R followed by the acetic acid,

Orange G and Light green solutions. Shake thoroughly.

Note: This solution keeps indefinitely.

I. Acetic Acid 0·2%

Technique:

1. Fix in solution A for 24 hours.

2. Wash in running tap water for six to eight hours.

3. Dehydrate, clear and embed in paraffin wax in the usual manner.

4. Cut sections, in the horizontal plane, 3 to 4μ in thickness.

5. Remove paraffin wax from sections with xylol.

6. Pass through the usual descending grades of alcohol to distilled water.

7. Immerse in Lugol's iodine for thirty minutes.

8. Transfer to the sodium thiosulphate solution until the sections have regained their natural colour (about two minutes).

9. Rinse thoroughly in distilled water.

10. Stain in the aldehyde-fuchsin solution from two to ten minutes, taking care not to overstain, which can be avoided by checking the slides (after rinsing in 95% alcohol) at intervals under the microscope: the staining should be stopped as soon as the beta cells stand out clearly in dark purple against a colourless or faintly purple background.

11. Rinse in two changes of 95% alcohol.

12. Immerse for five to ten minutes in a third change of 95% alcohol.

13. Rinse in 70% alcohol.

14. Rinse in distilled water.

15. Stain in Ehrlich Haematoxylin for three to four minutes.

16. Rinse in distilled water.

17. Differentiate by dipping three or four times in the acid alcohol solution F.

18. Blue in the lithium carbonate solution; or in running tap water for five to ten minutes.

19. Counterstain in the light green-orange-chromotrope (solution H) for 45 seconds.

20. Rinse quickly with 0·2% acetic acid.

21. Rinse in 95% alcohol.

22. Immerse for two minutes in each of two changes of absolute alcohol.

23. Blot slides carefully.

24. Immerse in xylol for two minutes.

25. Immerse in another lot of xylol for five minutes.

26. Mount in D.P.X. or Clearmount.

Results:

Granulation of beta cells, selectively stained dark purple with the aldehyde-fuchsin. The cells of the pas intermedia and the Herring bodies of the neutral lobe should have little or no affinity for aldehyde-fuchsin. The delta cells are stained green, and the acidophilic granules varying shades of orange. Nuclear chromatin, purplish brown to reddish brown. Nucleoli are tinged bright red, by the chromotrope 2R. The non-granular cytoplasm, greyish green or unstained. Coagulated contents of the cytoplasmic vacuoles, orange.

Reference: Halmi, Nicholas S. (1952).

ALDEHYDE FUCHSIN - PERIODIC ACID - SCHIFF' COMBINED

For staining the pituitary

Solutions required:

A.	Mercuric chloride	25 gm.
	Distilled water	500 ml.
B.	Solution A	100 ml.
	Chrome alum	5 gm.
	Formalin (formaldehyde 40%) . .	5 ml.

Note: Solution B should be made up freshly in quantities sufficient for the day's use.

C. Iodine crystals, A.R. .. .. 1 gm.
Potassium iodide, A.R. .. .. 2 gm.
Distilled water .. .. .. 100 ml.

Dissolve the potassium iodide in about 5 ml. of distilled water, then shake in the iodine until dissolved; add the rest of the distilled water and shake thoroughly.

D. Basic fuchsin .. .. .. 10 gm.
Absolute alcohol .. .. 100 ml.

Place in a 250 ml. flask and plug the neck with non-absorbent cotton wool.

Heat on a water bath to boiling point.

Allow to cool to room temperature; then filter.

E. Solution D .. .. .. 5 ml.
Absolute alcohol .. .. 65 ml.
Distilled water .. .. .. 30 ml.
Paraldehyde .. .. .. 0·75 ml.
HCl, conc. .. .. .. 1·25 ml.

Solution E can be prepared in the stoppered bottle in which it is to be stored. After mixing, the solution should be left to stand for three days at room temperature by which time the reaction will have been completed and the red colour of the fuchsin will have disappeared, marking the completion of the reaction. Alternatively, the reaction takes place in twenty-six hours if the solution is kept at 37° C in an incubator.

F. Periodic acid .. .. .. 1 gm.
Distilled water .. .. .. 100 ml.

G. *Sensitized Schiff reagent*
Hydrochloric acid N/1 .. .. 20 ml.
Distilled water .. .. .. 80 ml.
Basic fuchsin .. .. .. 1 gm.
Sodium metabisulphite .. .. 2 gm.

(i) Shake in a closed bottle or stoppered flask at intervals over a period of fifteen to twenty minutes until the colour of the solution is pale yellow.

(ii) Add 1–2 gm. of decolorizing charcoal; shake well; then filter: the filtrate should be colourless.

(iii) Place 1 ml. of solution C (iodine solution) in a small tube.

(iv) Draw up and hold 2 ml. of Schiff's solution, prepared as above, in a pipette graduated in 0·1 ml.

(v) Drop Schiff's reagent from the pipette into the tube containing the 1 ml. of iodine test solution, cautiously, shaking the tube after each addition, until the iodine is decolorized and note what volume of the Schiff reagent is required. Best results are obtained when 0·8–2 ml. of the Schiff reagent is required to decolorize 1 ml. of the iodine solution. To achieve this it may be necessary, depending upon how recently the Schiff reagent has been prepared, to reduce the amount of sulphur dioxide present in the solution by heating (in a fume cupboard) or by mixing with a Schiff solution already depleted by continued use.

H. Orange G	3 gm.
Distilled water	100 ml.
HCl, conc.	1 ml.

Note:

Elftman states that the pH of the orange G 3% aqueous should be adjusted to 2·0 with HCl. In my laboratories, using orange G (Michrome No. 411), it was found that a 3% aqueous solution of this dye (in distilled water at pH 7·0) registered a pH 8·75 and it was necessary to add 1 ml. of N/1 HCl to reduce the pH of the staining solution to 2·0. In other laboratories, the amount of acid necessary will vary according to the pH of the distilled water available, and possibly the make of dye used.

Technique:

1. Remove the pituitary from the animal and fix in solution B (freshly prepared) at room temperature overnight, although the time may be prolonged up to forty-eight hours.
2. Transfer to 70% alcohol.
3. Dehydrate, clear, and embed in paraffin wax as usual.
4. Cut sections at 5 μ and attach them to slides.
5. Remove paraffin wax with xylol.
6. Carry through the usual graded alcohols into water.
7. Treat with 1% iodine in 70% alcohol for ten minutes.

8. Treat with 5% aqueous sodium thiosulphate for five minutes.

9. Wash thoroughly in water for five minutes.

10. Rinse in distilled water.

11. Wash in 70% alcohol.

12. Immerse in the aldehyde-fuchsin (solution E) for thirty minutes to one hour, depending upon the age of the stain.

13. Rinse in 95% alcohol.

Note: It may be necessary to acidify the alcohol slightly to remove background staining which will be present if the staining solution has been used before it is fully ripe.

14. Transfer to 1% periodic acid (solution F) and leave therein for fifteen minutes.

15. Immerse for two minutes in each of three changes of distilled water.

16. Immerse in the sensitized Schiff reagent (solution G) for twenty minutes.

17. Rinse in tap water.

18. Immerse in acidified orange G (solution H) for ten minutes to stain the acidiphils.

19. Rinse quickly in water.

20. Dehydrate through the usual graded alcohols.

21. Mount in Michrome mountant, or proceed as follows:

22. Clear in xylol.

23. Mount in D.P.X., Clearmount, or Michrome mountant.

Results:

Thyrotrophs: deep purple. Gonadotrophs: red. Acidiphils: orange. Nuclei: unstained.

Notes:

(*a*) It is stated that the absence of a nuclear stain greatly contributes to the crispness of cytological detail.

(*b*) The authors, who developed the procedure for routine evaluation of rat and mouse pituitaries, state that it has also proved useful for tissues in general, but for some tissues a counterstain other than orange G, which was found of particular value for the pituitary, may be more useful.

Reference: Elftman, H. (1959).

ALDEHYDE THIONIN - LUXOL FAST BLUE - PAS

For the simultaneous and specific demonstration of thyrotroph, gonadotroph and acidophil cells in the anterior hypophysis

Solutions required:

A. Potassium permanganate, 2% aqueous
B. Sulphuric acid, 0·5% aqueous
C. Solution A } equal volumes
   Solution B } of each
D. Potassium bisulphite, 2% aqueous
E. Aldehyde thionin
F. Luxol fast blue .. .. .. 0·1 gm.
   Alcohol, 95% .. .. .. 100 ml.
   Glacial acetic acid .. .. 0·5 ml.
G. Lithium carbonate 0·05% aqueous
H. Periodic acid, 1% aqueous
I. Schiff reagent

Notes:

(*a*) Solution E gives best results for pituitary staining after it has undergone a lengthy period of ripening.

(*b*) Solution F is used at a temperature of 57° C (see step 14, below).

Technique:

1. Fix material in a saturated solution of mercuric chloride in 10% formalin.
2. Embed in paraffin wax in the usual manner.
3. Cut sections at 5 μ and fix them to slides.
4. Remove paraffin wax with xylol.
5. Take through the usual graded alcohols down to water.
6. Treat for the removal of mercuric precipitate.
7. Immerse in solution C for two minutes.
8. Bleach in solution D for about one minute.
9. Wash well in running water.

10. Stain in the well-ripened aldehyde thionin (solution E) for ten minutes.
11. Rinse in water.
12. Immerse in 70% alcohol for a few seconds.
13. Immerse in 95% alcohol for a few seconds.
14. Stain in the luxol fast blue (solution F) for thirty minutes maintaining the temperature at 57° C for the whole of this period.
15. Rinse in 95% alcohol.
16. Rinse in 70% alcohol.
17. Rinse in water.
18. Differentiate in the lithium carbonate (solution G).
19. Immerse for a few seconds in each of four changes of 70% alcohol.
20. Wash in water.
21. Immerse in the periodic acid solution for ten minutes.
22. Rinse in water.
23. Immerse in Schiff's reagent for thirty minutes.
24. Wash in tap water for ten minutes.
25. Dehydrate through the usual graded alcohols.
26. Clear in xylol and mount in Canada balsam in xylol, D.P.X., Cristalite or Clearmount.

Results:

Thyrotrophs are stained intense blue-black by the aldehyde thionin, the granules of the thyroidectomy being particularly well shown. Gonadotrophs are easily distinguishable, stained the clear red of the PAS technique, as are also the basement membranes of the blood vessels. Acidiphils are stained a clear intense blue-green. Chromophobes are unstained or very lightly stained by the luxol fast blue, depending upon the degree of differentiation which has been carried out.

Notes:

(*a*) The authors state that the whole pituitary presents a brilliant appearance of clarity, enabling cell counts to be performed easily by individuals unversed in the morphology of pituitary cells, purely by reference to their colour. The secretions of the pars intermedia and pars posterior are also brilliantly stained. It is also stated that although other stains (e.g. orange G)

can be applied for the chromophobes, in the authors' hands luxol fast blue appeared to be the most satisfactory.

(*b*) The authors also state that as permanganate oxidation appears to be essential for staining with aldehyde thionin and permanganate will not materially alter the sulphate groups of polysaccharides, it is possible that some other mechanism must be involved to account for the specific staining encountered.

Permanganate oxidation, although freeing glycol groups, is said to destroy them. As the authors point out, it must be supposed that the oxidation does not proceed to the point at which destruction of the groups so freed makes any appreciable difference to the colour formed, since the colour of leuco fuchsin is restored presumably by such groups (1:2-glycol).

(*c*) Readers are referred to the original paper, which carries two photomicrographs, for more detailed information.

Reference: Paget, G. E. & Eccleston, Enid (1960).

ALIZARIN RED, S

For calcium deposits in cartilagenous and embryonic bone

Solutions required:

A. Alizarin Red, S, aqueous 1%

B. Polychrome Methylene Blue (Unna)

Technique:

Tissues are fixed in 80–90% alcohol and embedded in paraffin wax.

1. Sections are brought down to distilled water; then stained in Solution A for five to sixty minutes, according to the material.
2. Wash with distilled water, followed by 95% alcohol at 60° C.
3. Counterstain with Solution B for one to three minutes.

Results:

Cartilage: intense violet. Calcium: red. Nuclei: blue. Cytoplasm, etc.: yellow.

The method is particularly suitable for pathological specimens.

For bone staining in small vertebrates (Dawson's method)

Solutions required:

A. Potass. hydroxide 1% aqueous

B. Alizarin Red, S .. .. .. 0·1 gm.
Potass. hydroxide .. .. 10 gm.
Distilled water .. .. .. 1 litre

C. *Mall's solution:*
Glycerin .. .. .. 20 ml.
Distilled water .. .. 79 ml.
Potass. hydroxide .. .. 1 gm.

Technique:

1. Whole specimens are fixed in 95% alcohol for at least three days.
2. Transfer to acetone and leave for several days to dissolve out the fats which would otherwise stain intensely and obscure the view of the bony structures.
3. Wash well with 95% alcohol; then immerse in 95% alcohol for twenty-four hours.
4. Immerse in Solution A from one to seven days, according to the size of the specimen, until the bones are clearly visible through the muscle.
5. Transfer to Solution B until the bones are stained the desired depth of colour; this takes from one to seven days, and the solution should be changed on the fourth day.
6. Clear in Solution C until no more colour comes out.
7. Pass into a mixture of equal parts of glycerin and water, and continue through increasing strengths of glycerin.
8. Store in pure glycerin.

Results:

Bones are stained red; soft tissue, transparent and unstained.

Notes:

If the initial clearing in potass. hydroxide solution has progressed to the proper stage only the bone will be stained, but otherwise soft tissue will also be stained.

The prolonged preliminary fixation in alcohol renders the tissue less liable to maceration in the potass. hydroxide solution.

Objects fixed in liquids other than alcohol may be stained by this method provided they are soaked in 90% alcohol for at least three days. The best preparations are made with fish, but amphibia and mammals have also been tried with a fair degree of success, although there is not the same firm consistency about the flesh of a mammal or amphibian, prepared by this technique, as there is with that of a fish.

The technique is particularly suitable for demonstrating developing bone.

Reference: Dawson, A. B. (1926).

William's modification of Dawson's method

This technique is particularly suitable for mammalian embryos and mature specimens of Urodele amphibians; for distinguishing between bone and cartilage and for demonstrating the relative amount of ossification.

The removal of the viscera is unnecessary in the case of museum specimens.

Solutions required:

A. Toluidine Blue .. .. .. 0·25 gm.
Alcohol 70% .. .. .. 100 ml.
Hydrochloric acid 0·5% .. 2 ml.
Allow the solution to stand for twenty-four hours; then filter and store in a tightly corked bottle.

B. Potass. hydroxide 2% aqueous ..

C. Alizarin Red, S .. .. .. 0·001 gm.
Potass. hydroxide 2% aqueous .. 100 ml.
(This solution should be freshly prepared.)

D. Methyl salicylate 25% in cellosolve

E. Methyl salicylate 50% in cellosolve

F. Methyl salicylate 75% in cellosolve

Technique:

1. Wash specimens for twenty-four hours in 70% alcohol containing 0·2% of concentrated ammonia solution.

2. Stain for seven days in Solution A.

3. Harden and destain for seventy-two hours in four changes of 95% alcohol.

4. Macerate for five to seven days, depending on the size of the animal, in several changes of 2% aqueous potass. hydroxide.

(*Note:* This process is hastened by exposure to sunlight.)

5. Transfer to Solution C for about twenty-four hours when the bones should be well stained. If the specimen has been insufficiently macerated the soft tissue will be slightly stained, in which case the specimen may be destained rapidly in acid alcohol (1% sulphuric acid in 95% alcohol).

6. Dehydrate by leaving the specimen in three changes of cellosolve for six hours in each. Instead of cellosolve, 50%, 80% and 90% alcohol, followed by three changes of benzol may be used for dehydration. Small embryos require less time in the dehydrating fluids.

7. Clear by transferring to solutions of 25%, 50% and 75% methyl salicylate in cellosolve for twenty-four hours in each.

8. Store in methyl salicylate.

Note: If glycerin is used for clearing the technique has to be modified as follows:

Omit stage 6 and transfer directly from the Alizarin Red S solution into a series of 50%, 70% and 80% glycerin for twenty-four hours in each; then store in pure glycerin.

Results:

Soft tissues: transparent. Osseous tissue: deep blue. Cartilage: dark blue.

Note: The relative degree of ossification and chondrogenesis which has taken place is indicated by the intensity of the stains. Bone and cartilage may be stained separately by omitting stage 2, or stage 5 for cartilage.

Reference: Williams, T. W. (1941).

For foetal specimens

The technique is particularly suitable for mammalian embryos, for demonstrating minute bones and foetal ossification.

Solutions required:

A. Alizarin Red, S, 1% aqueous (freshly prepared) .. .. 1 litre
Potass. hydroxide 1% aqueous .. 1 ml.

B. Potass. hydroxide .. .. 10 gm.
Water .. .. 800 ml.
Glycerin .. .. 200 ml.

Note: For small specimens 5 gm. potass. hydroxide is sufficient.

Technique:

Fix in 95% alcohol for at least two weeks after making a midline abdominal incision to allow penetration of the fixative.

1. Rinse in tap water.

2. Immerse for at least four weeks in 1% potass. carbonate.

3. Immerse for at least ten days in 1% aqueous potass. hydroxide until the bones are clearly visible through the soft tissue.

Note: Formalin-fixed specimens require four to six weeks in 1% potass. hydroxide. Should the specimen become too soft it may be hardened by immersing for twelve to twenty-four hours in a mixture consisting of equal volumes of glycerin, water and 95% alcohol before returning to the clearing solution. Potass. hydroxide 0·5% may be used during the last few days of the clearing.

4. Wash twenty-four hours in running tap water.

5. Stain one-half to six hours, according to the size of the specimen, in Solution A.

6. Wash for thirty minutes in running tap water.

7. Decolorize seven to fourteen days in Solution B.

8. Mount in a glass frame and dehydrate by passing slowly through alcohol-glycerin-water mixtures beginning with the proportions 1 : 2 : 7 and then in succession 2 : 2 : 6, 3 : 3 : 4, 4 : 4 : 2; and finally equal parts of alcohol and glycerin only.

9. Seal in the usual glycerin-alcohol mixture.

References:

Dawson, A. B. (1926).
Richmond, G. W. & Bennett, L. (1938).

For nervous tissues (Benda's method)

Solutions required:

A. Nitric acid, conc. .. .. 1 volume
Distilled water .. .. .. 10 volumes

B. Potass. dichromate 2%

C. Chromic acid 1%

D. Iron alum 4%

E. Alizarin Red, S, saturated in absolute alcohol .. .. .. 1 ml.
Distilled water .. .. .. 90 ml.

F. Toluidine Blue 0·1% aqueous

Technique:

1. Material is fixed in 90–95% alcohol for at least two days.
2. Pieces, which must not be thicker than 0·5 cm., are immersed in Solution A for twenty-four hours.
3. Transfer to Solution B for twenty-four hours.
4. Transfer to Solution C for forty-eight hours; then wash in water for twenty-four hours.
5. Dehydrate in the usual manner.
6. Clear in beechwood creosote for twenty-four hours; then in benzol for twenty-four hours.
7. Embed in paraffin wax via four graded mixtures of paraffin wax and benzol, the first at room temperature, the second at 38° C., the third at 42° C. and the fourth at 45° C.
8. Mount sections on slides: bring down to distilled water, mordant sections on slides with Solution D for twenty-four hours, then wash thoroughly in water.
9. Stain for two hours with Solution E; then rinse in tap water.
10. Flood slides with Solution F and warm gently until vapour is given off; or stain at room temperature for 24 hours.
11. Rinse in 1% acetic acid; then dry by blotting carefully.
12. Pass through absolute alcohol; then differentiate for about ten minutes in beechwood creosote; dry by blotting carefully, wash with xylol, and mount.

Reference: Benda, C. (1900, 1901).

Vital staining of neurological tissue in small vertebrates

Solution required:

Alizarin Red, S, 2% aqueous

Technique:

1. Paraffin sections are brought down to distilled water by the usual method.
2. Stain twenty-four hours in 2% aqueous Alizarin Red, S.
3. Differentiate thirty to sixty seconds in distilled water to which has been added three drops 1% calcium acetate per 10 ml.
4. Dehydrate: clear and mount.

Note: This is a general stain which also demonstrates Nissl bodies as well as other details.

THE ALLOCHROME METHOD

(Periodic Acid - Schiff - Haematylin - Aniline Blue)

For connective tissues

Solutions required:

A. Periodic acid, 1% aqueous ..

B. Basic fuchsin .. .. .. 1 gm.
Potassium metabisulphite .. 3·8 gm.
HCl, N/15 .. .. .. 200 ml.
Dissolve the basic fuchsin in the dilute hydrochloric acid in a flask; then add the potassium metabisulphite; plug the neck of the flask with cotton wool; shake at intervals over a period of one to two hours until the liquid assumes a clear pale yellow colour; then add:
Decolorizing carbon .. 1 gm.
Replace the cotton wool plug; shake for a few minutes; then filter. The filtrate should be colourless.

C. Potassium metabisulphite, 0·5% aqueous

D. Weigert's haematoxylin No. 1

E. Weigert's haematoxylin No. 2

F. Picric acid, saturated, aqueous .. 100 ml.
Aniline blue, aqueous .. .. 0·025 g.

Technique:

1. Fix pieces of tissue in any desired fixative, and embed in paraffin wax.
2. Fix sections to slides and dewax with xylol.
3. Pass through the usual graded alcohols, using the iodine and sodium thiosulphate procedure for the removal of mercurial precipitate, if tissues have been fixed in a mercury-containing fixative.
4. Wash with water.
5. Oxidize in the periodic acid solution for ten minutes.
6. Wash for five minutes in running water.
7. Immerse in solution B (Schiff's reagent) for ten minutes.
8. Immerse for two minutes in each of two changes of the potassium metabisulphite (solution C).
9. Wash for five minutes in running tap-water.
10. Stain for two minutes in a freshly prepared mixture consisting of equal volumes of Weigert's haematoxylin No. 1 and No. 2.
11. Wash in running tap-water for four minutes.
12. Stain for six minutes in solution F (picro-aniline blue).
13. Differentiate in two changes each of 95% alcohol and absolute alcohol.
14. Wash in a mixture consisting of equal parts of absolute alcohol and xylol.
15. Mount in D.P.X., Cristalite, Clearmount or Emexel.

Results:

Nuclei: black or dark grey. Collagen: blue. Cytoplasm and muscle cells: greyish green to yellowish green. Chromaffin cells: partly red and partly blue. Amyloid: pink to light purplish red (*Note:* if the amount of aniline blue in reagent F is increased to 0·4 gm., amyloid is stained blue-violet). Muscle reticulum and epithelial basement membranes: blue or dark violet, and occasionally red. Lymphoid tissue reticulin: blue. Particularly striking results are given in certain chronic nephropathies. Casts are coloured deep purple, intact convulated tubules stain a light yellowish green with red to purple cuticular margin and deep purplish red basement membrane. Connective tissue between both cortical and medullary tubules is stained bright, clear blue.

Note:

Lillie designated this procedure as the " Allochrome " method denoting the change of colour of collagen, and some related substances, from red to blue, during the process (allochrome = of different or changing colour) which is a sequence of Schiff's leucofuchsin, picro aniline blue.

Reference: Lillie, R. D. (1951).

AMMONIACAL SILVER CARBONATE

For vascular reticulum, tumour cells, connective tissues around tumour, in abnormal brain tissue

Solutions required:

A. Pyridin, pure .. .. .. 2 volumes
Glycerin, pure .. .. 1 volume

B. *Ammoniacal Silver Carbonate*
Ammonia solution is added drop by drop to 10 ml. silver nitrate 10·2% until the precipitate formed is almost redissolved, leaving a slightly opalescent solution to which is then added 10 ml. sodium carbonate 3·1% solution and sufficient distilled water to make the volume up to 100 ml.

C. *Reducing solution*
Sodium carbonate anhydrous 1 gm.
Formalin .. .. .. 1 ml.
Distilled water .. .. .. 103 ml.

D. Brown gold chloride 0·2% aqueous.

E. *Intensifying solution*
Oxalic acid 2% aqueous .. 100 ml.
Formalin .. .. .. 1 ml.

F. Sodium hyposulphite 10% aqueous.

Technique:

The material is fixed in 10% formalin or in Bouin and embedded in paraffin wax.

1. Bring sections down to distilled water and immerse in Solution A for twenty-four hours.

2. Wash with 95% alcohol, then with distilled water.

3. Immerse in Solution B for two and a half hours at 40° C.

4. Wash with distilled water; then reduce in Solution C for five minutes, afterwards washing in tap water.

5. Tone for five minutes in Solution D at 30° C.; then wash in tap water.

6. Intensify by immersing in Solution E for five minutes; then rinse in tap water.

N.B.—The above stages must be carried out in the darkroom.

7. Fix in Solution F.

(*Note:* Fixation should be completed in fifteen to twenty minutes.)

8. Wash in tap water; dehydrate; clear and mount.

Results:

Tumour cells: reddish to greyish violet. Vascular reticulum: black.

Reference: Conn, H. J. & Darrow, Mary A. (1947).

Important.—The tissues must not be allowed to come into contact with mercuric chloride, as even a trace will ruin the preparation.

ANILINE BLUE - FUCHSIN ACID

For elementary bodies in animal sections

Solutions required:

A.	Picric acid, saturated alcoholic,	10 ml.
	Formalin	25 ml.
	Absolute alcohol	65 ml.
	Glacial acetic acid	5 ml.
B.	Aniline blue, water soluble	1 gm.
	Distilled water	65 ml.
	Methyl alcohol, pure	35 ml.
	Glycerin, pure	5 ml.
	Oxalic acid 3% aqueous	2 ml.

C. Acid fuchsin 1% aqueous .. 100 ml.
Oxalic acid 3% .. .. .. 2 ml.

Technique:

1. Pieces of tissue are fixed for twenty-four hours in Solution A; washed, dehydrated, cleared and embedded in paraffin wax as usual.

2. Sections, not thicker than 5μ, are fixed to slides, de-waxed and taken through descending grades of alcohol down to water as usual.

3. Stain for one half to one hour in the aniline blue (Solution B) in a stoppered staining jar.

4. Rinse well with distilled water.

5. Drain and blot carefully to remove excess water.

6. Rinse in absolute alcohol.

7. Stain for twenty minutes in the acid fuchsin (Solution C).

8. Pour off; drain and blot carefully to remove excess liquid.

9. Dehydrate with absolute alcohol; clear in xylol and mount in cristalite or in Canada balsam.

Results:

Elementary bodies in cells associated with the following viruses are stained scarlet: borna, zoster, rabies and pseudo rabies.

Reference: Nicolau, S. & Kopciowska, L. (1937).

ANILINE BLUE - EOSIN B

A simple and rapid technique for spermatozoa, which is particularly suitable for dog and human semen

Solutions required:

A. Ether .. .. 1 volume
Absolute alcohol .. .. .. 1 volume

B. Aniline blue aqueous .. .. 2 gm.
Eosin, B .. .. 1 gm.
Phenol 1% aqueous .. .. 20 ml.
Distilled water .. .. .. 60 ml.

Technique:

1. Fresh semen is allowed to stand for about one half to one hour until it liquifies.
2. Prepare thin even smears of the liquified semen on scrupulously clean and dry slides or coverslips.
3. Fix for 3 minutes in a mixture consisting of equal volumes of ether and absolute alcohol; then allow to dry in the air.
4. Flood smears with solution B and warm over a steam bath or hot plate while the stain is allowed to act for 5–7 minutes at 40–60° C.
5. Pour off excess stain.
6. Wash the preparation thoroughly with distilled water.
7. Drain and allow to dry thoroughly in air.
8. Mount in a neutral synthetic resin such as D.P.X., Clearmount or Cristallite.

Results:

Sperm structure	*Stain reaction*
Galea capitis	Pale bluish-grey; sharply outlined
Cell membrane	Bluish-grey; sharply outlined
Nuclear membrane (shell)	Bluish-grey; sharply outlined
Acrosome	Slate blue
Nucleus	Pink
Neck	Sharply outlined (dark blue), inside colourless
End knobs	Dark blue
Middle piece	Sheath, dark blue; centre dark pink
Axial filament	Dark blue
Tail	Dark blue

Notes:

It is suggested that the technique which has been tried out only on dog and human semen, might be applied to other species.

The technique gives good differentiation and preservation of cytological structure, reliable fixation and staining and undistorted and easily recognizable detail, upon which assays of semen for fertility depend.

Casarett states that there is a tendency for abnormal forms of spermatozoa to stain more intensely than the formal forms.

Reference: Casarett, George W. (1953).

ANILINE CRYSTAL VIOLET - GRAM'S IODINE

For epithelial fibres

Solutions required:

A. Aniline crystal violet.

B. Gram's iodine.

C. Aniline xylol.

Technique:

1. Material should be fixed in absolute alcohol and embedded in paraffin wax.

2. Sections, not more than 5μ thick, are fixed to slides and brought down to distilled water in the usual manner.

3. Stain for ten to fifteen minutes in aniline crystal violet.

4. Wash well in running water.

5. Stain with Gram's iodine for ten to thirty seconds.

6. Wash in water; drain; then blot carefully but thoroughly to remove water.

7. Differentiate with aniline xylol, controlling at frequent intervals by examination under the microscope.

8. Wash well with xylol; mount in balsam or D.P.X.

Results:

Epithelial fibres are stained blue.

ANILINE CRYSTAL VIOLET - LITHIUM CARMINE - IODINE

For fibrin and for Gram-positive organisms in animal tissues

Solutions required:

A. Lithium carmine

B. Crystal violet .. .. .. 1 gm.
Aniline oil.. .. .. .. 3 ml.
Absolute alcohol .. .. 10 ml.
Dissolve and filter.

C. Crystal violet 2% aqueous.

D. Solution B .. .. .. 3 ml.
Solution C .. .. .. 27 ml.
This mixture should be prepared immediately before use.

E. Gram's iodine.

F. Aniline oil.. .. .. .. 1 volume
Xylol .. .. 1 volume

Technique:

1. Fix material in absolute alcohol, Carnoy or alcohol-formalin and embed in paraffin wax.

2. Fix sections to slides; de-wax and pass through descending grades of alcohol down to distilled water in the usual way.

3. Stain in the lithium carmine solution for two to five minutes.

4. Wash thoroughly in distilled water.

5. Immerse in Solution D for five to ten minutes.

6. Rinse in distilled water; drain well and blot carefully.

7. Cover with Gram's iodine solution and allow the stain to act for five to ten minutes.

8. Pour off the excess iodine solution and blot carefully with filter paper.

9. Differentiate with the aniline xylol solution until no more purple coloration comes out.

10. Drain, and blot carefully.

11. Rinse with several changes of xylol.

12. Mount in balsam or in cristalite.

Results:

Fibrin and Gram-positive organisms are blue to blue-black while nuclei are red.

References:

Cowdry, E. V. (1952).
Mallory, F. B. (1938).
Weigert, C. (1887).

ANILINE BLUE - ORANGE G - ACID FUCHSIN

(After Mallory)

For connective tissue

Solutions required:

A. Acid fuchsin 0·5% aqueous.

B. Aniline Blue - Orange G.

Technique:

Tissues are fixed in Zenker and embedded in paraffin wax, Celloidin or L.V.N.

1. Mount sections on slides and bring down to 90% alcohol; then treat with iodine in the usual way to remove mercuric deposits.

2. Bring down to distilled water and stain for one to ten minutes in Solution A; then without washing:

3. Stain for twenty minutes to one hour or longer in Aniline Blue - Orange G; then remove excess stain with several changes of 95% alcohol.

4. Dehydrate with absolute alcohol; clear in xylol and mount in Cristalite.

Note: If Celloidin or L.V.N. sections are used the staining time may be shortened and 95% alcohol should be used for decolorizing and dehydration; terpineol for clearing.

Results:

Collagenous fibrils: intense blue. Ground substances of cartilage, bone, mucus, amyloid: varying shades of blue. Nuclei, myoglia, neuroglia fibrils, axis cylinders, fibrin, nucleoli: red. Blood corpuscles and myelin: yellow. Elastic fibrils: pale pink or pale yellow, or unstained; fibriloglia: red or unstained.

Note: By omitting the acid fuchsin the collagenous fibres are more sharply defined.

Reference: Mallory, F. B. (1900).

AZAN STAIN (HEIDENHAIN)

A general purpose stain

Solutions required:

A.	Azocarmine B	0·5 gm.
	Distilled water	100 ml.
	Glacial acetic acid	1 ml.

Dissolve by warming; cool and filter.

B.	96% alcohol	100 ml.
	Aniline oil	0·1 ml.
C.	95% alcohol	99 ml.
	Glacial acetic acid	1 ml.
D.	Phosphotungstic acid	5 gm.
	Distilled water	75 ml.
	Methyl alcohol	25 ml.
E.	Aniline Blue, water soluble	0·5 gm.
	Orange G	2 gm.
	Glacial acetic acid	8 ml.
	Distilled water	100 ml.

Dissolve by warming; cool and filter.

For staining, dilute 1 volume of this solution with 3 volumes of distilled water.

Technique:

1. Zenker-, Bouin- or Carnoy-fixed tissues are stained from forty-five to sixty minutes at 55° C. in Solution A; then at room temperature for five to ten minutes.

2. Wash in distilled water; then differentiate in Solution B until cytoplasm is pale pink, and nuclei are red and clear.

3. Rinse for one half to one minute in Solution C.

4. Transfer to Solution D for about one to three hours or until the connective tissue is completely decolorized; then wash quickly in distilled water.

5. Stain for one to two hours in the diluted Solution E, examining at ten- or fifteen-minute intervals to prevent over-staining.

6. Wash quickly in distilled water; then differentiate in 95% alcohol followed by absolute alcohol.

7. Clear and mount.

Results:

Collagen stained deep blue; reticulum, deep blue; chromatin, red; muscle tissue, reddish to orange; erythrocytes, red; neuroglia, reddish; mucin, blue.

Reference: Heidenhain, M. (1915).

AZO CARMINE - MALLORY STAIN

For Islets of Langerhans

Solutions required:

A. Azocarmine, B, aqueous 0·1% .. 100 ml.
Boil for about 5 minutes; then cool and add 2 ml. glacial acetic acid. Then warm to 60 °C. and filter at that temperature.

B. 90% alcohol 99 ml.
Aniline Oil 1 ml.

C. Iron alum 5% aqueous.

Aniline Blue - Orange G (Mallory):

D. Aniline blue, aqueous .. .. 0·5 gm.
Orange G. .. .. 2 gm.
Distilled water .. .. .. 100 ml.

E. Solution D .. .. 1 volume
Distilled water .. .. 2 to 3 volumes

Technique:

1. Fix thin slices of pancreas in Bouin for eight to ten hours.

2. Wash in distilled water, dehydrate, clear and embed in paraffin wax.

3. Cut section 4μ in thickness.

4. Fix section to slides; dewax and take down through the usual grades of alcohol to distilled water.

5. Stain in solution A for about forty-five to sixty minutes at 56° C.

6. Rinse quickly in distilled water and blot very carefully.

7. Destain in solution B until acinous tissue is almost colourless and B cells show red against pink background of A cells.

8. Rinse briefly with distilled water and treat with 5% iron alum solution for 5 minutes or more.

9. Rinse again and stain two to twenty minutes in solution E until the collagenic tissue appears deep blue under the microscope.

10. Rinse and blot carefully.

11. Differentiate and dehydrate in absolute alcohol.

12. Clear in xylol and mount.

Results:

Cytoplasm of A cells: rich yellow orange; of B cells: bright red; and of D cells: sky blue.

Note: **It is stated that it can be demonstrated that there is no gradation between A and B cells by first staining with Neutral Gentian (Bensley) decolorizing, then restaining by the above technique.**

References:

Gomori, G. (1939).
Cowdry, E. V. (1952).

AZOCARMINE - HAEMATOXYLIN - ACID GREEN - ORANGE G

For differential cell analysis of the rat anterior hypophysis

Solutions required:

A. *Zenker - Formol*

Potassium dichromate	25 gm.
Mercuric chloride	50 gm.
Ringer's solution (i.e. 0·9% saline)..	1 litre.

Add 1 ml. neutral formaldehyde 40% solution per 10 ml. of the above solution immediately before using.

B. Lugol's iodine

C. Sodium thiosulphate 0·5% aqueous

D. Delafield Haematoxylin

E. Alcohol 95% .. .. .. 999 ml.
Aniline oil .. .. 1 ml.

F. Azocarmine G or B 1% aqueous .. 100 ml.
Glacial acetic acid .. .. .. 4 ml.

G. Glacial acetic acid .. .. .. 10 ml.
Alcohol 90% .. .. .. 90 ml.

H. Phosphotungstic acid 5%

I. Acid green L extra .. .. .. 0·1 gm.
Orange G .. .. 0·5 gm.
Clove oil .. .. 100 ml.

N.B. Use fresh stain for each batch of about twenty slides.

Technique:

1. Fix in solution A for six to twelve hours.

2. Wash six to twelve hours in running water.

3. Dehydrate by immersing for thirty minutes in each of the following: 30%, 50%, 70%, 80% and 95% alcohol.

4. Immerse in absolute alcohol, two changes, for one hour in each.

5. Immerse in a mixture consisting of equal parts of absolute alcohol and cedarwood oil for one hour.

6. Cedarwood oil, one to sixteen hours.

7. Xylol, for fifteen minutes.

8. Infiltrate in paraffin wax 56–58° C. (four changes before finally embedding).

9. Fix sections, 4μ in thickness, to slides and remove paraffin wax by immersing in two changes of xylol for three minutes each.

10. Immerse for three minutes in each of two changes of absolute alcohol.

11. In 95% alcohol for three minutes.

12. Distilled water for three minutes.

13. Lugol's Iodine for three minutes.

14. Sodium thiosulphate solution for three minutes when the sections should have been restored to their natural colour.

15. Stain in Delafield Haematoxylin for thirty seconds.
16. Wash in tap water for three minutes.
17. Immerse in distilled water for three minutes.
18. Immerse in 80% alcohol for three minutes.
19. Aniline alcohol (Solution E) for fifteen minutes.
20. Stain in Azocarmine for forty-five minutes.
21. Rinse in distilled water.
22. Differentiate in aniline alcohol for two to three minutes.
23. Wash in acid alcohol (solution G) for thirty to sixty seconds.
24. Immerse in phosphotungstic acid solution for one hour.
25. Dehydrate by passing through 70%, 95% and absolute alcohols (two minutes in each).
26. Counterstain in acid green - orange G solution for five minutes.
27. Clear in xylol for one minute.
28. Immerse for half an hour in each of two changes of xylol, to remove completely all traces of clove oil which would otherwise cause further decolorisation.
29. Mount in D.P.X. or Clearmount.

Results:

Alpha granules: purplish red. Beta cell granules: light green. Nuclear membranes are sharply defined and mitochondria are orange red. Erythrocytes: brilliant orange. Golgi apparatus shows as negative image in both alpha and beta cells. Chromophobes show little or no cytoplasm, which is colourless to pale green.

Note: A method of counting the cells is given in the original paper.

Reference: Briseno-Castrejon, B. & Finerty, J. C. (1949).

ALLOXAN - NINHYDRIN - SCHIFF - LUXOL FAST BLUE

(W. M. Shanklin & M. Issidorides, 1960)

Solutions required:

A. Ninhydrin 0·5% or alloxan 1%
in absolute alcohol

B. Schiff reagent

C. Sodium bisulphite M/1 (100 gm./litre $NaHSO_3$)

D. Solution C .. .. .. 10 ml.
Tap water .. .. .. 200 ml.

D. Luxol fast blue (Michrome No. 331) .. .. 1 gm.
Alcohol 95% .. .. .. 1 litre

Technique:

1. Sections of material that have been fixed in 10% formalin are passed through two changes of xylol.
2. Pass through two changes of absolute alcohol.
3. Incubate at 37° C for five to twenty-four hours in ninhydrin or alloxan (solution A.)
4. Wash gently under the tap for one to two minutes.
5. Immerse in Schiff's reagent for one-half to one hour.
6. Pass sections through three jars of solution D, for ninety seconds in each, with frequent agitation.
7. Wash in tap water for five minutes.
8. Dehydrate in 95% alcohol.
9. Counterstain in the luxol fast blue solution for five minutes.
10. Pass through absolute alcohol.
11. Clear in xylol and mount in Canada balsam in xylol or D.P.X.

Results:

Human brain: protein material in the nucleus supraopticus of the hypothalamus and in the hypoglossal nucleus is stained red by alloxan-Schiff treatment, while the Nissl bodies are stained blue by the luxol fast blue.

Notes

(*a*) The authors (Shanklin & Issidorides) state that Lillie (1954) in describing Yasuma & Ichikawa's (1953) technique observes that counterstains in his hands had not been found successful so far. In the method described above, however, Shanklin and Issidorides found luxol fast blue highly satisfactory as a counterstain for the alloxan-Schiff method.

(*b*) It should be mentioned that alloxan is considerably less expensive than ninhydrin.

References:
Shanklin, W. M. & Issidorides, M. (1960).
Lillie, R. D. (1954).
Yasuma, A. & Ichikawa, T. (1953).

BAUER - FEULGEN STAIN

For Glycogen, etc.

Solutions required:

A. Chromium trioxide 4% aqueous

B. Feulgen's fuchsin (Schiff's reagent)

C. Sodium metabisulphite 10·4%
aqueous .. .. 5 ml.
Tap water .. .. 95 ml.

Technique:

1. Fix fresh material immediately in alcohol and embed in paraffin wax.

2. Dewax sections; pass through the usual descending grades of alcohol to distilled water.

3. Immerse in solution A for one hour.

4. Wash in running water for five to ten minutes.

5. Immerse in the Feulgen fuchsin for ten to twenty minutes.

6. Agitate the slides gently for about two minutes in each of three changes of solution C.

7. Wash in running water for ten minutes.

8. Stain nuclei, if desired, in haemalum, for two to five minutes.

9. Dehydrate as usual; clear in xylol and mount.

Results:

Glycogen: intense reddish-violet. Nuclei: pale mauve to navy blue (if haemalum is used).

Reference: Bauer, H. (1933).

BENZIDINE METHOD

(After Pickworth)

For haemoglobin

Solutions required:

A.	Benzidine base, extra pure	2 gm.
	Methyl alcohol, pure	150 ml.
	Glacial acetic acid	2 ml.
	Sodium nitroprusside	0·2 gm.
B.	Ether	50 ml.
	Methyl alcohol, pure	100 ml.
	Hydrogen peroxide, 3%	50 ml.
C.	Neutral red, 1% aqueous	

Technique:

1. Fix pieces of tissue in 10% neutral formalin and embed in paraffin wax or celloidin or Waterwax, or cut frozen sections.
2. Take sections through to water.
3. Wash in 70% alcohol.
4. Wash in absolute methyl alcohol.
5. Immerse in reagent A, in a Coplin jar, for ten minutes.
6. Wash well with reagent B to remove excess benzidine.
7. Wash in running water for fifteen minutes.
8. Counterstain in neutral red for three minutes.
9. Wash in distilled water.
10. Dehydrate quickly through the usual graded alcohols.
11. Clear in xylol.
12. Mount in Cristalite or in D.P.X. or Emexel.

Results:

Haemoglobin and certain oxidase granules in leucocytes appear dark blue. Nuclei: red.

Note:

Lillie (1948) states that a further identification of the haemoglobin lies in the fact that with acid formaldehyde fixation (pH 3·0–3·5), or with acid treatment of the sections for a sufficient

time after neutral formalin fixation, red corpuscles and haemoglobin may sometimes give the Prussian blue reaction of Perls.

References:
Lillie, R. D. (1948).
Perls, M. (1867).
Pickworth, F. A. (1934).

BIEBRICH SCARLET - ETHYL VIOLET - HAEMATOXYLIN

A modification of Bowie's stain for pepsinogen granules of the body chief cells in the gastric glands

Solutions required:

A. Delafield Haematoxylin, aqueous

B. Ethyl Violet - Biebrich Scarlet .. 0.5 gm.
20 % Ethyl alcohol .. .. .. 50 ml.

C. Solution B. .. .. 0·5 gm.
Absolute alcohol .. .. .. 20 ml.

Distilled water .. .. 80 ml.

N.B.—This solution must be freshly prepared each time it is required for use.

D. Clove Oil .. .. 1 volume
Toluol .. .. 1 volume

Technique:

1. Fix in Regaud's fluid for five days in a dark phial placed in a larger amber bottle which should be wrapped round with a thick cloth and kept in a dark room. Change the fixative daily.

2. Dehydrate with normal propyl alcohol (*see* page 514).

3. Transfer to paraffin wax which should be changed three times before the block is finally cast.

4. Cut sections 5 to 6μ in thickness and fix to slides as usual.

5. Remove wax with two changes of xylol.

6. Pass through absolute alcohol and the usual descending grades of alcohols down to distilled water.

7. Stain in the Haematoxylin solution for 1 minute.

8. Wash and blue in tap water.

9. Remove excess water by draining and blotting very carefully.

10. Stain with solution B for ten to fifteen minutes, or longer (up to twenty-four hours).

11. Rinse briefly in distilled water, then drain and carefully remove excess water by blotting.

12. Differentiate in solution D (clove-toluol), controlling under the microscope, for about ten to fifteen minutes.

13. Rinse with two changes of toluol.

14. Mount in permount-toluene or in Clearmount or Cristalite.

Results:

Zymogen granules: dark violet. Parietal cells, scarlet, and nuclei, blue. The parietal cells are distinctly contrasted from the pepsinogen cells.

Reference: Cambel, P. & Sgouris, J. (1951).

BISMARCK BROWN - METHYL GREEN

For mucin, cartilage, and goblet cells in embryonic tissue, trachea and intestine

Solutions required:

A. Bismarck brown 1% aqueous.

B. Methyl green 0·5% aqueous.

Technique:

Tissues are fixed in Bouin or Zenker and embedded in paraffin wax.

1. Sections are brought down to distilled water; then stained five to ten minutes in Solution A.

2. Wash with 95% alcohol.

3. Stain with Solution B until the preparation appears dark green to the naked eye.

4. Dehydrate with 95% and absolute alcohol; then clear in xylol, and mount.

Results:

Cartilage: dark brown. Mucin: light brown. Nuclei of all cells: green.

Notes:

The authors state that the method is essentially the same as List (1885).

Reference: Conn, H. J. & Darrow, Mary A. (1947), Section A: "Animal Histology," p. 5.

BIONDI - EHRLICH - HEIDENHAIN STAIN

For chromatin, nucleoli, mucin, etc.

Solution required:

Biondi - Ehrlich - Heidenhain	0·9 gm.
Distilled water	100 ml.

Dissolve by warming and stirring in a beaker.
When cool add:

Chloroform	0·25 ml.

Technique:

1. Fix tissues in saturated aqueous mercuric chloride and embed in paraffin wax in the usual manner.

2. Fix sections to slides; de-wax with xylol and pass through absolute alcohol followed by 90% and 70% alcohol.

3. Treat for the removal of mercuric precipitate by the standard technique.

4. Immerse in the staining solution from six to twenty-four hours.

5. Rinse directly with 95% alcohol.

6. Dehydrate with absolute alcohol.

7. Clear in xylol and mount.

Results:

Chromatin is stained bluish green, while nucleoli are red; mucin is stained green; erythrocytes, orange. Cytoplasm and connective tissue elements are in varying shades of red.

References:
Ehrlich, P. & Lazarus, A. (1898).
Heidenhain, M. (1892).
Pfluger Arch. (1888).

BEST'S CARMINE

For glycogen

Solutions required:

A. Ehrlich haematoxylin.

B. Best's carmine stock solution .. 10 ml.
Methyl alcohol, pure .. .. 15 ml.
Strong ammonia solution .. 10 ml.

Note: This solution should not be prepared until it is required for immediate use.

C. Celloidin 1% in equal volumes of absolute alcohol and ether.

D. Absolute (ethyl) alcohol .. .. 80 ml.
Absolute (methyl) alcohol .. 40 ml.
Distilled water .. .. .. 100 ml.

Technique:

1. Tissues are fixed in Bouin Fluid and embedded in Celloidin or in paraffin wax. If Celloidin sections are employed proceed as from stage 5 (below). If paraffin sections are used the procedure is as follows:

2. Float sections on the slide with 70% alcohol; flatten out; then remove excess alcohol with filter paper and blot carefully but thoroughly.

3. Remove paraffin wax with xylol in the usual manner.

4. Wash with absolute alcohol as usual.

5. Transfer the slide to a stoppered staining jar containing 1% Celloidin (Solution C, above), for fifteen minutes.

6. Transfer to a stoppered jar containing 70% alcohol, after rapidly wiping off the Celloidin from the back of the slides. This operation must be carried out quickly so that the Celloidin is not allowed to dry. Leave in the alcohol from ten to fifteen minutes.

7. Transfer to Ehrlich haematoxylin and allow the stain to act from two to ten minutes, differentiating if necessary with acid alcohol, controlling under the microscope.

8. Rinse in water, and without " blueing " in tap water, transfer to Best's carmine solution (recipe as above) and allow the stain to act for five to ten minutes.

9. Differentiate in Solution D (above) from one to five minutes until the stain ceases to come away from the section.

10. Transfer to a mixture consisting of equal volumes of ether and absolute alcohol to dissolve out Celloidin and to dehydrate.

11. Clear with xylol and mount.

Results:

Glycogen is stained as brilliant red granules, while nuclei are blue.

Reference: Best, F. (1906).

BENZIDINE

For brain capillaries

Solutions required:

A. Benzidine base, pure .. .. 1 gm.
Acetic acid 2·5% aqueous .. 200 ml.

B. Sodium nitroprusside 1% aqueous

C. Solution A .. .. .. 20 ml.
Solution B .. .. .. 10 ml.
Distilled water .. .. .. 70 ml.
Mix well and filter.

N.B.: This mixture should be prepared immediately before use.

D. Distilled water .. .. .. 100 ml.
Hydrogen peroxide 20 vols. .. 1·5 ml.

Technique:

1. Tissue should be fixed for one to three weeks in 10% formalin and frozen sections, 200 to 300μ should be employed.

2. Immerse in several changes of distilled water for a total period of two hours.

3. Immerse in Solution C for half an hour at 37° C., agitating at frequent intervals.

4. Wash in several changes of distilled water.

5. Immerse in Solution D for half an hour at 37° C. agitating frequently.

6. Wash in distilled water.

7. Dehydrate by passing through ascending strengths of alcohol, beginning with 70%, in the usual manner.

8. Clear in xylol and mount.

Results:

Blood vessels are stained black against a background which is almost colourless.

Note:

Cowdry (1952) states that this is a simplified Lephen–Pickworth peroxidase method, due to Campbell and Alexander.

References:

Mallory, F. B. (1938), p. 257.
Cowdry, E. V. (1952), p. 56.

BRILLIANT CRESYL BLUE

For reticulated cells and platelets

Solutions required:

A. Brilliant Cresyl Blue 0·3% absolute ethyl alcohol.

B. Leishman stain or Wright's stain.

Technique:

1. Place a drop of 0·3% Brilliant Cresyl Blue stain in absolute alcohol on a slide and allow it to dry.

2. A drop of blood 2 to 3 mm. in diameter is placed on another slide and brought in contact with the dried stain; the two slides are then manipulated hinge-like until all the stain has gone into solution and the blood appears blue-black. Allow the slides to come into contact to spread the drop; then separate the slides and allow the films to dry.

3. Counterstain with Leishman or Wright by the standard technique.

Results:

Reticulum of immature red cells is stained clear cut blue; background, pale blue or pink. Blood platelets: pale blue or lilac.

Note: The counterstain may be omitted if it is desired only to count the platelets.

The number of red cells per cm. should be determined separately in a haemocytometer, and the ratio of platelets to red cells computed from the stained preparation.

For reticulum

Stock solution:

Brilliant Cresyl Blue	.. ..	1·5 gm.
Normal saline (0·85% NaCl)	..	100 ml.

Technique:

1. Mix a small quantity of the stock solution of the stain with 140 times its volume of normal saline solution.

2. Mix the blood in a white-cell counting pipette in the proportion of 1 volume blood to 20 vols. of the diluted staining solution. Shake the mixture for five minutes in the pipette, and place in a blood counting cell.

3. The fresh preparations are sealed with petroleum jelly to prevent drying, and are counted immediately. At least 1,000 should be counted for each test.

Result:

Reticulum only stained (blue).

Reference: Robertson, O. H. (1917).

CARBOL ANILINE FUCHSIN

For Negri bodies

Solutions required:

A. Basic fuchsin .. .. .. 0·5 gm.
Distilled water .. .. .. 80 ml.
Absolute alcohol .. .. .. 20 ml.
Aniline oil.. .. .. .. 1 ml.
Phenol .. .. 1 gm.

B. Methylene Blue (Loeffler).

Technique:

1. Fix tissues in Zenker's Fluid for twenty-four hours; wash in running water for two or three hours; dehydrate; clear; embed in paraffin wax in the usual manner.

2. Sections, 4 to 5μ in thickness, are stained from ten to thirty minutes in Solution A; then washed with distilled water.

3. Stain with Methylene Blue (Loeffler) for fifteen to sixty seconds; then wash with water.

4. Dehydrate and differentiate for a few seconds in absolute alcohol; then clear in xylol and mount.

Results:

Negri bodies are stained crimson against a blue background.

Note: The method is stated to be excellent for Borrel bodies also.

Reference: Goodpasture, E. W. (1925).

CARBOL CRYSTAL VIOLET

For fibrin network in blood smears

Solution required:

Carbol Crystal Violet

Technique:

Note: To retain the normal arrangement of the filaments in the fibrin network, the slides must be slowly and carefully placed in, and taken out of the reagents.

1. Fresh thick blood smears are made on scrupulously clean slides and placed in a moist chamber, immediately, to prevent drying.

Note: The moist chamber may consist of a petri dish the lid of which is lined with two sheets of filter paper, moistened with warm water to accelerate clotting.

2. After the blood has coagulated and the fibrin framework has formed (this takes about six to ten minutes) take the slides out of the moist chamber and transfer to distilled water to haemolyse the red corpuscles: this takes about five to thirty minutes.

3. Change the water when it is tinged red.

4. When the haemolysis is complete: that is when all traces of red colour has disappeared from the slides and the fibrin network appears as a whitish film on the slide, remove slides and carefully blot away excess water, but do not blot network owing to risk of displacement.

5. Stain for eight minutes in carbol crystal violet.

6. Rinse in distilled water and carefully blot off excess water.

7. Pass through three or four changes of dioxane and mount quickly in D.P.X. or Cristallite or Clearmount, before the network drys.

Results:

Fibrin network is stained violet. White cells appear as irregularly shaped black dots. Platelets appear as black dots about the size of a pin head.

Reference: Badertscher, J. A. (1952), *Stain Tech.*, **27**, no. 4, 217–20.

CARBOL FUCHSIN

For acid fast lipofuscins

Solutions required:

A.	Carbol fuchsin (Ziehl Neelsen) . .	5 ml.
	Distilled water	45 ml.
B.	Absolute alcohol	35 ml.
	HCl conc.	0·5 ml.
	Distilled water	14·5 ml.
C.	Haematoxylin (Ehrlich) . .	

Technique:

1. Fix pieces of tissue in 10% neutral formalin.
2. Embed in paraffin wax or celloidin or cut frozen sections.
3. Take sections down to distilled water in the usual way.
4. Immerse in reagent A for three to four hours at 60° C.
5. Wash well in running water.
6. Rinse in distilled water.
7. Differentiate in solution B, controlling under the microscope until the red cells are faint pink in colour.
8. Wash well in distilled water.
9. Counterstain in the haematoxylin for five minutes.
10. Wash and "blue" in tap water or in lithium carbonate solution.
11. Wash in distilled water.
12. Dehydrate through the usual graded alcohols.
13. Clear in xylol.
14. Mount in Clearmount or in D.P.X. or Emexel.

Results:

Acid fast lipofuscins are stained a brilliant red, while lipoproteins are pink, and nuclei are blue or dark blue.

References:
Gomori, G. (1952).
Lillie, R. D. (1948).
Pearse, A. G. Everson (1953).

CARBOL FUCHSIN (Ziehl Neelsen)

For Nissl Bodies

Solutions required:

A.	Carbol fuchsin (Ziehl Neelsen) ..	10 ml.
	Acetic acid 0·5%	10 ml.
B.	Acetic acid 1%	10 ml.
	Formalin	0·1 ml.

Technique:

1. Small pieces of tissue are fixed in 10% formalin or in 95% alcohol or in formol-saline for at least twenty-four hours, and

afterwards washed, dehydrated, cleared and embedded in paraffin wax in the usual manner.

2. Stain sections for three to four minutes with the carbol fuchsin (Solution A).

3. Wash quickly in distilled water; then destain in Solution B.

4. Wash in distilled water; then dehydrate.

5. Clear in xylol; then mount.

Results:

Nissl bodies and nucleoli are stained dark red; remainder unstained.

Reference: Dei Poli, G. & Pomerri, G. (1938).

CARBOL FUCHSIN - BORREL BLUE

For Leprosy and for T.B.

Solutions required:

A. Carbol fuchsin (Ziehl Neelsen).

B. Sulphuric acid 5%.

C. Hydrochloric acid .. .. 1 ml.
Alcohol 70% .. .. .. 99 ml.

D. Borrel's Blue .. .. .. 5 ml.
Distilled water .. .. .. 20 ml.

Technique:

1. Material should be fixed in saturated aqueous solution of mercuric chloride and embedded in paraffin wax.

2. Fix sections to slides and treat them for the removal of mercuric precipitate by the standard method.

3. Immerse in carbol fuchsin in a staining jar for thirty minutes to an hour in the incubator at 37° C.

4. Decolorize in Solution C.

5. Decolorize in 70% alcohol (neutral) for two or three minutes until the sections appear faintly pink to the naked eye.

6. Counterstain in Borrel's Blue (diluted as above: Solution D) for one or two minutes.

7. Rinse in distilled water; drain and carefully blot away excess water.

8. Dehydrate and differentiate the Borrel Blue, controlling by examination under the microscope.

9. Clear in xylol and mount.

Results:

T.B. or leprosy, bright red; other bacteria, blue; cells and cell debris, varying shades of blue; cell nuclei, blue.

N.B.: For demonstrating leprosy, differentiation of the carbol fuchsin (stages 4 and 5) must be very carefully carried out, as this organism is more easily completely decolorized than T.B.

References:
Carleton, H. M. & Leach, E. H. (1947), pp. 320–321.
Neelsen, F. (1883).
Ziehl, F. (1882).

CARBOL FUCHSIN - HAEMATOXYLIN

For tubercle bacilli in mammalian tissue

Solutions required:

A. *Alum Haematoxylin:*

Potash alum	20 gm.
Haematoxylin	1 gm.
Thymol	1 gm.
Distilled water	400 ml.

B. Carbol fuchsin (Ziehl Neelsen) 1 volume
Distilled water .. .. .. 3 volumes

Technique:

Tissues are fixed in Zenker or Flemming and embedded in paraffin wax, L.V.N. or Celloidin.

1. Paraffin sections are brought down to distilled water; then stained one to five minutes in Solution A.

2. Differentiate if necessary with acid alcohol, controlling under the microscope, till nuclear detail is sharp and clearly defined; wash thoroughly in water.

3. Stain with Solution B for five minutes, heating till steam rises; or stain at room temperature overnight.

4. Decolorize for twenty seconds in acid alcohol; then wash thoroughly in water to which two or three drops of ammonia have been added to remove the acid.

5. Dehydrate with 95% and absolute alcohols.

6. Clear and mount.

If Celloidin or L.V.N. sections are employed, clear in terpineol or origanum oil; blot carefully on slide and mount.

Results:

Tubercle bacilli: bright red. Nuclei: blue.

Reference: Cowdry, E. V. (1952).

CARBOL FUCHSIN - HAEMATOXYLIN - PICRO ACID FUCHSIN

For M. leprae in sections

Solutions required:

A. Carbol fuchsin (Ziehl Neelsen).

B. Hydrochloric acid, conc. .. 3 ml.
Absolute alcohol .. .. 97 ml.

C. Potassium permanganate 1% aqueous.

D. Oxalic acid 2% aqueous.

E. Haematoxylin (Ehrlich).

F. Picric acid, saturated, aqueous .. 50 ml.
Acid fuchsin, aqueous 1% .. 10 ml.
Distilled water .. .. 40 ml.

Technique:

1. Pieces of tissue are fixed for three to seven days in a mixture consisting of equal volumes of 10% formalin and absolute alcohol, and paraffin sections are employed.

2. Stain sections in the carbol fuchsin solution in a stoppered staining jar for three or four days.

3. Immerse in 10% formalin, of a slightly acid reaction, for five minutes.

4. Immerse in the acid alcohol for five minutes.

5. Flood the preparation with potassium permanganate and allow the reagent to act until the sections turn brown (this usually takes from two to five minutes).

6. Immerse in the oxalic acid for one minute.

7. Stain with Ehrlich haematoxylin solution for two minutes; then blue in tap water or in saturated lithium carbonate solution aqueous.

8. Stain in picro-acid fuchsin for two to five minutes; then without washing:

9. Dehydrate, clear and mount.

Results:

M. leprae: dark blue. Connective tissue fibres: red. Muscle: yellow. Nuclei: brown.

Reference: Fite, G. L. (1939).

CARBOL FUCHSIN - HAEMATOXYLIN ORANGE G

For demonstrating leprosy organisms together with neurokeratin of the myelin sheath

Solutions required:

A. Lugol's iodine.

B. Carbol fuchsin (Ziehl Neelsen).

C. Absolute alcohol .. .. .. 35 ml.
Distilled water .. .. .. 65 ml.
Hydrochloric acid concentrated.. 0·5 ml.

D. Ehrlich's haematoxylin.

E. Strong ammonia solution (sp. gr. 0·880) .. .. 1 ml.
Distilled water .. .. .. 99 ml.

F. Orange G aqueous 1%.

Technique:

1. Pieces of tissue are fixed in Zenker; washed; then transferred to a mixture of Lugol's iodine and 80% alcohol (equal volumes of each) for six to twenty-four hours.

2. Transfer to 80% alcohol for twelve to twenty-four hours.

3. Immerse in 95% alcohol for two to six hours.

4. Transfer to a mixture consisting of equal volumes of absolute alcohol and xylol, for half an hour.

5. Immerse in xylol for half an hour.

6. Immerse in two changes of paraffin wax before casting the block and finally sectioning.

7. Fix sections to slides and remove wax with xylol.

8. Pass through absolute, 90% and 70% alcohol.

9. Stain for half an hour in carbol fuchsin (Solution B).

10. Rinse in distilled water.

11. Partially differentiate with the acid alcohol (Solution C, above).

12. Rinse well with distilled water.

13. Stain for one to two minutes with Ehrlich haematoxylin solution.

14. Differentiate in the acid alcohol (Solution C).

15. Rinse well with distilled water.

16. Immerse in the ammonia solution (Solution E above) for a few seconds.

17. Rinse well with distilled water.

18. Stain with the Orange G solution for two to three minutes.

19. Dehydrate rapidly with two changes of acetone.

20. Clear in xylol and mount.

Results:

Leprosy organisms and neurokeratin are stained red, while nuclei are blue and cytoplasm is yellow.

Reference: Campbell, H. (1929).

CARBOL FUCHSIN - METHYL GREEN

For demonstrating hyaline substance

Solutions required:

A. Carbol fuchsin (Ziehl Neelsen) .. 5 ml.
Distilled water .. .. .. 45 ml.

B. Methyl green, 1% in 5% aqueous phenol

Technique:

1. Material which has been fixed in any of the standard fixatives is embedded in paraffin wax.
2. Fix sections to slides: then bring down to distilled water as usual.
3. Stain in Solution A for fifteen to forty-five minutes.
4. Wash with distilled water; drain off excess; then blot carefully.
5. Dehydrate rapidly with absolute alcohol.
6. Differentiate and counterstain in Solution B for two or three minutes.
7. Wash quickly with absolute alcohol.
8. Clear in xylol and mount.

Results:

Hyaline substance is stained bright red while nuclei are light green.

Note: Carleton & Leach attributed the method to Russell.

References:

Carleton, H. M. & Leach, E. H. (1947), pp. 307–308.
Schmorl, C. G. (1928), p. 219.

CARBOL THIONIN - PICRIC ACID

(After Schmorl)

For demonstrating bone canaliculi

Solutions required:

A. *Decalcifying solution:*
Formalin 10% .. .. .. 100 ml.
Nitric acid, conc. .. .. 15 ml.

B. Carbol thionin (Nicolle).

C. Picric acid 1% aqueous.

Technique:

1. Formalin-fixed specimens are placed in a large volume of Solution A, which is changed once or twice a day. The time required for complete decalcification will depend, of course, on the thickness and the nature of the specimen. The bones of young animals usually take from twenty-four to forty-eight hours, but in other cases as long as a week may be necessary. Decalcification is complete when the bone has become flexible and can easily be punctured with a needle.
2. Make Celloidin or frozen sections.
3. Rinse in water for ten minutes.
4. Stain with Solution B for 10 minutes; then rinse in distilled water.
5. Immerse in Solution C for half to one minute; then wash in water.
6. Differentiate with 70% alcohol for five to ten minutes until the stain ceases to come out of the sections.
7. Dehydrate with 96% alcohol; clear in origanum oil; then mount in balsam.

Results:

Ground substance, yellow to brown; bone canaliculi, dark brown to black; cells, red; ground substance of cartilage, brilliant purple.

Note: Carbol thionin (Nicolle) deteriorates after a few weeks and this stain is therefore best when freshly prepared.

References:

Carleton, H. M. & Leach, E. H. (1947), pp. 213–214.
Schmorl, G. (1928).

CARMINE - METHYLENE BLUE (Schultz Stain)

For demonstrating sodium urate in animal tissue

Solutions required:

A.	Distilled water	64 ml.
	Lithium carbonate	0·5 gm.

Carmine .. .. 1 gm.
Ammonium chloride .. .. 2 gm.

Boil for a few minutes; allow to cool; then make up to the original volume and add:

Strong ammonia solution .. 6 ml.
Filter before use.

B. Solution A .. .. .. 15 ml.
Pure methyl alcohol .. .. 12·5 ml.
Strong ammonia solution (sp. gr. 0·880) .. .. 2 ml.
Distilled water .. .. .. 5·5 ml.

C. Methylene Blue 1% in absolute alcohol.

D. Picric acid, saturated aqueous .. 27 ml.
Sodium sulphate saturated aqueous .. .. .. 3 ml.

Technique:

1. Fix thin slices of the material in absolute alcohol.

2. Immerse for one-and-a-half to two hours in each of three changes of acetone.

3. Transfer to a mixture of equal volumes of acetone and benzol for half an hour.

4. Immerse in pure benzol for one half to one hour; then embed in paraffin wax.

5. Fix sections to slides and de-wax with xylol.

6. Pass through absolute, followed by 90% alcohol.

7. Immerse in Solution B in a grooved staining jar for five minutes, rocking gently, but continuously, during the period of staining.

8. Rinse thoroughly with absolute alcohol.

9. Stain for half a minute in the methylene blue solution.

10. Rinse with absolute alcohol.

11. Stain for fifteen to thirty seconds in Solution D keeping the slides in motion by rocking.

12. Dehydrate thoroughly with absolute alcohol; clear in xylol and mount in balsam or cristalite.

Results:

Nuclei are stained greyish blue, while cytoplasm is yellowish; uric acid crystals are deep greenish blue; monosodium urate, brilliant green.

References:

Schultz, A. (1931), pp. 174–180.
Lillie, R. D. (1948), p. 252.

CELESTIN BLUE - CHROMOTROPE 2R

A substitute for haematoxylin-eosin, for simple diagnostic or photographic purposes, emphasizing the staining of collagen and reticulum

Solutions required:

A. Celestin blue (as solution B, page 166)

B. Alcohol 70% .. .. .. 100 ml.
Hydrochloric acid, conc. .. .. 2 ml.

C. Phosphomolybdic acid 1%

D. Chromotrope 2R 1% in absolute alcohol

Technique:

1. Fix tissues in Zenker or Bouin (exactly as described on page 166, stage 1).
2. Stain section in the celestin blue solution for fifteen minutes.
3. Remove any cytoplasmic staining with solution B.
4. Wash with water for one minute.
5. Mordant with 1% phosphomolybdic acid solution for one to two minutes.
6. Wash well with water.
7. Dehydrate; then stain for two minutes in the chromotrope 2R solution.
8. Dehydrate; clear in xylol, and mount in D.P.X.

Results:

Nuclei: bluish purple. Cytoplasm, pink; collagenous elements, bright red.

Note: For photographing this stain the best filters to use are those giving a spectral transmission of 5,600 to 6,000 A.U.

Reference: Lendrum, A. C. (1935).

CELESTIN BLUE - ORCEIN - LIGHT GREEN

For the study of skin lesions, etc.

Solutions required:

A. *Rubens - Duval Orcein:*

Orcein	0·1 gm.
Alcohol 70%	100 ml.
Nitric acid, conc.	2 ml.

B. Celestin Blue .. .. .. 0·5 gm.
Iron alum 5% .. .. .. 100 ml.

Shake the dye with the iron alum solution in a flask; then boil for three minutes.

Allow to cool; filter; then add:

Sulphuric acid, concentrated	2 ml.
Glycerine	14 ml.

C. Eosin yellowish aqueous 1% .. 2 volumes
Gallic acid 0·5% aqueous .. 1 volume

D. Phosphomolybdic acid 1% aqueous.

E. *Masson's Light Green:*

Light green 2% aqueous	100 ml.
Glacial acetic acid	2 ml.

Technique:

1. Fix tissues in Zenker or Bouin; if the latter is used then picric acid must be removed by washing de-waxed and dehydrated sections on slides with saturated lithium carbonate solution. If Zenker is employed; then mercuric precipitate must be removed after fixation.

2. Wash; dehydrate; clear; embed in paraffin wax as usual.

3. Fix sections to slides; remove paraffin wax with xylol.

4. Pass through descending grades of alcohol down to distilled water in the usual manner.

5. Stain for one half to two hours in Solution A, in a stoppered grooved staining jar in the incubator or for twenty-four hours at room temperature.

6. Rinse well with distilled water.

7. Stain the nuclei with celestin blue (Solution B) for fifteen minutes.

8. Wash with running water for twenty minutes.

9. Stain muscle and epidermis for two minutes with eosin (Solution C).

10. Decolorize the collagen somewhat in water or in 30% alcohol.

11. Immerse in phosphomolybdic acid for two minutes.

12. Wash well in distilled water.

13. Stain collagen with the light green solution.

14. Dehydrate rapidly.

15. Clear in xylol; mount in D.P.X.

Results:

Elastin: light brown. Nuclei: bluish purple. Muscle and epidermis: red. Collagen: green.

Reference: Lendrum, A. C. (1935).

CHLORAZOL BLACK

A general-purpose stain, which can be used for whole tissues as well as for sections. The stain requires no mordant or differentiation, and it may be employed in aqueous or alcoholic solution. A saturated solution in 70% alcohol stains ordinary sections in fifteen to thirty minutes; the stain does not fade.

The stain is particularly suitable for staining embryo; kidney; intestine for demonstrating epithelial cells; chromatin; nucleoli; muscle fibres.

Solution required:

Chlorazol Black, saturated in 70% alcohol.

Technique:

1. Tissues should be fixed in Zenker and embedded in paraffin wax.

2. Bring sections down to 70% alcohol and remove mercuric precipitate in the usual manner.

3. Stain in a freshly prepared, unfiltered, saturated solution of Chlorazol Black in 70% alcohol for five to ten minutes.

4. Drain off excess dye; dehydrate; clear in xylol and mount.

Results:

Embryo, epithelial cell tissues: outlined in black. Chromatin: black. Nuclei: black. Muscle fibres: intense black. Lymphocytes: intense black. Blood cells: yellowish green. Cytoplasm: greenish grey.

Kidney and intestine: varying shades of green, grey and black. Blood cells: light green.

Nuclei and chromosomes are stained black; cytoplasm and secreted products grey; chitin, green; glycogen, red.

Notes:

(*a*) Benzyl alcohol may also be used as a solvent, in which case results are somewhat different.

(*b*) If it is desired to differentiate chlorazal black, dilute "Milton" (a proprietary antiseptic) may be used for the purpose.

(*c*) The stain may be incorporated with Lactophenol.

(*d*) In an earlier paper (Cannon, 1937) terpineol was suggested in error as the differentiating agent.

Reference: Cannon, H. G. (1941).

CHLORAZOL FAST PINK

As an *in vivo* stain for unmineralized bone and tooth matrix in experimental animals

Solution required:

Chlorazol fast pink 5% in physiological saline.

Technique:

1. Two adult litter-mate rabbits (A and B) were intravenously injected with 10 ml. of the above solution.

2. Ten days after injection, fluorine was administered to rabbit B to induce the lesions of skeletal fluorosis.

3. A third litter-mate (C) was subjected to fluorine for the same period of time, but not injected with the dye until two hours before sacrifice.

4. All three animals were killed six weeks after administration of fluorine.

Note: Skeletal fluorosis was induced by adding sodium fluoride to the drinking water to produce a fluoride concentration of 500 parts per million.

5. The long bones of the rabbits were dissected.

Results:

The long bones of the non-fluoretic rabbit (A) were found to be stained red upon their periosteal and endosteal surfaces; little dye was found in the middle of the cortical compacta.

The fluorotic rabbits (B and C) were found to have developed exostoses upon the middle surfaces of the long bones. In rabbit B the surfaces of the bone had taken up the dye before the exostoses had developed, and the original cortical bone was separated from the more recently formed fluorotic exostoses by a red line. The exostoses themselves were unstained.

In rabbit C, however, the exostoses bone had formed upon the long bones before the dye had been injected, and it was found to be heavily stained.

Notes:

(*a*) The author states that apparently the age of osteoid tissue does not affect the dye uptake and the stainability of the matrices depended solely upon their being non-mineralized and he goes on to describe experimental work in support of this.

(*b*) Other experimental work, is also described, in which culture filtrates of *Clostridium histolyticum* containing varying amounts of three proteinases, the β, γ, and δ toxins (Hobbs, 1958) were used for digesting undecalcified bone and the results indicate that chlorazol fast pink stains collagen of the bone matrix without denaturing it with respect to the B toxin of *Cl. histolyticum*, which is a specific collagenase. The author cites the works of Oakley and Warrack (1950, 1958), who showed that while denatured forms of collagen (gelatine and azocoll) are attacked by

the β, γ, and δ toxins of *Cl. histolyticum*, native collagen is attacked by the β toxin only.

(*c*) The author confirms the results of Evans and Prophet (1950) in similar experiments on dentine with the enzymes of *Cl. histolyticum*.

(*d*) In the original paper, to which reference should be made for further information, it is suggested that possibly the method could be extended by use of different coloured dyes structurally related to chlorazol fast pink. In this connection I would suggest the following dyes which are structurally related to chlorazol fast pink:

Chlorazol sky blue FF (Michrome No. 91)
Chlorazol violet N (,, ,, 456)
Chlorazol green (,, ,, 675)

Another dye of the same group (disazo), which might be worth trying is

Chlorazol black E, Michrome No. 92

Reference: Weatherell, J. A. (1960).

CHRYSOIDIN

For the selective staining of mast cell granules

Note: The author (Kiyoshi Harada) states that smears of abdominal fluid, mesentery and skin of mouse were fixed in methyl alcohol, ethyl alcohol, 20% formalin and 4% basic lead acetate solutions, dehydrated with alcohol and embedded in paraffin wax. Also, cock's comb, umbilical cord of pig, cartilage matrix and chick embryo, goblet cells of mouse intestine and juxtaglomerular granules of mouse kidney were used for the contrast materials.

Method 1

Solution required:

Chrysoidin Y, 0·5% aqueous

Technique:

1. Remove paraffin wax from sections with xylol, and take down to 80% alcohol in the usual way.
2. Stain in the chrysoidin solution for five to ten minutes.

3. Rinse in distilled water.
4. Dehydrate in 96% alcohol and two changes of absolute alcohol.
5. Clear in xylol, and mount in Canada balsam in xylol or in a synthetic mountant such as D.P.X. or Cristalite.

Results:

Mast cell granules: deep brown to black in type I cell; brown to yellow in type II cell. Nuclei and other tissue elements: slightly yellowish. Glands in subcutaneous tissues: yellow.

Note: The author states that in using simple aqueous solutions of various basic dyes in investigating the staining of tissue containing acid polysaccharides, it was found that only chrysoidin stained the mast cell granules selectively.

Method 2

Chrysoidin-eosin-light green-haematoxylin

Solutions required:

A. Alum haematoxylin (Carazzi)

Haematoxylin	0·1 gm.
Potash alum	5 gm.
Distilled water	80 ml.
Glycerine	20 ml.
Potassium iodate	0·02 gm.

B.

Light green SF, 1% aqueous ..	1 ml.
Distilled water	49 ml.

or:

C.

Eosin, 1% aqueous	0·5 ml.
Tap water	49·5 ml.

Note: Solution C contains 1:10,000 of eosin, and this may be further diluted, to as low as 1:50,000, with tap water, if desired.

D. Chrysoidin Y, 0·5% aqueous

Technique:

1. Take sections down to 80% alcohol as usual.
2. Stain in the alum haematoxylin for five to ten minutes.

3. Wash in tap water.
4. Stain for three minutes in either solution B or C.
5. Rinse in water.
6. Stain in the chrysoidin solution for five to ten minutes.
7. Rinse in water.
8. Dehydrate with 96% alcohol and two changes of absolute alcohol; then clear and mount.

Results:

Mast cell granules: brown. Neuclei: greyish. Epidermis: slightly yellow. Glands: yellow. Connective tissue: green or red.

Method 3

Chrysoidin - periodic acid - Schiff

Solutions required:

A. Periodic acid, 0·7% aqueous*
   * See note (*d*), page 173
B. Schiff's reagent
C. Sodium bisulphite, 0·52% aqueous
D. (**Optional**) Alum haematoxylin (Carazzi)
E. Chrysoidin Y, 0·5% aqueous

Technique:

1. Immerse in solution A for ten minutes.
2. Rinse in water.
3. Immerse in the Schiff's reagent for fifteen to thirty minutes.
4. Immerse for one minute in each of three changes of the sodium bisulphite solution.
5. Wash in running water for ten minutes.
6. (**Optional**) Stain in the haematoxylin solution for five to ten minutes.
7. Wash in water.
8. Stain in the chrysoidin solution for ten minutes.
9. Rinse in water.
10. Dehydrate with 96% alcohol and two changes of absolute alcohol.
11. Clear in xylol and mount in Canada balsam in xylol or in a neutral synthetic medium.

Results:

Mast cell granules: brown. Nuclei: grey (weakly yellowish). Epidermis: slightly yellow. Glands: yellowish. Connective tissue: intense purple.

In type II cells of abdominal fluids, the granule or its halo of the outer layer is stained weakly with the periodic acid–Schiff procedures.

Notes:

(*a*) The author states that according to Lison (1953) it has been shown that metachromasia of tissue elements is produced by the presence of ribonucleic acid, polysaccharide sulphate ester, metaphosphate, heparin, hyaluronic acid and occasionally deoxyribonucleic acid.

(*b*) It is also stated in the original paper, to which readers are referred for more detailed information, that the mechanism of chrysoidin staining of the mast cell granules is not due to metachromasia but to strong basophilia of mast cell granules, while that of a metachromatic dye consists of two factors: basophilia and metachromasia.

(*c*) In his observation of the staining with the combination periodic acid–Schiff–chrysoidin, Harada claims that it is clearly proved that mast cell granules are PAS negative, but intergranular cytoplasm is often slightly PAS negative, and accordingly the PAS reaction is negative in type I mast cells, but a proportion of the granules or their halos in the outer layer of the cytoplasm of type II cells are stained weakly purple.

(*d*) In the original paper, solution A is given as: "0·69% potassium periodate in 0·3% nitric acid." This amounts to the same as 0·7% periodic acid, which solution is simpler and quicker to prepare when the solid periodic acid is readily available.

References:
Harada, K. (1957).
Lison, L. (1953).

CONGO RED

For Amyloid in tissues

Solutions required:

A. Congo Red 1% in distilled water.

B. Lithium carbonate saturated aqueous

C. Delafield or Ehrlich haematoxylin.

Technique:

1. Formalin or alcohol-fixed material may be embedded in Celloidin or in paraffin wax, or frozen sections may be employed.

2. Sections are mounted on slides and brought down to distilled water as usual.

3. Stain in the Congo Red solution for ten to thirty minutes.

4. Immerse in the lithium carbonate solution for fifteen seconds.

5. Decolorize in 80% alcohol until stain ceases to come away in clouds.

6. Wash in running water for fifteen minutes; then stain with Ehrlich or Delafield haematoxylin for five to ten minutes.

7. Wash in tap water; dehydrate in the usual manner; clear in xylol and mount.

Note: If Celloidin sections are used dehydration should be carried out with isopropyl alcohol in place of ethyl alcohol.

Results:

Amyloid, red; nuclei, blue.

References:

Bennhold, H. (1922).
Cowdry, E. V. (1952).
Hass, G. M. (1942).
Taran, A. G. (1936/7).

CONGO RED - ANILINE BLUE - ORANGE G

For elastic fibres

Solutions required:

A. Aluminium chloride, 2% aqueous.

B.

Congo Red	2 gm.
Sodium citrate	2·5 gm.
Glycerin	1 ml.
Distilled water	97 ml.

C. Aniline Blue, aqueous .. .. 1·5 gm.
Orange G .. .. .. 2·25 gm.
Resorcinol .. .. .. 3 gm.
Phosphomolybdic acid 1% aqueous 100 ml.

Technique:

Tissues should be fixed in 10% formalin, and frozen sections should be employed.

1. Wash sections in water; then immerse them in Solution A for ten minutes.

2. Wash with water and drain; then stain in the Congo Red solution for ten minutes.

3. Wash with tap water; then plunge the slide into a dish of tap water and agitate it there for ten seconds.

4. Wash again with tap water; then stain from five to ten minutes in the Aniline Blue-Orange G solution (Solution C above).

5. Rinse carefully in tap water; drain well and blot.

6. Dehydrate in absolute alcohol; clear in origanum oil; wash in xylol and mount.

Results:

Elastic fibres: bright red. Fibrin: dark blue. Erythrocytes: yellowish orange.

Reference: Krajian, A. A. (1941).

CONGO RED - EHRLICH HAEMATOXYLIN
For eleidin and keratohyalin

Solutions required:

A. Congo Red 0·05% aqueous.

B. Ehrlich haematoxylin.

Technique:

1. Material should be fixed in absolute alcohol and embedded in paraffin wax.

2. Sections not more than 5μ thick are mounted on slides and brought down to distilled water as usual.

3. Stain for five to ten minutes in the Congo Red solution.

4. Rinse in distilled water; then stain for five to ten minutes in Ehrlich haematoxylin.

5. Blue in tap water in the usual manner.

6. Dehydrate; clear in xylol and mount.

Results:

Eleidin is stained red, while nuclei and keratohyalin are blue.

Reference: Carleton, H. M. & Leach, E. H. (1947), p. 287.

CONGO RED

A simple stain for beta cells of the hypophysis

Solutions required:

A. Congo red .. .. 1 gm.
Distilled water .. .. .. 100 gm.

B. Lithium carbonate, saturated aqueous

C. Haematoxylin (Harris)

D. Absolute alcohol .. .. 70 ml.
Distilled water .. .. .. 30 ml.
HCl, conc. .. .. .. 1 ml.

E. Tap water substitute (Scott), as recipe, page 546

Technique:

1. Fix material in 10% formalin and embed in paraffin wax.

Note: After adequate fixation the time the material is left in formalin, within reasonable limits, does not affect the result.

2. Cut sections at 6μ and attach them to slides.

3. Remove paraffin wax with xylol.

4. Wash in absolute alcohol.

5. Wash in 90% alcohol.

6. Wash in 70% alcohol.

7. Wash in distilled water.

8. Immerse in congo red solution for twenty minutes.

9. Immerse in saturated aqueous lithium carbonate (solution B) for one minute.

10. Wash in running water for ten minutes.

11. Stain in Harris' haematoxylin for one to two minutes.

12. Differentiate the nuclei in solution D (acid alcohol), controlling by observation under the microscope.

13. Blue in tap water substitute (solution E) for five minutes.

14. Rinse in distilled water.

15. Rinse in 70% alcohol.

16. Rinse in 90% alcohol.

17. Dehydrate in absolute alcohol.

18. Clear in xylol.

19. Mount in D.P.X. or Clearmount or Cristalite.

Results:

Granules of beta cells: deep orange-red. Granules of the alpha cells: pale yellow. Cytoplasm of chromophobes: unstained. Nuclei: blue or blue-black.

Notes:

(*a*) It is claimed that this simple method, employing congo red, provides differential staining of high specificity which is not dependent upon the use of a special fixative or fixation times.

(*b*) The author states that the method has been well tried in his laboratories over a period of a year on a wide range of materials from autopsies and experimental animals, and the positive staining by congo red consistently coincides with that given by other accepted stains for beta cells.

(*c*) It is mentioned by the author (Kerenyi) that congo red was tried by Trautman (1916) as a stain for the hypophysis as a result of which it was suggested at that time as a specific stain for alpha cells, which proved to be an error, and in consequence, Trautman's method never became generally used.

(*d*) For further information and photomicrographs, the original paper should be consulted.

Reference: Kerenyi, N. (1959).

CRESOFUCHSIN - CARMINE - ANILINE BLUE - ORANGE G

For demonstrating the various components of the Hypophysis

Solutions required:

A. Cresofuchsin, 1% in absolute alcohol containing 2% of conc. HCl

B. Alum carmine (Mayer).

C. Orange G .. .. .. 2 gm.
Phosphomolybdic acid .. .. 1 gm.
Distilled water .. .. .. 100 ml.

D. Phosphomolybdic acid 5% aqueous.

E. Aniline Blue 0·2% aqueous.

Technique:

1. Fix in 10% formalin; harden and dehydrate in graded alcohols; clear in chloroform: embed in paraffin wax.

2. Sections are brought down to 70% alcohol and stained for two to twenty-four hours in Solution A.

3. Wash quickly with distilled water; then stain with Solution B for three hours, afterwards washing with distilled water.

4. Differentiate and stain the acidophil cells for five minutes with Solution C; then rinse in distilled water.

5. Immerse in Solution D for two minutes; then blot dry.

6. Stain ten to twenty minutes with Solution E.

7. Rinse in distilled water; differentiate with 75% alcohol until no more stain comes out; then dehydrate; clear in xylol and mount.

Results:

Chief cells: blue to grey. Pregnancy cells, blue with small bright yellow granules; basophiles with coarse reddish blue granules. Epithelium of the pars intermedia and pars tuberalis: variable. Collagen fibre: intense blue. Glia fibres, blue-grey; axons, occasionally black.

Reference: Berblinger & Bergdorf (1935).

CRESYL FAST VIOLET

For the demonstration of *Cryptococcus Neoformans* in polarized light

Solutions required:

A. Sodium acetate, 1% aqueous

B. Cresyl fast violet .. .. .. 0·1 gm.
Acetic acid, 1% aqueous .. 30 ml.
Solution A .. .. .. 20·5 ml.
Distilled water .. .. .. 67·5 ml.

Technique:

1. Fix pieces of tissue in 10% formalin and embed in paraffin wax in the usual manner.
2. Fix sections to slides, then dewax with xylol.
3. Take sections into distilled water through the usual descending grades of alcohol.
4. Stain in solution B (buffered cresyl fast violet, pH 3·5) for fifteen to thirty minutes.
5. Differentiate in 95% alcohol, controlling by microscopic examination while the section is still wet, until the background is clear.
6. Dehydrate quickly in three changes of absolute alcohol.
7. Clear in xylol or toluene.
8. Mount in D.P.X. or Clearmount.
9. Examine in polarized light.

Results:

The spherical cytoplasm of the fungi appears as a pink luminous centre, surrounded by brilliantly birefringent spinous radiations of the capsule.

Reference: Klatzo, I. (1958).

Notes:

(*a*) The author states that in his experience the intensity of birefringence of the organism in sections stained by routine histological and special fungal techniques is frequently too low to be of any value in diagnostic problems, but with the buffered cresyl fast violet technique, the appearance of the organisms is so distinct and characteristic that even single isolated cells can be identified easily under the low power.

CRESYL FAST VIOLET

A durable Nissl stain for frozen and paraffin sections

(After R. C. Fernstrom, 1958)

Solutions required:

A. Albrecht's alcoholic gelatine (recipe page 527)

B. Cresyl fast violet .. .. .. 5 gm.
Distilled water .. .. .. 100 ml.

C. Acetic acid, 1% aqueous

D. Solution B .. .. .. 5 ml.
Solution C .. .. .. 5 ml.
Distilled water .. .. .. 90 ml.

Note: This solution should be prepared as and when required for immediate use.

E. Celloidin 0·5% in equal volumes of absolute alcohol and ether

F. Absolute alcohol .. .. 50 ml.
Chloroform .. .. .. 50 ml.

Technique:

(*a*) *Frozen sections*

1. Frozen sections are cut at 25–50μ and stored in 10% formalin.
2. Rinse in distilled water.
3. Immerse in Albrecht's alcoholic gelatine for at least five minutes.
4. Transfer the sections, by means of a fine camel hair brush to slides.
5. Drain off excess liquid by tilting the slide and carefully blotting around the edges of the section.
6. Leave the preparation exposed to the atmosphere until it is almost dry.
7. Gently blot with filter paper.
8. Immerse at once in 95% alcohol.
9. Dehydrate in absolute alcohol.
10. Immerse in solution E (0·5% celloidin) for at least one minute.

11. Expose the preparation to the air for a few seconds.
12. Immerse for at least twenty minutes in solution F.
13. Pass through the usual descending grades of alcohol into distilled water.
14. Proceed as steps 7–15, below.

(*b*) *Paraffin sections*

1. Fix sections to slides and remove paraffin wax with xylol as usual.
2. Wash in two changes of absolute alcohol.
3. Coat with the celloidin solution (solution E).
4. Expose the preparation to the air for a few seconds.
5. Immerse in solution F for at least twenty minutes.
6. Pass through the usual ascending grade of alcohol in distilled water.
7. Immerse in the acidified cresyl fast violet (solution D) for three to ten minutes.

Note: The solution can be used several times but it should be renewed when signs of any loss of colour intensity are observed.

8. Rinse in distilled water.
9. Agitate the slides in 70% alcohol until the stain begins to come out of the sections in clouds.
10. Rinse quickly in 95% alcohol.
11. Immerse in chloroform for twenty minutes or more.
12. Differentiate with 95% alcohol, controlling by microscopic examination while the preparation is still wet, until the desired coloration is attained.
13. Dehydrate in absolute alcohol.
14. Immerse in normal butyl alcohol (absolute) for one to five minutes.
14. Clear by immersion in at least two lots of xylol.
15. Mount in D.P.X. or Clearmount.

Notes:

It is claimed that the technique offers at least two advantages over other Nissl techniques:

(*a*) Continuous exposure to light in the laboratory for almost a year produced no noticeable fading or colour change.

(*b*) The method greatly simplifies the staining of nerve cell bodies in frozen sections.

Reference: Fernstrom, R. C. (1958).

CRESYL FAST VIOLET

A chromatin test for the detection of chromosomal sex

(After K. L. Moore, 1960)

Solutions required:

A. Absolute alcohol } equal
   Ether } volumes

B. Cresyl fast violet CNS .. .. 1 gm.
   Alcohol, 50% .. .. .. 100 ml.

Technique:

1. Take scrapings, from the inside of the cheek, with a spatula.

2. Spread the scraping on a slide.

3. Fix immediately in the alcohol–ether mixture, for at least fifteen minutes.

Note: The author (Moore) states that the usual causes of poor smears are: (*a*) not scraping firmly enough, with the result that the number of cells obtained is insufficient, and (*b*) allowing the smear to dry before fixation, resulting in poor nuclear detail.

4. Immerse the preparation in 70% alcohol for two minutes.

5. Immerse in 50% alcohol for two minutes.

6. Wash for one minute in each of two changes of distilled water.

7. Stain in the cresyl fast violet solution for seven to eight minutes.

8. Differentiate by dipping the slide quickly about five to seven times in 95% alcohol.

9. Dehydrate in absolute alcohol for one minute.

10. Mount in Clearmount or Michrome mountant, or proceed as follows:

11. Wash in xylol.

12. Mount in Clearmount or Michrome mountant or D.P.X. or, if these are not available, in neutral Canada balsam in xylol, although this is liable to cause fading of the stain.

13. Examine under the oil immersion objective with a strong source of light.

Results:

The sex chromatin is visible only in the nucleus from the female.

Notes:

(*a*) The author (Moore) states that a minimum of one hundred nuclei, selected at random should be examined for the presence of sex chromatin. The sex chromatin is most definite and easiest to recognize when it is located adjacent to the nuclear membrane. It is about one micron in diameter and is usually round or plano-convex. In occasional smears the sex chromatin is flattened against the nuclear membrane, and in consequence it is less conspicuous than usual. Unmistakable sex chromatin is visible in 30 to 60% of nuclei of chromosomal females, but nuclei of chromosomal males rarely present a mass of chromatin which can be recognized as sex chromatin.

(*b*) The author observes that it is difficult to differentiate a female infant with congenital adrenogenital syndrome from a male with pseudohermaphroditism, and it is important to make this distinction early to prevent metabolic disturbances: knowledge of chromosomal sex is a helpful guide in cases of doubt.

(*c*) The original paper should be consulted for more detailed information, including a number of colour plates. Moore's 1959 paper should also be consulted.

References:

Moore, K. L. (1960).
Moore, K. L. (1959).

CRESYLFAST VIOLET - TOLUIDINE BLUE - THIONIN (EHRLICH)

A non-fading tri-basic stain for nerve cells and Nissl granules, in normal and pathological tissues

Solutions required:

A.	Cresylfast violet, CNS	2 gm.
	Toluidine blue	1 gm.
	Thionin (Ehrlich)	0·5 gm.
	Ethyl Alcohol 30%	200 ml.

B. Distilled water .. .. .. 200 ml.
Sulphuric or nitric acid, conc. .. 0·5 ml.

Technique:

1. Formalin fixed material is embedded in paraffin wax, and sections, 4μ in thickness are fixed to slides with glycerin albumen.
2. Remove wax with xylol.
3. Rinse with absolute alcohol.
4. Pass through 95% alcohol.
5. Pass through 80% alcohol.
6. Immerse slides for five to ten seconds in the staining solution at 80–90° C.
7. Differentiate for one second in solution B.
8. Dip and agitate slides in a beaker of cold distilled water for one second.
9. Differentiate further in 80% and 95% alcohol for one to two seconds in each.
10. Immerse in 80% alcohol for one second.
11. Dip and agitate the slides in the still warm solution A for one to two seconds.
12. Return to 80% alcohol for one second.
13. Repeat steps 11 and 12.
14. Rinse in distilled water.
15. Dehydrate by immersing for one second in each of 80%, 95% and absolute alcohol.
16. Immerse in xylol for one minute.
17. Immerse in a fresh lot of xylol for three minutes.
18. Mount and examine.

Results:

Neurons stand out distinctly against a pale background, and can be followed for a considerable distance. The cytons are stained dark purple emphasizing the blue tint, while the dendrite and axon processes and endings present a somewhat lighter shade, bluish to reddish. Granules in the cell body as well as in the protoplasm processes appear purple or reddish. Nuclei and nucleoli are well differentiated.

Reference: Spoerri, Rosette (1948).

DAHLIA ACETIC

For mast cell granules in sections

Solution required:

Distilled water	100 ml.
Absolute alcohol	50 ml.
Glacial acetic acid	12·5 ml.
Dahlia	10 gm.

Dissolve by heating in a flask, lightly plugged with cotton-wool, on a water bath. Allow to cool; then filter.

Technique:

1. Fix tissues in absolute alcohol and embed in Celloidin.
2. Immerse sections in the staining solution for twelve hours.
3. Differentiate in 95% alcohol.
4. Clear in origanum oil and mount in balsam or in cristalite.

Results:

Granules of mast cells are stained reddish violet

Reference: Romeis, B. (1948), p. 321.

DIGITONIN REACTION

For cholesterol

Reagents required:

A. Digitonin, saturated in 70% alcohol
Note: Digitonin is only slightly soluble.

B.

Paraffin wax, M.Pt. 52–56° C	95 gm.
Glyceryl monostearate	5 gm.

C.

Bismuth trichloride	0·2 gm.
Acetyl chloride	1 ml.
Nitrobenzene, anhydrous	100 ml.

D.

Acetyl chloride	10 ml.
Nitrobenzene, anhydrous	90 ml.

Technique:

1. Fix small pieces of tissue in reagent A for twenty-four to forty-eight hours.

2. Transfer to a porcelain plate or a watch glass and leave exposed to the air until the alcohol has evaporated.

3. Embed in reagent B.

4. Cut sections at 6 to 10μ and mount them on slides.

5. Without removing the wax from the sections on the slides, immerse them in reagent C for fifteen to forty-five minutes.

6. Rinse rapidly in reagent D.

7. Dewax the sections with benzene, and clear in benzene.

8. Mount in paraffin liquid.

Results:

Cholesterol assumes a brown colour, but its esters are uncoloured.

The preparations are stable for some considerable time.

Note:

If the colour is not considered strong enough, it may be intensified as follows:

From stage 6 above, proceed as follows:

(*a*) Wash in a mixture consisting of 1 volume of absolute alcohol and 3 volumes of nitric acid, conc., to remove excess bismuth.

(*b*) Wash rapidly in absolute alcohol.

(*c*) Immerse in a dilute solution of ammonium sulphide for a few seconds.

(*d*) Wash in absolute alcohol.

(*e*) Clear in benzene or xylol.

(*f*) Examine in paraffin liquid or mount in D.P.X. (Lendrum & Kirkpatrick).

References:

Brunswik, H. (1922).
Lison, L. (1936).

DOPA REAGENT

For melanoblasts

Solutions required:

A. Dopa reagent (3:4-dihydroxy-phenylalanin) .. .. 0·2 gm.
Cold distilled water .. .. 200 ml.

Note: This solution, which deteriorates fairly rapidly at normal temperatures, should be kept in a refrigerator. When the solution turns dark red it is useless and should be discarded.

B. Buffer tablet pH 7·4 .. .. 1 tablet
Distilled water (cold) .. .. 100 ml.

C. Solution A .. .. .. 25 ml.
Solution B .. .. .. 8 ml.

Note: This solution must be freshly prepared as required.

Technique:

Tissues should be fixed not longer than two to three hours in 5% formalin, or frozen sections of fresh material may be employed.

1. Rinse with distilled water from four to five seconds; then immerse in Solution C for three to four hours, controlling under the microscope at intervals, until melanoblasts are stained black.

Note: This solution is likely to overstain if it becomes sepia brown.

2. Wash with distilled water; dehydrate; clear; mount

Result:

Melanoblasts: black.

Notes:

(*a*) After dehydration (in stage 2) 1% crystal violet in absolute alcohol may be employed as a counterstain, if desired.

(*b*) " Dopa " is an abbreviation for dihydroxyphenylalanin.

Reference: Laidlaw, G. F. (1932).

ELASTIN STAIN

(Weigert)

Any fixative may be used except Susa, and tissues may be embedded in paraffin wax or in Celloidin or in L.V.N.

Preparation of the Staining Solution:

Triturate 1 gm. of Weigert elastin stain and 5 gm. clean, dry silver sand with 100 ml. absolute alcohol and 2 ml. pure hydrochloric acid until all the stain has gone into solution; then filter.

Note: The staining solution deteriorates after two or three weeks.

The nuclei may be stained with Orth's lithium carmine prior to the following procedure if no other counterstain is desired.

Technique:

1. Sections are brought down to 90% alcohol and stained one half to twelve hours according to depth of staining desired. The slides should be stained in a jar or in a Petri dish, sections face downwards, to prevent a deposit forming on the sections.

2. Wash off excess stain with 95% alcohol, and if necessary differentiate in acid alcohol for a few minutes.

3. Wash quickly with 70% alcohol; then thoroughly with water.

4 Counterstain with Van Gieson, Ehrlich haematoxylin or Safranin for about five minutes.

5. Differentiate, if necessary, in 95% alcohol.

6. Dehydrate; clear in xylol and mount.

Note: If Celloidin or L.V.N. sections are used clear in origanum oil or in terpineol after 95% alcohol.

Results:

Elastic fibres, dark blue or black. Nuclei, brilliant red (if Orth's carmine is used) or bluish black (with haematoxylin). Collagen, pink to red; other tissue elements, yellow (if Van Gieson is used).

Reference: Weigert, C. (1898).

ELASTIN STAIN (Sheridan)

This stain has an advantage over Weigert's elastin stain in that the solution may be kept for reasonably long periods without deterioration.

The staining procedure is the same as for Weigert's elastin stain.

Results:

Elastic fibres are stained green to greenish black.

Reference: Sheridan, W. F. (1929).

ELASTIN - TRICHROME STAIN

For the demonstration of elastic, smooth muscle and collagenic fibres with equal clarity, particularly in the walls of blood vessels

Solutions required:

A. *Weigert's elastin stain*

Weigert's elastin stain powder ..	1 gm.
Hydrochloric acid, conc., pure ..	2 ml.
Absolute alcohol	100 ml.

Dissolve the stain by boiling for two minutes in a flask, plugged lightly with cotton-wool, on a water bath. Allow to cool; then filter; make the volume up to 100 ml. with absolute alcohol; then add the acid. Alternatively the solution may be prepared as described on page 188.

Note: This solution deteriorates after three or four weeks.

B. Ehrlich haematoxylin.

C. *Ponceau-acid fuchsin (Masson):*

Acid fuchsin	0·3 gm.
Ponceau de xylidine	0·7 gm.
Distilled water	100 ml.
Glacial acetic acid	1 ml.

D. Phosphotungstic acid 3% aqueous.

E. Light Green 1% aqueous.

Technique:

1. Paraffin sections are mounted on slides and brought down to distilled water in the usual manner; then immersed in Weigert's elastin stain in a staining jar for one hour.

2. Wash rapidly in acid alcohol; then dehydrate and differentiate in absolute alcohol until the sections appear only faintly red.

3. Immerse in 70% alcohol, followed by distilled water.

4. Stain in Ehrlich haematoxylin for eight to ten minutes; then differentiate in water for five minutes.

5. Stain in Ponceau-acid fuchsin for five minutes.

6. Wash thoroughly in 3% phosphotungstic acid; then immerse in the phosphotungstic acid for ten minutes.

7. Wash thoroughly in distilled water; then stain with Light Green for two to five minutes; then without washing:

8. Flood the preparation with 1% acetic acid and allow it to act for three minutes; pour off excess; then without washing:

9. Dehydrate; clear; mount in D.P.X.

Results:

Elastic tissue stained blue-black; smooth muscle, red; collagen, green.

Reference: Mendeloff, J. & Blechman, H. (1943).

EOSIN B - ANILINE BLUE (Rhodocyan technique)

A one-step staining method for anterior pituitary

(After G. G. Glenner & R. D. Lillie, 1957)

Solutions required:

A. Eosin B, aqueous 10%

B. Aniline blue, aqueous 10%

C. Citric acid, M/10

D. Disodium hydrogen phosphate, M/5

E.	Solution A	0·8 ml.
	Solution B	0·2 ml.
	Solution C	1·1 ml.
	Solution D	0·9 ml.
	Distilled water	37 ml.

Technique:

1. Slices of tissue 3–4 mm. in thickness are fixed in neutral buffered 10% formalin or Lillie's (1954) "B-5" fixative for six to twenty-four hours. Zenker-formalin can be used instead, but acetic-Zenker and Bouin's fixatives are stated to give poor results.

2. Wash in running water; dehydrate; clear; and embed in paraffin wax as usual.

3. Cut sections at 5μ, fix them to slides; dewax and pass through the usual graded alcohols down to distilled water.

4. Immerse in a Coplin jar containing solution E at room temperature, then immediately transfer the whole to an oven at 60° C, and leave the stain to act at that temperature for an hour.

5. Wash in running water for five minutes.

6. Dehydrate and clear through 50%, 80% and anhydrous acetone, acetone xylol (1:1), xylene (two changes), and mount in a synthetic medium such as Cristalite, Emexel, Clearmount, or D.P.X.

Note: The authors suggest cellulose caprate as a mountant, but this product does not appear to be available in Britain, except, as far as I am aware, in very small quantities, and at a cost which would be considered wholly prohibitive by the vast majority of biologists.

Results:

Beta-cell granules: blue-black. Acidiphil granules: dark red. Chromophobe granules: slate grey to pale pink. Colloid: red to bluish violet. Erythrocytes: orange. Collagen: blue.

Notes:

(*a*) It is stated that no differentiation is necessary, and that the above results are consistently obtained.

(*b*) The authors state that the method can be modified for duodenal enterchromaffin cells and alpha cells of the pancreatic

islets by adjusting the buffer to pH 3·6 and staining for only three minutes at 60° C.

(*c*) The name "Rhodocyan" (rose–dark blue) was given to the technique by the authors (Glenner & Lillie).

(*d*) Using the modification mentioned above (note *b*) the results are:

Duodenal enterchromaffin cells: blue-black. Goblet cells and mucin of gastric mucous cells: light blue. Paneth cell granules: blue-black. Alpha cells of pancreatic islets: red. Beta cells: purple. Collagen and reticulum: deep blue. Striated and smooth muscle: purple-violet. Neurokeratin: pale pink. Endoneurium: blue. Axis cylinder: grey. Eosinophil granules: red. Internal root sheath of hair follicle: orange-red. External root sheath: violet.

(*e*) In the original paper 1% stock solutions of eosin B and aniline blue are stipulated, but since a 1% aqueous solution of either stain is liable to attack by air-borne micro-organisms, which bring about their decomposition in a relatively short time, I suggest dilutions be made as and when required from 10% solutions, which are immune to attack by moulds and other micro-organisms and remain stable for several years.

(*f*) For more detailed information regarding the Rhodocyan technique readers should consult the original paper which carries three photomicrographs.

References:
Glenner, G. G. & Lillie, R. D. (1957).
Lillie, R. D. (1954) p. 41.

EOSIN - LIGHT GREEN - CHROMIC ACID

For demonstrating tryptophane-containing proteins

Solutions required:

A. Chromium trioxide, 4% aqueous
B. Phosphomolybdic acid, 1% aqueous
C. Eosin, yellowish, 1% aqueous
D. Light green SF, yellowish, 1% aqueous

Technique:

1. Immerse sections in solution A for thirty minutes.
2. Wash in running water for twenty to thirty minutes.

3. Rinse in distilled water.
4. Immerse in solution B (phosphomolybdic acid) for twenty minutes.
5. Wash in distilled water.
6. Rinse in tap water.
7. Immerse in eosin (solution C) for thirty minutes.
8. Wash in tap water.
9. Immerse in light green SF (solution D) for ten minutes.
10. Rinse quickly with tap water.
11. Dry rapidly but carefully with filter paper.
12. Dehydrate with two changes of absolute isopropyl alcohol.
13. Clear in xylol.
14. Mount in Emexel, D.P.X., Clearmount or Canada balsam in xylol.

Results:

The author (Hrsel) found after a number of tests on proteins having different isoelectric points and varying tryptophane contents, that the eosin stained tryptophane-containing proteins, while the light green was taken up by amino groups. However, ribonuclease, which is devoid of tryptophane, was stained by the eosin, while pepsin, which is known to contain tryptophane, was not stained by the eosin. Chromium trioxide (chromic acid) reacts with tyrosine, cysteine, cystine and proline and very intensely with tryptophane. Root meristems of plants stained by the author (Hrsel) exhibited a strong reaction in the nucleoli.

Reference: Hrsel, I. (1957).

EOSIN AZUR 2 - HAEMATOXYLIN (Maximow)

For demonstration of inflammatory changes in haemopoietic tissues

Solutions required:

A. Azur 2 eosin .. .. .. 0·1 gm.
Distilled water .. .. .. 100 ml.
Heat to boiling point, then allow to cool.
B. Solution A (as above) .. .. 10 ml.
Distilled water .. .. .. 50 ml.
C. Ehrlich haematoxylin.

Technique:

1. Formalin-fixed material (sections or smears) are stained from five to ten minutes with Ehrlich haematoxylin.

2. Pour off excess stain; immerse the preparation in tap water until it appears blue to the naked eye; then wash thoroughly with distilled water and drain well.

3. Stain for eighteen to twenty-four hours in Solution B (as above) in a staining jar. If a staining jar is not available, place the slide, resting face downwards, on two pieces of thin glass rod, so that any precipitate formed is not deposited on the preparation.

4. Differentiate in 95% alcohol until dense blue clouds cease to come away from the preparation, and the red corpuscles and collagen are pink.

5. Immerse the preparation in three changes of absolute alcohol, followed by two changes of xylol; then mount.

Results:

Cartilage stained purple; basophil leucocytes and mast cell granules, purple to violet; nuclei, blue; erythrocytes, pink; cytoplasm, pink to blue; eosinophil granules and secretion granules, pink.

Reference: Maximow, A. J. (1924).

ERIE GARNET - AZUR A

A rapid polychromatic stain for fresh tissue diagnosis in the operating theatre

Solutions required:

A. Azur A, 1% aqueous

B. Erie garnet B, 0·5% aqueous

C. Solution A (freshly filtered) .. 4 volumes
Solution B (freshly filtered) .. 1 volume

Add solution B rapidly to solution A and immediately filter the mixture.

D. Glucose, 40% aqueous

Technique:

1. The piece of tissue, selected for microscopic examination from freshly excised material taken in the operating theatre, should preferably not be much over 7 mm. in diameter, and about 3 to 4 mm. in thickness: if the piece of tissue is too large, the size of the section cut will be difficult to handle without folding.

2. Cut sections at 10 to 15μ at a temperature of -10° to $-0{\cdot}1^{\circ}$ C on the freezing microtome, and float them on to distilled water.

3. Transfer sections to solution C by means of a glass rod and leave therein for ten to fifteen seconds.

Note: The staining time varies with different tissues, but the time specified above usually gives the most satisfactory results.

4. Lift the sections, still on the same glass rod, out of the staining solution and float them through two changes of distilled water.

5. Transfer, still on the same glass rod, to clean slides.

6. Place a large drop of the 40% glucose on a coverslip and immediately invert it over the section: allow half to one minute to elapse before examining under the microscope.

Results:

Cytoplasm is stained magenta red by the erie garnet while nuclei and other basophilic elements are stained polychromatically in shades of blue and violet by the azur A.

Notes:

(*a*) The author, Charles F. Geschickter, of the Garvan Cancer Research Laboratories, Johns Hopkins Hospital and University, Baltimore, Maryland, U.S.A., in his paper (1930), cited below, proposed the technique as an aid to the diagnosis of benign and malignant tumours.

(*b*) It is inadvisable to keep a stock solution consisting of a mixture of solutions A and B above, as suggested in the original paper, because azur A, being a basic dye, will combine to form a water-insoluble, compound dye, with erie garnet (which is an acid dye), which will be thrown out of solution. For this reason, I would suggest that two separate stock solutions (A and B above) be kept and mixed as and when required.

(*c*) The original author states that the combination azur A–erie garnet produces colour reactions closely simulating haematoxylin and eosin.

(*d*) Readers are referred to the original paper for more detailed information.

Reference: Geschickter, C. A. (1930).

ERYTHROSIN - WATER BLUE - HAEMATOXYLIN

For vaginal smears to facilitate analysis of the menstrual cycle

Solutions required:

A. Haematoxylin (Ehrlich)

B. Erythrosin, 0·5% aqueous

C. Phosphomolybdic acid, 2%

D. Water blue, 2% aqueous

Technique:

1. Fix wet smears in a mixture consisting of equal volumes of absolute alcohol and ether for five to fifteen minutes.
2. Rinse successively in 90%, 70%, and 50% alcohols.
3. Rinse in distilled water.
4. Stain for twenty minutes in Ehrlich's haematoxylin.
5. Rinse in distilled water.
6. Differentiate in 0·5% hydrochloric acid.
7. Wash in running tap water for a few seconds.
8. Blue in saturated aqueous lithium carbonate.
9. Wash well in tap water.
10. Immerse in the erythrosin solution for fifteen minutes.
11. Wash with tap water.
12. Mordant in the phosphomolybdic acid for five minutes.
13. Wash well with tap water; then rinse with distilled water.
14. Stain with water blue (solution D) for about twenty minutes.
15. Wash well with tap water.
16. Dry thoroughly in the air and mount in D.P.X. or other neutral synthetic mountant.

Results:

Nuclei: dark blue. Cytoplasm: red, blue or pale purple, depending upon the condition of the cell. The proportion of the cells with either red or blue cytoplasm was found to depend on the stage of the menstrual cycle. Pale purplish cytoplasm was found in smears made half an hour before menstrual bleeding started.

The author (C. Van Duijn, jnr.) claims that the method facilitates cycle diagnosis.

Reference: Van Duijn, jnr., C. (1956).

ETHYL VIOLET - BIEBRICH SCARLET

For pepsinogen granules in gastric mucosa

Solutions required:

A. *Stock solution.*
Ethyl violet-Biebrich scarlet 1%
in 20% alcohol.

B. *Staining solution.*

Stock solution (as above) ..	0·5 ml.
Alcohol 20%	100 ml.

Technique:

1. Tissues should be fixed in Regaud's fluid, washed in running water, dehydrated, cleared and embedded in paraffin wax in the usual manner.

2. Fix sections to slides, dewax and pass through descending grades of alcohol down to distilled water.

3. Stain for twenty-four hours in Solution B in a covered staining jar.

4. Drain and wipe off excess liquid.

5. Differentiate for about half an hour with a mixture consisting of equal volumes of clove oil and xylol, controlling by examination under the microscope at intervals.

6. Wash well with several changes of xylol; mount in cristalite.

Results:

Pepsinogen of the pepsin-forming cells is stained violet, while parietal cells are red.

Reference: Bowie, D. J. (1935/6).

THE FALGIC ACID REACTIONS

(M. A. MacConaill & E. Gurr)

1. FALG METHOD

A simple universal polychrome staining method applicable to animal and plant tissues.

Solutions required:

A.	Acid fuchsin (Michrome No. 5)	0·5 gm.
	Acetic acid, 4% aqueous ..	100 ml.
	(See note (*a*), page 199.)	
B.	Light green (Michrome No. 240)	0·125 gm.
	Water	100 ml.

Technique:

1. Stain sections or smears in solution A for five minutes.
2. Wash in water for five minutes.
3. Stain in solution B for five minutes.
4. Wash in water for five minutes.
5. Dehydrate through the usual graded alcohols.
6. Clear in xylol.
7. Mount in D.P.X., Emexel, Clearmount or Canada balsam in xylol.

Results:

A polychrome picture in shades of red, violet and blue is produced. Nuclei and chromosomes can easily be distinguished even with a × 10 objective. Erythrocytes are stained scarlet. Neurokeratin of myelinated nerve fibres; odontoblastic fibres of dentine; the most superficial fibres of stratum corneum of skin and other cornified epithelia are also stained red. Both in animal and in plant cells, the nucleus as a whole either exhibits a bright blue staining or it is unstained (clear nucleoplasm). The nucleolus, in the resting stages between mitosis, is, however, stained a bright red. During mitosis, the bright red nucleolus disappears and is replaced by mauve or violet chromosomes. The colour of these chromosomes contrasts so markedly with the blue of the cytoplasm, that a mitotic nucleus can be distinguished, as stated above, even with a × 10 objective.

(*a*) The acidified fuchsin solution is unreliable after it has been kept in stock for a week or so. The reason for this is explained on page 85. However, unacidified concentrated solutions (5 or 10%) of the dye remain stable indefinitely. It is recommended, therefore, that a 10% solution of acid fuchsin in distilled water be kept as the stock solution, and dilutions made from this in quantities sufficient for one week's use, as follows:

Acid fuchsin, 10% aqueous ..	5 ml.
Distilled water	91 ml.
Glacial acetic acid	4 ml.

Notes:

(*b*) It was found by the authors that the Falg method applied to neurological tissues gave results that were pedagogically equivalent to the Weigert–Pal method, but were obtained in a matter of only a few minutes as compared with a few weeks required for the performance of the latter method.

(*c*) It should be noted that tissues to be stained by any of the compound fuchsinic acid techniques (Falg, Trifalgic acid, Falgog, Falgose and basic and standard Faviols, etc.) should be fixed in 10% formaldehyde which has not been neutralized with metallic compounds. However, Professor MacConaill has since found that instead of pure formalin solution, acetic acid-formalin or Susa may be used for fixation. If Susa is used care must be taken to remove all the mercuric chloride from the sections before staining.

(*d*) If after staining with the acid fuchsin the preparations are immersed in a bath of 2% aqueous acetic acid for five minutes before staining with light green, the subsequent picture is in green and red only, and not in the typical Falg colours. The authors found that this is due to blockage of the amino groups of acid fuchsin through union with the acetate radicals thereby preventing the formation of the falgic acids to which the polychrome effects of Falg staining are due. Compounds formed by the union of acid fuchsin and acetic acid have since been isolated in the dry form (see page 85). The highest homologue, nono-fuchsinic acid, will not unite with light green at all.

(*e*) The Falg method was developed during the course of an investigation which was originally intended as a continuation of

the earlier studies of MacConaill (1949, 1951) on the erythrophile substances, found in the nervous system, including the so-called neurokeratin.

(*f*) Reference should be made to pages 76–90 of this book as well as to the authors' original papers for information on the mechanism of the Falg reaction and other theoretical aspects which may be of interest.

2. TRIFALGIC ACID METHOD (one-stage Falg method)

Solutions required:

Trifalgic acid (Michrome No. 818)	1 gm.
Water	100 ml.

Technique:

1. Stain sections or smears in the trifalgic acid solution for five minutes.
2. Wash in running water for five minutes.
3. Dehydrate through the usual graded alcohols.
4. Clear in xylol.
5. Mount in D.P.X. or Exemel or Canada balsam in xylol.

Results:

As with the Falg (two-stage) method, given above, but the colours are fainter.

Notes:

(*a*) The trifalgic acid method cannot be regarded as a satisfactory substitute for the Falg (two-stage) method in histology.

(*b*) Trifalgic acid was originally isolated in the solid state to confirm a hypothesis, but has since been found of value in protein-electrophoresis (Bodman, 1960).

(*c*) Readers should refer to pages 76–90 for information regarding the mechanism of the staining reactions.

3. FALGOG METHOD

For demonstrating erythrocytes, minute blood vessels and other strongly erythrophile elements of animal and plant tissues.

Solutions required:

*A. Acid fuchsin 0·5% (Michrome No. 5) in 4% aqueous acetic acid
(See note (*a*), page 199.)

B. Light green (Michrome No. 240), 0·125% aqueous

C. Acetic acid, 2% aqueous

D. Orange G (Michrome No. 411), 0·5% aqueous

Technique:

1. Stain sections or smears in solution A for five minutes.
2. Wash in running water for five minutes.
3. Stain in the light green (solution B) for five minutes.
4. Wash in running water for five minutes.
5. Immerse in the dilute acetic acid (solution C) for five minutes.
6. Wash in running water for five minutes.
7. Immerse in the orange G (solution D) for five minutes.
8. Wash in running water for five minutes.
9. Dehydrate through the usual graded alcohols.
10. Clear in xylol.
11. Mount in D.P.X. or Emexel or Canada balsam in xylol.

Results:

The orange G obliterates the Falg colours but leaves the strongly erythrophile (MacConaill, 1949, 1951) elements stained red according to their degree of erythrophilia. The red blood corpuscles stand out very clearly, facilitating the tracing of minute blood vessels throughout their course.

Notes:

(*a*) Without the protective acid bath (step 5) all the colours are obliterated by the orange G.

(*b*) Steps 1–4 constitute the Falg reaction (as page 198). The acid bath (step 5) curbs the decolorizing effects or orange G so that the strongly erythrophile elements remain stained (red).

(*c*) The picture obtained with the Falgog method (or the Falgose, described below) is superior to that produced by simple staining with acid fuchsin. Probably the orange G and glucose combine with and decolorize (reduce) definite falg-tissue compounds (chemical), thereby producing sharper pictures of the erythrophile elements.

(*d*) While the Falgose method is recommended for the usual microscopic sections (i.e. up to a thickness of 100μ) the Falgog method is preferable for thicker sections (over 100μ), including thick pieces of tissue (e.g. omentum). The reason for this is that the stronger Orange G solution used in the Falgog method tends to stain many tissues. In thick sections the stronger Orange G is apparently necessary to ensure the removal of colour from moderately and weakly erythrophile elements. Instead of being entirely removed the violet colour may be reduced to a faint blue or green. This, however, has no effect on the clarity of the picture of the blood vessels, and may in fact be useful.

(*e*) Reference should be made to pages 76–90 of this book and to the authors' original papers for an explanation of the mechanism of the staining reactions, and for other information.

FALGOSE METHOD

For demonstrating erythrocytes, minute blood vessels, and other strongly erythrophile elements of animal and plant tissues

Solutions required:

As in the Falgog method, plus the following:

E. Glucose, anhydrous .. .. 1 gm.
Water .. .. 100 ml.

F. Equal volumes of solution D (Orange G) and E

Technique:

Exactly as in the Falgog method except solution F (above) is used in place of solution D.

Results:

Similar to those given by the Falgog method: see " Results " and " Notes ", page 201–202.

Notes:

Readers should refer to pages 76–90 of this book, and to the authors' original papers listed in the bibliography, for further information.

References:

MacConaill, M. A. & Gurr, E. (1960*a*).
Gurr, E. & MacConaill, M. A. (1960*b*).

FAVIOLIC ACID REACTIONS

1. BASIC FAVIOL METHOD

A simple universal polychrome staining method applicable to animal and plant tissues.

A.	Acid fuchsin (Michrome No. 5)	0·5 gm.
	Acetic acid, 4% aqueous	100 ml.
	(See note, page 199.)	
B.	Violamine 3B (Michrome No. 161)	0·5 gm.
	Water	100 ml.

Technique:

1. Stain sections or smears in solution A for five minutes.
2. Wash in running water for five minutes.
3. Stain in solution B (violamine) for five minutes.
4. Wash in running water for five minutes.
5. Dehydrate through the usual graded alcohols.
6. Clear in xylol.
7. Mount in D.P.X. or Emexel or Canada balsam in xylol.

Results:

Similar to those produced by the Falg technique but the violet and blue colours are deeper than the corresponding shades obtained by using the Falg method.

Notes:

(*a*) Among strongly erythrophile elements are the nucleoli of cells, probably because of their histone component: red blood corpuscles and neurokeratin. The oldest fibres formed by fibroproteins, e.g. keratin and collagen fibres, are strongly erythrophile, the youngest fibres being weakly erythrophile. Indeed, there appears to be a tendency for erythrophila to increase with age in all erythrophilic tissues.

As a general rule, not yet certain, the Falg method is preferable for adult gland tissues.

(*b*) The faviolic acids have the remarkable property that they intensify the reaction of already birefringent material to polarized light. As a consequence of this, polarized light can now be used for thinner sections than previously, and for the detection of birefringent elements at the limits of the optical microscope.

(*c*) A further remarkable property of the faviolic acids is that they intensify the differentiation of structures under phase-contrast. Normally stains reduce this contrast.

(*d*) Reference should be made to pages 76–90 of this book and to the authors' original papers for further information on the mechanism of the Faviol staining.

2. STANDARD FAVIOL METHOD

A simple universal trichrome staining method applicable to animal and plant histology and cytology.

Solutions required:

A. Acid fuchsin (Michrome No. 5) 0·5 gm.
Acetic acid, 4% aqueous .. 100 ml.
(See note (*a*), page 199.)

B. Acetic acid, 2% aqueous ..

C. Violamine 3B (Michrome No. 161), 1% aqueous .. .. 12·5 ml.
Sun yellow G (Michrome No. 720), 1% aqueous .. .. 12·5 ml.
Water .. .. 75 ml.
(See notes (*a*) and (*d*), pages 205–6.)

Technique:

1. Stain sections or smears in the acid fuchsin (solution A) for five minutes.
2. Wash in running water for five minutes.
3. Immerse in the dilute acetic acid (solution B) for five minutes.
4. Wash in running water for five minutes.
5. Stain in solution C for five minutes.
6. Wash in running water for five minutes.
7. Dehydrate through the usual graded alcohols.
8. Clear in xylol.
9. Mount in D.P.X. or Emexel or Canada balsam in xylol.

Results:

Strongly erythrophile (fuchsinophile) elements: red. Moderately erythrophile elements: violet; all others yellow. Mitotic figures (red) are easily distinguished against the yellow background, even with a medium-powered objective (× 10).

Notes:

(*a*) Should the depth of the violet-stained (moderate erythrophile) elements be regarded as insufficient, then the strength of the violamine in solution C can be doubled.

(*b*) Sun yellow G, whose structure can be seen on page 62, if used without the violamine, has an effect like that of orange G on Falg and Faviol-stained preparations. It is, however, an intensely yellow dye, so that after erasing all the Falg or basic Faviol colours everywhere except in the strongly erythrophile elements, it takes their place so that specimens so treated would be stained yellow everywhere.

(*c*) The standard Faviol method is particularly useful when photomicrographs have to be taken in black and white monochrome. Such photographs show the strongly erythrophile elements as full black; moderately erythrophile as dark grey; and the yellow-stained areas appear as light grey.

(*d*) The violamine 3B and Sun yellow G solutions should not be mixed to form solution C in large quantities at a time, because a reaction takes place between the two dyes and after a week solution C becomes unreliable, resulting in the loss of yellow

staining and overstaining in violet. The mixture of these two dyes, however, should be made twenty-four hours before use.

(*e*) Better pictures are obtained without a colour filter than with one.

(*f*) The sun yellow is used really as an adjunct to the basic Faviol technique. In the basic Faviol technique the use of a yellow filter is recommended as this makes the red-stained (i.e. erythrophile) elements more distinct. In the standard Faviol technique, however, the yellow background imparted by the Sun yellow G affords an excellent contrast for the red-stained, strongly erythrophile elements.

(*g*) For further information readers are referred to the authors' original paper, and to pages 76–90 of this book.

Reference: MacConaill, M. A. & Gurr, E. (1960*b*, 1961, 1962).

FEULGEN - NAPHTHOIC ACID HYDRAZIDE

(A. G. E. Pearse)

For deoxyribonucleic acid

Reagents required:

A.	2-Hydroxy-3-naphthoic acid hydrazide	0·1 gm.
	Alcohol, 50%	100 ml.
	Glacial acetic acid	5 ml.
B.	Tetrazotized *o*-dianisidine ..	0·1 gm.
	Veronal buffer solution, pH 7·4 ..	100 ml.

Technique:

1. Take sections down to distilled water, then rinse briefly in N/1 HCl.
2. Immerse in N/1 HCl at 60° C for the optimum time required for hydrolysis, which is dependent on the fixative used.
3. Rinse briefly in cold N/1 HCl.
4. Rinse in distilled water.
5. Rinse in 50% alcohol.
6. Immerse for one to three minutes in reagent B at 0° C.
7. Dehydrate through the usual graded alcohols.
8. Clear in xylol and mount in D.P.X.

Results:

DNA: bluish purple. Cytoplasmic and other proteins may be stained red, especially if they are strongly basic.

Note:

The principle of the reaction is that the 2-hydroxy-3-naphthoic acid hydrazide, which replaces Schiff's reagent, not only combines with aldehydes and ketones, but couples with the diazotized *o*-dianisidine to give a purplish blue dye. Moreover, the hydrazide combines with tissue proteins to form pinkish coloured protein hydrazides.

Reference: Pearse, A. G. Everson (1951).

FONTANA STAIN

For argentaffine granules

Solutions required:

A. Silver diaminohydroxide (as page 415).

B. Sodium thiosulphate 5% aqueous.

Technique:

1. Tissues are fixed in 10% neutral formalin, washed, dehydrated in alcohol, cleared in cedarwood oil, and embedded in paraffin wax as usual.

2. Fix sections to slides, bring down to distilled water and wash thoroughly in two or three changes of neutral, freshly distilled water.

3. Immerse in the silver oxide (Fontana) solution for twelve to twenty-four hours in the dark in a covered, scrupulously clean vessel.

4. Wash in neutral, freshly distilled water for one minute.

5. Immerse for one minute in the sodium thiosulphate.

6. Immerse in tap water for ten minutes.

7. Counterstain, if desired in carmalum.

8. Dehydrate; clear in xylol and mount.

Results:

Argentaffine granules: black.

References:

Jacobson, W. (1939).
Fontana (1912).

FONTANA STAIN - SILVER NITRATE

For reticular and collagen fibres

Solutions required:

A. Strong ammonia solution (sp. gr. 0·880) .. .. 10 ml.
Distilled water .. .. .. 90 ml.

B. Potassium permanganate 0·5% aqueous.

C. Oxalic acid 1·5%.

D. Silver nitrate 5% aqueous.

E. Fontana stain (silver diaminohydroxide, as page 415).

F. Aniline oil.. .. .. .. 1 volume
Xylol .. .. 1 volume

Technique:

1. Frozen sections not thicker than 10μ are fixed in 10% formalin and afterwards washed in three changes of water for fifteen minutes in each.

2. Immerse in Solution A at 60° C. for fifteen minutes, in an oven.

3. Rinse well in three changes of distilled water.

4. Immerse in the potassium permanganate for three or four minutes.

5. Rinse with distilled water for about ten or twenty seconds.

6. Decolorize with the oxalic acid solution until the brown colour just disappears; then wash well in distilled water.

7. Immerse in silver nitrate solution in the dark for an hour.

8. Wash well with two changes of distilled water in the dark.

9. Immerse in Fontana's stain for fifteen minutes at 60° C. in the dark.

10. Wash rapidly in three changes of distilled water.

11. Immerse in 30% formalin for two or three minutes at 60° C.

12. Wash thoroughly in running tap water; then transfer to slides.

13. Blot away excess water.

14. Dehydrate with two changes of absolute alcohol.

15. Clear in the aniline-xylol (Solution F, above).

16. Wash with xylol; mount in Clearmount.

Results.

Reticulum is stained black, while collagen is brown.

N.B.: Sections must be handled with glass needles throughout this technique, as contact with metal instruments will ruin the preparations.

Reference: Krajian, A. A. (1933).

FUCHSIN ACID, ACETIC

A simple and selective stain for both alpha and beta cell granules of the anterior part of the pituitary

Solutions required:

Acid fuchsin, 1% aqueous	.. 100 ml.
Acetic acid, glacial ..	.. 1 ml.

Technique:

1. Fix material in Helly's fluid for six to twenty-four hours, and embed in paraffin wax as usual.

2. Fix sections to slides; dewax and carry down through the alcohols to distilled water as usual.

3. Immerse in the acetic acid fuchsin solution for one hour.

4. Wash in running water.

5. Examine under the microscope while the preparation is still wet; if the beta cells are not bluish violet, return to the acetic acid fuchsin solution for a further period of one hour, then wash in running water and re-examine under the microscope.

Note: A staining time of from one to as much as twelve hours is stipulated in the original paper, and it may, therefore, in some cases, be necessary to repeat steps 3, 4, and 5 above, before the desired results are obtained.

6. Dehydrate through the usual graded alcohols.

7. Clear in toluol and mount in Canada balsam, Cristalite, Clearmount or D.P.X.

Results:

Alpha cell granules: red. Beta cell granules: blue-violet.

Reference: Petrovitch, A. (1953).

FUCHSIN ACID - LIGHT GREEN

A rapid method for staining mitochondria

Solutions required:

A. Hydrochloric acid N/1

B. Fuchsin acid, 1% aqueous

C. Light green SF, 1% aqueous

Technique:

1. Paraffin sections of formalin-fixed material are mounted on slides and dewaxed with xylol as usual.

2. Pass the slides through the usual graded alcohols into water.

3. Hydrolyse for three minutes in the hydrochloric acid (solution A), at 60° C.

4. Rinse in water.

5. Stain in the fuchsin acid solution for thirty seconds.

6. Rinse in water.

7. Counterstain in the light green solution for one to three minutes.

8. Rinse in water.

9. Dehydrate with 95% and absolute alcohol.

10. Clear in xylol.

11. Mount in D.P.X. or in Canada balsam in xylol.

Results:

The mitochondria stand out sharply stained purplish red with green peripheral wall. Chromatin and collagen are green. Muscle tissue: purplish. Erythrocytes: brilliant red.

Note:

It may be of interest to compare the results obtained with this technique with those obtained using the same dyes, but under simpler conditions and without hydrolysis, in the techniques of MacConaill & Gurr, pages 198–200.

Reference: Novelli, A. (1959).

FUCHSIN ACID (Altmann) - METHYL GREEN

For mitochondria in leucocytes of fixed blood smears

Solutions required:

A. Potassium dichromate .. .. 15 gm.
Distilled water .. .. .. 100 ml.
B. Iodine 0·5% in 70% alcohol
C. Sodium thiosulphate 0·5% aqueous
D. Altmann's acid fuchsin
E. Methyl green, 1% aqueous

Technique:

1. Make smears of fresh, unoxalated blood on scrupulously clean slides, and fix by placing them at once in Helly's fluid and leaving therein for twenty-four hours.
2. Post-chrome in solution A for forty-eight hours.
3. Immerse in solution B for two minutes.
4. Rinse in 70% alcohol.
5. Immerse in the sodium thiosulphate solution for one minute.
6. Wash in running water for several hours or overnight.
7. Transfer to distilled water.
8. Take a slide from the distilled water and blot or wipe away the water from all parts of the slide except that covered by the smear.
9. Place the slide over the corner of a tripod, flood with solution D and heat the preparation over a flame until fumes appear.

10. Pour off excess stain and wash with water.
11. Stain for a few seconds with methyl green by allowing a few drops of the solution to flow over the slide.
12. Dehydrate in two changes of absolute alcohol.
13. Clear in xylol and mount in Cristalite or D.P.X.

Results:

Mitochondria, erythrocytes, and the specific granules of eosinophilic leucocytes are stained red. Nuclei are stained by the methyl green, while the cytoplasm also takes this dye but to a lesser extent.

Notes:

(*a*) The use of oxalated blood is not to be recommended due to the alteration produced, by the commonly used oxalates, in the cytoplasm and nuclear structure of the leucocytes.

(*b*) Fixation of the blood smears before they are dry is absolutely essential, according to the author, to obtain well differentiated preparations.

(*c*) For further information readers are referred to the original paper which shows two photomicrographs.

Reference: Andrew, W. & Johnson, H. (1956).

FUCHSIN ACID - PICRIC ACID

(After Altmann)

For mitochondria

Solutions required:

A. *Altmann's Fluid:*

Potassium dichromate 5% aqueous 1 volume
Osmic acid 2% aqueous .. 1 volume

Note: Although the penetration power of this fixative is poor, it is very satisfactory for surface fixation.

B. Acid fuchsin .. .. .. 20 gm.
Aniline water .. .. .. 95 ml.

C. Picric acid saturated in absolute alcohol.

Technique:

1. Small pieces of tissue, not more than 2 mm. in diameter, are fixed for twenty-four hours in Altmann's Fluid.

2. Wash for an hour in running water; then dehydrate; clear, and embed in paraffin wax in the usual manner.

3. Sections, not thicker than 4μ, are brought down to distilled water; then stained for six minutes in Solution B.

4. Pour off excess stain; then blot section carefully; then differentiate and counterstain by flooding the preparation with Solution C.

5. Rinse quickly in 95% alcohol; then dehydrate with absolute alcohol; clear in xylol, and mount.

Results:

Mitochondria are stained crimson against a vivid yellow background.

Reference: Altmann, R. (1904).

FUCHSIN ACID - TOLUIDINE BLUE - AURANTIA

For mitochondria

Solutions required:

A. *Champy's Fluid:*

Potassium dichromate 3% ..	7 ml.
Chromic acid 1%	7 ml.
Osmic acid 2%	4 ml.

B. Pyroligneous acid .. .. 1 volume
Chromic acid 1% .. .. 2 volumes

C. Potassium dichromate 3%.

D. Acid fuchsin .. .. .. 10 gm.
Aniline water .. .. .. 100 ml.

E. Toluidine blue 0·5% aqueous.

F. Aurantia 0·5% in 70% alcohol.

Technique:

1. Tissues are fixed in Champy's Fluid for twenty-four hours; then washed in running water for at least an hour.

2. Immerse in Solution B for twelve to twenty hours; then wash in distilled water for thirty minutes.

3. Mordant in Solution C for three days; then wash in running water for twenty-four hours.

4. Dehydrate; clear; embed in paraffin wax.

5. Sections, 3 to 5μ in thickness are taken down to water in the usual manner; then stained for six minutes by flooding the slide with Solution D, and heating gently till vapour rises.

6. Rinse in distilled water; then counterstain in Solution E.

7. Rinse with distilled water; then stain with Solution F for thirty to fifty seconds.

8. Differentiate with 95% alcohol; dehydrate; clear in xylol and mount.

Results:

Mitochondria are stained red; nuclei, blue. Background, yellow.

Reference: Kull, H. (1913).

GALLOCYANIN - ORCEIN - ACID ALIZARIN BLUE - ALIZARIN VIRIDINE

A general stain for animal tissues

Solutions required:

A. Gallocyanin .. .. .. 0·1 gm.
Chrome alum 5% aqueous .. 100 ml.

Boil for ten minutes. Allow to cool; then make up the volume to 100 ml., filter and add five or six drops of formalin.

B. Orcein 0·5% in 70% alcohol .. 99 ml.
Hydrochloric acid conc. .. 1 ml.

C. Acid Alizarin Blue SWR.. .. 5 gm.
Aluminium sulphate 10% aqueous 100 ml.

Boil for ten minutes. Cool and filter. Make up the volume to 100 ml. with distilled water and add five or six drops of formalin.

D. Phosphomolybdic acid 5%.

E. Alizarin viridin .. .. .. 0·2 gm.
Buffer solution pH 5·8 .. 100 ml.

Technique:

1. Fix tissues in 10% formalin and embed in paraffin wax in the usual manner.
2. Fix sections to slides and take down to distilled water as usual.
3. Stain nuclei intensely by immersing the slides in the gallocyanin solution in a staining jar, examining the preparations under the microscope at intervals over a period of twenty-four hours, to ascertain the depth of staining.
4. Wash with two changes of distilled water.
5. Stain elastic fibres in the orcein solution for ten minutes to half an hour in a grooved, covered staining jar.
6. Wash well with distilled water.
7. Stain muscle in the acid alizarin blue solution for seven minutes.
8. Wash with distilled water.
9. Differentiate in the phosphomolybdic acid solution for about thirty minutes, controlling by examination under the microscope at intervals.
10. Wash with two changes of distilled water.
11. Stain collagen in the alizarin viridin for seven minutes.
12. Drain and blot thoroughly but carefully.
13. Rinse with 96% alcohol; followed by carbol xylol.
14. Wash well with two or three changes of xylol, and mount.

Results:

Nuclei: dark brown. Muscle and epithelium: pale violet. Erythrocytes and elastic fibres are stained a rich brown, while mucus, collagen are in varying shades of green; myelin sheaths, pink; and axis cylinders, dark blue.

Reference: Buzaglio, J. H. (1934).

GIEMSA STAIN

For malarial parasites, rickettsia, etc., in sections

Solution required:

Giemsa stain	1 ml.
Distilled water, buffered to pH 7·2	20 ml.

N.B.: This mixture should be freshly prepared immediately before use.

Technique:

1. Fix small pieces of tissue in 10% formalin, Regaud or Zenker.
2. Dehydrate; clear and embed.
3. Bring down paraffin sections to distilled water in the usual manner.
4. Stain for eighteen to twenty-four hours in the diluted Giemsa (as above).
5. Wash in distilled water; differentiate quickly in 0·5% acetic acid until the section is pink; then wash with distilled water.
6. Blot and dry in air and mount.

Results:

Nuclei are stained dark red; erythrocytes, pink. Malaria parasites, bluish red with red chromatin.

Reference: Giemsa, G. (1904).

GIEMSA - WRIGHT STAIN

A permanent stain for differentiating the structures, particularly Nissl bodies and cytons, of the spinal cord

Solution required:

Wright's stain	5 volumes
Giemsa stain	1 volume

Technique:

1. Material should be fixed in neutral formalin 10%.
2. Wash, dehydrate, clear, and embed in paraffin wax in the usual manner.

3. Fix sections to slides; dewax; pass through the usual descending grades of alcohol, down to distilled water.

4. Flood the sections with a measured volume of the above staining solution and allow it to act for two minutes.

5. Add an equal volume of distilled water and mix with stain by rocking the slides gently. Allow this diluted stain to act for two minutes.

6. Pour off excess stain and immerse the slides in fresh distilled water for one minute.

7. Transfer immediately into 80% alcohol and leave therein for fifteen seconds.

8. Dehydrate rapidly in 95% and absolute alcohol.

9. Clear in xylol and mount.

Results:

Cytons and Nissl granules are stained deep blue. Nuclei of blood-vessel structures and neuroglia are light blue. Elastic fibres of blood vessels: deep blue. Erythrocytes: pink. Neuroglia fibres: light red.

Note: The proportion of the Giemsa stain regulates the intensity of the cyton stain.

Reference: Hansburg, L. (1935).

GIEMSA STAIN

For blood, malaria parasites, trypanosomes, etc., in smears

Note: Best results are obtained by buffering the distilled water to 7·2.

Rapid method for films:

1. Fix air-dried films for three minutes in pure methyl alcohol.

2. Stain for five minutes in a mixture consisting of one part of Giemsa stain and two parts of distilled water.

3. Wash with distilled water for one half to one minute; then blot and dry in air.

Rapid method for spirochaetes:

1. Air-dried films are fixed by heat by drawing through the flame.

2. Allow the slide to cool; then flood the slide with a freshly prepared mixture consisting of 10 drops of Giemsa stain to 10 ml. distilled water.

3. Heat the slide gently till steam rises; allow to cool for about twenty seconds; then pour off the stain and repeat the process five or six times.

4. Wash with distilled water; blot dry and mount.

Slow method for films, for demonstrating spirochaetes, trypanosomes, etc.:

1. Air-dried films are fixed for three minutes in pure methyl alcohol.

2. A fresh mixture is prepared by diluting Giemsa stain in the proportion of 10 drops of stain to 10 ml. distilled water. The slide is then placed in a staining jar and left to stain in the diluted Giemsa for sixteen to twenty-four hours; if a staining jar is not available, place a piece of thin glass rod in a Petri dish; lay the slide with one end resting on the rod, film face downwards, in the Petri dish, and pour in sufficient diluted stain to cover the film; then line the Petri dish lid with two sheets of moist filter paper to prevent evaporation, and cover the preparation.

3. Wash with distilled water; blot and dry.

Method for bacterial smears, throat exudate, etc.:

1. Thin, air-dried, unfixed smears are covered with undiluted Giemsa stain for thirty seconds; then a quantity of distilled water, equivalent to five to ten times the volume of stain used, is added and mixed with the stain by gently rocking the slide.

2. Allow the diluted stain to act for two to five minutes; then wash with distilled water; blot and dry.

Thin blood film method:

1. Air-dried films are fixed for five minutes in pure methyl alcohol; then blotted and dried in air.

2. Stain for fifteen to forty-five minutes in a mixture consisting of one part of Giemsa stain and twenty-five parts of distilled water.

3. Wash in distilled water; blot and dry in air.

Results:

Nuclei of the leucocytes are stained reddish purple, while the rest of the leucocytes appear similar to Leishman-stained preparations. Cytoplasm of plasmodia: blue. Chromatin: red.

Thick film method (for demonstrating malaria parasites)*:*

1. A film is prepared by spreading 3 to 5 drops of blood in a circle about 15 mm. diameter over a slide; then without fixation it is allowed to dry on a level surface for eighteen to twenty-four hours at room temperature protected from dust; or for two to three hours in an incubator at 37° C.

2. Stain for forty minutes in a mixture consisting of one part of Giemsa stain and fifty parts of distilled water.

3. Wash for five to ten minutes in distilled water; blot and dry in air.

Results:

Malaria parasites: chromatin, clear red; cytoplasm, clear blue.

Treponema pallidum.

Solutions required:

A. Giemsa stain.

B. Sodium carbonate, A.R. 1% in distilled water.

Technique:

1. A lesion is rubbed roughly, until it bleeds, with a swab which has been damped with absolute alcohol.

2. While bleeding is taking place, swab the chancre at intervals. After a few minutes a clear fluid exudate will appear from the abraded surfaces.

3. A drop of the clear fluid exudate (free from blood) is taken from the periphery of the lesion with a platinum wire loop, transferred to a scrupulously clean slide, and spread into a film.

4. Allow to dry in the air; then fix in absolute alcohol for fifteen minutes.

5. Pour off any excess alcohol, and allow the last traces to evaporate away in the air.

6. Stain for fifteen to thirty minutes in a mixture consisting of 10 ml. distilled water buffered to 7·2, and 0·5 ml. Giemsa stain.

7. Rinse in running tap water for a few seconds holding the slide with the film facing downwards.

8. Blot dry, and examine.

Results:

The spirochaetes are stained reddish violet. Nuclei of the leucocytes reddish purple, while the rest of the leucocytes and pus cells appear similar to Leishman-stained preparation. Cytoplasm of plasmodia, blue; chromatin, red. Erythrocytes: yellowish pink.

Reference: Giemsa, G. (1904).

GIEMSA STAIN - MAY-GRUNWALD STAIN

For blood and parasites

Solutions required:

A. May-Grunwald stain.

B. Giemsa stain.

Technique:

1. Unfixed air-dried films are stained for three minutes in May-Grunwald stain; then an equal volume of distilled water is added and mixed with the stain by rocking the slide. The diluted stain is then allowed to act for one minute; then drained off, without washing.

2. Stain for ten to fifteen minutes in a mixture consisting of 10 drops of Giemsa stain in 10 ml. distilled water.

3. Differentiate for about five seconds, with distilled water, examining under the microscope to ensure that differentiation is complete.

4. Blot and dry in air; mount.

Results:

As Giemsa stain but with more intense colouring.

References:
Giemsa, G. (1904).
May & Grünwald (1902).
Pappenheim, A. (1912, 1917).

GOLD CHLORIDE - SUBLIMATE

(After Cajal)

For neuroglia fibres; for astrocytes in central nervous system

Solutions required:

A. Neutral formalin .. .. .. 15 ml.
Ammonium bromide .. .. 2 gm.
Distilled water .. .. .. 85 ml.

B. Gold chloride (brown or yellow) 1% aqueous.

C. Mercuric chloride 5% aqueous.

D. Sodium thiosulphate 10% aqueous.

Technique:

1. Fresh pieces of tissue are fixed for two to twenty-one days in Solution A.

2. Frozen sections are cut 15 to 30μ thick.

3. Rinse in several changes of distilled water.

4. Immerse sections, flattened out and not lying on top of one another, for three to four hours in a freshly prepared mixture consisting of:

Solution B .. .. .. 5 ml.
Solution C .. .. .. 5 ml.
Distilled water .. .. .. 30 ml.

(*Note:* 3 ml. of the mixture is required for each section)

until the astrocytes are stained dark against a relatively light background; the reaction should be controlled by microscopic examination of a section while still wet.

5. Wash in distilled water; then fix in Solution D.
6. Wash thoroughly in tap water.
7. Dehydrate; clear and mount.

Results:

Astrocytes: black. Nerve cells: red. Nerve fibres: unstained. Background: light brownish purple, or unstained.

References:
Cajal, S. Ramon y (1913, 1916, 1920).
Conn, H. J. & Darrow, M. A. (1947).
Jones, Ruth McClung (1950), pp. 407–413.

GOLGI METHOD (Rapid)

For nerve cells

Solutions required:

A. Potass. dichromate 3% .. .. 40 ml.
Osmic acid 1% .. .. .. 10 ml.
This solution must be freshly prepared.

B. Silver nitrate 0·75%.

Technique:

1. Immediately the animal is killed, tissues are cut into slices about 2 mm. thick and fixed in Solution A for one to three days depending upon the size of the pieces.
2. After removing excess fixative by blotting, the tissues are rinsed in Solution B until no more precipitate is formed.
3. Transfer to a fresh lot of Solution B and leave for two days or longer.
4. With a camel-hair brush carefully brush the precipitate from the surface of the tissue; then wash well in distilled water.
5. Dehydrate by immersing for one to four hours, depending upon the size of the pieces, in each of the following: two lots of 95% alcohol; two lots of absolute alcohol; one lot of ether-alcohol (equal vols. ether and absolute alcohol).
6. Embed in Celloidin or L.V.N.
7. Sections 60–100μ are mounted on slides and covered with Canada balsam in benzol or cristalite, without cover glasses (*see* L.V.N. technique).

Results:

Background, dull yellow. Nerve cells and their processes, black. Blood vessels, black.

Reference: Golgi, C. (1875).

GRAM'S IODINE

For bacteria in sections

Solutions required:

A. Carbol gentian violet.
B. Gram's iodine.
C. Carbol fuchsin (Ziehl Neelsen) .. 1 volume
Distilled water .. .. .. 9 volumes
D. Picric acid, saturated, aqueous.

Technique:

1. Pieces of tissue are fixed in 10% formalin; dehydrated; cleared and embedded in paraffin wax.
2. Fix sections to slides; dewax and take down to distilled water in the usual manner.
3. Stain in Solution A for about two minutes.
4. Pour off excess stain and without washing add Gram's iodine and allow the stain to act for one minute.
5. Differentiate in pure acetone until colour ceases to come out of the sections.
6. Counterstain in the carbol fuchsin (Solution C) for about a minute.
7. Pour off excess stain, and drain, without allowing the sections to dry; then without washing:
8. Cover the sections with the picric acid solution, pouring off after one half to one minute.
9. Dehydrate and clear with pure acetone for about fifteen seconds.
10. Clear in xylol and mount.

Results:

Gram-positive organisms are stained violet, while Gram-negative are red. Nuclei are stained pink, while cytoplasm is yellow.

GREENSTEIN'S FIVE-DYE STAIN

(After Greenstein, 1961)

A general purpose stain for sections and smears

Solutions required:

Stock solutions:

A.	Haematoxylin	1 gm.
	95% alcohol	50 ml.
	Glycerin	50 ml.
	Iron alum	15 gm.
	Ferrous sulphate	15 gm.
	Distilled water	100 ml.

Dissolve the haematoxylin in the alcohol. Measure out the glycerin and pour it into a 250 ml. bottle. Rinse the measuring cylinder out with the haematoxylin solution, to remove adhering glycerin. Add the haematoxylin solution to the glycerin in the stock bottle. Dissolve the iron alum and the ferrous sulphate in the water, by shaking: do not heat. Pour this solution into the stock bottle. Shake well.

B.	Ponceau 2R	1 gm.
	Distilled water	100 ml.
	Glacial acetic acid	1 ml.
C.	Acid fuchsin	0·5 gm.
	Distilled water	100 ml.
D.	Phosphomolybdic acid 1% aqueous	
E.	Fast green FCF	2 gm.
	Glacial acetic acid	1 ml.
	Distilled water	99 ml.
F.	Picric acid, saturated aqueous.	
*G.	Solution A	8 parts
	Solution B	3 parts
	Solution C	1 part
*H.	Solution D	1 part
	Solution E	1 part
	Solution F	1 part

* Filter before use.

Technique:

1. Carry sections through to distilled water as usual, removing mercuric precipitate if a mercury-containing fixative has been used.
2. Immerse in solution G for three to five minutes, agitating occasionally.
3. Wash thoroughly in tap water.
4. Immerse in solution H for two to three minutes, with agitation.
5. Rinse briefly in distilled water until the water runs clean.
6. Treat with two changes of 95% alcohol.
7. Dehydrate in absolute alcohol.
8. Clear in xylol.
9. Mount in a neutral synthetic mountant such as DPX, or Clearmount, etc.

Results:

Nuclei: dark greyish purple to greyish brown.
Cytoplasm: light greyish yellow to greyish brown.
Collagen: bright green.
Erythrocytes: orange-yellow.
Muscle cytoplasm: greyish green to olive brown.
Mucus: pale green to turquoise.

Reference: Greenstein, J. S. (1961).

Notes:

(*a*) The author (Greenstein) states that another method added to the many earlier variants of Mallory, Masson and Heidenhain connective tissue stains appears to be justified if it is simpler, more dependable or more versatile and can be successful even in the hands of inexperienced technicians. It is claimed that this method, which employs only two staining solutions and fewer steps than many routine haematoxylin–eosin methods, gives results comparable with those obtained by skilled workers with more exacting methods.

(*b*) It is stated that the method can be applied to vaginal and cervical smears fixed in ether-alcohol (1 : 1), or isopropyl alcohol for two minutes.

(*c*) For further information the original paper should be consulted.

HAEMALUM - EOSIN

For demonstrating collagenous tissue

Solutions required:

A. Haemalum (Mayer).

B. Eosin, bluish 0·2% in 20% alcohol.

Technique:

1. Paraffin sections are fixed to slides, dewaxed and taken through descending grades of alcohol to distilled water in the usual manner.
2. Stain for five to ten minutes with the haemalum solution, examining under the microscope at intervals until a satisfactory degree of staining has been achieved.
3. Rinse for a few seconds in tap water.
4. Stain for one or two seconds with the eosin solution.
5. Rinse for a few minutes in running tap water.
6. Pass through 70%, 90% and absolute, alcohol.
7. Clear in xylol and mount.

Results:

Collagen: deep pink. Smooth muscle: pink. Cytoplasm: pale pink. Nuclei: blue.

Reference: Kornhauser, S. I. (1930).

HAEMATEIN ACID

(J. R. Baker)

For phospholipids

Solutions required:

A. *Formol calcium fixative*

Formalin (40% formaldehyde) . .	10 ml.
Calcium chloride, anhydrous, 1% aqueous	10 ml.
Distilled water	80 ml.

Keep a few chips of marble in the stock bottle to maintain neutrality.

B. *Dichromate calcium*

Potassium dichromate	5 gm.
Calcium chloride, anhydrous, 10% aqueous	10 ml.
Distilled water	90 ml.

C. *Acid haematein*

Sodium iodate, 1% aqueous ..	1 ml.
*Haematoxylin, 1% aqueous ..	5 ml.
Distilled water	43 ml.

Shake or stir well; then heat to boiling point; cool and add:

Glacial acetic acid	1 ml.

* *Note:* The 1% haematoxylin should not be more than seven to fourteen days old.

The acid haematein (solution C) should be prepared in a quantity sufficient only for one day's use, as it does not keep for more than a few hours.

D. *Borax-ferricyanide differentiator*

Potassium ferricyanide, 0·5% aqueous	50 ml.
Borax, 0·5% aqueous	50 ml.

Keep solution D in a dark bottle.

Technique:

1. Fix small pieces of tissue in solution A for six to eighteen hours.

2. Transfer to the dichromate–calcium (solution B) and leave therein for eighteen hours at 22° C.

3. Transfer to a fresh lot of dichromate–calcium and leave therein for twenty-four hours at 60° C.

4. Wash well in distilled water.

5. Cut frozen sections at 10μ.

Note: If desired, tissues can be first embedded in gelatine (as in the Sudan black technique, page 424).

6. Mordant in dichromate–calcium (solution B) for one hour at 60° C.

7. Wash well in distilled water.

8. Stain in the acid haematein solution for five hours at 37° C.

9. Wash in distilled water.

10. Immerse in the borax-ferricyanide solution for eighteen hours at 37° C.

11. Wash in water.

12. Mount in Aquamount or glycerine jelly, sealing the edges of the coverslip with two coats of Laktoseal.

Results:

Phospholipids and nucleoproteins are stained dark blue to almost black. Mucin: dark blue. Fibrinogen: pale blue. Cytoplasm: pale yellow.

Note:

Substances stained dark blue or black by this technique, and which take the stain just the same after the pyridine extraction test (page 431) are not phospholipids. Substances that stain dark blue to black with the acid haematein, but which are removed by pyridine extraction, are phospholipids. Kanwar (1961), however, observes that this is not necessarily so in all cases.

Reference: Baker, J. R. (1946).

HAEMATOXYLIN CHROME ALUM

(After Gomori)

For lipofuscins

Solutions required:

A. Sulphuric acid, 5% aqueous

B. Potassium permanganate, 1% aqueous .. .. ..

C. Solution A .. .. .. 3 ml.
Solution B .. .. .. 47·5 ml.
Water .. .. 49·5 ml.

D. Oxalic acid, 1% aqueous

E. Haematoxylin, 10% in absolute alcohol

F. Chrome alum, 3%

G. Potassium dichromate, 5%

H. *Chrome alum haematoxylin*

Solution E (haematoxylin)	5 ml.
Distilled water	45 ml.
Solution F (chrome alum)	50 ml.
Solution A (sulphuric acid)	1 ml.
Solution G (potass. dichromate)	2 ml.

Note: Solution H will keep for several weeks without deterioration.

I. Alcohol, 70%	99 ml.
HCl conc.	1 ml.

J. Biebrich scarlet, 1% aqueous

Technique:

1. Fix pieces of tissue in 10% neutral formalin, and embed in paraffin wax or in celloidin, or cut frozen sections.
2. Take sections through to water.
3. Oxidize in solution C for one to two minutes.
4. Bleach in solution D for one minute.
5. Wash well in running water.
6. Stain in solution H for ten minutes.
7. Differentiate in acid alcohol (solution I).
8. Wash and " blue " in tap water, or in 1% lithium carbonate solution.
9. Wash in distilled water.
10. Counterstain in the Biebrich scarlet solution.
11. Wash in running water.
12. Dehydrate through the usual graded alcohols.
13. Clear in xylol or benzene.
14. Mount in D.P.X. or Clearmount or Canada balsam in xylol.

Results:

Lipofuscins: dark blue. Nuclei: purple. Cytoplasmic structures: scarlet.

Reference: Gomori, G. (1952).

HAEMATOXYLIN - AZOPHLOXINE

For muscle, connective tissue, ganglion cells, etc.

Solutions required:

A. *Lavdowsky's Fixative:*

Formalin (40% formaldehyde) ..	10 ml.
Glacial acetic acid	2 ml.
Alcohol 95%	50 ml.
Distilled water	40 ml.

B. Haematoxylin (Delafield, Harris or Ehrlich)

C. Azophloxine .. .. .. 0·2 gm.
Acetic acid 0·2% .. .. .. 100 ml.
Add about 1 ml. chloroform as a preservative.

D. Acetic acid 0·2% aqueous.

E. Orange G. .. .. 2 gm.
Phosphotungstic acid .. .. 4 gm.
Distilled water .. .. .. 100 ml.
Fast Green FCF .. .. .. 0·2 gm.

Technique:

1. Fix material in Lavdowsky's mixture or in 10% formalin and embed in paraffin wax.

2. Stain in Harris, Ehrlich or Delafield Haematoxylin for five to ten minutes.

3. Blue in tap water or 1% lithium carbonate solution.

4. Stain in the azophloxine solution for two minutes.

5. Rinse in 0·2% acetic acid.

6. Stain in the orange G-fast green solution for one minute.

7. Differentiate for about five minutes with 0·2% acetic acid.

8. Rinse in distilled water.

9. Remove excess distilled water by draining and blotting round the edges of the sections carefully, but do not allow to dry completely.

10. Dehydrate directly with two or three changes of absolute alcohol, or with cellosolve.

Results:

Connective tissue: green. Striated muscle: brick red. Smooth muscle: reddish violet. Nerves: blue-grey. Ganglion cells: violet. Erythrocytes: orange. Cardiac conductive tissue is easily distinguishable from cardiac muscle as it takes a lighter shade of staining.

Note: Azophloxine is used here as a substitute for ponceau de xylidine in Goldner's modification of Masson's technique.

The stain is also suggested in place of Eosin as a counterstain for use with haematoxylin. The advantages of using azophloxine are that it gives clear and delicate pictures and it does not overstain, if the recommended procedure is followed. When azophloxine is to be used merely as a counterstain for haematoxylin the procedure is as follows:

I. Proceed as steps, 1, 2, 3, 4, and 5 (above).
II. Rinse in distilled water.
III. Dehydrate with three changes of absolute alcohol; then clear and mount.

Reference: Halpern, M. H. (1954).

HAEMATOXYLIN - BASIC FUCHSIN

For haemofuscin, melanin and haemosiderin in animal tissues

Solutions required:

A. Haematoxylin (Ehrlich).
B. Basic fuchsin 0·5% in 50% alcohol.

Technique:

Tissues may be fixed in Zenker or in absolute alcohol or in 10% formalin. Paraffin or Celloidin sections may be employed. If Zenker's fixative is used it will be necessary to remove mercury deposits in the usual manner.

1. Stain for five to ten minutes in Ehrlich haematoxylin.
2. Wash well in tap water, then several times in distilled water.
3. Stain from five to twenty minutes in the basic fuchsin solution; then pour off excess stain and wash well in distilled water.
4. Differentiate in 95% alcohol; then dehydrate in absolute alcohol; clear in xylol and mount in balsam.

Results:

Nuclei, blue; melanin and haemosiderin remain unstained in their natural brown colours: haemofuscin, bright red.

References:

Lillie, R. D. (1948), p. 130.
Mallory, F. B. (1938).

HAEMATOXYLIN - BIEBRICH SCARLET - PICRO ANILINE BLUE

For differential staining of connective tissue and muscle

Solutions required:

A. Haematoxylin (Weigert) A.
B. Haematoxylin (Weigert) B.
C. Biebrich scarlet, 0·2% aqueous 100 ml.
Glacial acetic acid .. .. 1 ml.
D. Picric acid, saturated aqueous .. 100 ml.
Aniline blue, water soluble .. 0·1 gm.
E. Acetic acid 1% aqueous.

Technique:

1. Tissues should be fixed in 10% formalin and paraffin sections employed.
2. Stain for five minutes in a freshly prepared mixture consisting of equal parts of Weigert's Haematoxylin A and B.
3. Wash in tap water.
4. Stain for three to five minutes in the acetic Ponceau, S (Solution C).
5. Rinse in distilled water.
6. Stain for three to five minutes in the picro aniline blue (Solution D).
7. Wash for three or four minutes in 1% acetic acid solution.
8. Dehydrate in ascending strengths of alcohol and clear in xylol in the usual manner.
9. Mount in acid balsam.

Results:

Connective tissue, glomerular basement membrane and reticulum: blue. Muscle and plasma: pink. Erythrocytes: bright red.

Reference: Lillie, R. D. (1940).

HAEMATOXYLIN - EOSIN

For general staining

Solutions required:

A. Haematoxylin (Delafield or Ehrlich)

B. Eosin, yellowish, 1%, aqueous.

Technique:

1. Tissues should be fixed in Zenker, Bouin or 10% formalin and embedded in paraffin wax.
2. Sections are brought down to distilled water; then stained with Delafield haematoxylin for ten minutes.
3. Wash and immerse in tap water for about five minutes until the section appears blue to the naked eye.
4. Wash rapidly with distilled water; then stain for one to two minutes with 1% eosin yellowish, aqueous.
5. Wash quickly with distilled water; then dehydrate with 95% and absolute alcohol.
6. Clear in xylol and mount.

Results:

Nuclei are stained blue; cytoplasm, pink.

Notes:

Lee (1913) states that Delafield haematoxylin is frequently attributed erroneously to Grenacher or Pruden. Conn & Darrow (1947) state that the Delafield haematoxylin-eosin method was introduced by Pruden (1885) in a note without a title.

References:
Delafield, F. (1885).
Ehrlich, P. (1886).

HAEMATOXYLIN (Ehrlich)

For keratohyalin

Solutions required:

A. Haematoxylin (Ehrlich).

B. Potass. permanganate 0·1%.

Technique:

1. Material should be fixed in 10% formalin and embedded in paraffin wax.

2. Fix sections to slides and bring down to distilled water as usual.

3. Stain in Ehrlich haematoxylin for ten minutes.

4. Pour off excess stain; then rinse and blue in tap water.

5. Immerse in potassium permanganate 0·1% for ten seconds; then wash well with water.

6. Dehydrate; clear; mount in balsam.

Results:

Keratohyalin is stained blue-black while the other elements are unstained or faintly stained.

Reference: Cowdry, E. V. (1952).

HAEMATOXYLIN - GENTIAN VIOLET - IODINE

(Gram–Weigert method)

For demonstrating Gram-positive bacteria and fibrin in sections

Solutions required:

A. Haematoxylin (Delafield).
B. Aniline gentian violet
C. Lugol's iodine.
D. Aniline oil .. .. 20 ml.
   Xylol .. .. 10 ml.
E. Erythrosin 5% in absolute alcohol.

Technique:

1. Tissues may be fixed in 10% formalin or in Zenker, if the latter is used then mercuric precipitates must be removed from the sections by the standard technique.

2. Fix sections to slides; dewax with xylol and pass through descending grades of alcohol to water in usual manner.

3. Stain in the haematoxylin solution for five to twenty minutes.
4. Rinse quickly in acid alcohol.
5. Immerse in a large volume of tap water for two to five minutes.
6. Stain in the aniline gentian violet for two to five minutes.
7. Pour off excess stain, and without washing, blot the slide carefully.
8. Flood with Lugol's iodine and allow the solution to act for two to five minutes.
9. Pour off excess, and without washing, blot dry carefully.
10. Decolorize for a few seconds with Solution D.
11. Flood the preparation with erythrosin (Solution E, above) and allow the stain to act from one half to one minute.
12. Pour off excess stain and wash the preparation with Solution D.
13. Rinse well with xylol; drain off excess and blot dry carefully.
14. Mount in balsam or Cristalite or Clearmount.

Results:

Nuclei: blue. Fibrin and Gram-positive organisms: purplish blue.

References:

Weigert, C. (1887).
Mallory, F. B. (1938), p. 272.

HAEMATOXYLIN (Heidenhain) - EOSIN

A general tissue stain

Solutions required:

A. Iron alum .. .. .. 3 gm.
Distilled water .. .. .. 100 ml.
Dissolve by shaking.

B. Haematoxylin 10% in absolute alcohol (which has been ripened for three months or longer) .. 5 ml.
Distilled water .. .. .. 95 ml.

C. Eosin yellowish 1% aqueous.

Technique:

Fix in Zenker. Embed in paraffin wax.

1. Sections are brought down to distilled water, then mordanted in Solution A for one half to three hours.

2. Wash in tap water; stain one to three hours in Solution B; then rinse in tap water.

3. Differentiate in Solution A, controlling by examination under the microscope.

4. Wash in running water for five to ten minutes.

5. Stain with Solution C for one to three minutes.

6. Wash in tap water; dehydrate; then clear in xylol and mount.

Results:

Nuclei are stained black; cytoplasmic structures, pink.

Reference: Heidenhain, M. (1892).

HAEMATOXYLIN

For the identification of lipines

Solutions required:

A. Potassium bichromate 5% aqueous.

B. Haematoxylin (Kultschitzky)

C. *Weigert's borax ferricyanide*

Potassium ferricyanide	2·5 gm.
Borax	2 gm.
Distilled water	100 ml.

Technique.

1. Tissues are fixed for twelve to twenty-four hours in 10% formalin in normal saline.

2. Wash for several hours in running water.

3. Make frozen sections and collect them in distilled water.

4. Immerse in Solution A for twenty-four to forty-eight hours at 37° C.

5. Wash in several changes of distilled water, handling the sections with care (as they become brittle after immersion in Solution A).

6. Immerse in Solution B for four to six hours at 37° C.

7. Wash in distilled water.

8. Differentiate in Solution C, controlling under the microscope, until the ground cytoplasm is changed from black to yellow. This process takes several hours.

9. Wash thoroughly in five or six changes of distilled water; then mount in glycerine jelly or Aquamount.

Result:

Lecithin and other lipines are stained black to *deep* blue (light blue coloration should not be taken as positive). Lipids and other tissue constituents are colourless.

Reference: Dietrich, A. (1910).

HAEMATOXYLIN

(After Kultschitzky)

(Weigert's modification)

For finer studies of cortical architecture and for total brain sections

Solutions required:

A. *Weigert's Secondary Mordant:*

Cupric acetate neutral, normal ..	5 gm.
Fluorchrome	2·5 gm.
Distilled water	100 ml.
Boil; allow to cool; then add:	
Glacial acetic acid	5 ml.

B. Haematoxylin (Kultschitzky).

C. Lithium carbonate, saturated, aqueous .. .. .. 100 ml.
Potassium ferricyanide 1% aqueous 10 ml.

Technique:

1. After fixing material in 10% formalin mordant for four to five days in Solution A.

2. Dehydrate in ascending grades of alcohol in the usual way, and embed in Celloidin.

3. Immerse for twelve to twenty-four hours in the haematoxylin (Solution B).

4. Differentiate in Solution C from four to twelve hours, controlling by examination under the microscope at intervals, and changing the differentiating fluid three or four times.

5. Wash thoroughly in distilled water.

6. Dehydrate with 95% alcohol.

7. Clear in terpineol.

8. Drain well and blot carefully.

9. Mount in balsam or D.P.X.

Results:

Finest myelin sheaths are stained a deep black.

References:
Carleton, H. M. & Leach, E. H. (1947), pp. 235–276.
Lillie, R. D. (1948), pp. 170–175.
Kultschitzky, N. (1889, 1890).
Weigert, C. (1903).

HAEMATOXYLIN - PHLOXINE - ANILINE GENTIAN VIOLET

For actinomyces in sections

Solutions required:

A. Ehrlich haematoxylin.

B. Phloxine 3% aqueous.

C. Aniline gentian violet.

D. Gram's iodine.

Technique:

1. Tissues are fixed in 10% formalin, washed, dehydrated, cleared and embedded in paraffin wax in the usual manner.

2. Fix sections to slides; dewax and bring down to distilled water as usual.

3. Stain with Ehrlich haematoxylin five to ten minutes; then blue and wash in lithium carbonate saturated aqueous.

4. Stain for fifteen to twenty-five minutes in the phloxine solution; then wash with distilled water.

5. Stain in aniline gentian violet for about ten minutes.

6. Rinse in distilled water.

7. Immerse in Gram's iodine solution for one minute.

8. Wash in distilled water.

9. Decolorize with aniline oil until the stain ceases to come out of the sections.

10. Rinse well in several changes of xylol and mount.

Results:

Branched forms are stained blue, while clubs appear red.

Reference: Mallory, F. B. (1938), p. 279.

HAEMATOXYLIN, PHOSPHOTUNGSTIC

For Pleuropneumonia organisms in sections of lung

Solution required:

Phosphotungstic acid haematoxylin (Mallory).

Technique:

1. Pieces of tissue should be fixed in Bouin, Carnoy or absolute alcohol and embedded in paraffin wax in the usual manner.

2. Fix sections to slides; dewax, pass through descending grades of alcohol down to distilled water, as usual.

3. Stain in Mallory's phosphotungstic acid haematoxylin in a stoppered staining jar for twenty-four hours, without treatment with the usual potassium permanganate and oxalic acid solutions.

4. Pour off excess stain; drain well without washing; then carefully blot dry.

5. Dehydrate quickly in absolute alcohol.

6. Clear in xylol and mount.

Results:

The organisms appear as masses of mycelia which are stained deep blue.

Reference: Turner, H. W. (1935).

HAEMATOXYLIN - PICRO FUCHSIN

For keratin, collagenic fibres, etc.

Solutions required:

A.	Distilled water	47·5 ml.
	Ferric chloride, hydrated 4% aqueous	2 ml.
	Hamaetoxylin 10% in absolute alcohol	0·4 ml.
B.	Picric acid, saturated, aqueous ..	20 ml.
	Acid fuchsin 1% aqueous	0·5 ml.
C.	Picric acid, saturated in absolute alcohol.	

Technique:

1. Tissues are fixed in Bouin and embedded in paraffin wax.
2. Sections about 8μ in thickness are fixed to slides, dewaxed with xylol and taken through the usual descending grades of alcohol to distilled water.
3. Stain for two to three minutes in solution A.
4. Differentiate and counterstain for about ten to fifteen seconds in solution B, controlling under the microscope, until only the nuclei are stained a greyish colour with the haematoxylin.
5. Rinse immediately in distilled water.
6. Dehydrate by dripping solution C onto the slide.
7. Clear with Terpineol.
8. Mount directly with Michrome mountant, or rinse with xylol, then mount with Clearmount or Cristalite.

Results:

Chromatin: black to grey. Muscle: yellow. Connective tissue: red. Keratinized regions: bright yellow. Cytoplasm: yellow.

Reference: Margolena, L. A. & Dolnick, E. H. (1951).

HAEMATOXYLIN - PICRO PONCEAU S

A selective stain for collagen and connective tissue in place of Haematoxylin - Van Gieson

Solutions required:

A. Haematoxylin (Heidenhain or Ehrlich).

B. *Picro Ponceau S (Curtis):*

Ponceau S 1% aqueous	.. ..	10 ml.
Picric acid 1% aqueous	.. ..	86 ml.
Acetic acid 1% aqueous	.. ..	4 ml.

Technique:

Proceed exactly as for Haematoxylin (Ehrlich) - Van Gieson or Haematoxylin (Heidenhain) - Van Gieson (pages 243–4).

Results:

Identical with Haematoxylin - Van Gieson.

Note: It is claimed that unlike Van Gieson, Picro Ponceau does not fade when mounted in Canada balsam.

References:
Carleton, H. M. & Leach, E. H. (1947).
Curtis, F. (1905).
Leach, E. H. (1946).

HAEMATOXYLIN (Weigert) SCARLET R

For demonstrating fatty acids crystals, soaps and neutral fats in fat necrosis

Solutions required:

A. Formalin 10% saturated with calcium salicylate.

B. Copper acetate 10% aqueous.

C. Weigert haematoxylin, A.

D. Weigert haematoxylin, B.

E.

Borax 0·2% aqueous	.. ..	1 litre
Potassium ferricyanide	.. ..	2·5 gm.

F. *Scarlet R* (*Herxheimer*).

Acetone	50 ml.
Alcohol, 70%	50 ml.
Scarlet R	1·5 gm.

Heat on a hot water bath; then allow to cool before filtering.

Technique:

1. Fix tissues in Solution A; wash in running water and cut frozen sections.

2. Mordant the sections in Solution B for three to twenty-four hours; then wash in water.

3. Immerse in mixture of equal parts of Solutions C and D for twenty to forty-five minutes.

4. Differentiate in Solution E, examining under the microscope at intervals.

5. Wash well with distilled water.

6. Stain with Solution F for about five minutes.

7. Rinse quickly with 70% alcohol.

8. Rinse with distilled water.

9. Mount in neutral glycerine jelly or in Aquamount.

Results:

Neutral fats are stained red, whilst fatty acids are deep blue black, haemoglobin, calcium and iron may also be stained.

Note: Calcium salicylate is added to the formalin fixative to convert soaps, which are sodium and potassium salts of fatty acids, into insoluble calcium soaps. If it is desired to demonstrate how much, if any, soap is present in addition to fatty acids, compare stained sections of two pieces of the same material, fixing one piece in Solution A and the other in ordinary 10% formalin.

References:
Lillie, R. D. (1948), pp. 161–2.
Fischler, F. (1904).

HAEMATOXYLIN (Heidenhain) - VAN GIESON STAIN

A selective stain for collagen and connective tissue, superior to Haematoxylin (Ehrlich) - Van Gieson

Solutions required:

A. Haematoxylin (Heidenhain) A.

B. Haematoxylin (Heidenhain) B.

C. Van Gieson stain (Picro fuchsin)

Technique:

1. Fix pieces of tissue in Bouin, Carnoy, Susa or 10% formalin.

2. Fix sections to slides; dewax and take down to water in the usual way, after removing mercurial precipitate if Susa has been used as the fixative.

3. Immerse in solution A for one half to one hour.

Note: If a fixative other than Bouin, Carnoy, Susa or formalin has been used it will be necessary to increase the time in solution A and in solution B up to twelve hours or longer: the time varies for different fixatives.

4. Rinse in water.

5. Stain in solution B for a time exactly equal to step 3.

6. Rinse in water.

7. Differentiate with solution A, controlling by examination under the microscope, after the preparation has been rinsed briefly in water.

8. Wash gently in running water for about five minutes to remove all traces of solution A (iron alum).

9. Stain for three to five minutes in Van Gieson.

10. Rinse for a few seconds in water.

11. Examine, while still wet, under the microscope.

12. Continue the staining with Van Gieson, or continue the differentiation with water, whichever is necessary.

13. Drain and draw off excess water by means of a filter paper applied carefully to the edges of the section, but do not allow the preparation to dry completely.

14. Dehydrate with absolute alcohol only.

15. Clear in xylol.

16. Mount in D.P.X., Cristalite or Clearmount.

Results:

Nuclei: dark brown to black. Collagen fibres: bright red. Erythrocytes, muscle, epithelia and other tissues: yellow.

Note: Van Gieson stain fades if mounted in Canada balsam, but the degree of fading can be reduced by the use of D.P.X., or Cristalite or Clearmount.

References:

Carleton, H. M. & Leach, E. H. (1947), pp. 128–129.
Van Gieson, J. (1889).

HEXAZONIUM PARAROSANILINE - ALPHANAPHTHYL PHOSPHATE

For the localization of acid phosphatase

Solutions required:

A. Formalin (formaldehyde 40%) .. 10 ml.
Calcium chloride .. .. 1 gm.
Distilled water .. .. .. 90 ml.

B. Sodium acetate, trihydrate, A.R. 9·714 gm.
Sodium diethyl barbiturate (soluble barbitone) .. .. 14·714 gm.
Boil about 500–600 ml. of distilled water to drive off the dissolved CO_2; then cool.
Dissolve the two solids in about 450 ml. of the CO_2-free distilled water; then make up the volume to 500 ml. exactly, with CO_2-free water.

C. Pararosaniline chloride .. .. 4 gm.
Hydrochloric acid, 2N .. .. 100 ml.

D. Sodium nitrite .. .. .. 4 gm.
Distilled water .. .. .. 100 ml.

E. Sodium alphanaphthyl phosphate 0·02 gm.
Distilled water .. .. .. 13 ml.
Solution B .. .. .. 5 ml.

F. Solution C .. .. .. 0·8 ml.
Solution D .. .. .. 0·8 ml.

Drop the 0·8 ml. of solution C into a test tube and chill to 0°–10° C. Add the 0·8 ml. of solution D a drop at a time, from a fine pipette, shaking the tube after each addition.

G. Add the contents of the test tube (solution F) to the whole of solution E.

Using a pH meter, adjust the pH of solution G, which is the incubating medium, to 6·5 with N/1 NaOH (about 0·6 ml. will be required).

Technique:

1. Fix material for at least twenty-four hours, in solution A.

Note: It is stated that a longer period of fixation (forty-eight to seventy-two hours) is permissible, but this results in a small decrease in activity. Fresh material is preferable, but autopsy material of twenty-four hours gives fairly good results.

2. Cut frozen sections and mount on albuminized slides that have been dried at room temperature for one to two hours.

3. Immerse in the freshly prepared solution G, at room temperature, for five to thirty minutes, depending on the tissue.

4. Rinse in distilled water.

5. Dehydrate in alcohol.

6. Clear in xylol.

7. Mount in D.P.X., Clearmount or Exemel, or Canada balsam in xylol.

Results:

Sites of acid phosphatase: deep brownish red.

Notes:

(*a*) The freshly prepared hexazonium salt (in solution G) is sufficiently stable at acid pH to permit incubation at room temperature without change of the incubating medium for sixty minutes.

(*b*) The colour of the hexazonium compound is reddish at acid pH, becoming brownish towards neutral.

(*c*) This new diazonium compound, introduced by Davis and Ornstein (1959), "hexazonium pararosaniline," was successfully used in the demonstration of esterases at microscopic and electron microscopic levels.

(*d*) Besides the very good localization, the advantages of the method described above are: α-naphthyl phosphate is water-soluble; it is more readily hydrolysed than the phosphate esters of naphthol AS or indoxyl derivatives; the reagents are commercially available; and the method works easily.

(*e*) Readers are referred to the original paper for more detailed information.

Reference: Barka, T. (1960).

HICKSON'S PURPLE

A general stain suitable for class work

Solution required:

Hickson purple, saturated aqueous.

Technique:

1. Bring sections down to water as usual.
2. Stain in Hickson's purple for ten to twenty minutes.
3. Dehydrate, clear and mount.

Results:

Leucocytes, purple; erythrocytes, distinct red. The rest of the tissues purple.

Reference: Cannon, H. G. (1941).

HICKSON'S PURPLE - VICTORIA GREEN

A general stain, particularly suitable for class work

Solutions required:

A. Hickson's Purple saturated aqueous.

B. Victoria green, G. saturated in 70% alcohol.

Technique:

1. Fix sections to slides; dewax and take through the alcohols down to distilled water as usual.
2. Stain for ten minutes in the Hickson's purple.
3. Rinse in distilled water.
4. Stain in the victoria green for half to one hour.
5. Rinse in 70%, followed by 90% alcohol.
6. Clear in xylol and mount.

Results:

Nuclei are sharply defined, purple. Erythrocytes sharply defined, stained vivid green, against a general blue-purple background.

Note: This method is an improvement on Hickson purple used alone.

Reference: Cannon, H. G. (1941).

HITCHCOCK AND EHRICH'S MIXTURE

For plasma cells, etc.

Technique:

1. Fix in Zenker-acetic acid, corrosive sublimate, but not in Müller or formalin.

2. Paraffin sections are brought down to 90% alcohol; then passed through a solution of iodine in 90% alcohol.

3. The iodine is removed by passing through the graded alcohols to water and finally washing for fifteen minutes in running water.

4. Flood with the stain and allow it to act for fifteen to thirty seconds; then pour off the stain and wash rapidly in water.

5. The preparation is then passed directly into absolute alcohol, where it is allowed to remain only as long as the stain continues to be washed out in clouds.

6. Clear in xylol and mount.

Results:

Plasma cells: cytoplasm, brilliant crimson: nuclei, bluish-green. Other cells appear in lighter shades of green and crimson.

Notes:

(*a*) Sometimes the brilliancy of the stain is enhanced by re-staining.

(*b*) Readers requiring more detailed information should consult the original paper which shows seven photomicrographs.

Reference: Hitchcock, C. H. & Ehrich, W. (1930).

INDOLE REACTION (Ehrlich)

For tryptophane

Solution required:

Ehrlich's indole reagent

Technique:

1. Frozen or paraffin sections are taken to distilled water.

2. Immerse in the reagent for one to two hours at 60° C, in a stoppered staining jar (which should be only partially filled to allow room for expansion and vaporization of the alcohol contained in the reagent).

3. Take the jar out of the oven and allow it to cool for about ten minutes.

4. Rinse the sections in absolute alcohol.

5. Clear rapidly in xylol.

6. Mount in D.P.X., Cristalite, or Clearmount.

Results:

The presence of tryptophane or other substances containing the indole group is indicated by a bluish or reddish violet coloration.

Notes:

(*a*) The method cannot be regarded as specific for tryptophane without confirmation with other methods, and the colour produced by this reaction is insufficiently intense to demonstrate accurate localization.

(*b*) It is advisable to protect the sections, with 0·5% celloidin solution (as page 509, steps 10–15), before processing.

References:
Ehrlich, P. (1901).
Lison, L. (1936).

JANUS GREEN, B - NEUTRAL RED

For supravital staining of blood

Solutions required:

A. Janus green, B vital stain 0·4% in neutral absolute alcohol.

B. Neutral red chloride, vital stain 0·25% in neutral absolute alcohol.

C. Solution A .. .. 0·07 ml.
Solution B .. .. 1·75 ml.
Absolute alcohol, neutral .. .. 10 ml.

Note: Solutions A and B are stable, but solution C deteriorates after a few hours, and it should, therefore, be prepared as and when required, for immediate use.

Technique:

1. Scrupulously clean, dry slides are flooded with solution C.
2. Drain and leave to dry.
3. Place a small drop of blood on each slide.
4. Cover the blood with scrupulously clean, dry coverslips and allow the blood to spread.

5. Seal the edges of the coverslips with soft paraffin wax (M.P. 38° C.), and examine under the microscope.

Results:

Basophilic granules: brilliant scarlet. Eosinophilic granules: yellow to light orange. Neutrophilic granules: salmon colour. Mitochondria: small blue dots or rods. Nuclei: unstained.

Note: The proportion of the two stains may be varied to suit the particular specimen; for instance, specimens very rich in cells, such as leucaemic blood, need more concentrated mixtures of the stains.

Reference: Lightwood, Hawksley & Bailey (1935).

JENNER STAIN

For blood-forming organs

Solutions required:

A. *Formol-Saline.*

Formalin, conc. (i.e. 40% formaldehyde)	100 ml.
Sodium chloride, A.R.	8·5 gm.
Distilled water	1 litre
Acid sodium phosphate, monohydrate, A.R.	4 gm.
Anhydrous disodium phosphate, A.R.	6·5 gm.

B. Jenner stain.

Technique:

1. Fix pieces of tissue for two or three days in Solution A.
2. Dehydrate in ascending grades of alcohol as usual; clear; embed in paraffin wax.
3. Fix sections, not exceeding 5μ in thickness, to slides; dewax; pass through the usual descending grades of alcohol to distilled water which has been buffered to pH 7·0.
4. Stain for forty-five minutes in a grooved, stoppered staining jar, with a mixture consisting of equal volumes of Jenner stain and distilled water, buffered to pH 7.0.

5. Differentiate and dehydrate with absolute alcohol.
6. Clear in xylol and mount in Cristalite or D.P.X.

Results:

Neutrophile granules are stained pink. Oxyphile granules, brownish red. Basophile granules, purple. Nucleoli (plasmosomes), pink. The cytoplasm of partially haemoglobinated precursors of erythrocytes is stained in varying shades of reddish violet, while mature erythrocytes are deep pinkish orange.

References:

Carleton, H. M. & Leach, E. H. (1947), p. 208.
Jenner, L. (1899).

JENNER STAIN

For cytological examination of blood

Technique:

1. Air-dried, unfixed blood films are stained for three minutes, face downwards to prevent precipitate depositing on the film and obscuring the picture.
2. Wash with distilled water until the film appears pink to the naked eye.
3. Blot dry and examine.

Results:

Neutrophile granules: pink. Oxyphile granules: brownish red. Basophile granules: purple. Nucleoli (plasmosomes): pink. Cytoplasm of partially haemoglobinated precursors of erythrocytes: varying shades of purple. Mature erythrocytes: deep pinkish orange.

Reference: Jenner, L. (1899).

JENNER STAIN - GIEMSA STAIN

For the polychromatic staining of blood-forming organs

Solutions required:

A. Formol saline.
B. Jenner Stain.

C. Giemsa stain .. .. .. 1 ml.
Distilled water (buffered to pH 7·0) 20 ml.

D. Acetic acid 0·08% aqueous

Technique:

1. Fix material in Solution A (above) from twelve to forty-eight hours.

2. Dehydrate in the alcohols and clear as usual; embed in paraffin wax and cut sections not exceeding 5μ in thickness.

3. Fix sections to slides and remove wax with xylol.

4. Wash well with two changes of pure methyl alcohol.

5. Stain sections with a measured volume of Jenner stain, which should be freshly filtered.

6. Cover the slides with a Petri dish lid lined with two or three sheets of moistened filter paper (this is to prevent the evaporation of the alcohol and the consequent formation of a precipitate on the sections), and allow the stain to act for three minutes.

7. Add a volume of distilled water (buffered to pH 7·0), equal to that of the stain, to the slides, which should now be gently rocked to ensure thorough mixing of the stain and water.

8. Allow this diluted stain to act for one minute.

9. Pour off excess stain; then without washing, immerse the slides in a stoppered staining jar containing diluted Giemsa stain (Solution C above) and leave the stain to act for forty-five minutes.

10. Rinse and differentiate in Solution D.

11. Rinse thoroughly in distilled water.

12. Dehydrate quickly in 95% alcohol, followed by two changes of absolute alcohol.

13. Clear in xylol and mount in Cristalite.

Results:

Erythrocytes are stained orange. Cytoplasm of lymphocytes and blastocytes are blue. Nuclei: deep blue to violet. Mast cell granules: violet to violet-red.

Reference: Pappenheim, A. (1912).

J.S.B. STAIN

A rapid, water-soluble stain for malaria parasites

(After Jaswant Singh & Bhattacharji, 1944)

Note: Both J.S.B. No. 1 and No. 2 stains can be purchased ready prepared, both in the powder and the liquid forms, but for those workers who prefer and have the time to make their own, the following recipes are given:

J.S.B. Stain No. 1

Preparation of the dry stain

Methylene blue (zinc free), extra pure	0·5 gm.
Tap water	400 ml.
Dissolve thoroughly, then add:	
Sulphuric acid, 1% aqueous	3 ml.
Potassium dichromate, 0·5% aqueous	100 ml.

Technique:

1. Mix thoroughly; a heavy amorphous precipitate of purple coloured methylene blue chromate forms.

2. The resultant mixture is heated very gently, under a reflux condenser, over a very low flame, on a water bath, or on an electric hotplate.

Note: It is stated that the duration of heating is always guided by the appearance of a deep blue colour, and not by any fixed time factor.

Sometimes, although the solution may appear blue while boiling, it may turn green on shaking or cooling, in which case it should be boiled again for a further period. However, four to six hours boiling over a flame or five to six hours on a water bath is usually needed.

3. When the solution turns blue, it should be cooled to room temperature, then filtered, and the precipitate collected.

4. The precipitate is left to dry at room temperature in the air, or in a dessicator.

5. The dried precipitated dye is then ground to a homogeneous powder with:

1·75 gm. of disodium hydrogen phosphate (Na_2HPO_4, $2H_2O$)

in a glass mortar, and stored in a well-closed glass tube until required for preparing the staining solution.

Notes:

(*a*) The yield of the dye powder (without sodium hydrogen phosphate) is between 0·35 gm. and 0·5 gm.

(*b*) When only very small quantities of the stain are required it is recommended that one-fifth of the total yield of the dye should be mixed with 0·35 gm. of the sodium hydrogen phosphate and dissolved in 100 ml. of distilled water, and the remainder of the dry dye, which keeps well, should be stored until required for use.

(*c*) J.S.B. stain No. 1 solution requires about two weeks to mature before it is ready for use.

Solutions required:

A. *J.S.B. stain No. 2*

Eosin, water soluble	0·2 gm.
Tap water	100 ml.

Note: Add a few drops of chloroform as a preservative, to inhibit the growth of air-borne micro-organisms, otherwise the stain is liable to deteriorate within a few days.

B. *Buffered wash water, pH* 6·2–6·6

(i) Disodium hydrogen phosphate (Na_2HPO_4, $2H_2O$) 0·22 gm.

Potassium dihydrogen phosphate (KH_2PO_4) .. .. 0·74 gm.

or

(ii) Simply dissolve 1 Michrome buffer tablet, pH 6·5, in 100 ml. distilled water

C. *J.S.B. stain No. 1*

(i) Prepare the solution in accordance with notes (*b*) and (*c*) above

or

(ii) J.S.B. stain (Michrome) powder .. .. .. 0·45 gm.
Distilled water .. .. 100 ml.

Technique:

1. Take three Coplin jars; fill one with solution A, another with solution B, and the third with solution C.

2. Dip smears in the jar containing solution A (eosin) for one to three seconds.

3. Remove excess eosin by dipping the smears into solution B (buffered wash water).

4. Immediately transfer to solution C and leave therein for forty to forty-five seconds.

5. Wash by dipping, in the same jar of solution B as used at stage 3, three or four times.

6. Dry and examine under the oil immersion objective.

Results:

Similar to Giemsa stained preparations.

Notes:

(*a*) Manwell (1945) concluded that this stain was found to be superior in most respects to any of the commonly used processes for staining blood and blood parasites. The authors (Jaswant Singh, A. P. Ray and C. P. Nair) state that J.S.B. stain has completely replaced Leishman and Giemsa stains in their laboratories in India. However, my own experience is that Giemsa, May–Grünwald, Leishman and Wright, all of which are used in vast quantities, appear to be favoured by a large majority of the biologists in many countries.

(*b*) It is stated that solutions of J.S.B. stain are not difficult to make up, are relatively inexpensive, and keep well for weeks or months, even in hot weather.

(*c*) The preparations are said to be somewhat less resistant to fading than the usual blood stains such as Giemsa, Wright, etc., but they will stand much more exposure to light than they would ordinarily receive.

(*d*) For more detailed information, readers are referred to the original papers, particularly the second one cited below.

References:

Jaswant, Singh & Bhattacharji, L. M. (1944).
Jaswant, Singh, Ray, A. P. & Nair, C. P. (1953).
Manwell, R. D. (1945).

KH STAINS

(Edward Gurr)

For keratin, vaginal epithelium, etc.

Solutions required:

A. Trichloric acid, 1% in 80% alcohol
B. KH1 or KH8 or KH9 stain

Technique:

1. Fix material in solution A and embed in paraffin wax as usual.
2. Fix sections to slides and carry through to 70% alcohol in the usual way.
3. Rinse briefly with distilled water.
4. Immerse in the KH staining solution for five minutes.
5. Rinse in 70% alcohol.
6. Rinse in 90% alcohol.
7. Dehydrate with absolute alcohol.
8. Clear in xylol.
9. Mount in D.P.X. or Clearmount or Cristalite.

Results:

Stain	*Keratin*	*Collagen*
KH1	Purple	Blue
KH8	Reddish orange	Blue
KH9	Brilliant red	Pale blue

Reference: Gurr, E. (1958*a*), pp. 37–39.

LEISHMAN STAIN

For blood, malaria parasites, trypanosomes, etc., in smears

This stain offers a simple and precise method of staining blood for diagnostic purposes.

Best results are obtained by buffering the distilled water to pH 6·6–7·0.

Technique for blood films:

Fixation is unnecessary unless the films are to be kept in stock for any length of time, in which case they should be fixed for five minutes in pure methyl alcohol at room temperature.

Thin film method:

1. Air-dried films are stained, without fixing, for one minute with five to ten drops of the stain; then double the quantity of distilled water (i.e. ten to twenty drops) is added and mixed by rocking the slide gently.

2. Allow this diluted stain to act for five to ten minutes; then pour off.

3. Wash gently with distilled water; then differentiate by flooding the slide with distilled water and allowing the water to remain on the slide for about one half to one minute, until the film appears pink to the naked eye.

4. Pour off; blot gently and dry in air.

Results:

Similar to Wright's stain.

Thick film method:

This method should be employed in searching for blood parasites when negative results have been obtained by the thin film method.

1. A film is prepared by spreading three to five drops of blood in a circle about 15 mm. diameter over a slide; then without fixation, it is allowed to dry at room temperature from twelve to twenty-four hours, protected from dust. The time required for

drying may be very considerably shortened by placing the slide in an incubator at 37° C.

2. Remove the haemoglobin by placing the film face downwards in a dish of distilled water at room temperature.

3. Fix in acid alcohol for five to fifteen minutes.

4. Wash well with distilled water; blot gently and dry in air.

5. Stain in accordance with the method described above for thin films.

Results:

Similar to Wright's stain.

Reference: Leishman, W. B. (1901).

LEAD HAEMATOXYLIN - ACID FUCHSIN
(M. A. MacConaill)

A differential stain for neurokeratin

Solutions required:

A.	Lead nitrate	2 gm.
	Glacial acetic acid	8 ml.
	Acid fuchsin	0·5 gm.
	Water	92 ml.
B.	Haematoxylin	1 gm.
	Acetic acid 4% aqueous	100 ml.
C.	Solution A	1 volume
	Solution B	1 volume

Note: This solution should be prepared as and when required: it deteriorates after one day.

D.	Ammonium acetate 6% aqueous	10 ml.
	Ammonium molybdate, saturated, aqueous	70 ml.
	Water	80 ml.

Note: All the above solutions must be made without the application of heat. Tap water may be used: the solutions must be filtered.

Technique:

1. Material should be fixed in 10% formalin and embedded in paraffin wax. Sections are cut 6 to 12μ in thickness.

2. Fix sections to slides; remove paraffin wax and take down to 70% alcohol by the usual stages.

3. Pass through 30% alcohol; then stain in Solution C for five minutes.

4. Rinse in two changes of tap water.

5. Immerse in Solution D for one to two minutes.

6. Wash in running water for two to five minutes to remove the unchanged molybdate.

7. Dehydrate; clear in xylol and mount.

Note: A deep yellow filter is of great help in microscopic examination, although not necessary.

Results:

Nuclei, dark blue; nucleoli of neurones, red; axial substances of nerve fibres, dark to pale blue; cuticular substance (including myelotheca) of nerve fibres, red; neurilemma (of Glees), purplish red.

Notes:

(*a*) To eliminate all myelin, sections should be passed through Cellosolve after the alcohols. The same precaution should be observed when preparing tissue for embedding.

(*b*) The essence of this technique is that the lead haematoxylin reduces the ammonium molybdate to form a blue lake, which makes it possible to employ only the minimum exposure to haematoxylin, thereby leaving the erythrophile ("Fuchsinophile") parts of the neurone red.

(*c*) The Falg technique, introduced later (MacConaill & Gurr, 1959, 1960*a*, see pages 198–203 of this book) is simpler to perform and is considered to give more satisfactory results than the above method.

Reference: MacConaill, M. A. (1949, 1951).

LEISHMAN STAIN

For general differentiation of blood corpuscles; malarial parasites; trypanosomes, etc. in sections.

This stain is extensively used by British workers who generally prefer it to Wright's stain which is used extensively in America.

Solutions required:

A. Formol saline, neutral, buffered.

B. Leishman stain.

C. Acetic acid 0·08% aqueous.

Technique:

1. Fix pieces of tissue in Solution A for sixteen to forty-eight hours.

2. Dehydrate in the usual ascending grades of alcohol; clear; and embed in paraffin wax.

3. Fix sections, not exceeding 5μ in thickness to slides; remove wax with xylol; pass through descending grades of alcohol down to neutral distilled water.

4. Stain for five to ten minutes in freshly prepared mixture consisting of one volume of Leishman's stain and two volumes of neutral distilled water, in a stoppered staining jar.

5. Rinse with neutral distilled water.

6. Differentiate with the acetic acid solution, controlling by examination under the microscope, until the protoplasm of the cells is pink, and only nuclei are blue.

7. Wash with neutral distilled water.

8. Dehydrate quickly with absolute alcohol; clear in xylol; mount in Cristalite, D.P.X. or Clearmount.

Results:

Erythrocytes: yellowish red. Polymorphonuclears: dark purple nuclei, reddish violet granules, pale pink cytoplasm. Eosinophiles: blue nuclei, red to orange-red granules, blue cytoplasm. Basophiles: purple to dark blue nuclei, dark purple to black granules. Lymphocytes: dark purple nuclei, sky blue cytoplasm. Platelets:

violet to purple granules. Malarial parasites and Leishmania: chromatin, red; cytoplasm, blue. Trypanosomes: chromatin, red.

Note: The timing of the staining either before or after dilution may be altered to suit individual requirements. Staining effects similar to Giemsa are obtained by staining for ten minutes in Leishman stain diluted with twice its volume of distilled water buffered to pH 6·5.

Reference: Leishman, W. B. (1901).

LEUCO PATENT BLUE

For the identification of haemoglobin.

Solutions required:

A. Patent blue AF54 .. .. 1 gm.
Distilled water .. .. .. 100 ml.

Dissolve; then add:

Zinc metal powder .. .. 10 gm.
Glacial acetic acid .. .. 2 ml.

Boil until the blue colour completely disappears. Allow to cool; shake with about 1 gm. of decolorizing carbon; then filter. The liquid which should then be quite colourless is stored in a well stoppered bottle.

B. Solution A .. .. .. 10 ml.
Glacial acetic acid .. .. 2 ml.
Hydrogen peroxide 3% .. 1 ml.

N.B.: This solution must be freshly prepared and filtered before use.

C. Safranin 0·1% aqueous .. 99 ml.
Glacial acetic acid .. .. 1 ml.

Technique:

1. Fix tissue blocks, not more than 3 to 5 mm. in thickness in 10% formalin buffered to pH 7·0 for 24 to 28 hours (prolonged fixation should be avoided).

2. Embed in paraffin wax as usual and cut section 5 to 6μ in thickness.

3. Take sections down to water as usual.

4. Stain in solution B for three to five minutes.

5. Wash briefly in water.

6. Counterstain for thirty to sixty sections in the safranin solution.

7. Rinse briefly with water.

8. Dehydrate as usual.

9. Clear in xylol.

10. Mount in Clarite or other synthetic mountant such as D.P.X., Clearmount, etc.

Results:

Haemoglobin, dark blue-green; background light pink.

Note: Blood and tissue smears fixed with methyl alcohol may also be stained by applying the stains as prescribed above.

References:

Dunn, R. C. (1946).
Gurr, E. (1958*a*).

LEVADITI'S STAIN

For Treponema pallidum in sections

Solutions required:

A.	Silver nitrate	2·5% aqueous
B.	*Reducing solution:*	
	Pyrogallic acid	3 gm.
	Formalin	5 ml.
	Distilled water	100 ml.

Technique:

Tissues about 1 mm. thick should be fixed for twenty-four hours in 10% formalin and embedded in paraffin wax *after staining.*

1. After rinsing tissues in tap water, immerse in 95% alcohol for twenty-four hours.

2. Immerse in distilled water until the tissue sinks to the bottom of the jar.

3. Transfer to Solution A for 3 to 6 days at 37° C. in the dark, changing the solution every twenty-four hours.

4. Wash in distilled water; then immerse in Solution B for twenty-four to seventy-two hours in the darkroom at room temperature.

5. Wash in distilled water; then dehydrate with 80%, 95%, and absolute, alcohol.

6. Clear in cedarwood oil and embed in paraffin wax.

7. Sections are cut 5μ in thickness and mounted after removal of the paraffin wax.

Results:

Treponema: jet black. Tissue: yellow to brown.

References:
Conn, H. J. & Darrow, M. A. (1947), pt. 3, sect. B, p. 14.
Levaditi, C. & Manouelian (1906).
Mallory, F. B. (1938), p. 293.

LIGHT GREEN - ACID FUCHSIN

(After Alzheimer)

For demonstrating neuroglia changes

Solutions required:

A. Osmic acid 2% aqueous .. 20 ml.
Chromic acid 1% aqueous .. 75 ml.
Glacial acetic acid .. .. 0·5 ml.

B. Acid fuchsin 25% aqueous.

C. Picric acid, saturated, alcoholic 15 ml.
Distilled water .. .. .. 30 ml.

D. Light green 10% aqueous.

Technique:

1. Fix thin slices of the material in 10% formalin for twenty-four hours to three days.

2. Wash for twenty-four hours in running water.

3. Immerse very thin slices of the material in a comparatively large volume of Solution A which should be changed once or twice if it blackens.

4. Wash for several hours in running water.

5. Pass through ascending grades of alcohol.

6. Clear in the usual manner and embed in paraffin wax.

7. Sections not more than 2 to 4μ in thickness are fixed to slides.

8. Dewax with xylol.

9. Rinse thoroughly with absolute alcohol and pass through the usual descending grades of alcohol down to distilled water.

10. Stain for an hour at 60° C. with the acid fuchsin solution.

11. Allow the preparation to cool to room temperature; then wash with water.

12. Immerse in Solution C (picric acid) from one second to two minutes.

13. Rinse in two changes of water.

14. Stain from one half to one hour in the Light Green solution.

15. Rinse quickly in absolute alcohol.

16. Rinse in xylol.

17. Mount in Canada balsam or Cristalite or D.P.X.

Results:

Migrating astrocytes, of varying shades of green and sometimes containing fuchsinophile granules of brown stained lipoid inclusions. Lipoid contents of perivascular phagocytes are brown to black. Neuroglia fibres and erythrocytes: red. Medullary sheaths are unstained. Connective tissue: deep green. Nerve cells are pale green with red stippling, while nerve-cell nuclei are a darker green with bright red nuclei.

Notes:

(*a*) The material must be fresh and only small pieces should be employed.

(*b*) Sections stained by this technique should appear lilac in colour to the naked eye.

(*c*) It is advantageous to experiment in order to determine the optimum staining time in the picric acid and the light green, as results vary according to the material to be stained.

Reference: Mallory, F. B. (1938).

LIGNIN PINK

For whole mounts of marine invertebrates, particularly for crustaceans limbs, ostracod appendages, Medusa of Obelia, etc., as well as for demonstrating chitin

Solutions required:

A. *Sea water Bouin:*

Sea water saturated with picric acid	75 ml.
Formaldehyde 40%	25 ml.
Glacial acetic acid	5 ml.

B. Lignin pink saturated in distilled water or in Benzyl alcohol.

Technique:

1. Specimens are fixed from eighteen to forty-eight hours, according to the material, in Solution A.

2. Wash out the fixative with 50% alcohol, followed by 70% alcohol until the yellow coloration, due to the picric acid, is completely extracted.

3. Wash in running water to remove the alcohol.

4. Immerse in solution B for fifteen minutes or longer.

Results:

With the aqueous solution of the stain

Medusa of Obelia and limbs of crustaceans are stained deep carmine colour. The finest structures of ostracod appendages, uniform pink, but a better effect can, however, be obtained by staining the specimen for a longer period (up to sixteen hours) with a solution of the dye in benzyl alcohol: the final result in this case is a definite purple for the exoskeleton, while the other tissues are carmine colour.

Note: Overstaining with lignin pink is impossible, and it will not wash out with alcohol.

Reference: Cannon, H. G. (1941).

LIPID CRIMSON METHOD

For lipids

Reagents required:

A. Lipid crimson (Michrome No. 931) 1 gm.
Alcohol, 70% .. .. .. 100 ml.

B. Haematoxylin (Ehrlich)

Technique:

1. Fix material in 10% formaldehyde and cut sections on a freezing microtome, or use freehand sections.

2. Stain in reagent A for ten to fifteen minutes.

3. Wash rapidly in 50% alcohol.

4. Wash in distilled water.

5. Stain in the haematoxylin solution for five to ten minutes.

6. "Blue" in tap water or in 1% lithium carbonate solution.

7. Wash in distilled water.

8. Mount in Aquamount.

Results:

Lipids: brilliant crimson. Nuclei: blue to bluish black.

Notes:

(*a*) This method gives better results than Sudan 3 or Sudan 4 or Scarlet R, the colour being much more intense and the smaller lipid-particles or droplets are readily visible.

(*b*) Bodman (1960) employs lipid crimson in gel electrophoresis for staining lipo-protein complexes.

Reference: Gurr, E. (1958*a*).

LITHIUM SILVER

(After Laidlaw)

For staining skin and tumours

Solutions required:

A. Iodine 1% in absolute alcohol.

B. Sodium thiosulphate 5% aqueous.

C. Potassium permanganate 0·5% aqueous.

D. Oxalic acid 5% aqueous.

E. *Lithium silver:*

Dissolve 12 gm. silver nitrate in 20 ml. distilled water in a 500 ml. stoppered bottle; then add 230 ml. lithium carbonate, saturated, aqueous, and shake well. Transfer to a 250 ml. measuring cylinder; cover with a watch glass and allow to stand undisturbed until the precipitate formed measures about 70 ml. Pour off the clear liquid and transfer the precipitate to another vessel. Wash precipitate with three or four changes of distilled water, decanting after each washing so that the precipitate remaining measures 70 ml. Add a diluted ammonia solution (15 ml. strong ammonia solution, sp. gr. 0·880 diluted with 35 ml. distilled water) a little at a time until the fluid precipitate is almost clear. Filter through a Whatman No. 40 filter paper.

F. Formalin 1% in tap water.

G. Gold chloride (yellow) 0·5% in distilled water.

Technique:

1. Fix tissues in 10% formalin for three days.

2. Dehydrate, clear, embed in paraffin wax in the usual manner.

3. Fix sections to slides, dewax and take down to water as usual.

4. Wash in running water for five minutes.

5. Immerse in the iodine solution for three minutes.

6. Pour off excess iodine and immerse in the sodium thiosulphate solution for three minutes.

7. Rinse in tap water; then immerse in the potassium permanganate solution for three minutes.

8. Rinse in tap water.

9. Immerse in the oxalic acid solution for five minutes.

10. Wash in running tap water for ten minutes.

11. Immerse in three changes of distilled water for three or four minutes in each.

12. Stain in an oven for five minutes with the lithium silver solution heated to 50° C.

13. Rinse the slide back and front with distilled water to remove all traces of excess lithium silver.

14. Immerse slide in a jar of 1% formalin.

15. Rinse both sides of the slide with distilled water to remove all traces of the formalin solution.

16. Immerse in the yellow gold chloride solution in a Coplin staining jar for ten minutes.

17. Rinse both sides of the slide with distilled water to remove all traces of excess gold chloride.

18. Flood the slides with oxalic acid and allow this reagent to act for ten minutes.

19. Rinse in distilled water.

20. Flood the sections with the sodium thiosulphate solution changing the solution every time it becomes turbid over a period of ten minutes.

21. Wash well in running water; then drain.

22. Dehydrate in ascending grades of alcohol, clear in xylol and mount.

Results:

Collagen is stained a reddish purple, while reticulum appears as black threads.

Reference: Laidlaw, G. F. (1929).

LORRAIN SMITH - DIETRICH STAIN

For lipids

Solutions required:

A. Potass. dichromate 5% aqueous.

Haematoxylin (Kultschitzky)

B. Haematoxylin 10% in absolute alcohol (ripened three months or longer) .. .. .. 10 ml.
Acetic acid 2% aqueous .. 90 ml.

C. Potass. ferricyanide .. .. 2·5 gm.
Borax 2% aqueous .. .. 100 ml.

Technique:

Material is fixed in 10% formalin and frozen sections are employed.

1. Mordant sections twenty-four to forty-eight hours in Solution A at 37° C.; then wash thoroughly in distilled water.

2. Immerse in Solution B at 37° C. for four to five hours; then wash in distilled water.

3. Differentiate overnight in Solution C.

4. Wash thoroughly in distilled water; drain and mount in Aquamount.

Results:

Lipids: blue-black.

References:
Dietrich, A. (1910).
Mallory, F. B. (1938), p. 123.
Smith, J. Lorrain & Mair, W. (1908).

LUGOL'S IODINE

For the identification of glycogen in tissues

Solution required:

Lugol's iodine.

Technique:

1. Thin slices of tissue are fixed in absolute alcohol; then dehydrated; cleared, and embedded in paraffin wax in the usual manner.

2. Float sections on slides with 70% alcohol and flatten by warming gently, on a warm surface (but not with a direct flame).

3. Remove excess 70% alcohol by blotting very carefully but thoroughly.

4. Treat with xylol; then with absolute alcohol.

5. Stain in Lugol's iodine solution for ten minutes; then pour off excess stain and carefully blot the preparation thoroughly dry.

6. Clear and differentiate with origanum oil, controlling by examination under the microscope.

7. Mount in origanum balsam.

Results:

Glycogen, reddish brown; tissue constituents, pale yellow.

Reference: Langerhans, T. (1890).

LUXOL FAST BLUE

As a selective stain for alpha cells in human pituitary

Solutions required:

A. Luxol fast blue (Michrome No. 331) .. .. 0·1 gm.
Absolute alcohol .. .. 95 ml.
Distilled water .. .. .. 14·5 ml.
Glacial acetic acid .. .. 0·5 ml.

Note: This solution, which is stable for several months at least, should be filtered before use.

B. Lithium, carbonated, 0·05% aqueous

C. Solution A .. .. .. 5 ml.
Distilled water .. .. .. 95 ml.

D. Erythrosin .. .. .. 1 gm.
Tap water .. .. .. 100 ml.

Note: If this solution is to be kept in stock for more than a few days, add a few drops of chloroform to prevent decomposition by air-borne micro-organisms.

Technique:

1. Fix adult pituitaries in Bouin's fluid or in 10% formalin and embed in paraffin wax as usual.
2. Cut sections at 5μ and attach them to slides.
3. Dewax with xylol.
4. Rinse with absolute alcohol.
5. Wash with two changes of 95% alcohol.
6. Immerse sections in the luxol fast blue (solution A) in an incubator at 55° C. for two to four hours.
7. Wash off excess stain with 95% alcohol.
8. Rinse with distilled water.
9. Begin the differentiation by immersing the preparation in 0·05% lithium carbonate (solution C) for three to five seconds.

Note: Considerable care has to be exercised to obtain the correct degree of differentiation as this is a very delicate process.

10. Continue the differentiation in four changes of 70% alcohol.

Note: The final lot of alcohol should be free of stain.

11. Wash with distilled water.
12. Check the degree of differentiation by microscopic examination, and if further differentiation is necessary, repeat steps 10 and 11 quickly.
13. Wash preparations in tap water.
14. Counterstain in the erythrosin (solution D) for five to ten seconds.
15. Wash in tap water.
16. Wash in 90% alcohol.
17. Dehydrate in absolute alcohol.
18. Clear in xylol.
19. Mount in D.P.X. or Clearmount.

Results:

Alpha cells: blue. Colloid: pale blue.

Notes:

(*a*) The authors used adjacent sections from the same human pituitary tissue block and stained them by (i) the periodic acid–Schiff method, (ii) Masson trichrome method, and (iii) the luxol fast blue method described above to prove, by comparison of the sections stained by these different techniques, that Luxol fast blue stains the alpha cells an intense blue but does not stain the beta cells.

(*b*) Whereas beta cells stained by PAS or by Masson trichrome methods exhibited fine granules, granules were not seen in Luxol fast blue-stained alpha cells.

(*c*) The authors state that their findings throw no further light on the suggestion of Klüver (1944) that the staining reaction of Luxol fast blue is due to porphyrin, or to that of Pearse (1955) that it is due to phospholipids.

(*d*) The authors also state that their studies demonstrate that Luxol fast blue is highly selective for alpha cells in the human and a series of other mammalian pituitaries (cat, cow, dog, horse, jackal, pig, rabbit, and rat), and that their conclusion is diametrically opposed to that of German & Schwarz (1958).

(*e*) For more detailed information and photomicrographs, the original paper should be consulted.

Reference: Shanklin, William M., Nassar, Tamir K., Issidorides, Marrietta (1959).

LUXOL FAST BLUE

For gross brain sections

Solutions required:

A.	Luxol fast blue	1 gm.
	Alcohol, 95%	1 litre
	Acetic acid, 5% aqueous ..	5 ml.
B.	Lithium carbonate, saturated aqueous	
C.	Solution B	10 ml.
	Distilled water	1 litre

Technique:

1. Fix brains in 10% formalin, preferably by perfusion, and sliced to the desired thickness as soon as they are firm enough.
2. Store the sliced brain in the fixative, for a minimum time of two weeks for human material, or in the case of laboratory animals, for at least one week.
3. Wash the slices in water for at least six hours.
4. Dehydrate for one hour in each of two changes of 95% alcohol.
5. Stain in solution A for sixteen to eighteen hours at 45–55° C.
6. Remove excess stain by immersing the preparations in 95% alcohol.
7. Rinse in distilled water.
8. Differentiate in several changes of solution C.
9. Refine the differentiation in a number of changes of 70% alcohol.
10. Rinse in distilled water.
11. Store in 10% formalin.

Results:

Tracts of white matter stain brilliant blue, contrasting strongly with cellular areas of grey matter, stained very pale green.

Notes:

The author states that absence of myelin is evident in pathological demyelinated regions of adult brain and in the non-myelinated areas of very young animals.

Reference: Dziabis, M. D. (1958).

LUXOL FAST BLUE

For increasing the contrast of sectioned material for phase contrast microscopy

Solutions required:

A. Luxol fast blue 2% in 95% alcohol

B. Ferric chloride 29% aqueous ($FeCl_3$)

C. Solution B .. .. .. 4 ml.
Hydrochloric acid, conc. .. 1 ml.
Alcohol, 95% .. .. .. 95 ml.

D. Solution A .. .. .. 1 volume
Solution C .. .. .. 1 volume

Note: Solution D should be prepared only as and when required for immediate use.

Technique:

1. Fix material in Bouin, Helly, Zenker, Susa–Heidenhain, Susa with picric acid, or Flemming, and embed in paraffin wax in the usual manner.
2. Cut sections at 4 to 5μ (thicker sections are of little value for phase contrast microscopy), and fix them to slides.
3. Dewax with xylol, and wash with absolute alcohol followed by 90% and 70% alcohols.
4. If a mercury-containing fixative has been used, treat for the removal of mercurial precipitate in the usual way, then return to 70% alcohol, otherwise this step should be omitted.
5. Immerse for one and a half hours in solution D.
6. Rinse in 70% alcohol.
7. Dehydrate through the alcohols; clear in xylol and mount in a neutral synthetic resinous medium such as D.P.X. (Lendrum & Kirkpatrick), or Cristalite, Clearmount, or Emexel, etc.

Results:

Observed with dark-contrast medium objectives, cellular and nuclear membranes, chromatin and cytoplasmic structures appear black against a green background.

Notes:

(*a*) The author (J. A. Green) states that although the phase contrast microscope was designed primarily for the study of living material, it has proved to be a useful tool for the study of unstained sectioned material, and that it is, in addition, an excellent substitute for counterstaining to show cellular detail after periodic acid–Schiff, elastin stains, Feulgen stain and a very large number of other highly selective staining procedures. However, when the phase contrast microscope is employed with

unstained sections, two troublesome features are frequently met with; i.e. (i) a halo of light around isolated cells and cellular structures and, (ii) insufficient contrast, which is particularly troublesome in studying epithelial sheets, cell boundaries being difficult to discern. Light filters of different wavelengths were tried without success, but the staining technique described above, which according to the author was discovered by an accident, was found to increase contrast and eliminate halo-ing.

(*b*) Structures well defined by this technique, which is stated to be of no value for conventional microscopy, include terminal bars, epithelial tonofibrils, brush borders, striated borders, zymogen granules, cytoplasmic vacuoles, filamentous mitochondria of renal tubules, and according to the author, many others too numerous to mention.

(*c*) For further information readers should consult the original paper, and J. A. Green's Luxol fast scarlet method (1957) which is described on page 280 of this book.

(*d*) I would suggest that Luxol fast orange, Luxol fast red, and Alcian blue might also be tried out along the same lines as Luxol fast scarlet C and Luxol fast blue.

Reference: Green, J. A. (1956).

LUXOL BRILLIANT GREEN BL

A nuclear stain for phase-contrast microscopy

Solutions required:

A. *David's mordant*

Potash alum	5 gm.
Mercuric chloride	1·25 gm.
Tannic acid	4 gm.
Distilled water	90 ml.

B.

Luxol brilliant green BL	0·25 gm.
Alcohol, 95%	100 ml.

Technique:

1. Fix material in 10% formalin and embed in paraffin wax as usual.

2. Cut sections at 4 to 5μ and fix them to slides.

3. Remove paraffin wax with xylol.

4. Carry to distilled water, through the usual descending grades of alcohol.

5. Immerse in solution A for one and a half hours.

6. Wash well in tap water.

7. Wash in 70% alcohol.

8. Immerse in the luxol fast green solution for one hour, in a covered jar, to prevent evaporation of the alcohol.

9. Rinse in 95% alcohol.

10. Dehydrate with absolute alcohol.

11. Mount in Clearmount or Michrome mountant or proceed as follows:

12. Rinse in xylol.

13. Mount in Clearmount, Michrome mountant, D.P.X., or Emexel.

Results:

When viewed with dark contrast medium objectives, nucleoli, nuclear chromatin and nuclear membranes appear black against a background of green cytoplasm, while certain cytoplasmic structures, such as the secretion granules of pancreatic acinar cells, appear black also. The contrast of epithelial cells although increased was not of the same high degree as in the case of luxol fast blue (see page 273).

Notes:

The authors found that 10% formalin, Bouin and Zenker's fixative gave equally good results. The materials used were salivary gland, pancreas, lymph node, liver, spleen, uterus, and ileum. It is suggested by the authors that the technique might also be applied with advantage in the study of a wider variety of organs. Other fixatives might also be used, besides the three mentioned above as having been tried by the authors.

Reference: Wood, M. L. & Green, J. A. (1958*b*).

LUXOL FAST BLUE - CRESYL FAST VIOLET

(After Klüver and Barrera, 1954)

For the combined staining of cells and fibres in the nervous system, obviating the need for chromate treatment and haematoxylin

Solutions required:

A.	Luxol fast blue 0·1% in 95% alcohol	100 ml.
	Acetic acid 10% aqueous	0·5 ml.
B.	Lithium carbonate 1% aqueous ..	5 ml.
	Distilled water	95 ml.
C.	Cresyl fast violet, CNS 0·1 to 0·25% aqueous	120 ml.
	Acetic acid 1% aqueous	1 ml.
D.	Xylol	1 volume
	Terpinol	3 volumes

Technique:

Material should be fixed in 10% formalin.

Paraffin or frozen sections give somewhat better results than Celloidin. Affixed Celloidin give better results than loose Celloidin sections.

(a) **Frozen sections**

1. Cut sections 25μ in thickness and place them in distilled water.

2. Immerse in 70% alcohol for ten to fifteen minutes.

3. Stain from five to twenty-four, but preferably not less than sixteen hours, in the Luxol fast blue solution, in a stoppered jar in an oven at 40° C.

Note: For staining four sections of the brain stem of a monkey, for example, 20 to 25 ml. of the stain should be used and then discarded.

4. Immerse in 95% alcohol and wash off the excess stain.

5. Wash in distilled water.

6. Immerse for two or three seconds, but no longer, in the lithium carbonate solution, as the first stage of differentiation.

7. Continue the differentiation in several changes of 70% alcohol until the grey and white matter can be distinguished, but taking care not to over-differentiate.

8. Wash in distilled water.

9. Immerse in the lithium carbonate solution for three to five seconds, but no longer.

10. Complete the differentiation by immersing in several changes of 70% alcohol, until the white matter is stained greenish-blue in sharp contrast with the colourless grey matter.

11. Wash thoroughly in distilled water.

12. Stain for one to two minutes in the cresyl fast violet solution, which should be warmed carefully and filtered before use.

13. Wash for two or three seconds in distilled water.

14. Differentiate in several changes of 95% alcohol until colour ceases to come away from the preparation and the alcohol is no longer tinted.

15. Clear in xylol-terpineol (Solution D).

16. Clear in xylol and mount.

(b) Paraffin sections

1. Cut sections 15 to 20μ in thickness and fix to slides.

2. Remove paraffin wax with xylol and pass through absolute alcohol.

3. Rinse with several changes of 95% alcohol.

4. Stain with the Luxol fast blue solution for five to twenty-four hours, but preferably not less than sixteen hours, at 57° C. in an oven, taking precautions to prevent the loss of alcohol through evaporation from the staining solution.

5. Proceed exactly as at Stage 4 in the technique given above for frozen sections, except at Stage 12 the cresyl violet should be allowed to act for six minutes.

(c) Celloidin sections (loose)

1. Cut sections 15 to 30μ in thickness and place them into 75% alcohol.

2. Stain from five to twenty-four hours, but preferably not less than sixteen, in the Luxol fast blue solution at 57° C. in an oven,

taking precautions to prevent the loss of alcohol by evaporation from the staining solution.

2. Proceed exactly as at Stage 4 in the technique given for frozen sections, except at Stage 12 the staining time for the cresyl fast violet should be increased to three minutes.

(d) Celloidin sections (affixed)

1. Cut sections 15 to 30μ in thickness, keeping the microtome knife and tissue continually flooded with 75% alcohol.

2. Place sections on slides, which have previously been smeared with glycerine albumen.

3. If necessary flatten out the sections on the slides by rolling with a piece of glass tubing half an inch in diameter and about two inches in length, or a small glass phial will serve the purpose.

4. Drop on sufficient clove oil to cover the sections and leave the oil to act for five minutes.

5. Remove the clove oil with 95% alcohol.

6. Remove the Celloidin with absolute alcohol.

7. Wash with 95% alcohol.

8. Proceed exactly as at Stage 2 in the technique given for frozen sections, except at Stage 12 the staining time for cresyl fast violet should be increased to six minutes.

Results:

Myelinated fibres are sharply contrasted greenish-blue against the reddish-coloured Nissl cells. The technique shows the Nissl picture and differentiates between the three types of glia cells: Myelin sheaths, neurons and glia nuclei are well demonstrated. Differentiation is also obtained between the three layers of medium-sized and larger blood cells, and capillary endothelium as well as mesothelial lining of Arachnoid membrane are sharply outlined. The finer fibres of the molecular layer of the cerebral cortex can be most effectively demonstrated in paraffin sections. Bacteria and pigments in nerve cells are more clearly demonstrated with this technique than with the usual Nissl stains.

Note: In the original paper it is stated that this cell-fibre stain has been employed for peripheral nerves as well as structures of the central nervous system in amphibians, birds and mammals (rat,

guinea-pig, rabbit, cat, monkey, chimpanzee, gorilla and man), and that with suitable counterstains Luxol fast blue will give excellent preparations of cochlea, adrenals and numerous extraneural tissues.

References:

Klüver, H. & Barrera, Elizabeth (1953).
Klüver, H. & Barrera, Elizabeth (1954).

LUXOL FAST SCARLET C

As a stain for phase contrast microscopy for increasing contrast of cellular structures, etc.

Solutions required:

A. Tannic acid, 1% aqueous
B. Luxol fast scarlet C 2% in 95% ethyl alcohol

Technique:

1. Fix material in any of the following fixatives: Bouin, Helly, Regaud, Zenker, Susa–Heidenhain, Flemming's fluid, or 10% formalin.
2. Embed in paraffin wax in the usual way, and cut sections at 4 to 5μ.
3. Fix sections to slides, dewax, and carry through the usual descending grades of alcohol into water.
4. Immerse in the tannic acid solution for six to twelve hours.
5. Rinse in tap water.
6. Wash with 70% alcohol.
7. Immerse in the Luxol fast scarlet solution for one hour, in a well-closed staining jar to prevent evaporation of the alcohol.
8. Dehydrate in absolute alcohol, clear in xylol and mount in D.P.X., Cristalite, Clearmount, or Emexel.

Results:

The sections, observed with dark contrast medium objectives, show cellular and nuclear membranes, chromatin and cytoplasmic structures, black against a light red background. Secretion granules and mitochondria of the pancreas and salivary glands are stated by the author (J. A. Green) to be well defined when their fixation has been satisfactory; while renal tubule cell structure, epithelial cell structure, cells in lymph nodes, and reticular

fibres show clearly, in epithelial sheets the cell boundaries are sharply delineated.

Notes:

(*a*) The author states that the presence of light halos and insufficient contrast often constitute a source of trouble when unstained sections are subjected to phase-contrast microscopy; the simple staining procedure described reduces haloing and affords increased contrast. According to the same author, staining for phase-contrast work appears to act advantageously by producing a change in the refractive index of the featureless cytoplasm, the visible cellular structures, or both, and it is probable that methods will be devised to increase the utility of the phase-contrast microscope, thereby facilitating the study of histological and cytological details in the same section.

(*b*) Readers should also refer to the Luxol fast blue method, which is also due to J. A. Green and is described on page 273.

Reference: Green, J. A. (1957).

MacCALLUM'S STAIN

For influenza bacilli and Gram-positive organisms in tissues

Solutions required:

A. *Goodpasture's stain:*

Alcohol 30%	100 ml.
Basic fuchsin	0·59 gm.
Aniline oil	1 ml.
Phenol crystals	1 gm.

B. Picric acid saturated aqueous.

C. Stirling's gentian violet.

D. Gram's iodine.

E. Equal volumes of xylol and aniline oil.

Technique:

Tissues should be fixed in Helly and embedded in paraffin wax.

1. Stain for ten to thirty seconds in Solution A; then wash in tap water.

2. Differentiate for a few seconds in formalin till the bright red colour changes to a clear deep pink; then wash with tap water.

3. Counterstain one to five minutes in Solution B until the section appears purplish yellow to the naked eye; then wash with tap water.

4. Differentiate in 95% alcohol until the section appears red; then wash in tap water.

5. Stain for about five minutes in Solution C; then wash in tap water.

6. Stain for one minute in Solution D; then, without washing, blot dry.

7. Treat in Solution E until no more colour comes out.

8. Pass through two changes of xylol; then mount.

Results:

Gram-positive organisms: blue. Gram-negative: red. Tissues: varying shades of red and purple.

Reference: MacCallum, W. G. (1919).

MALLORY'S STAIN - HAEMATOXYLIN

For the differential staining of the pancreatic islets

Solutions required:

A. Distilled water .. .. .. 100 ml.
Sulphuric acid conc. .. .. 1 ml.
Potassium Permanganate .. .. 1 gm.

B. Harris or Ehrlich haematoxylin.

C. Lithium carbonate, saturated aqueous.

D. Acid Fuchsin acetic (Mallory) 0·5% aqueous.

E. Phosphomolybdic Aniline Blue - Orange G (Mallory).

Technique:

1. Mammalian pancreases are fixed in Bouin and afterwards washed in 80% alcohol.

2. Dehydrate, clear, and embed in paraffin wax.

3. Cut sections 4 to 5μ in thickness.

4. Fix sections to slides: remove wax with xylol and pass through the usual grades of alcohol down to distilled water.

5. Immerse in solution A for about one half to one minute, until the sections appear uniform reddish brown in colour.

6. Rinse in distilled water.

7. Stain with Ehrlich or Harris Haematoxylin for five to ten minutes.

8. Blue in lithium carbonate solution.

9. Wash well in tap water.

10. Wash with distilled water.

11. Stain in Mallory's Acid Fuchsin, controlling under microscope until the A cells are red, and the B cells are pink.
If overstained, wash out with distilled water until the above effects are obtained.

12. Stain in solution E for twenty minutes to twelve hours according to the condition of the pancreas and degree of differentiation in the first stain.

Results:

Nuclei are stained dark violet; nucleoli, red. Cytoplasm in A cells contains red granules. Cytoplasm of B cells contains blue granules. Cytoplasm in acinar cells varies from red to pale violet with deep violet ergastoplasm. Canalicular cells: blue-grey. Connective tissue: blue. Erythrocytes: red. Mucus: azure blue.

Reference: Isaac, J. P. & Aron, C. (1952).

MALLORY STAIN - HAEMATOXYLIN

For differential staining of acidophils, basophils and chromophobes in mouse pituitary

Solutions required:

A. *Zenker - Formol:*

Zenker's Fluid	..	..	95 ml.
Formaldehyde	..	..	5 ml.

B. Formic acid 10% aqueous.

C. Iodine 0·5% in 70% alcohol.

D. Sodium thiosulphate 0·75% in 10% alcohol.

E. Harris haematoxylin.

F. Lithium carbonate satd. aqueous.

G. Acid fuchsin acetic (Mallory) 0·5% aqueous.

H. Phosphomolybdic acid 1% aqueous.

I. Mallory's Aniline blue-orange G.

J. Carbol xylol.

Technique:

1. Pituitary gland together with bone to which it is attached is fixed in solution A for 4–8 hours, or overnight in a refrigerator.

2. Wash in running tap water for 8 to 10 hours.

3. Decalcify by immersing in 10% formic acid for 24 hours.

4. Wash in running tap water for 2 to 4 hours.

5. Dehydrate, clear and embed in paraffin wax 56 to 58° C. in the usual way.

6. Cut sections about 4μ in thickness and mount on slides with glycerine albumen.

7. Dry the slides thoroughly in an oven at 56° C. for one to two hours, or overnight at room temperature.

8. Remove wax with xylol; then wash with two changes of absolute alcohol.

9. Wash with 90% followed by 70% alcohol.

10. Immerse for half to two minutes in solution C (Iodine).

11. Wash well with water.

12. Immerse in solution D (thiosulphate) for half to two minutes or until the natural colour of the sections is restored.

13. Wash well with tap water.

14. Rinse in distilled water.

15. Stain in Harris haematoxylin for 2–3 minutes.

16. Rinse in tap water.

17. Blue in the lithium carbonate solution for 1 minute.

18. Rinse well in distilled water.

19. Stain in the acid fuchsin solution for 1–2 minutes.

20. Rinse quickly in tap water.

21. Immerse in the phosphomolybdic acid solution for 2–5 minutes.

22. Without washing, pass the slides directly into Mallory's Aniline blue - orange G and leave therein for 1–2 hours.

23. Differentiate with 95% alcohol, controlling by examination under the microscope until the blue granules are intense, but the fuchsinophil granules are clearly visible.

24. Rinse with absolute alcohol.

25. Rinse with carbol xylol.

26. Immerse in two changes of xylol for 10 minutes in each.

27. Mount in Clearmount of D.P.X.

Results:

The nuclei of all cells are stained dark blue black. Granules of basophils: blue. Acidophil granules: brilliant red. Non-granular cytoplasm of chromophobes: light grey. Erythrocytes: orange to red. Bone: intense blue.

Reference: Gude, William D. (1953).

MALLORY STAIN - HAEMATOXYLIN (Ehrlich)

For Negri bodies in sections of brain

Solutions required:

A. Ehrlich haematoxylin.

B. Orange G. .. .. 0·5 gm.
Phosphotungstic acid, saturated aqueous .. .. 100 ml.

C.	Acid fuchsin	0·5 gm.
	Phosphotungstic acid	0·5 gm.
	Acetic acid 1%	100 ml.
D.	Phosphotungstic acid	2 gm.
	Phosphomolybdic acid	2 gm.
	Picric acid, saturated, aqueous	70 ml.
	Absolute alcohol	30 ml.
E.	Aniline blue, aqueous	1 gm.
	Distilled water	98 ml.
	Glacial acetic acid	2 ml.

Technique:

1. Fix paraffin sections to slides; dewax and take down to distilled water in the usual way.

2. Stain in Ehrlich haematoxylin for five minutes.

3. Blue in tap water, or in lithium carbonate solution, for two minutes.

4. Rinse in distilled water.

5. Stain for one minute in the orange G solution.

6. Wash in tap water until only the erythrocytes are stained yellow.

7. Rinse in distilled water.

8. Stain for ten minutes in the acid fuchsin solution.

9. Rinse in distilled water.

10. Differentiate for five minutes in solution D.

11. Rinse in distilled water.

12. Rinse in 1% acetic acid.

13. Dehydrate, clear and mount.

Results:

Negri bodies: purplish red with blue granulations. Cytoplasm of neurones: bluish. Nucleoli: dark purple. Erythrocytes: yellow.

Reference: Zlotnik, I. (1953).

MALLORY HEIDENHAIN STAIN (Jane E. Cason)

A rapid one-step method for connective tissue

Solution required:

Phosphotungstic acid crystals A.R.	1 gm.
Orange G.	2 gm.
Aniline blue, water soluble ..	1 gm.
Acid fuchsin	3 gm.
Distilled water	200 ml.

Technique:

1. Fix pieces of tissue in Zenker-formol for preference, although Bouin's fluid, formalin and alcohol have been used with success.
2. Embed in paraffin wax and cut sections 6μ in thickness.
3. Fix sections to slides and remove wax with xylol.
4. Pass through descending grades of alcohol and if Zenker-formol has been used as the fixative, treat with iodine and sodium thiosulphate as usual to remove mercurial precipitate.
5. Take down to tap water.
6. Immerse for five minutes in the staining solution.
7. Wash in running tap water for three to five minutes.
8. Dehydrate rapidly through the usual graded alcohols.
9. Clear in xylol and mount.

Results:

Appear to be the same as those listed by Mallory (1938), i.e. collageneous fibrils, intense blue. Ground-substance of cartilage and bone, mucus, amyloid, and certain other hyaline substances are stained in varying shades of blue. Nuclei, fibroglia, myoglia and neuroglia fibrils, nucleoli, axis cylinders and fibrin are stained red. Erythrocytes and myelin, yellow. Elastic fibrils are stained pale pink or yellow.

Reference: Cason, J. E. (1950).

MALLORY'S PHOSPHOTUNGSTIC ACID HAEMATOXYLIN

A general stain for vertebrate tissues

Solution required:

Haematoxylin 10% in absolute alcohol (ripened for three months or longer)	1 ml.
Phosphotungstic acid	2 gm.
Distilled water	100 ml.

Note: If ripened haematoxylin solution is not available, the following artificially ripened stain should be used:

Haematoxylin (dry) 0·1 gm., phosphotungstic acid 2 gm., distilled water 100 ml., potassium permanganate 1% aqueous 1·77 ml.

Technique:

1. Fix in Zenker. Embed in paraffin wax.
2. Bring sections down to distilled water.
3. Treat with iodine to remove mercuric precipitate.
4. Remove iodine with 0·5% aqueous sodium hyposulphite.
5. Wash thoroughly in running water.
6. Immerse for five to ten minutes in 0·25% potass. permanganate; then wash in tap water.
7. Immerse for ten to twenty minutes in 5% oxalic acid; then wash thoroughly with tap water.
8. Stain twelve to twenty-four hours in haematoxylin solution, prepared as above.
9. Wash in tap water; dehydrate with 95% and absolute alcohol.
10. Clear in xylol and mount.

Results:

Nuclei, centrioles, achromatic spindles, fibroglia, myoglia, neuroglia fibrils, fibrin, contractile elements of striated muscle: blue. Collagen, reticulum, ground substances of cartilage and bone: yellowish to brownish red. Coarse elastic fibrils: faint purple.

References:

Jones, Ruth McClung (1950), p. 244.
Mallory, F. B. (1900).

MALLORY HEIDENHAIN STAIN - HAEMATOXYLIN

For the differentiation of cells in the hypophysis and in the pancreatic islets

Solutions required:

A. Potassium permanganate, 0·3% aqueous

B. Sulphuric acid, 3% aqueous

C. Potassium metabisulphate, 4% aqueous

D. *Gomori's chrome haematoxylin*

Chrome alum, 3% aqueous	50 ml.
Haematoxylin, 1% aqueous	50 ml.
Potassium dichromate, 5% aqueous	2 ml.
Solution B (sulphuric acid, 3%)	3·35 ml.

E. Sulphuric acid 1% in 70% alcohol

F. *Jane E. Cason's modified Mallory–Heidenhain stain*

Acid fuchsin	3 gm.
Phosphotungstic acid	1 gm.
Orange G	2 gm.
Aniline blue, aqueous	1 gm.
Distilled water	200 ml.

Technique 1

For hypophysis

1. Slice large hypophysis, such as human, into three pieces in the planes for future sectioning; or dissect small ones from the sella turcica (these need not be divided).

2. Fix for four to twenty-four hours, depending on the size of the specimen, in Susa–Heidenhain, or for one to three days in formalin-sublimate, or in 10% formalin.

3. Embed in paraffin wax as usual and cut sections at 3 to 5μ.

4. Fix sections to slides and carry through to distilled water, but if a mercury-containing fixative has been used, treat for the removal of mercurial precipitate before transferring to water.

5. Oxidize, in a freshly prepared mixture consisting of equal volumes of solutions A and B, for two to four minutes.

6. Decolorize with solution C.

7. Stain the nuclei, in the haematoxylin solution, for five to ten minutes.

8. Wash and then differentiate in solution E.

9. Rinse well, in running tap water, to remove the acid.

10. Stain cellular granules in solution F for five minutes.

11. Wash in running tap water.

12. Differentiate in 95% alcohol for one to three minutes, controlling at intervals by microscopic examination.

13. Dehydrate rapidly with absolute alcohol; clear in xylol; mount in Canada balsam in xylol, or in a neutral synthetic medium such as D.P.X., Cristalite, Clearmount, or Emexel.

Technique 2

For pancreatic islets

Proceed as in the above technique, but with the following modifications:

Step 5. Oxidize for only one half to one minute.
Step 7. Increase the staining time to one hour.
Step 10. Increase the staining time to 10–15 minutes.
Step 12. Differentiate for three to five minutes.

Results:

Basophilic cells of the hypophysis stain a deep blue. Eosinophilic cells: carmine red. Chromophobe cells: grey. The greyish black chromatin structures of the nuclei are brought out with exceptional clarity. Erythrocytes are orange. Fuchsinophil colloid: carmine red. Fuchsinophobe colloid: greyish blue. Connective tissue: dark blue.

In pancreas, the protoplasm of the external epithelial secretory cells is greyish blue. The zymogen granulation: carmine red (or orange in some experimental animals). Alpha cells of the islets are either bright red or orange, and the beta cells are dark blue. In the different experimental animals, the granulations of the islet cells are stained various shades of red and the blue colour respectively, but the two types of cells are always easy to differentiate.

Notes:

(*a*) The authors (Róna & Morvay, of the Cancer Research Department, Medical University, Budapest, Hungary) state that the production of clear pictures of the cellular granules of the hypophysis and especially of the pancreatic islets is one of the most difficult histological tasks. Nearly all the recommended methods are lengthy and complicated and the accepted procedures often give unsatisfactory and variable results. These difficulties led to the development of the above techniques, which are considered to be reliable and relatively simple.

(*b*) The methods facilitate the study of the cell types in the hypophysis or in the pancreatic islets, and make it possible to estimate the granule content of the cells.

(*c*) The method works on human autopsy material provided fixation of hypophysis takes place within twenty-four hours, and pancreas within twelve hours, of death.

(*d*) For further information readers should consult the authors' original paper.

Reference: Róna, G. & Morvay, I. (1956).

MARSHALL RED - VICTORIA GREEN

A general stain, particularly suitable for class work

Solutions required:

A. Marshall red, saturated aqueous.

B. Victoria green saturated in 70% alcohol.

Technique:

1. Fix sections to slides; dewax and take down to distilled water in the usual manner.
2. Stain in the Marshall red solution for twenty minutes.
3. Rinse in distilled water.
4. Stain in the Victoria green solution for half an hour.
5. Rinse in 70% alcohol followed by 90% alcohol.
6. Dehydrate with absolute alcohol.
7. Clear in xylol and mount.

Results:

Myofibrils: sage green. Nuclei: bright carmine. The results vary somewhat, but the muscle fibres always appear greenish to greenish-grey, while the nuclei are red. White matter of spinal cord: yellowish-green. Cartilage: pink. Retina stands out well as the rods and cones appear bright bluish-green. Erythrocytes unstained.

Reference: Cannon, H. G. (1941).

MASSON'S TRICHROME STAIN

For connective tissues

Solutions required:

A. Iron alum 5% aqueous.

B. Regaud's haematoxylin solution.

C. Picric acid, saturated in 95% alcohol .. .. 20 ml.
Alcohol 95% .. .. .. 10 ml.

D. Ponceau fuchsin.

E. Phosphomolybdic acid 1% aqueous.

F. Aniline Blue 5% in 2% acetic acid.

Technique:

1. Fix pieces of tissue in Bouin's fluid for three days or in Regaud's fluid for one day.

2. Wash in running water; dehydrate; clear and embed in paraffin wax as usual.

3. Sections 5μ in thickness are fixed to slides; de-waxed and passed through descending grades of alcohol down to distilled water in the usual manner.

4. Mordant in Solution A for five minutes at 45° C. to 50° C.

5. Wash well in distilled water.

6. Stain for five minutes in Regaud's haematoxylin at 45° C. to 50° C.

7. Rinse in distilled water.

8. Differentiate in picric alcohol (Solution C above) controlling by examination under the microscope, while the preparation is still wet.

9. Wash in running tap water for a minute or so.

10. Stain for five minutes in the Ponceau fuchsin solution.

11. Rinse in distilled water.

12. Differentiate in the phosphomolybdic acid solution for five minutes.

13. Add 0·5 ml. of the acetic aniline blue (Solution F above) to the phosphomolybdic acid on the slide and mix by rocking the slide gently. Allow this mixture to act for five minutes.

14. Pour off excess liquid and rinse in distilled water.

15. Immerse in phosphomolybdic acid solution again, for five minutes.

16. Transfer to 1% acetic acid and leave therein for five minutes.

17. Wash in distilled water.

18. Dehydrate in 95% alcohol, followed by absolute alcohol; clear in xylol; mount.

Results:

Collagen, deep blue. Neuroglia fibrils, red. Nuclei, black. Argentaffin granules, black or red.

Reference: Masson, P. (1929).

MAXILON BLUE RL

For mucopolysaccharide structures

Solutions required:

A. Formol–calcium fixative (Baker, 1946) (*see* page 226)

B. Maxilon blue RL, 0·05% in distilled water

C. Tertiary butyl alcohol

Technique:

1. Fix slices of material for twenty-four hours in formalin–calcium.

2. After washing, dehydrate, clear, and embed in paraffin wax.

3. Cut sections at 6 to 7μ and fix them to slides.

4. Remove wax with xylol and pass through the usual descending grades of alcohol to distilled water.

5. Stain in the Maxilon blue RL solution for thirty to sixty seconds.

6. Wash in distilled water.

7. Remove the excess water from the slide with filter paper.

8. Immerse in two lots of anhydrous tertiary butyl alcohol for one minute in each

or

Rinse in 70% alcohol and dehydrate in two changes of absolute alcohol, for two minutes in each, but the tertiary butyl alcohol procedure is considered to be the more satisfactory one.

9. Clear in xylol.

10. Mount in D.P.X. or Clearmount or Canada balsam in xylol.

Results:

Acid mucopolysaccharide-containing elements are stained red to violet, while other basophilic structures are stained in various shades of blue.

Notes:

(*a*) The author states that his preliminary unpublished results point to the possibility of distinguishing between the various acid mucopolysaccharides present in tissue sections by varying the dye concentration.

(*b*) Maxilon blue RL is a basic dye of the mono-azo group. Its 0·05% aqueous solution, which is dark blue in colour, has a pH of 4·1.

(*c*) It is also stated that this is similar to the method suggested by Hempelmann (1940) with toluidine blue, which has apparently

not yet found confirmation by others (Glick, 1940). There is some experimental support, according to de Almeida, to Hempelmann's suggestion, since the binding strength of the chromotropes to the dye varies with the acidic character of the radical involved, in the order COOH < PO_4 < SO_4 (Bank & Bungenberg de Jong, 1939). The author also states that in the work of Kelley & Miller (1935) evidence can be found for the histological differentiation between metachromatic staining due to nucleoproteins and to mucins, and that there seems to be no reason why this concept could not be extended to the various acid mucopolysaccharides.

Reference: Almeida, D. F. de (1960).

MAY–GRUNWALD STAIN

This stain may be used alone for the cytological examination of blood smears but it is not suitable for parasites. The technique is the same as that for Jenner stain (page 250). The chief use of May–Grünwald stain is in conjunction with Giemsa stain (page 251).

Reference: May & Grünwald (1902).

METHYL BLUE - EOSIN (Mann)

For demonstrating the various types of cells in the anterior lobe of the pituitary and for the study of the relationship and development of the blood vessels

Solution required:

Methyl Blue-Eosin (Mann's stain).

Technique:

1. Paraffin sections of tissues which have been fixed in a fluid containing mercuric chloride are mounted on slides and treated by the standard technique for the removal of mercuric precipitate.

2. Bring the sections down to distilled water; then stain for a quarter of an hour to two hours (the longer time is required if it is desired to demonstrate anterior lobe of pituitary).

3. Wash and differentiate in tap water.

4. Dehydrate rapidly with two changes of absolute alcohol.

5. Clear in xylol; mount in xylol balsam and examine under the low power, as the stain is too diffuse for critical high power work.

Results:

Nuclei are stained blue; karyosomes, dark blue; plasmosomes, red; basophil cytoplasm, blue; oxyphil cytoplasm and oxyphil granules, red.

Note: Although this method is of considerable antiquity, it is still used to some extent, and the above details are given to satisfy requests received from time to time from biologists in various countries for details of this technique.

Reference: Mann, G. (1894).

METHYL GREEN

For amyloid

Solution required:

Methyl green 0·3% aqueous

Technique:

1. Fix tissues in absolute alcohol or in 10% formalin and cut frozen sections.

2. Immerse in the methyl green solution for five to ten hours.

3. Wash well in distilled water.

4. Mount in neutral glycerine, or in aquamount.

Results:

Amyloid is stained reddish violet, while other tissue elements are green.

Reference: Cowdry, E. V. (1952).

METHYL GREEN - PYRONIN Y

For differentiating ribonucleic and deoxyribonucleic acids

Solutions required:

A. Pyronin Y .. .. .. 1 gm.
Distilled water .. .. .. 50 ml.

Extract with chloroform by shaking the above solution in a separating funnel until the chloroform layer (lower layer) is colourless. About 60 ml. of chloroform will be required, using 20 ml. each for three extractions.

Note: The third extraction should be almost colourless, otherwise the extracting process will have to be continued with one or two additional 20-ml. lots of chloroform. If after that the chloroform layer is still coloured, the pyronin is unsuitable for this technique.

B. Methyl green OO .. .. 2 gm.
Distilled water .. .. .. 100 ml.

Extract with chloroform by shaking the above solution in a separating funnel until the chloroform layer is no longer coloured violet.

Note: Use about 50 ml. of chloroform for each extraction. A total of about 300 ml. of chloroform will be required.

C. Pyronin Y solution, chloroform extracted as above .. .. 12·5 ml.
Methyl green OO solution, chloroform extracted as above .. 7·5 ml.
Acetate buffer pH 4·7 .. .. 30 ml.

Technique:

1. Carry sections through to distilled water in the usual way.
2. Immerse in solution C for six minutes.
3. Blot carefully with filter paper.
4. Immerse for five minutes in each of two changes of normal butyl alcohol.
5. Immerse in xylol for five minutes.

6. Immerse in cedarwood oil for clearing, for five minutes.
7. Mount in D.P.X. or other synthetic neutral mountant.

Results:

Chromatin: clear green. Nucleoli: bright red. RNA of cytoplasm: red. Eosinophil granules and osteoid: red.

Notes:

(*a*) The quality of the pyronin Y, as the author demonstrates in his paper, is a very important factor in the success of this method. Only two brands of the dye were found satisfactory, and these were from British sources.

(*b*) It is also stated in the original paper that pyronin Y of American and of British manufacture appears to be different dyes. Differences were noted not only in the staining characteristics, such as slightly greater staining of protein by the American dye, complete elimination of the British but not the American by water, differentiation of the British but not the American by normal butyl alcohol, but also solubility in chloroform.

(*c*) Staining with pyronins alone indicated that the British samples of pyronin Y produced less staining of structures known to be devoid of nucleic acid than did any of the other pyronins studied. There appeared to be no staining of protein. Osteoid and eosinophil granules were the only nucleic acid-free structures observed by Kurnick to stain in mammalian tissues. Both polymerized and depolymerized DNA were stained when pyronin was used alone. However, in the methyl green–pyronin mixture (solution C, above), the methyl green competed successfully for the polymerized DNA.

(*d*) Kurnick shows that it is possible, in the animal tissues stained with the mixture (solution C, above), to interpret green staining as being due to polymerized DNA (except cartilage matrix); and red staining due to RNA (except partially depolymerized DNA, osteoid, and eosinophilic granules).

(*e*) As previously reported by the author (Kurnick, 1952), tertiary butyl alcohol is not satisfactory for differentiation.

(*f*) For more detailed information and colour plates, readers should consult the original paper.

Reference: Kurnick, N. B. (1955).

METHYL VIOLET 6B

For amyloid

Solution required:

Methyl Violet 6B aqueous 1%.

Technique:

1. Material should be fixed in alcohol and embedded in paraffin wax (although frozen sections may be employed).

2. Paraffin sections are fixed to slides and brought down to distilled water.

3. Stain for two to five minutes in the methyl violet 6B solution.

4. Rinse quickly in distilled water.

5. Differentiate with 1% acetic acid, controlling by examination under the microscope, until the amyloid appears reddish in colour.

6. Wash in distilled water.

7. Mount directly in Aquamount.

Results:

Amyloid substance is stained red, while cells and nuclei are in varying shades of blue.

Notes:

(*a*) The technique is not suitable for material which has undergone prolonged fixation in formalin.

(*b*) The stain is extracted from the amyloid by alcohol.

METHYL VIOLET - METANIL YELLOW

For typhus fever rickettsiae in lungs of mice

Solutions required:

A.	Methyl violet 10B	0·01% aqueous.
B.	Distilled water	90 ml.
	Acetic acid 1%	10 ml.
C.	Metanil yellow	0·01% aqueous.

Technique:

1. Fix pieces of lung in 10% neutral formalin.

2. Dehydrate, clear and embed in paraffin wax in the usual manner.

3. Fix sections to slides and take down to distilled water as usual.

4. Stain in the methyl violet solution for half to one hour.

5. Differentiate in solution B, controlling by examination under the microscope until the cytoplasm is decolorized.

6. Counterstain in the metanil yellow solution for a few seconds.

7. Dehydrate in acetone.

8. Mount in D.P.X. or Clearmount.

Results:

Rickettsiae are stained violet.

Reference: Nyka, W. J. (1945).

METHYL VIOLET - PYRONIN - ORANGE G (Bonney's Triple Stain)

For chromatin, connective tissue, keratin

Solutions required:

A. Methyl violet 6B (Jensen) 1% aqueous .. .. .. 25 ml.
Pyronin B 10% aqueous .. .. 10 ml.
Distilled water .. .. .. 65 ml.

B. Acetone .. .. 100 ml.
Orange G aqueous 2% .. .. About 10 ml.

Add orange G solution drop by drop to the acetone, with shaking, until the flocculent precipitate formed just redissolves with further addition of orange G solution.

Technique:

1. Small pieces of tissue are fixed in acetic-alcohol or in mercuric chloride.

2. Wash; dehydrate; clear; embed in paraffin wax.

3. If mercuric chloride has been used for fixation treat sections for the removal of mercuric precipitate by the standard method.

4. Take sections down to distilled water.

5. Immerse for two minutes in the methyl violet pyronin (Solution A above).

6. Pour off excess stain and carefully wipe the slide dry.

7. Flood the preparation with acetone-orange G solution.

8. Pour off after a few seconds.

9. Flood the preparation with a fresh lot of acetone-orange G solution and pour off after a few seconds.

10. Wash quickly in pure acetone.

11. Rinse with two lots of xylol.

12. Mount in balsam.

Results:

Cytoplasm, red; chromatin, violet; keratin, violet; connective tissue, yellow.

Reference: Bonney (1908).

METHYLENE BLUE - BASIC FUCHSIN

Rapid method of demonstrating Negri bodies in sections

Solutions required:

A.	Methylene blue	1 gm.
	Basic fuchsin	1·75 gm.
	Methyl alcohol, pure	100 ml.
	Glycerine, pure	100 ml.
B.	Potass. hydroxide N.20	6·25 ml.
	Distilled water	93·75 ml.
C.	Solution A	10 ml.
	Solution B	0·25 ml.

Note: Solution C should be freshly prepared as required.

Technique:

1. Blocks not exceeding 3 mm. in thickness of fresh tissue from the hippocampus major and cerebellum should be fixed in Zenker for twelve to twenty-four hours.

2. Remove mercurial precipitate with iodine in alcohol by the usual method (see page 496); then wash in running water for three to six hours.

3. Dehydrate in dioxane (*see* page 512) and embed in paraffin wax.

4. Sections not more than 4μ thick are mounted on slides and brought down to distilled water.

5. Flood slides with Solution C and steam gently for five minutes; then cool and wash rapidly in tap water.

6. Decolorize and differentiate each section separately by waving the slide gently in a jar of 90% alcohol until the section assumes a faint violet colour.

7. Dehydrate rapidly in 95% and absolute alcohol; clear in xylol and mount.

Results:

Negri bodies, deep red; granular inclusions, dark blue; nucleoli, bluish black; cytoplasm, bluish violet; erythrocytes, dull reddish brown.

Reference: Scheifstein, J. (1937).

METHYLENE BLUE - BASIC FUCHSIN

For rickettsia in sections

Solutions required:

A. Methylene blue 1% aqueous.

B. Basic fuchsin 0·5% aqueous, in phosphate buffer pH 7·0.

C. Citric acid 0·5% aqueous.

Technique:

1. Tissues are fixed in Regaud's fluid, washed in running water, dehydrated, cleared and embedded in paraffin wax as usual.

2. Fix sections to slides; dewax; pass through the alcohol down to distilled water in the usual manner.

3. Stain in the methylene blue solution for twelve to sixteen hours.

4. Decolorize with 95% alcohol.

5. Counterstain with the basic fuchsin solution for fifteen minutes.

6. Decolorize for one to three seconds in the citric acid solution.

7. Pour off excess citric acid; rinse in distilled water; drain and blot carefully.

8. Decolorize and dehydrate rapidly in absolute alcohol.

9. Clear in xylol and mount in Clearmount or D.P.X.

Results:

Rickettsia are stained a deep red; surrounding tissue, pink; background, light blue.

Reference: Zinsser, H., Fitzpatrick, F. & Hsi Wei (1939).

METHYLENE BLUE, LEUCO

For the differential cytophysiological diagnosis of cancerous and normal tissues

Solutions required:

A. *Hyposulphite leuco base of methylene blue (HLM)*

Methylene blue, 0·1% aqueous .. 10 ml.

Note: This should be prepared with fresh, double distilled water.

Sodium thiosulphate ($Na_2S_2O_3$, $5H_2O$), A.R. .. .. .. 0·8 gm.

Hydrochloric acid, pure, conc. (diluted 1:3 with distilled water) .. .. 4 drops

Note: **All apparatus used must be scrupulously clean and dry.**

(*a*) Add the sodium thiosulphate to the methylene blue solution in a flask; shake to dissolve; then add the acid.

(*b*) Heat carefully, agitating the solution, over a bunsen flame for twenty to thirty minutes, until a light grey sediment is thrown down.

(*c*) Allow the solution to cool for ten minutes.

(*d*) Filter through two thicknesses of filter paper.

(*e*) If the leuco base has been properly prepared it will be colourless or slightly yellowish, otherwise it will be useless.

(*f*) Should the filtered leuco base assume a blue colour it should be left to stand for a while in the dark.

(*g*) The leuco methylene blue solution should be prepared as and when required for immediate use; it will, however, keep in the dark for ten to twelve hours.

B. Acid fuchsin, 0·05% aqueous

Technique:

1. Cut frozen sections, 10–15μ in thickness, of fresh unfixed fragments of tumour or other pathological material and transfer to physiological saline solution.

Note: Pieces of normal or pathological tissues may be kept before use in physiological saline, in a refrigerator at $-2°$ C to $+2°$ C for a period not exceeding two days.

2. Transfer sections, by means of a pipette or a glass rod, to slides.

3. Draw off any excess saline solution from the slides with filter paper.

4. Spread sections over slides by means of needles.

5. Allow to dry at room temperature.

6. Besides the section of tumour or other pathological material, a section of normal tissue should be attached to the slide to serve as a control of the staining effects.

Note: When both sections on the slide are dry they are ready for staining immediately but they need not be stained for one or two days thereafter. The authors state that dried sections of malignant tumours preserve their typical staining capacity for two weeks, provided they are kept in a dry, dark place.

7. Cover the dried sections on the slide with the leuco methylene blue solution, which should be applied by means of a pipette.

Note: If the solution becomes bluish it must be changed.

8. Allow the staining solution to act until the section of the normal tissue stains a distinct blue while that of the malignant tissue remains colourless: this usually requires not more than two to three minutes.

9. Pour off the excess liquid.

10. Rinse with a large volume of fresh, double-distilled water.

11. Stain in solution B (acid fuchsin) for about two minutes, taking care not to overstrain.

12. Pour off excess stain; then dry sections thoroughly.

13. Mount in glycerine, glycerine jelly or Aquamount, and seal the edges of the coverslip with two coats of Laktoseal. Alternatively, sections may be mounted in Cristalite, Clearmount or D.P.X., but Canada balsam is not to be recommended as in time it will cause cancerous tissue to become blue.

In examining the slides it should be borne in mind that bright illumination may distort the staining effect of the leuco methylene blue which is affected by light.

Results:

The nuclei of normal cells stain dark blue while the nuclear structures are quite distinct.

Nuclei of cancerous cells are said to remain almost colourless or to assume a diffuse, hardly noticeable light blue or a slightly perceptible pale pink. The nucleoli of malignant cells are said to appear more intensely coloured than the almost colourless nuclei, although their blue colour is far below that of normal nucleoli. The cytoplasm of normal and malignant cells is stained distinctly red by the acid fuchsin. The authors state that the conclusion is indicated that in contrast to the nuclei or normal cells, those of malignant cells are unable to transform the colourless hyposulphite leuco base of methylene blue into the coloured form.

Notes:

(*a*) The authors, Roskin and Struve of the histological laboratory of M. Lomonossov University, Moscow, U.S.S.R., state that the application of Unna's Rongaliteweiss for cytodiagnosis

may involve certain difficulties, and the new technique, described above, using sodium thiosulphate (hyposulphite) in place of Rongalite, was developed by them in the belief that the rapid and differential staining of normal and malignant cells and tissues could be accomplished. The authors state that the procedure, which they refer to for short as the " HLM " (hyposulphite leuco methylene blue) method, may prove more than an aid in the routine histopathological practice, especially for the diagnosis of malignant cells in exudates and punctate, and it may become a useful tool for studying the properties of malignant and benign tumours, in precancerous states, etc.

(*b*) It is also stated in the original paper, to which readers are referred for further information, that the value of the method has been checked on extensive material at the Moscow Oncological Institute.

(*c*) The authors claim that with proper observance of the directions described above that the difference between malignant and normal tissue can also be observed macroscopically, normal tissue appearing blue, and malignant, red.

References:
Roskin, G. I. & Struve, M. F. (1947).
Unna, P. G. (1911).

METHYLENE BLUE POLYCHROME

(After Unna)

For mast cells in sections

Solution required:

Methylene blue, polychrome (Unna)	100 ml.
Potash alum	5 gm.

Technique:

1. Material is fixed in absolute alcohol and embedded in Celloidin.

2. Stain sections in a watch glass from three to sixteen hours in the methylene blue solution.

3. Rinse well in distilled water.

4. Dehydrate with 95% alcohol.
5. Clear in origanum oil.
6. Mount in Canada balsam or in Cristalite.

Results:

Mast cell granules are stained red; nuclei, blue.

Reference: Unna, P. G. (1910*b*), p. 72.

METHYLENE BLUE POLYCHROME - GLYCERINE ETHER

(After Unna)

For differentiating mast cells and plasma cells

Solutions required:

A.	Formalin (40% formaldehyde) ..	50 ml.
	Absolute alcohol	100 ml.
B.	Polychrome methylene blue (Unna)	
C.	Glycerine ether (Unna)	5 ml.
	Distilled water	35 ml.

Technique:

1. Fix tissues in Solution A; dehydrate; clear; embed in paraffin wax.
2. Immerse sections in the methylene blue solution for ten minutes.
3. Rinse in distilled water.
4. Differentiate in the glycerine ether mixture from one half to one minute until the sections appear to be deep sky blue to the naked eye (care should be taken that the sections are not over-differentiated).
5. Wash thoroughly in distilled water for a few minutes.
6. Fix sections to slides and carefully blot with filter paper.
7. Dehydrate rapidly with absolute alcohol.
8. Clear in xylol and mount.

Results:

Nuclei are stained blue, while mast cells are red and plasma are blue.

Reference: Unna, P. G. (1910*b*), p. 411.

MF4 STAIN

(Edward Gurr)

For keratin, etc.

Technique:

1. Fix smears, while still wet, in equal parts of ether and absolute alcohol for one minute.
2. Rinse with two changes of 80% alcohol, followed by two changes of 70% alcohol.
3. Rinse by dipping the slide seven times in rapid succession in a jar of distilled water.
4. Immerse in the staining solution for two minutes.
5. Rinse with 70%, followed by 90%, alcohol.
6. Dehydrate with absolute alcohol.
7. Clear in xylol and mount.

Results:

Cornified cells: orange to red. Non-cornified cells: blue.

Reference: Gurr, E. (1956, 1958*a*).

MILLON'S REAGENT

(J. R. Baker's modification)

For tyrosine

Solutions required:

A. *Formol saline*
Formaldehyde, 40% .. .. 10 ml.
Distilled water .. .. .. 83 ml.
Sodium chloride, 10% aqueous
Keep a few chips of marble in the stock bottle to maintain neutrality.

B. Sulphuric acid, 10% .. .. 100 ml.
Mercuric sulphate .. .. 10 gm.
Heat until the mercuric sulphate is dissolved; then cool and make up the volume to 200 ml. with distilled water.
The solution is stable.

C. Sodium nitrite, 0·25% aqueous

Technique:

1. Fix pieces of tissue in solution A overnight.

2. Embed in celloidin.

3. Cut sections at 20 to 30μ, or thinner for special purposes.

Note: Sections may be stored indefinitely in 70% or 80% alcohol.

4. Take sections through 50% alcohol to water.

5. Place 5 ml. of solution B in a 50 ml. beaker and add 0·5 ml. of the sodium nitrite solution, and mix well.

6. Place the sections in the liquid in the beaker and heat gently to boiling; then immediately turn off the heat and set the beaker aside to cool for two or three minutes.

7. Wash sections for at least two minutes in each of three lots of about 50 ml. of distilled water.

8. Mount in glycerine or Kaiser's glycerine jelly, or Aquamount, or dehydrate through the usual graded alcohols; then mount in Eurparal or Michrome mountant or Clearmount, or pass through 95% alcohol to origanum oil and mount in D.P.X.

Results:

Phenols are indicated by red, pink or orange colorations. In animal tissues the reaction will be due, in the vast majority of cases, to the presence of tyrosine-containing proteins.

Notes:

(*a*) The method is considered to be more rational than Millon's and most of the modifications of Millon's original method, and it produces a more intense coloration.

(*b*) The author (Dr. J. R. Baker) found that celloidin sections could be subjected to quite violent treatment without being injured. Boiling, to which paraffin and frozen sections cannot be subjected, intensifies the colour reaction in this technique and does not damage the celloidin sections.

Reference: Baker, J. R. (1956).

MILLON'S REAGENT

(R. R. Bensley & I. Gersh's modification)

For tyrosine

Reagents required:

A. Nitric acid (sp. gravity 1·42) .. 30·3 ml.
Distilled water .. .. .. 20·2 ml.

Shake thoroughly, in a stoppered bottle, then leave to stand for forty-eight hours.

B. Reagent A.. .. .. .. 10 ml.
Distilled water .. .. .. 90 ml.
Mercuric nitrate .. .. 18 gm.

Shake at intervals, in a stoppered bottle, over a period of several days; then filter.

C. *Millon's reagent* (*Bensley & Gersh*)
Reagent A.. .. .. .. 0·3 ml.
Reagent B .. .. .. 40 ml.
Sodium nitrite .. .. .. 0·14 gm.

D. Reagent A.. .. .. .. 10 ml.
Distilled water .. .. .. 39 ml.

Technique:

1. Place a section of fresh material on a slide.
2. Cover with a few drops of solution C (Millon's reagent) and leave it to act for about ten to fifteen minutes.
3. Rinse with reagent D.
4. Wash with water.
5. Examine under the microscope.

Result:

Tyrosine and tyrosine-containing proteins: orange to brick red.

Notes:

(*a*) Over-exposure to the action of the Millon reagent produces a marked reduction in the colour, and optimum time for a particular tissue has to be determined experimentally. It may be

necessary, however, in some cases, to increase the time of exposure to the Millon.

(*b*) The reaction of Millon's reagent is due to the hydroxyphenyl group of the tyrosine.

(*c*) Tyrosine, which occurs in most proteins, is the least water-soluble of all the proteins. It is readily soluble in dilute alkalis and mineral acids, but is insoluble in alcohol, acetone, ether and glacial acetic acid.

(*d*) The colour produced with Millon's reagent in the above method begins to fade after several hours, although material which is abundant in tyrosine will retain some of the colour for a few weeks.

References:

Bensley, R. R. & Gersh, I. (1933). *Anat. Rec.*, 57, 217–233.
Gurr, E. (1958*a*), pp. 52–53.

MUCICARMINE - METANIL YELLOW - HAEMATOXYLIN

For mucin and connective tissue

Solutions required:

A. Haematoxylin (Weigert) A.

B. Haematoxylin (Weigert) B.

C. Metanil yellow 0·25% aqueous.

D. Mucicarmine (Southgate).

Technique:

1. Fix material in 10% formalin.

2. Dehydrate, clear; embed in paraffin wax.

3. Fix sections to slides; de-wax with xylol and pass through the usual descending grades of alcohol.

4. Rinse with distilled water.

5. Stain sections for one minute in a freshly prepared mixture consisting of equal volumes of Solution A and B.

6. Wash in distilled water.

7. Immerse in Solution C for about two minutes.

8. Rinse quickly with distilled water.
9. Immerse in the mucicarmine solution for forty-five minutes.
10. Rinse quickly with distilled water.
11. Rinse quickly with 95% alcohol.
12. Dehydrate rapidly but thoroughly with absolute alcohol.
13. Clear in xylol and mount.

Results:

Mucin is stained red, while connective tissue is yellow, and nuclei are black.

Reference: Conn, H. J. & Darrow, M. A. (1947), pt. 1, Sect. B, p. 14.

MUCICARMINE (Mayer)

Solution required:

Mucicarmine, stock solution ..	1 volume
Alcohol 70%	10 volumes

Note: This diluted solution should be freshly prepared.

Technique:

Tissues should be fixed in absolute alcohol for five to eight hours and embedded in paraffin wax, Celloidin or L.V.N.

1. Bring paraffin sections down to distilled water; then stain for ten to twenty-five minutes in the above solution.
2. Wash rapidly with distilled water.
3. Dehydrate with 70%, 95% and absolute alcohol.
4. Paraffin sections are cleared in xylol; Celloidin or L.V.N. sections in terpineol or origanum oil.
5. Mount in Clearmount.

Results:

Mucin is stained red.

Reference: Mayer, P. (1896).

MUCICARMINE (Southgate)

This is used in accordance with the Mayer technique, but 1 volume of the stock solution is diluted with 9 volumes of distilled water instead of 10 volumes 70% alcohol.

Southgate's modification gives more uniform results than Mucicarmine prepared by Mayer's original recipe.

Reference: Southgate, H. W. (1927).

MUCIHAEMATEIN

For mucin

Solution required:

Haematein	0·2 gm.
Aluminium chloride	0·1 gm.
Glycerine, pure	40 ml.
Distilled water	60 ml.

Technique:

1. Material is fixed in absolute alcohol; cleared; and embedded in paraffin wax.
2. Sections are stained for ten minutes in the above solution.
3. Wash with distilled water.
4. Dehydrate by plunging the slide into two or three changes of 95% alcohol.
5. Pass through absolute alcohol; then clear in xylol; mount.

Results:

Mucus is stained blue, while the remainder is colourless.

Reference: Mayer, P. (1896).

NADI REACTION

For oxidase granules

Solutions required:

A. α-Naphthol .. .. .. 1 gm.
Distilled water .. .. .. 100 ml.
Place in a 250-ml. flask and boil until the α-Naphthol begins to melt; then add 40% potassium hydroxide

aqueous solution drop by drop until the solution becomes yellowish-blue in colour and the α-Naphthol is still not completely dissolved.

B. Cold tap water .. .. .. 100 ml.
*Dimethyl-p-phenylenediamine base .. .. 0·5 gm.

Place the water in a clean amber bottle, open the ampoule by filing a groove at one end then breaking in the usual manner. Tip the contents of the ampoule into the bottle; then replace the stopper and allow the bottle to stand, with occasional shaking, for twenty-four hours. Care should be taken that the Dimethyl-p-phenylenediamine base does not come into contact with the body.

This solution deteriorates after three or four weeks.

C. Gram's iodine solution.

D. Carmalum.

Technique:

1. Thin pieces of fresh tissue (not more than 3 mm. thick) are fixed for three to five hours in formalin-saline solution.

2. Make frozen sections and collect them in distilled water.

3. Immerse in a mixture consisting of equal volumes of Solutions A and B (*Note:* This mixture must be made and filtered immediately before use) for about five minutes until the sections turn blue.

4. Rinse sections rapidly in distilled water.

5. Immerse in Gram's iodine solution until the sections turn brown.

6. Transfer to distilled water to which two drops of lithium carbonate 0·5% aqueous have been added for each 100 ml. of distilled water, for a quarter to twenty-four hours until the sections have regained their blue colour.

7. Wash in distilled water then counterstain in carmalum for two to five minutes.

8. Mount in glycerine jelly or Apathy's medium or in Aquamount.

* This should be purchased in a sealed ampoule.

Results:

Oxidase granules are stained blue while nuclei are pink. Sometimes fat is stained also.

NAPHTHOL BLUE BLACK - HAEMATOXYLIN BRILLIANT PURPURIN - AZOFUCHSIN

For collagen, reticulum, smooth muscle, etc.

Solutions required:

A. Weigert's Haematoxylin A.

B. Weigert's Haematoxylin B.

C. Brilliant purpurin R.
in 1% acetic acid .. .. .. 30 ml.
Azofuchsin 1% in 1% acetic acid.. 20 ml.

D. Naphthol blue black .. .. 1 gm.
Picric acid, satd., aqueous .. 100 ml.

Technique:

1. Paraffin sections are fixed to slides and taken down to 70% alcohol in the usual manner.

2. Stain for six minutes in a freshly prepared mixture consisting of equal parts of solutions of A and B.

3. Wash in tap water.

4. Counterstain for five minutes in solution C.

5. Wash in 1% acetic acid.

6. Stain in solution D for five minutes.

7. Rinse in 1% acetic acid for two minutes.

8. Pass through the usual ascending grades of alcohol dehydrate in absolute.

9. Clear in Xylol.

10. Mount in D.P.X. or Clearmount.

Results:

Collagen, reticulum and basement membranes: dark green. Smooth muscle: brown. Nuclei: brownish black.

Reference: Lillie, R. D. (1945).

NAPHTHANIL DIAZO BLUE - H ACID

For histidine

Solutions required:

A. Gram's iodine .. .. .. 30 ml.
Ammonia solution (sp. gr. 0·880) 2 ml.
B. Veronal acetate buffer pH 9·2 .. 200 ml. (approx.)
C. Solution B .. .. .. 50 ml.
Naphthanil diazo blue B .. 0·05 gm.
(Michrome blue salt 250)
D. H acid (8-amino-1-naphthol-3:6-disulphonic acid) .. .. 1 gm.
Solution B .. .. .. 50 ml.

Technique:

1. Fix paraffin sections of 10% formalin-fixed tissues to slides.
2. Dewax with xylol and pass through the usual graded alcohols into distilled water.
3. Immerse in solution A at room temperature for twenty-four hours.
5. Rinse in water.
6. Rinse in 96% alcohol until the sections lose their yellowish colour.
2. Rinse in water, then in solution B (veronal-acetate buffer).
8. Immerse in reagent C for fifteen minutes at 0° C. to 4° C.
9. Wash in water.
10. Immerse for two minutes in each of three changes of the veronal-acetate buffer (solution B).
11. Immerse in solution D at 0° C. to 4° C. for fifteen minutes, agitating the slides gently at intervals.
12. Wash in running water.
13. Dehydrate with two or three changes of anhydrous acetone or rapidly in absolute alcohol.
14. Clear in xylol and mount in Cristalite, Clearmount, D.P.X. or Emexel.

Results:

Sites of histidine groups are brick-red to reddish-brown.

References:
Landing, B. H. & Hall, H. E. (1956).
Gurr, E. (1958*a*), pp. 15–19.

NAPHTHOL GREEN B - HAEMATOXYLIN

For connective tissue

Solutions required:

A. Weigert's haematoxylin, A.
B. Weigert's haematoxylin, B.
C. Eosin, yellowish, 1% in tap water.
D. Ferric chloride, hydrated 10%.
E. Naphthol Green B, 1% aqueous.
F. Equal volumes of acetone and xylol.

Technique:

1. Paraffin sections are mounted on the slide and brought down to distilled water in the usual manner.
2. Stain for six minutes in a freshly prepared mixture consisting of equal volumes of Weigert's haematoxylin A and B.
3. Wash thoroughly in tap water; then stain for three minutes in the eosin solution.
4. Wash in tap water; then immerse in the ferric chloride solution for five minutes.
5. Rinse well in distilled water; then stain for five minutes in the naphthol green solution.
6. Differentiate for two or three minutes in 1% acetic acid.
7. Drain well; then dehydrate with acetone, afterwards clearing in acetone-xylol (as above); then mount.

Results:

Connective tissue, green; muscle and cytoplasm, pink.

Reference: Lillie, R. D. (1945).

NAPHTHOCHROME GREEN

For the histochemical detection of beryllium

Solutions required:

A. Naphthochrome green G .. 0·25 gm.
(Michrome No. 938)

Distilled water .. .. .. 50 ml.

Note: This solution must be freshly prepared immediately before use.

B. Phosphate buffer solution pH 5·0

Note: This can be conveniently prepared merely by dissolving 1 Michrome buffer tablet pH 5·0 in 100 ml. of distilled water.

C. Acridine red .. .. .. 1 gm.
Distilled water .. .. .. 100 ml.

Technique:

1. Fix pieces of tissue in formol alcohol or formol saline.
2. Dehydrate through the usual graded alcohols.
3. Clear in xylol or cedarwood oil.
4. Embed in paraffin wax by the usual procedure.
5. Fix sections to slides and dewax as usual.
5. Carry through graded alcohols to water.
7. Prepare a mixture of equal volumes of solutions A and B sufficient to fill a Coplin jar (about 35 ml.).

Note: This mixture deteriorates after a few hours.

8. Immerse the slides in the stain in the Coplin jar for thirty minutes at 37° C. in the incubator. The intensity of the staining is poor at temperatures below 37° C.
9. Wash in distilled water.
10. Differentiate by direct immersion in absolute alcohol for thirty minutes.
11. Wash in distilled water.
12. Counterstain with acridine red (solution C) for five minutes.
13. Wash in distilled water.
14. Differentiate rapidly in absolute alcohol.
15. Mount in Clearmount:

or

16. Clear in xylol.
17. Mount in D.P.X. or Emexel or Clearmount.

Results:

Sites of beryllium deposits are indicated by a clear apple-green colorization against a red background.

Notes:

(*a*) This histochemical test was developed by Denz (1949) during an investigation of the toxic action of beryllium salts on experiment animals, and the technique was reported at the time as an example of the application of chemical methods to histological problems.

(*b*) The original paper, which should be consulted for more detailed information, deals with the effect of solubility of beryllium compounds, intravenous injections in animals, utilization of the method, etc.

(*c*) In the original paper Denz describes the dye as naphthochrome green B, Schultz No. 851; but the structural formula, the chemical name, and the Schultz reference he gives all relate to naphthochrome green G, not B. In fact, as far as I am aware, and this has been confirmed by searches through dyestuffs literature, there is no dye under the name of naphthochrome green B, and Schultz No. 851 relates to an anthraquinone dyestuff, known as alizarin direct blue B, according to the Colour Index (1924), but not according to the new edition. Naphthochrome green G is a triarylmethane dye.

Reference: Denz, F. A. (1949).

α-NAPHTHYL ACETATE - FAST BLUE B SALT

For the demonstration of esterase activity in leucocytes in human blood and bone marrow

Solutions required:

A. 10% Formalin in normal saline

B. Phosphate buffer (M/20), pH 7·4

C. α-Naphthyl acetate 1% in 50% acetone

D. Solution B .. .. .. 40 ml.
Solution C .. .. .. 0·8 ml.

Fast blue B salt (Michrome salt 250) .. .. 0·04 gm.

E. Methyl green, 1% aqueous

Technique:

1. Fix smears in solution A for five minutes.
2. Wash for five minutes in running water.
3. Immerse in solution D for fifteen to thirty minutes at room temperature.
4. Wash well in distilled water.
5. Counterstain with methyl green 1% aqueous, if desired.

Results:

Esterase activity is indicated by a brownish black precipitate within the cells.

Notes:

(*a*) The author states that in his experience, esterase is consistently demonstrable only in blood monocytes, whether normal or leukaemic, and that esterase activity has never been observed in neutrophils, even on prolonged incubation (25° C.).

(*b*) It is also stated that occasionally minute brown granules were encountered in lymphocytes, but it was never certain that these represented evidence of esterase activity.

(*c*) The original paper should be consulted for photomicrograph and further information.

Reference: Braunstein, H. (1959).

NEOTETRAZOLIUM CHLORIDE

For the demonstration of succinic dehydrogenase in ascites tumour cells

Solutions required:

A. Neotetrazolium chloride* 1% aqueous

**Note:* This is very costly, and extravagant quantities of the solution should not therefore be prepared.

B. Sodium succinate (Molecular weight 270·16) M/5

C. Phosphate buffer M/10, pH 7·4

Note: This can be prepared by simply dissolving one Michrome buffer tablet in 100 ml. of distilled water.

D. Saline solution (0·85% sodium chloride)

E. Neutral 10% formalin

Technique:

1. Pipette 0·667 ml. each of solutions A, B, and C into a centrifuge tube.

2. Remove 0·05 ml. of tumour ascites from peritoneal cavity of host animals with a glass capillary tube, and immediately place the fluid into the centrifuge tube containing the measured amounts of solutions A, B, and C, as prepared at step 1 above.

3. Shake the centrifuge tube gently to suspend tumour cells diffusely in the medium.

4. Incubate at 37° C. for two hours.

5. Centrifuge for three minutes at 700 revolutions per minute.

6. Decant the supernatant fluid.

7. Wash the precipitate with solution D.

8. (**Optional**) Add 2 ml. of solution E, as fixative, to the precipitate in the centrifuge tube and stir with a glass rod.

9. (**Optional**) After ten minutes' fixation, centrifuge for three minutes at 700 revolutions per minute.

Note: Fixation inhibits enzymatic activity.

10. Using scrupulously clean slides, prepare smears of the precipitate, or make squash preparations, with one drop each of the precipitate and glycerine, and seal edges of the coverslips with soft paraffin wax, petroleum jelly or Laktoseal.

Results:

Succinic dehydrogenase activity is demonstrated by the purple pigment which is deposited intracellularly.

Notes:

(*a*) The author (Hirono) states that any lipid droplets present intracellularly were always stained, and that when tumour cells were incubated in a medium lacking sodium succinate, crystalline precipitation was not observed.

(*b*) For more detailed information and photomicrographs readers are referred to the original paper.

Reference: Hirono, I. (1957).

NEUTRAL RED - FAST GREEN

For staining both Gram-positive and Gram-negative bacteria in sections

Solutions required:

A. Aniline crystal violet.

B. Gram's iodine.

C. Absolute alcohol . . . . 98 ml.
Glacial acetic acid . . . . 2 ml.

D. Twort's stain, modified (neutral red–fast green)

Technique:

1. Fix tissue in 5% formal-saline, dehydrate, clear; embed in paraffin wax. Cut sections 3μ in thickness.

2. Stain in aniline crystal violet for three to five minutes.

3. Pour off excess and blot, without washing.

4. Flood with Gram's iodine and allow the stain to act for three minutes.

5. Destain with the acetic acid alcohol (Solution C above) until no more colour comes away, and the sections assume a dirty straw colour.

6. Rinse quickly in distilled water.

7. Stain in neutral red-fast green diluted one part with three parts of distilled water, for five minutes.

8. Wash quickly with distilled water.

9. Decolorize with the acetic alcohol solution until no more red stain comes out.

10. Rinse quickly in absolute alcohol.

11. Clear in xylol and mount.

Results:

Nuclei are stained red, while cytoplasm is light green. Gram-positive bacteria: dark blue. Gram-negative bacteria: pink. Erythrocytes: green.

Reference: Ollett, W. S. (1947).

NILE BLUE SULPHATE - SAFRANIN

An histochemical technique for demonstrating phospholipids in frozen sections

Solutions required:

A. Formalin 10% .. .. .. 100 ml.
Calcium Chloride 1% .. .. 1 ml.
Calcium Carbonate .. .. .. 2 gm.
Shake well: filter before use.

B. Safranin 1% aqueous .. .. 100 ml.
Aniline Oil .. .. 2 drops

C. Nile blue sulphate, saturated aqueous 100 ml.
Sulphuric acid 0·5% .. .. 10 ml.
Boil for 2 hours under reflux condenser.
Filter before use.

D. Acetic acid 5% aqueous.

E. HCl conc. .. .. 0·5 ml.
Distilled water .. .. .. 99·5 ml.

Technique:

1. Fix material in solution A: then cut frozen sections, without embedding: or the material may be embedded, if desired, in gelatine or carbowax or waterwax.

Note: Frozen sections should not be stored in water for more than ten to fifteen minutes.

2. Stain in the safranin solution for five minutes.
3. Rinse in distilled water.
4. Stain in the nile blue sulphate for ninety minutes at 60° C.
5. Rinse in distilled water.
6. Immerse in acetone heated to 50° C. on a water bath.
7. Remove the acetone from the source of heat and allow the sections to remain in it for half an hour.
8. Differentiate in 5% acetic acid for thirty minutes.
9. Rinse thoroughly in distilled water.
10. Refine the differentiation in the 0·5% HCl (Solution D).
11. Wash in several changes of distilled water.
17. Mount in glycerine jelly or in Aquamount.

Results:

Phospholipids: blue. Nuclei: red.

Reference: Menschik, Z. (1953).

NILE BLUE

For the differentiation of melanins and lipofuscins

(After Lillie, 1956)

Solution required:

Nile blue A	.. 0·1 gm.
Sulphuric acid, conc. ..	.. 100 ml.

Technique:

1. Fix pieces of tissue in 10% formalin or other appropriate fixative, and embed in paraffin wax.
2. Mount sections on slides and dewax with xylol.
3. Pass through the usual graded alcohols to distilled water.
4. Stain in the Nile blue solution for twenty minutes.
5. Wash in running water for ten to twenty minutes.
6. Mount in glycerine jelly or in Aquamount.

Results:

Lipofuscins: dark blue or greenish blue. Melanins: dark green. Cytoplasm, muscle: pale green. Erythrocytes: greenish yellow to greenish blue. Myelin: green to deep blue. Nuclei: unstained or only faintly stained.

Notes:

The method is based on Professor Lillie's findings that:

(*a*) Melanins stain with basic dyes at pH levels below 1·0 and retain the stain when dehydrated and mounted in resinous media.

(*b*) Lipofuscins stain with Nile blue by two mechanisms, a fat solubility one which operates at pH levels below 1·0, and an acid-base mechanism operating at pH levels above 3·0.

When stained by the second of these mechanisms, lipofuscins retain their green stain after acetone or brief alcoholic extraction; but when the first mechanism is applied they are promptly decolorized.

Reference: Lillie, R. D. (1956*a*, 1956*b*, 1955).

NILE BLUE SULPHATE

For demonstrating fatty acids and neutral fats

Solution required:

To 100 ml. saturated aqueous Nile Blue sulphate add 0·5 ml. conc. sulphuric acid; then boil under a reflux condenser for two hours; allow to cool; then use as follows:

Technique:

1. Fix small pieces of tissue in 10% formalin.

2. Frozen sections are stained for about ten minutes to half an hour at 37° C.; or overnight at room temperature.

3. Differentiate in 2% acetic acid.

4. Rinse in distilled water; mount in Farrant.

Results:

Free fatty acids: blue. Neutral fats: red.

References:

Cain, A. J. (1947).
Smith, J. Lorrain (1907).

NILE BLUE - PICRO FUCHSIN

(After Murray-Drew)

For bacteria and actinomyces in pathological tissues

Solutions required:

A. Picro fuchsin (Van Gieson).

B. Nile Blue sulphate aqueous 1%.

Technique:

1. Formalin-fixed material is embedded in paraffin wax, or frozen sections may be employed.

2. Take sections down to distilled water in the usual manner then stain in picro fuchsin for two or three minutes.

3. Wash with distilled water.

4. Stain in Nile Blue sulphate for four to twenty-four hours.

5. Rinse in distilled water until the washings are tinted pale blue.

6. Drain off excess water; then blot the preparation carefully but thoroughly.

7. Dehydrate rapidly in absolute alcohol; then clear in xylol.

8. Differentiate in clove oil for five to fifteen minutes (for paraffin sections) or for several hours in the case of thick frozen sections.

9. Rinse in two or three changes of xylol to remove clove oil; then mount.

Results:

Bacteria and chromatin are stained blue; collagen fibres, red; mast cell granules, blue-black; fibrin, blue or reddish orange; erythrocytes and keratin, yellow.

Reference: Drew & Murray (1919).

NINHYDRIN

For amino groups

Solutions required:

A. Celloidin 0·5% in equal volumes of absolute alcohol and ether

B. Ninhydrin (triketohydrindene hydrate), 0·5% aqueous

Technique:

1. Fix pieces of fresh material in 10% formalin for two to four hours.

2. Wash in water and cut frozen sections.

3. Wash in 70% alcohol, followed by 90% and absolute alcohol.

4. Coat sections on slides with the celloidin solution, then allow the solvent to evaporate for a few minutes.

5. Immerse sections in solution B (Ninhydrin) for one minute at 90° C.

6. Wash in water.
7. Mount in Aquamount or glycerine or glycerine jelly.

Results:

Alpha-amino acids and proteins: blue to violet.

Notes:

(*a*) According to Serra (1947), the reaction is given not only by all amino acids (except proline and hydroxyproline), peptides and proteins, but by amines, aldehydes, sugars with free aldehyde and keto groups, and by ammonia and ammonium salts.

(*b*) Readers are referred to Serra's (1946) paper for more detailed information.

References:
Serra, J. A. (1946).
Pflugers, W. (1926).
Ruhemann (1910).

ORANGE G - CRYSTAL VIOLET

(After Bensley)

For secretion antecedents of serous or zymogenic cells.

Solutions required:

A. Osmic acid 2% .. .. 4 ml.
Potassium dichromate 5% .. 4 ml.
Glacial acetic acid .. .. 1 drop
Distilled water .. .. .. 2 ml.

B. *Neutral gentian*
Orange G 8% aqueous .. 25 ml.
Crystal violet 4% aqueous .. 25 ml.

Mix thoroughly by shaking until almost complete precipitation takes place; then allow the preparation to stand for an hour, afterwards collecting the precipitate on a filter. Wash the precipitate on the filter with about 250 ml. distilled water; then dry and dissolve it in 25 ml. absolute alcohol.

C. Solution B (as above) .. .. 10 ml.
Alcohol 20% .. .. Sufficient to impart a rich port-wine colour.
Allow to stand for twenty-four hours; then filter.
D. Absolute alcohol .. .. 1 volume
Clove oil .. .. .. 3 volumes

Technique:

1. Tissues are fixed in Solution A (as above) for twenty-four hours, and afterwards embedded in paraffin wax in the usual manner.
2. Stain sections for twenty-four hours in Solution C (as above); then pour off excess stain, and blot the preparation carefully.
3. Dehydrate by immersing in two or three changes of acetone.
4. Clear in xylol; then differentiate in Solution D (as above), controlling under the microscope.
5. Rinse in two changes of xylol; then mount in balsam.

Results:

Zymogen granules, violet; granules of acidophil cells are stained orange-red, while those of basophil cells are violet; background, brown.

Reference: Bensley, R. R. (1911).

ORCEIN

For staining myelin sheaths of human dental and paradental tissues, etc.

Solutions required:

A. Formic acid, 10% aqueous
B. Sodium citrate, 25% aqueous
C. Solution A .. .. .. 1 volume
Solution B .. .. .. 1 volume
D. Orcein (Unna–Taenzer), page 329

Technique:

1. Fix adult teeth in 10% formalin, for at least seven days, after cutting the radicular apex to facilitate penetration of the fixative into the pulp.

2. Decalcify in solution C.
3. Wash in water for twenty-four to forty-eight hours.
4. Immerse in 10% formalin for three to five days.
5. Cut frozen sections at 10 to 15μ and drop them into distilled water.
6. Immerse in the orcein solution for seven days.
7. Immerse the sections in 95% alcohol for two to twenty-four hours or until a satisfactory degree of differentiation has been obtained, which is determined by microscopic examination at intervals.
8. Immerse the sections in distilled water for about an hour.
9. Dehydrate and clear.
10. Mount in Canada balsam in xylol or in Exemel or D.P.X.

Results:

Myelin sheaths appear reddish brown in the dental pulp. Fibres emerging from Raschkow's plexus can be followed almost to the odontoblast layer, while Schmidt–Lantermann incisures, infundibular spaces and axons remain relatively unstained.

Note:

The author (Fuentes) states that his results appear to suggest that orcein can be used in a manner similar to that described above as a selective stain for myelin sheaths, and that trials made with sciatic nerve, spinal dorsal roots and cord in adult rabbit have yielded similar results.

Reference: Fuentes, A. (1960).

ORCEIN - METHYLENE BLUE

For elastic fibres and connective tissue

Solutions required:

A. Orcein (Unna-Taenzer)

Orcein	1 gm.
70% alcohol	100 ml.

Heat on a water bath to dissolve; cool; filter; then add:

Hydrochloric acid, conc. ..	1 ml.

Shake well.

B. Unna's polychrome methylene blue.
This should be diluted 1 : 10 or 1 : 15 for use.

1. Fix tissues (any fixative may be employed); dehydrate; clear; and embed.

2. Paraffin sections are brought down to 70% alcohol in the usual manner; flood with freshly filtered orcein solution, prepared as above, and warm gently for ten to fifteen minutes, until the solution thickens. Alternatively, the sections may be stained overnight at room temperature.

3. Wash thoroughly with 70% alcohol.

4. Wash thoroughly with distilled water.

5. Stain in diluted polychrome methylene blue until the nuclei are bright blue to blue-black; the time necessary may be ascertained by examining under the microscope at intervals.

6. Differentiate in 95% alcohol, followed by absolute.

7. Clear in xylol and mount.

Results:

Elastic fibres: deep brown. Nuclei: bright blue or blue to black. Connective tissue: pale brown.

Note: If it is desired only to stain the elastic fibres, omit No. 5, and treat sections with acid alcohol for a few seconds before washing with distilled water.

References:

Mallory, F. B. (1938), p. 171.
Unna, P. G. (1891).

ORCEIN - ANILINE BLUE - ORANGE G

A differential stain for elastic fibres, collagen, keratin, etc.

Solutions required:

A.	Orcein	1 gm.
	Alcohol 70%	100 ml.
	Hydrochloric acid, conc.	0·6 ml.
B.	Alcohol 50%	49 ml.
	Hydrochloric acid, conc.	0·5 ml.

C. *Mallory's Aniline Blue - Orange G.*

Aniline blue, aqueous	0·5 gm.
Orange G	2 gm.
Phosphomolybdic acid 1%.. ..	100 ml.

D. Orange G 0·1% in absolute alcohol.

Technique:

1. Fix material in Bouin and embed in paraffin wax.
2. Sections, about 8μ in thickness, are fixed to slides, dewaxed with xylol and passed through the usual descending grades of alcohol to distilled water.
3. Stain for one and a half hours in the orcein solution in a closed staining jar.
4. Differentiate with solution B, controlling under the microscope, until most of the pink is extracted from the sections.

Note: The duration of the differentiation will vary according to the nature of the material and to the thickness of the sections.

5. Wash thoroughly with running tap water.
6. Wash with distilled water.
7. Immerse in solution C diluted with an equal volume of distilled water, for one to two minutes.
8. Rinse with 95% alcohol.
9. Rinse with two lots of solution D.
10. Rinse quickly with absolute alcohol.
11. Clear in xylol and mount.

Results:

Elastic fibres: red. Collagen: blue. Muscle fibres: pale orange to dirty yellow. Cytoplasm: varying shades of yellow. Erythrocytes: golden yellow. Kerantinized material: bright yellow.

Reference: Margolena, L. A. & Dolnick, E. H. (1951).

ORCEIN - ANILINE SAFRANIN

For elastic and connective tissue fibres

Solutions required:

A. Orcein (Unna) $\frac{1}{2}$ to 1% in 80% alcohol .. .. 100 ml.
Hydrochloric acid, conc. .. 1 ml.

B.	Safranin O, water soluble	1 gm.
	Aniline water	48 ml.
	Absolute alcohol	52 ml.

Technique:

1. Sections are mounted on slides and brought down to 90% alcohol in the usual manner. (If the tissues have been fixed in a fluid containing mercury, the mercurial deposit is removed by the standard technique; the sections are then taken from 70% to 90% alcohol, then direct into the orcein stain.)

2. Stain from twenty to sixty minutes with the orcein solution in a stoppered staining jar.

3. Rinse in acid alcohol until stain ceases to come out of the preparation.

4. Rinse in 70% alcohol; then in distilled water.

5. Stain in aniline safranin (Solution B) for five minutes; then rinse in water.

6. Differentiate by dipping into 90% alcohol then examining rapidly under the microscope. Repeat this process until the cell nuclei are well brought out, stained clear bright red.

7. Dehydrate by rinsing quickly in absolute alcohol; then clear in xylol and mount in balsam or D.P.X.

Results:

Elastic fibres are stained dark to reddish brown; cell nuclei, bright red; ground substance of hyaline cartilage, yellow.

Reference: Carleton, H. M. & Leach, E. H. (1947), pp. 125–126.

ORCEIN - GIEMSA STAIN

For syphilitic tissue, particularly dermatological specimens

Solutions required:

A.	*Orcein (Unna-Tänzer):*	
	Orcein	0·5 gm.
	Alcohol 70%	100 ml.
	HCl, concentrated	0·6 ml.
B.	Absolute alcohol	100 ml.
	HCl, concentrated	0·5 ml.

C. Giemsa stain .. .. .. 0·25 ml.
Distilled water .. .. .. 100 ml.

Note: Solution C should be prepared freshly, as required, from Giemsa stain.

D. Alcohol 95% .. .. .. 100 ml.
Eosin 1% in 90% alcohol .. 1 ml.

Technique:

1. Paraffin sections are brought down to 70% alcohol.

2. Stain for one half to one hour in Solution A; then rinse for two to five minutes in distilled water.

3. Wipe off excess water; dip into 95% alcohol for a few seconds; then decolorize with absolute alcohol for five to twenty-five minutes, or until the sections assume a pale brown colour and the elastic fibres stand out, deep purple to black, under the low-power objective.

4. Decolorize in Solution B until the background is almost colourless. This usually takes two to seven minutes.

Note: Decolorization must not be extended more than ten minutes, as otherwise the thin elastic fibres will become destained.

5. Immerse in tap water for five to ten minutes.

6. Stain for two to twelve hours with Solution C until the epithelial and other cells are deep blue; connective tissue, greyish pink or greyish blue or blue.

7. Wipe off excess stain and dehydrate and decolorize in Solution D, controlling under the microscope.

Note: Decolorization must be stopped when the connective tissue has lost all trace of blue and has assumed a rose tint. The blue tinge is removed fairly rapidly in Solution D. The epidermis should remain bright blue.

8. Immerse in two changes of absolute alcohol for two minutes in each. Clear in xylol, and mount.

Results:

Nuclei: deep blue. Cytoplasm of the epidermis, muscle cells and connective tissue cells: light blue. Plasma cells: dark greyish blue. Eosinophilic granules: bright red. Mast cell granules: metachromatic (varying shades of) purple. Neutrophilic granules: only

faintly stained. Erythrocytes: reddish brown. Collagenous fibres: pale rose to brownish pink. Elastic fibres: dark brown to black. Senile degenerated connective tissue (collacin, elacin and collastin): various shades of dark grey and blue. Cartilage: metachromatic (varying shades of) purple. Decalcified bone: light brown. Keratin: blue (poorly stained). Stratum lucidum, dark red; keratin layer may be light blue or light pink or colourless depending upon the tissue and the degree of decolorization. Inner root sheath of the hair: deep blue. Melanin granules: green to black. Other pigments: unstained. Bacteria and mycelia: deep blue. Demodex folliculorum in hair follicles: brown with blue granulations.

Reference: Pinkus, H. (1944).

ORCEIN - PICRO FUCHSIN

For elastic and collagen fibres

Solutions required:

A. Orcein (Unna) 1% in 80% alcohol 100 ml.
Hydrochloric acid, conc. .. 1 ml.

B. Picro-fuchsin (Van Gieson).

Technique:

1. Sections are mounted on slides and brought down to 70% alcohol in the usual manner. If tissues have been fixed in a fluid containing mercury, the mercurial precipitate is removed by the standard technique.

2. Immerse in orcein solution (recipe as above) for half an hour or longer if necessary; then rinse in acid alcohol.

3. Rinse in 70% alcohol; then in water.

4. Stain with picro fuchsin (Van Gieson) for three to five minutes.

5. Rinse rapidly (not more than a few seconds) in water.

6. Dehydrate rapidly; clear, then mount.

Results:

Collagen fibres are stained red; elastic fibres, brown; erythrocytes, epithelia, muscle, etc., yellow.

Reference: Carleton, H. M. & Leach, E. H. (1947) p. 126.

ORCINOL - NEW FUCHSIN

A selective stain for elastic tissue

Solutions required:

A. New fuchsin .. .. .. 2 gm.
Orcinol .. .. 4 gm.
Distilled water .. .. .. 200 ml.

Boil for five minutes; then add:
Ferric chloride ($FeCl_3$), 15% .. 25 ml.
and boil for a further period of five minutes.

Allow the solution to stand until cold; then collect the precipitate by filtration: washing and drying is stated to be unnecessary.

Dissolve the precipitated orcinol–new fuchsin in 100 ml. of 95% alcohol and use this as the elastin stain.

Technique:

1. Material may be fixed in 10% formalin or saturated aqueous mercuric chloride or Zenker, etc.
2. Fix sections to slides, dewax and pass through absolute alcohol as usual.
3. If a mercury-containing fixative has been used, treat sections for the removal of mercurial precipitate by the standard method, afterwards rinsing with 90% alcohol.
4. Stain in the orcinol–new fuchsin for fifteen minutes at 37° C.
5. Differentiate for five minutes in each of three changes of 70% alcohol.
6. Dehydrate in absolute alcohol; clear in xylol, and mount in D.P.X., or Cristalite, or Clearmount, etc.

Results:

Elastic fibres: deep violet. Collagen: unstained.

Notes:

(*a*) The authors reported that they used the stain on human skin and aorta, and the following tissues of the mouse: liver, kidney, spleen, stomach, duodenum, colon, pancreas, heart, testes, seminal vesicles, ovary, uterus, aorta, pituitary, salivary gland, bone, striated muscle, and thyroid; and in all cases only elastic tissue was stained.

(*b*) Haematoxylin and eosin, or a safranin counterstain may be employed between steps 5 and 6.

(*c*) For more detailed information readers should consult the original paper which shows two photomicrographs.

Reference: Fullmer, H. M. & Lillie, R. D. (1956).

OSMIC ACID

For demonstrating the interstitial cells of the testis

Solutions required:

A. *Champy's fluid*
Osmic acid, 2% .. .. .. 4 volumes
Potassium dichromate, 3% .. 7 volumes
Chromium trioxide, 1% .. 7 volumes

B. Pyroligneous acid .. .. 1 volume
Chromium trioxide, 1% .. 2 volumes

C. Potassium dichromate, 3% ..

Technique:

1. Fix small pieces of tissue in solution A for twenty-four hours.
2. Wash in distilled water for half an hour.
3. Immerse in solution B for twenty hours.
4. Immerse in solution C for three days at 37° C., changing the solution every twenty-four hours.
5. Wash in running water for twelve to twenty-four hours.
6. Dehydrate; clear; embed in paraffin wax.
7. Cut sections 5μ in thickness and fix them to slides.
8. Dewax with xylol, and carry through the usual descending grades of alcohol to distilled water.
9. Mount in Farrants' medium, Aquamount or glycerine jelly.

Results:

Interstitial cells: conspicuous black granules of varying size in the cytoplasm. Remainder of testis: yellow.

Notes:

(*a*) See Threadgold's Sudan black method also.

(*b*) The osmic acid technique demonstrates the development of secretory granules, but it does not show cellular detail and all stages of cytomorphosis of the interstitial cell as clearly as the Sudan black method.

(*c*) Interstitial cells, actively synthesizing secretion droplets, are revealed by the presence of black lipid droplets of various sizes, in the cytoplasm; readers should consult the original paper for more detailed information and photomicrographs.

(*d*) Steps 3 and 4 may be omitted, but counterstaining will then be necessary to reveal cellular detail.

(*e*) Counterstaining with Ehrlich's haematoxylin or with eosin in 30% alcohol may be tried, but both stains tend to obscure the results, although the eosin is stated to give a fair contrast.

Reference: Threadgold, L. T. (1957).

OSMIC ACID

A rapid technique for staining fat in frozen sections

Solutions required:

A. Osmic acid 1% aqueous.
Note: Store in an amber bottle.

B. Eosin, yellowish, 1% aqueous.

Technique:

1. Tissues are fixed as follows:

Place 22·5 ml. distilled water in a 50-ml. beaker and heat to about 90° C.; then add 2·5 ml. formalin; raise to boiling point; drop in a thin piece of the tissue; then place the beaker in an oven at 60 to 65° C. for ten minutes.

2. Cut frozen sections 10μ thick, and place them in another 50-ml. beaker.

3. Boil Solution A in a large test-tube and pour onto the sections, then transfer to an oven at 60° C. for five minutes.

4. Wash sections in a dish of cold tap water after pouring back the osmic acid, which may be used again, into the stock bottle.

5. Counterstain in Solution B for one minute.

6. Wash quickly in tap water; transfer sections to slides; drain and mount in glycerine jelly, or Aquamount.

Results:

Fat globules: black or greyish black against a red background.

CAUTION: Osmic acid vapour is injurious to the eyes.

OSMIC ACID - ALPHANAPHTHYLAMINE - ALCIAN BLUE

For the simultaneous demonstration of normal and degenerating myelin

Solutions required:

A. 4% Formalin-saline

B. Osmic acid (osmium tetroxide), 1% aqueous

C. Potassium chlorate, 1% aqueous

D. Solution A .. .. .. 1 volume
Solution B .. .. .. 3 volumes

E. α-Naphthylamine, saturated aqueous

F. Alcian blue .. .. .. 2 gm.
Distilled water .. .. .. 95 ml.
Glacial acetic acid .. .. 5 ml.

Technique:

1. Cut frozen sections of unfixed tissue, on a cryostat, at 5μ; or cut formalin-fixed tissue, on the freezing microtome, at 10 to 15μ.

2. Immerse the sections for eighteen hours in solution D.

Note: The vessel into which the sections are to be placed should be tightly stoppered to prevent volatilization of osmium tetroxide.

3. Wash the sections in distilled water for thirty minutes.

4. Immerse sections in the α-naphthylamine (solution E) in the incubator at 37° C.; 15μ sections for ten to fifteen minutes; 5μ sections for twenty minutes.

Note: The α-naphthylamine solution should be warmed to about 37° C. before the sections are placed in it.

5. Wash in distilled water for five minutes.

6. Counterstain in the Luxol fast blue (solution F) for fifteen to sixty seconds.

7. Mount in glycerine jelly, or better in Aquamount, and seal the edges of the coverslips with Laktoseal.

Results:

The following are stained *black:*

Degenerating myelin, depot fat, fat droplets in fatty liver, and lipid in the zona fasciculata of the adrenal.

The following are stained *red*:

Normally myelin of the central or peripheral nervous systems, sphingomyelin storage granules in Niemann–Pick's disease, and lipid droplets in the zona glomerulosa of the adrenal.

The following are stained *light red*:

The erythrocyte envelope, and cerebroside storage granules in Gaucher's disease.

The following are stained in *shades of blue*:

The stored lipid granules, which are probably ganglioside, of Tay–Sach's disease; glial fibres and connective tissues.

The axon is unstained.

Notes:

(*a*) The author states that from chemical evidence it may be deduced that since normal myelin is hydrophilic, it is permeable to electrolytes such as potassium chlorate, and that the chlorate absorbed prevents the reduction of osmium tetroxide (to lower oxides which are black) by the unsaturated groups of normal myelin lipids. Osmium tetroxide itself is soluble in both hydrophilic and hydrophobic lipids. Therefore, osmium tetroxide is permeable to unsaturated hydrophobic lipids which will not accept the potassium chlorate, and it is these lipids that reduce the osmium tetroxide to give the black stain observed in the sections.

(*b*) The author states that the red reaction due to osmium-α-naphthylamine chelate indicates that lipids so stained are hydrophilic.

(*c*) It is claimed that this method (the OTAN method) rests upon a physico-chemical difference between classes of lipids, and for this reason the method may be regarded as histochemical, or histophysical.

(*d*) The original paper should be consulted for more detailed information and tables.

References:
Adams, C. W. M. (1959).
Carleton, H. M. and Leach, E. H. (1947), p. 126.

PAPANICOLAOU STAIN EA36

For improved differentiation of the cells of vaginal smears

Staining solutions:

A. Ehrlich or Harris haematoxylin.

B. *Papanicolaou stain OG6*

Orange G	..	..	0·2 gm.
Alcohol 95%	..	..	100 ml.
Phosphotungstic acid	..	..	0·015 gm.

C. Papanicolaou stain, EA36, recipe as solution page 341.

Place in a flask, plug the neck lightly with cotton-wool; then heat, on a waterbath until dissolved. Cool, then filter.

Technique:

1. Wet smears are fixed in a mixture of equal volumes of ether and absolute alcohol for five to fifteen minutes.

2. Rinse successively in 90%, 70% and 50% alcohols and distilled water.

3. Stain in Ehrlich's haematoxylin for five to ten minutes.

4. Rinse in distilled water; differentiate in 0·5% hydrochloric acid.

5. Rinse in distilled water; then leave for one minute in dilute lithium carbonate solution (three drops saturated lithium carbonate to 100 ml. distilled water).

6. Rinse thoroughly in distilled water and wash successively in 50%, 70%, 95% alcohol.

7. Stain for one minute in Orange G solution (prepared as above).

8. Rinse thoroughly in 95% alcohol to remove all excess stain.

9. Stain for two minutes in Papanicolaou stain; then rinse five to ten times in each of three jars of 95% alcohol.

10. Rinse in absolute alcohol; clear in xylol and mount.

Results:

Nuclei: violet. Erythrocytes: orange. Cornified cells: red, pink or orange. Basophile cells: blue or green.

Reference: Papanicolaou, G. N. (1942).

PAPANICOLAOU STAIN EA36

For paraffin sections of keratinizing epithelia

(After P. L. Johnson & M. N. Klein, 1956)

Solutions required:

A. Harris haematoxylin

B. Orange G .. .. 0·25 gm.
Alcohol, 95% .. .. .. 100 ml.
Phosphotungstic acid, 1% aq. 0·15 ml.
(*see solution F below*)

C. Light green SF, 0·5% in 95% alcohol

D. Bismarck brown Y, in 95% alcohol

E. Eosin yellowish, 0·5% in 95% alcohol

F. Phosphotungstic acid, 10% aqueous

G. Lithium carbonate, saturated, aqueous

H. *Papanicolaou stain EA36*
Solution C .. .. .. 45 ml.
Solution D .. .. .. 45 ml.
Solution E .. .. .. 10 ml.
Solution F .. .. .. 2 ml.
Solution G .. .. .. 1 drop
Mix well, then filter

Technique:

1. Fix material in 10% formalin, or 80% alcohol, or 1% trichloracetic acid in 80% alcohol, or Carnoy's fluid, or Bouin, and embed in paraffin wax in the usual way.
2. Fix sections to slides; dewax with xylol; then take down to distilled water through the usual graded alcohols.
3. Stain in Harris haematoxylin for six minutes.
4. Rinse thoroughly in running water, for two to three minutes.
5. Rinse in distilled water.
6. Wash successively with 50%, 70%, 80%, and 95% alcohols.
7. Stain for five minutes in orange G.
8. Rinse well in two changes of 95% alcohol.
9. Stain with solution H (Papanicolaou stain EA36) for two and a half minutes, with occasional agitation.
10. Rinse thoroughly in each of three jars of 95% alcohol. (Do not use the jars that were used at step 8, above.)
11. Dehydrate thoroughly with two or three changes of absolute alcohol.
12. Clear in xylol and mount in Cristalite, Clearmount, D.P.X. or Emexel.

Results:

Keratin: bright orange, while compact collagen appears orange-red.

The authors state that the method has been pre-eminently successful in the identification of keratin *per se*. As a typical example, a section of well cornified oral mucous membrane with long epithelial pegs showed basal cells that stained green. Successive layers of the stratum germinativum displayed a variety of colours ranging from deep red to bright orange of the superficial keratin. The corium presented a loosely textured lamina propria characteristically stained green. The deeper segments of the more compactly collagenous submucosa stained orange-red.

Notes:

(*a*) The authors state that the Papanicolaou technique was applied by them to various keratinizing epithelia, including: hairless skin, hairy skin, feathers, nails, claws, hooves, and well-cornified oral mucosa.

(*b*) It is stated in the original paper, to which readers are referred, for further information, that the enhanced nuclear stain and better cytoplasmic differentiation renders tissue morphology more conspicuous and more readily interpreted than with the routine haematoxylin and eosin methods.

(*c*) Further application of the procedure to cyclic changes in the vaginal mucosa as well as to pathological lesions are being investigated by the authors (Johnson & Klein).

(*d*) Readers are also referred to Aascher, Turner & De Boer's methods, in which Papanicolaou stain and MF4 stain are used for paraffin sections of vaginal epithelium.

References:
Johnson, P. L. & Klein, M. N. (1956).
Papanicolaou, G. N. (1942).
Aascher, A. W., Turner, C. J. & De Boer, C. H. (1956).
Gurr, E. (1958*a*), pp. 19–23.

PARALDEHYDE FUCHSIN - HAEMALUM - PICRO INDIGOCARMINE

For elastic, collagenous and muscle fibres, etc.

Solutions required:

A. *Gomori's fluid*

Potassium permanganate, 2·5% aqueous	12·5 ml.
Sulphuric acid, 5% aqueous ..	12·5 ml.
Distilled water	75 ml.

B. Potassium metabisulphite, 2% aqueous

C. Haemalum (Mayer)

D. *Gomori's paraldehyde fuchsin* (**stock solution**)

i. Shake 1 gm. basic fuchsin with 200 ml. distilled water, at room temperature, in a flask.
ii. Raise to boiling point and allow the solution to boil for one minute; then allow it to cool to room temperature.
iii. Filter; then add 2 ml. each of concentrated HCl and paraldehyde. Shake well; then plug the neck of the flask with non-absorbent cotton wool.

iv. Leave the preparation to stand until the red colour has disappeared and no further precipitate is formed. This usually takes about four days, during which time the preparation should be checked at intervals by shaking the liquid then placing a drop on filter paper. The red colour of the fuchsin gradually decreases in intensity, while the precipitate in the middle of the filter paper spots will be observed in increasing density.

v. Collect the precipitate by filtration, and discard the liquid, which is often violet in colour.

vi. Wash the precipitate, on the filter, with distilled water; then drain and dry.

vii. Make a saturated solution of the precipitate in 75% alcohol. This stock solution can be kept in the light and at room temperature and it will remain stable for at least a year.

E. *Gomori's paraldehyde fuchsin* (**staining solution**)

Solution D	5 ml.
Alcohol, 70%	25 ml.
Acetic acid, 50% aqueous ..	0·4 ml.

F. Picro indigocarmine

Technique:

1. Dewax the sections and take them down to water through the usual graded alcohols.
2. Oxidize in Gomori's fluid (solution A) for 20 to 30 seconds.
3. Rinse quickly in tap water.
4. Bleach in solution B (sodium metabisulphite).
5. Wash in running tap water for 30 seconds.
6. Stain the nuclei with haemalum for one minute.
7. Wash in running tap water for one minute.
8. Stain in paraldehyde fuchsin (solution E) for two minutes.
9. Rinse quickly in tap water.
10. Stain in picro indigocarmine for 20 seconds.
11. Rinse in tap water.
12. Dehydrate through the usual graded alcohols.
13. Clear in xylol.
14. Mount in D.P.X., Clearmount, Cristalite, or other synthetic neutral resinous media.

Results:

Elastic fibres, mast cells, matrix of cartilage: intense purplish red. Acidophilic cytoplasm and muscle fibres: green. Basophilic cytoplasm: grey. Chromatin: black or brown. Collagenous fibres: blue.

Notes:

In the same paper Gabe describes a similar procedure for demonstrating alpha and beta cells of the pancreas as well as neurosecretions in arthropods and vertebrates.

Reference: Gabe, M. (1953).

PARALDEHYDE - NEW FUCHSIN

For the demonstration of beta cells in pancreatic islets and their tumours

Solutions required:

A. Chromic chloride, A.R. .. 5 gm.
Glacial acetic acid .. .. 5 ml.
Distilled water .. .. .. 100 ml.

Note: This solution cannot be relied upon after six months.

B. *Paraldehyde new fuchsin**
New fuchsin (Michrome No. 624) 1 gm.
Alcohol, 70% .. .. .. 100 ml.
Dissolve; cool to room temperature, then add the following mixture:
HCl, conc. .. .. .. 2 ml.
Paraldehyde .. .. .. 2 ml.

Shake well, then allow the stain to remain at room temperature for twenty-four hours, or until the solution is a deep purple colour.

Technique:

1. Material should be fixed in Bouin's fluid or in buffered neutral 10% formalin.
2. Cut sections at 5μ or less and fix them to slides as usual.
3. Remove paraffin wax with xylol, then pass through the usual descending grades of alcohol into distilled water.
4. Mordant in solution A for 72 hours.
5. Rinse thoroughly in distilled water.

* The solution, which is unreliable after about a week, should be stored in the refrigerator.

6. Rinse in 70% alcohol.

7. Stain in solution B for six hours.

8. Rinse in 70% alcohol.

9. Wash in running tap water for five to ten minutes.

10. Rinse in distilled water.

Note: If desired, a counterstain may be applied at this stage.

11. Rinse in 95% alcohol for thirty seconds.

12. Immerse in absolute alcohol for thirty seconds.

13. Clear in two changes of xylol and mount in Clearmount, Cristalite or D.P.X.

Results:

The beta cells in the pancreatic islets of man, dog, frog, guinea pig, rabbit and mouse are stained purple.

Notes:

(*a*) It was found that the staining of any species that had been fixed in Zenker's or Helly's fluids was capricious, and no staining was observed in tissues fixed in fluids containing alcohol. The beta granules are known to be alcohol soluble.

(*b*) The authors state that as opposed to the argentaffin granules of carcinoid tumours of the intestine, the beta granules in islet tumours are not readily demonstrable. Most of the techniques used to stain beta cells in non-neoplastic islets do not colour a significant number of cells in tumours arising from pancreatic islets. Moreover, the unstained cells will not accept the counterstains commonly used in such techniques and appear as alpha cells.

(*c*) It is stated that many types of counterstains can be used including Van Gieson, light green–orange (Halmi, 1952), phloxine (Fisher & Haskell, 1954), Gomori's trichrome (Gomori, 1950*b*), iron haematoxylin–metanil yellow, light green, fast green FCF, etc.

(*d*) The authors state that Gomori's (1950*a*) technique failed to stain the beta granules consistently in non-neoplastic islets and did not stain any of the beta cells in islet tumours, and the introduction

of the chromium chloride acetic acid (solution A, above) did not produce a positive stain with his method.

(*e*) In the authors' experience a negative result was obtained on tissue which had previously stained strongly if the paraldehyde used in preparing solution B had not been obtained from a freshly or recently opened bottle.

(*f*) It is also stated that a staining time of over six hours is not critical and although the normal islets in some tissues are stained adequately in five minutes, a period of six hours was found to be the optimum time for the evaluation of tumours.

(*g*) Readers are referred to the original paper for more detailed information.

Reference: Sieracki, J. C., Michael, J. E. & Clark, D. A. (1960).

PASINI'S STAIN (Improved)

For differentiation of connective tissue

Solutions required:

A. Iron alum 2·5% aqueous.

B. *Pasini's stain:*

Unna's aniline blue-orcein ..	10 ml.
Eosin bluish 2% in 50% alcohol..	12 ml.
Acid fuchsin	0·3 gm.
Neutral glycerine..	5 ml.

Technique:

1. Tissues should be fixed in Heidenhain's susa mixture and embedded in L.V.N. Sections are cut 3μ in thickness.

After removal of mercuric precipitate in the usual manner sections are mordanted in Solution A for twenty-four hours.

2. Transfer to Solution B for three to ten minutes.

3. Transfer to 95% alcohol and agitate for about one minute or until the colour ceases to come out in clouds.

4. Immerse in absolute alcohol for one minute; then blot, clear and mount.

Results:

Collagen fibres: deep blue. Cytoplasm: red. Epithelial cells, centroiles, basal bodies, nuclear structure: brilliant red. Erythrocytes: yellowish red. Connective tissue: blue. Secretory bodies: varying according to their nature. Slime of goblet cells: azure blue. Connective tissue wandering cells, smooth and striated muscle: well defined.

Reference: Pasini (1905).

PERACETIC ACID - ORCEIN - HALMI STAIN

For connective tissues

Solutions required:

A. *Peracetic acid* (*Greenspan*, 1946)

Glacial acetic acid	95·6 ml.
Hydrogen peroxide, 30%	259 ml.
Sulphuric acid, conc.	2·2 ml.

Leave the solution to stand for one to three days; then add 0·04 gm. disodium hydrogen phosphate as a stabilizer. Store in a refrigerator at 0° to 5°C.

Note: Lillie (1954) states that he has kept such solutions in the icebox for months, and a single Coplin jar of this reagent may be used for eight to ten groups of nine slides before discarding, but a positive control should be included, at least in the later groups.

B. Orcein (Unna–Taenzer)
as recipe, page 329.

C. *Modified Mayer's alum haematoxylin* (*Lillie*, 1954)

Haematoxylin	2·5 gm.
Ammonia alum	25 gm.
Glycerin	150 ml.
Distilled water	350 ml.
Sodium iodate	0·25 gm.
Glacial acetic acid	10 ml.

Acid salicylate .. .. .. 0·17 gm.

Dissolve the haematoxylin in 100 ml. of the water and place in a flask of 1 litre capacity. Dissolve the ammonia alum in 200 ml. of the water and put aside.

Measure out the glycerin and pour it into the flask containing the haematoxylin solution.

Use the alum solution now for rinsing out the glycerin adhering to the inside of the measuring cylinder; then pour the solution into the flask containing the haematoxylin.

Dissolve the potassium iodate in about 20 ml. of the remaining distilled water and add the solution to the haematoxylin.

Shake the contents of the flask well to ensure thorough mixing. Filter into the stock bottle.

Shake the acid salicylate with the remaining 30 ml. or so of the distilled water, and add to the stock bottle.

Shake the bottle again thoroughly. The solution is ready for immediate use.

D. Light green SF yellowish .. 10 gm.
Distilled water .. .. .. 100 ml.

E. Orange G .. .. .. 5 gm.
Distilled water .. .. .. 100 ml.

F. Phosphotungstic acid .. .. 10 gm.
Distilled water .. .. .. 100 ml.

G. Halmi stain, modified
Solution D .. .. .. 2 ml.
Solution E .. .. .. 20 ml.
Solution F .. .. .. 5 ml.
Acetic acid 1·3% aqueous .. 75 ml.

Note: Solution G keeps for at least two weeks.

H. Absolute alcohol, 95% .. .. 25 ml.
Glacial acetic acid .. .. 75 drop

Technique:

1. Fix material either in Lillie's aqueous neutral calcium 10% formalin for eighteen to twenty-four hours, or in 95% alcohol for the same time.
2. Decalcify mineralized tissues in 5% formic acid.
3. Embed in paraffin wax and cut sections at 6μ.
4. Fix sections to slides and remove paraffin wax with xylol.
5. Wash in absolute alcohol.
6. Oxidize in peracetic acid (solution A) for thirty minutes.

7. Wash in running water for two minutes.

8. Rinse in distilled water.

9. Stain in the orcein solution in a covered jar for fifteen minutes at 37° C.

10. Differentiate in three changes of 70% alcohol for a total time of five minutes.

11. Wash in distilled water.

12. Stain in the Lillie–Mayer alum haematoxylin (Solution C) for four minutes.

13. Rinse in water.

14. Differentiate in acid alcohol for thirty seconds.

15. Blue in running tap water.

16. Counterstain in the modified Halmi stain (solution G) for twenty seconds.

17. Rinse briefly in the acetic alcohol (solution H).

18. Rinse in 95% alcohol.

19. Dehydrate in absolute alcohol.

20. Mount in Michrome mountant or Clearmount, or proceed as follows:

21. Clear in xylol.

22. Mount in D.P.X. or Cristalite or Clearmount.

Notes:

(*a*) The material used by the author (Fullmer) comprised six embryos ranging in age from two to six months as well as the upper posterior alveolar processes of several individuals ranging from four months to seventy-nine years in age. Also sections from several extracted adult teeth were used.

(*b*) It is claimed that the technique demonstrates elastic and oxytalan fibres as well as fibrils in the mucous connective tissues previously undescribed histochemically. Readers should consult the original paper for further information.

References:

Fullmer, H. M. (1959).
Lillie, R. D. (1954).
Greenspan, F. P. (1946).
Halmi, N. S. (1952).

PERIODIC ACID - SCHIFF REACTION (PAS)
For polysaccharides

Solutions required:

A. Periodic acid .. .. .. 0·4 gm.
Distilled water .. .. .. 45 ml.
Sodium acetate M/5 .. .. 5 ml.

B. Distilled water .. .. .. 10 ml.
Periodic acid .. .. .. 0·4 gm.
Sodium acetate M/5 .. .. 5 ml.
Absolute alcohol .. .. 35 ml.

Note: This solution, which deteriorates after a few days, should be kept in an amber bottle.

C. *Reducing rinse:*
Potassium iodide .. .. 1 gm.
Sodium thiosulphate .. .. 1 gm.
Distilled water .. .. .. 20 ml.

Dissolve; then add with stirring:

Absolute alcohol .. .. 30 ml.
Hydrochloric acid 2N .. .. 0·5 ml.

Note: A percipitate of sulphur is slowly formed and this may be allowed to settle out, or the solution may be used immediately.

The solution loses its acid reaction on keeping for some time, and it should be tested with litmus paper; if the reaction is no longer acid a few drops of N/2 hydrochloric acid should be added until an acid reaction is obtained.

D. Schiff's reagent

E. *Sulphite wash water:*
Distilled water .. .. .. 45·5 ml.
Hydrochloric acid, pure, conc. 0·5 ml.
Potassium metabisulphite .. 0·2 gm.

Technique:

Note: It is advisable to protect preparations with celloidin coating (method, p. 509).

Any fixative may be used, but alcohol was recommended by Hotchkiss (1948) for glycogen and other easily soluble polysaccharides. If the material is fixed in alcohol, then solution B should be used instead of solution A in the following technique.

1. Press sections on to slides without the use of glycerine albumen.
2. Pass sections or smears into alcohol as usual.
3. Coat with celloidin, if desired.
4. Immerse preparations in solution A or B for five minutes.
5. Pour off excess solution A or B.
6. Wash well with 70% alcohol.
7. Immerse in reagent C for five minutes.
8. Wash well with 70% alcohol.
9. Immerse in solution D for fifteen to forty-five minutes.
10. Wash with two or three changes of solution E.
11. Wash thoroughly with running water for 5–10 minutes.
12. Dehydrate through the usual graded alcohols.
13. Clear in xylol.
14. Mount in D.P.X., Clearmount, or other synthetic neutral mountant.

Results:

The following are among the substances that are stained red and are, therefore, described as being strongly " PAS-positive ":

Gastric mucin	Liver
α-Glyceryl phosphate	Serine
Chitin	Umbilical cord polysaccharide
Glycogen	Pneumococcus type III polysaccharide
Hyaluronic acid	

The following are among the substances that take up the stain with moderate intensity and are, therefore, described as being moderately " PAS-positive ":

Glucuronic acid	Pneumococcus type I polysaccharide
Starch	Pneumococcus type II polysaccharide

The following are among those weakly stained (weakly " PAS-positive "):

Adenosine	Cellulose
Adenylic acid (muscle)	Serum albumen
Egg albumen	Xanthosine
Glucose-1-phosphate	

The following are among those substances which do not take up the stain at all and are, therefore, classed as " PAS-negative ":

Deoxyribonucleic acid Inositol Ribonucleic acid

Tryptophane is coloured brown (not red or pink), and is " PAS-negative ".

Notes:

(*a*) The action of the periodic acid is to break the carbon bond through 1,2-glycol groups or HO—C—C—NH_2 groups, converting these into dialdehydes, which are then available for reaction with Schiff reagent. A positive Schiff reaction after treatment with periodic acid or other oxidants is considered to be specific for adjacent hydroxyl (or hydroxyl and amino) groups.

(*b*) Readers requiring more detailed information regarding the chemistry of the reaction should consult any one of the following works: Gomori, G. (1952), Gurr, E. (1958*a*), or Pearse, A. G. Everson (1960). Davenport, H. A. (1960), and Lillie, R. D. (1954) also give a great deal of information on the application, limitations and modifications of the PSA technique.

(*c*) The confirmatory tests described below may be applied, if desired, after the PAS reaction.

References:
Hotchkiss, R. D. (1948).
McManus, J. F. A. (1946).

Diastase digestion test

For differentiating between glycogen, mucin and starch

Solution required:

Diastase (animal or malt) .. 1 gm.
Phosphate buffer solution pH 7·0, 100 ml.

Technique:

1. Unstained, control sections of material which have already been demonstrated as PAS-positive are taken though alcohols to distilled water.

2. Immerse in the above reagent for fifteen to thirty minutes at 15° to 37°C.

3. Rinse in distilled water.

4. Treat exactly as the original sections, which were found to be PAS-positive.

Results:

If the substance which was PAS-positive in the original sections is not stained after the digestion test, it is either glycogen or starch.

Notes:

(*a*) Instead of the diastase solution, saliva may be used.

(*b*) Sections used in the digestion test should, for preference, not be more than 10μ thick, and they should not be coated with celloidin as this will prevent the penetration of diastase. If celloidin embedded material is used, the celloidin should be removed from the sections before the diastase test is applied.

References:

Gomori, G. (1952).
Lillie, R. D. & Greco, J. (1947).

Iodine test

For differentiating between glycogen and starch

Solutions required:

A. Lugol's iodine

B. Iodine crystals .. .. .. 2·5 gm.
Absolute alcohol .. .. 100 ml.

C. Origanum oil

Technique:

1. Carry sections down to 70% alcohol in the usual way.

2. Stain in Lugol's iodine solution for ten minutes.

3. Pour off excess stain, then blot dry.
4. Dehydrate in alcoholic iodine (solution B).
5. Drain, then blot dry.
6. Clear and differentiate in origanum oil.
2. Mount in LPM (see page 523).

Results:

Glycogen assumes a mahogany colour, while starch is stained blue. Background: pale yellow.

Notes:

The Best's carmine method (page 150) may also be applied: it is considered to be highly specific for glycogen, and while it may stain mucin, fibrin, etc., these are coloured so faintly compared with the staining of glycogen that confusion is unlikely.

References:
Langerhans (1890).
Pearse (1953).

PERIODIC ACID - SCHIFF - CELESTIN BLUE

For human and animal pituitary glands, demonstrating both muco-protein precursors of the gonadotrophins

Solutions required:

A. Helly Fixative containing 5% of neutralized formalin.

B. Lugol's iodine.

C. Sodium thiosulphate 5% aqueous.

D. Periodic acid as solution A, page 351.

E. Reducing rinse (*Solution C as page* 351).

F. Schiff's reagent

G. *Celestin blue* (*Lendrum and Mcfarlane*)

Celestin blue	0·25 gm.
Iron alum	2·5 gm.

Distilled water .. .. .. 50 ml.
Glycerine .. .. 7 ml.

Dissolve the alum in the water: then add the celestin blue, and boil for three minutes. Cool and filter; then add the glycerine.

H. Toluidine blue 0·5% aqueous.

I. Orange G .. .. 2 gm.
Phosphotungstic acid 5% aqueous .. 100 ml.

Allow to stand for 48 hours; filter before use.

Technique:

1. Human and larger animal glands are bisected in the horizontal plane with a sharp knife, and the two halves are fixed and embedded separately. Rat hypophyses are fixed *in situ* after removal of the brain and overlaying meninges, by immersing the whole base of the skull in a beaker of the fixative.

2. Fix in Helly for eighteen hours (man, goat, sheep), or two to four hours (rat).

3. Wash from six to twelve hours in running water.

4. Dehydrate, clear and embed in paraffin wax.

5. Cut sections 4·5μ (rat), or 5·5μ (man).

6. Fix sections to slides; remove paraffin wax with xylol and pass through the usual descending grades of alcohol to distilled water.

7. Immerse in solution B for three minutes.

8. Immerse in solution C (sodium thiosulphate) for three minutes by which time the natural colour of the sections should have been restored and mercurial precipitate removed from the fixative.

9. Wash well with water.

10. Rinse with 70% alcohol.

11. Immerse in periodic acid (solution D) for five minutes.

12. Wash with 70% alcohol.

13. Immerse in Hotchkiss' reducing rinse (solution E) for one minute.

14. Wash with 70% alcohol.

15. Immerse in Feulgen's Fuchsin for ten to thirty minutes.

16. Wash in running water for ten to thirty minutes.

17. Stain in the celestin blue solution one half to three minutes.

18. Rinse in water.

19. Stain in the toluidine blue (solution H) for one half to three minutes.

20. Wash in running water for five minutes.

21. Stain in the phosphomolybdic orange G (solution I) for ten seconds.

22. Wash in water for five to thirty seconds, until a yellow tinge is just visible to the naked eye.

23. Dehydrate through the usual graded alcohols.

24. Clear in xylol and mount.

Note: In place of Toluidine blue, Iron Haematoxylin may be used at step 19 *in which case it will be necessary to differentiate quickly before washing in running water* (*step* 20).

Results:

Beta granules in the cyanophils and a number of granules and vesicles in cells which stain as chromophobes by other methods, are magenta to deep red: the colloid is magenta. Alpha granules of acidophils, orange yellow, and erythrocytes are a shade more yellow.

Nuclei: are blue-black.

Note:

Compared with other methods, finer differences in the cytology of the cyanophils can be appreciated. Cell counts can readily be carried out, and the counts are more accurate, giving more definite and clearer results than those obtained by older methods: for instance cells appearing by Mallory and other histological methods, to be chromophobes, are found to belong to cyanophil series.

The method has been applied with good cytological results to the hypophysis of sheep and goats.

Reference: Pearse (1950).

PERIODIC ACID - SCHIFF - HAEMALUM - AURANTIA

For the differential staining of gastric mucosa

(Marks & Drysdale's 1957 method)

Solutions required:

A. Periodic acid, 0·5%
B. Schiff's reagent
C. Acid haemalum (Mayer)
D. Lithium carbonate, saturated, aqueous
E. Aurantia, 0·5% in 50% alcohol.

Technique:

1. Sections of 10% formalin-fixed material are taken down to water as usual.
2. Immerse in solution A for two minutes.
3. Wash in running water for two minutes.

Note: This prevents connective tissue from reacting as Schiff positive.

4. Immerse in Schiff's reagent for five minutes.
5. Wash in running tap water for five minutes.
6. Stain in acid haemalum for five minutes.
7. Rinse in water.
8. Blue in solution D.
9. Wash for five minutes in running water.
10. Immerse in the aurantia solution for ten seconds.
11. Wash in water.
12. Differentiate with 95% alcohol, controlling by microscopic examination, until the aurantia is retained only by the oxyntic cells.
13. Dehydrate, clear, and mount.

Results:

Mucous cells: homogeneous red cytoplasm. Peptic cells: coarse blue granules in regular reticular framework. Oxyntic cells: fine closely packed yellow granules. Erythrocytes: brilliant yellow.

Notes:

This is a modification of Zimmermann's method. The mucicarmine employed in Zimmermann's technique is replaced by periodic acid–Schiff reagent.

References:

Marks, I. N. & Drysdale, K. M. (1957).
Zimmermann (1925).

PERIODIC ACID - SILVER - ORCEIN - ANILINE BLUE

For the selective demonstration of elastin, reticulum and collagen

(After G. L. Humason & C. C. Lushbaugh, 1960)

Solutions required:

A. Pyridine, pure, redistilled .. 50 ml.
Alcohol, 95% .. .. .. 50 ml.

B. Periodic acid .. .. .. 0·5 gm.
Distilled water .. .. .. 100 ml.

C. Silver nitrate, 20%

D. Sodium carbonate, 10%

E. Solution C .. .. .. 5·1 ml.
Distilled water .. .. .. 4·9 ml.

F. Ammonium hydroxide, specific gravity 0·880 .. .. .. 5 ml.
Distilled water .. .. .. 45 ml.

G. Solution D .. .. .. 3·1 ml.
Distilled water .. .. .. 6·9 ml.

H. Solution E .. .. .. 10 ml.

Add a little of solution F drop by drop, shaking the flask after each addition until the white precipitate formed just redissolves, leaving the solution slightly opalescent.

Add and shake in 10 ml. of solution G.

Make up the volume to 100 ml. with distilled water.

I. Solution F .. .. .. 0·5 ml.
Distilled water .. .. .. 99·5 ml.

J. Formalin, 5% .. .. .. 50 ml.
Solution D .. .. .. 0·1 ml.

Note: This solution should be freshly prepared, sufficient for one day's use.

K. Gold chloride (brown or yellow) 0·5 gm.
Distilled water .. .. .. 100 ml.

L. Sodium thiosulphate .. .. 5 gm.
Distilled water .. .. .. 100 ml.

M. Orcein .. .. 1 gm.
Alcohol, 70% .. .. .. 100 ml.
Hydrochloric acid, conc. .. 0·6 to 1 ml.

N. Phosphomolybdic acid, 5% aqueous.

O. *Koneff's aniline blue* (*Koneff*, 1936, *modified*)
Aniline blue, water-soluble .. 0·1 gm.
Oxalic acid .. .. .. 2 gm.
Phosphomolybdic acid .. .. 15 gm.
Distilled water .. .. .. 100 ml.

Note: Dissolve the phosphomolybdic acid in the water first; add the oxalic acid and dissolve; then the aniline blue.

P. Phosphomolybdic acid .. .. 5 gm.
Distilled water .. .. .. 100 ml.

Q. Acetic acid, glacial .. .. 1 ml.
Distilled water .. .. .. 99 ml.

Technique:

1. Fix in a mercuric chloride-containing fluid for preference, although satisfactory results can be obtained with material fixed in 10% formalin.
2. Embed in paraffin wax and cut sections at 6 to 10μ.
3. Attach sections to slides with glycerin albumen or Haupt's fixative.
4. Remove paraffin wax with xylol.
5. Wash in absolute alcohol.
6. Wash in 95% alcohol.
7. Immerse in pyridin–alcohol (solution A) for fifteen minutes.
8. Rinse in 95% alcohol for two to three seconds.

9. Rinse in 70% alcohol.

10. Wash in running water for five minutes.

11. Treat for the removal of mercuric deposits if a mercuric chloride-containing fixative has been used.

12. Immerse in the periodic acid (solution B) for ten to fifteen minutes.

13. Wash in running water for five minutes.

14. Immerse in distilled water for three to five minutes at 35° to 37° C.

15. Immerse in ammoniacal silver (solution H) in an incubator at 35° to 37° C. for one and a half to two hours.

16. Wash in solution I (ammoniated water) for a few seconds to remove excess silver precipitates.

Note: It is essential that the sections should not be allowed to come into contact with metals, and the use of metal instruments must therefore be avoided when transferring slides. Silver solutions should remain colourless.

17. Immerse in the " buffered " formalin (solution J) for five minutes.

Note: Use a fresh solution each time.

18. Wash in running water for five minutes.

19. Tone in gold chloride (solution K).

20. Fix in sodium thiosulphate (solution L).

21. Wash in running water for five minutes.

22. Immerse in the orcein (solution M) in a stoppered staining jar in an incubator at 37° C. for fifteen minutes, or at room temperature for an hour.

23. Rinse in 70% alcohol for two to three seconds.

24. Rinse in distilled water for two to three seconds.

25. Immerse in 5% phosphomolybdic acid (solution N) for ten to fifteen minutes.

26. Rinse in distilled water for two or three seconds.

27. Immerse in aniline blue (solution O) for two to three minutes.

28. Rinse in distilled water for a second or so.

29. Immerse in acidulated water (solution Q) for five minutes or longer.

30. Wash with 95% alcohol.

31. Dehydrate with absolute alcohol.

32. Clear in xylol.

33. Mount in neutral Canada balsam in xylol, or in D.P.X., Clearmount, or Cristalite.

Results:

Reticulum: black. Elastin: red. Collagen: blue. Colloid: brown or grey. Nuclei: brownish black. Erythrocytes: light orange. Smooth muscle: light buff. Striated muscle: greyish blue or mauve. Epithelium: light greyish brown. Cellular debris: light greyish brown.

Notes:

(*a*) The technique is a modification of the Lewis & Jones (1951) recommended staining procedure for diagnosis of invading carcinomas, diseases of the cardiovascular system, tuberculosis and other inflammatory reactions.

(*b*) The authors (Humason & Lushbaugh) claim that their modification, described above, has resulted in better defined and more easily interpreted stain for the three connective tissue components; that there is less background colour than in the original method and sections, even as thick as 20 to 25μ show clear detail and exhibit a three-dimensional effect.

(*c*) Periodic acid oxidation is used in this technique in place of potassium permanganate, because the authors found the former to be more selective in its action, thereby producing a superior histological picture of connective tissue with even the smallest fibres well defined.

(*d*) In this technique the specific staining of collagen is strengthened by treatment of the sections with phosphomolybdic acid before staining with aniline blue: this also reduces cytoplasmic staining to a minimum.

(*e*) To prevent the sections from becoming detached from the slides during processing in the various solutions they were first dried thoroughly on albuminized slides on a warming plate or in an oven overnight, followed by fifteen minutes or more in a mechanical hot-air dryer. If a mechanical dryer is not available, the authors advise careful warming over a small flame until the paraffin wax around the sections just begins to melt. It is recommended that no attempt should be made to stain the sections on the same day that they are mounted on the slides.

(*f*) For more detailed information and colour plates, the original paper should be consulted.

Reference: Humason, G. L., Lushbaugh, C. C. (1960).

PEROXIDASE REACTION

For blood films

Solutions required:

A. Copper sulphate crystals .. 0·5 gm.
Distilled water .. .. .. 100 ml.

B. Benzidine base, pure .. .. 0·2 gm.
Distilled water .. .. .. 200 ml.
Filter and add 4 drops hydrogen peroxide (20 vols.) to the filtrate; store in a dark bottle.

C. Safranin .. .. 1% aqueous

Technique:

1. Air-dried blood films are flooded with Solution A for one minute. Pour off; then without washing and while still wet:

2. Flood with Solution B and leave for two minutes; then rinse in tap water.

3. Counterstain in Solution C for one minute.

4. Wash with tap water; blot and dry.

Results:

Peroxidase granules, blue. Nuclei of leucocytes, orange-red.

Note: Unmounted specimens keep for many months without fading.

Reference: Sato, A. & Shoji, K. (1927).

PHLOXIN - CHROME HAEMATOXYLIN

For differentiating cells of pancreatic islets

Solutions required:

A. Potassium permanganate, 1% aqueous

B. Sulphuric acid, 5% aqueous

C. Solution A .. .. .. 47·5 ml.
Solution B .. .. .. 3 ml.
Distilled water .. .. .. 49·5 ml.

D. Sodium metabisulphite, 3% aqueous

E. Gomori's chrome alum haematoxylin

F. Alcohol, 70% .. .. .. 99 ml.
HCl, conc. .. .. .. 1 ml.

G. Phloxin, 0·5% aqueous

H. Phosphotungstic acid, 5% aqueous

Technique:

1. Fix pieces of tissue in Bouin or Formalin–Zenker, and embed in paraffin wax.
2. Cut sections no thicker than 4μ and fix them to slides.
3. Dewax with xylol.
4. Pass through the usual graded alcohols into water.
5. Post-fix in Bouin for twelve to twenty-four hours.
6. Wash sections thoroughly in tap water until excess picric acid is removed.
7. Immerse in solution C for about one minute.
8. Decolorize with solution D.
9. Wash in water.
10. Stain in the haematoxylin for ten to fifteen minutes, controlling by microscopic examination at intervals until the beta cells are prominent, stained blue.
11. Differentiate in solution F for about one minute.
12. Wash in running tap water until the sections are clear blue.
13. Counterstain with the phloxine solution for five minutes.
14. Rinse in tap water.
15. Immerse in the phosphotungstic acid solution for one minute.
16. Wash in running tap water for five minutes, when the sections should regain their red colour.
17. Differentiate in 95% alcohol: if the section is overstained with the phloxine and the alpha cells do not stand out clearly, rinse for a few seconds in 80% alcohol.
18. Dehydrate with absolute alcohol.

19. Clear in xylol, and mount in D.P.X., or Cristalite on Canada balsam.

Results:

Alpha cells: red. Beta cells: blue. Delta cells are stained pink to red and are indistinguishable from alpha cells. Acinar zymogen granules: red to colourless in pancreas. In hypophysis, the alpha cells are pink; beta cells greyish blue, and not easily distinguishable from chromophobes. Nuclei reddish purple to bluish violet. Erythrocytes: deep pink. Smooth muscle: pink. Collagen is unstained. Goblet cell mucin appears coarsely granular and dark, stained slightly greenish-blue.

Note: The method is said to afford easy distinction of alpha and beta granules in pancreatic islets on material fixed twelve hours post mortem.

Reference: Bell, E. T. (1946).

PHLOXIN - HAEMATOXYLIN

For hyaline

Solutions required:

A. Ehrlich haematoxylin solution.

B. Phloxin 0.5% in 25% alcohol.

C. Lithium carbonate 0·1% aqueous.

Technique:

1. Stain in Ehrlich haematoxylin for five minutes.
2. Wash in water; then stain one half to one minute in the phloxine solution.
3. Wash in tap water; then decolorize in the lithium carbonate solution.
4. Wash in tap water; then dehydrate in the usual manner and mount in balsam.

Results:

Fresh hyaline appears as red droplets and threads; while older hyaline is pink to colourless; nuclei, blue.

Reference: Mallory, F. B. (1938), p. 207.

PHLOXIN - METHYLENE BLUE

Rapid smear technique for Negri bodies in brain tissue

Solutions required:

A. Phloxin 2% aqueous.

B. Methylene Blue (Loeffler).

Technique:

1. The brain to be examined should be removed as quickly as possible; then small segments, 3 to 4 mm. thick, are cut from Ammon's horn perpendicular to its long axis and placed in a Petri dish. Cut away adjacent tissue, leaving only the horn.

2. Place a segment, cut surface downwards, on the small end of a new one-inch cork; then with a matchstick, wipe peripheral tissue downward and outward, so that the segment is more firmly attached to the cork and the grey matter containing the pyramidal cells bulges upwards. Press this gently against a scrupulously clean slide, and make a smear by repeating this process along the whole length of the slide. This operation should be carried out rapidly before the tissue commences to dry.

3. Fix immediately by immersing in pure methyl alcohol for five to ten minutes.

4. Rinse in running water; then stain in the phloxin solution from two to five minutes.

5. Wash in running water; then stain in methylene blue (Loeffler) for ten to thirty seconds.

6. Decolorize in 80% alcohol; then dehydrate in 95% alcohol and two changes of absolute alcohol.

7. Clear in xylol and mount in balsam.

Note: The slides should be handled with forceps throughout to prevent the preparation being spoiled by coming into contact with the fingers.

Results:

Pyramidal cells, blue; Negri bodies, bright red to reddish brown.

Reference: Dawson, J. R. (1934/5).

PHLOXIN - METHYLENE BLUE - AZUR B

For normal and pathological animal tissues

Solutions required:

A. Phloxin .. .. 0·5 gm.
Acetic acid 0·2% aqueous .. .. 100 ml.
Filter before use.

B. Methylene blue .. .. .. 0·25 gm.
Azur B .. .. 0·25 gm.
Borax.. .. .. 0·25 gm.
Distilled water .. .. .. 100 ml.

C. Acetic acid 0·2% aqueous.

Technique:

1. Fix sections to slides: remove paraffin and pass through the usual descending grades of alcohol to distilled water.
2. Immerse in solution A for one to two minutes.
3. Rinse well in water.
4. Stain for one half to one minute in solution B.
5. Destain partially in solution C.
6. Differentiate in three washes of 95% alcohol.
7. Dehydrate with two changes of absolute alcohol.
8. Clear in two changes of xylol.
9. Mount in synthetic medium (such as D.P.X. or Clearmount, etc.).

Results:

Nuclei and bacteria are stained blue with some metachromasia. Collagen and other tissue elements are bright pink to red. Erythrocytes, bright scarlet.

Note:

This is a rapid modification of Mallory's original method reducing the staining time from one hour or more to one to two minutes, and permitting good staining with formalin fixed tissues which is not possible with Mallory's original method which was designed for Zenker-fixed material. Colophony differentiation is obviated, and Phloxin is not washed out as in the original Mallory technique.

Reference: Thomas, John T. (1953).

PHLOXIN - TARTRAZINE

A general histological stain and for the demonstration of inclusion bodies

Solutions required:

A. Haemalum (Mayer).
B. Calcium chloride 0·5% aqueous. 100 ml.
Phloxine .. .. 0·5 gm.
C. Tartrazine, saturated in cellosolve.

Technique:

1. Stain for five to ten minutes with the haemalum, examining under the microscope at intervals, until the desired depth of staining has been attained.
2. Wash and blue in tap water or in saturated aqueous lithium carbonate.
3. Stain in the phloxine solution for half an hour.
4. Rinse quickly in water.
5. Drain off excess water and replace with tartrazine solution (Solution C, as above), using a dropping bottle to control the differentiation.

Note: The tartrazine replaces the phloxine from collagen. As tartrazine is readily soluble in water, slight overstaining is recommended before dehydration.

6. Rinse in 60% alcohol followed by 95% alcohol.
7. Dehydrate with absolute alcohol.
8. Clear in xylol and mount.

Results:

Kurloff bodies in guinea pig's lung are well shown. Inclusion bodies of a number of virus-containing tissues show retention of phloxine.

Notes: (*a*) Fixatives containing mercuric chloride give the best results.

(*b*) The author (Landrum) claims that this technique, in which use is made of a stable phloxine solution, gives a brilliant demonstration of certain inclusion bodies and is superior to Masson's erythrosin-saffron, which deteriorates fairly rapidly.

Reference: Lendrum, A. C. (1947).

PHOSPHOTUNGSTIC ACID-EOSIN-HAEMATOXYLIN

(After Massignani and Malferrari, 1961)

For Negri bodies

Solutions required:

A. Harris haematoxylin, without acetic acid, or Carazzi haematoxylin.

B. HCl, conc. .. .. 1 ml.
Distilled water .. .. .. 200 ml.

C. Lithium carbonate, saturated, aqueous.

D. Solution C .. .. 1 ml.
Distilled water .. .. .. 200 ml.

E. Eosin, yellowish, water soluble .. 1 gm.
Phosphotungstic acid .. .. 0·7 gm.

Grind together with a mortar and pestle, then add 10 ml. distilled water and grind into a paste. Pour in 90 ml. of absolute alcohol, in lots of about 10 ml. at the time, and grind until all the solid has gone into solution. Pour the solution into a 250 ml. measuring cylinder. Rinse the dye off the pestle into the mortar with not more than 50 ml. absolute alcohol. Transfer the washings to the dye solution in the cylinder. Now rinse out the mortar with 50 ml. absolute alcohol and add the rinsings to the cylinder. Make up the volume of the stain in the cylinder to 200 ml., then transfer it to a beaker and add two drops of solution C. Stir for ten minutes with mechanical stirring (a domestic type (kitchen) mixer will serve the purpose). Restore the volume of the mixture to 200 ml. with absolute alcohol.

This preparation may then be used for preparing either solution F or G as below:

F. Solution E, merely filtered.

G. Centrifuge solution E for forty minutes at 1,500 revolutions per minute, in a refrigerated centrifuge.

H. As solution E but distilled water is used instead of absolute alcohol. Centrifuge the suspension so obtained, then dissolve the precipitate in absolute alcohol. Add this alcoholic solution to the supernatant to produce the staining solution.

Notes:

(i) Solutions F, G and H, above, are referred to as Eosin solutions, Nos. 1, 2 and 3 respectively. Only one of them is required in the technique given below.

(ii) It is stated in the original paper that it is necessary that the two reagents (eosin and phosphotungstic acid) are ground together in the solid state, otherwise the dye-mordant combination takes twenty-four hours to form.

Technique:

1. Slices of hippocampus (Ammon's horn) from brains of cows, dogs and cats with street rabies virus, and hippocampus from brains of mice, inoculated or infected with human rabies, as well as normal material (to be used as controls), are fixed for twenty-four hours in a mixture of one volume of absolute alcohol and two volumes of saturated aqueous mercuric chloride.

2. Rinse thoroughly in 70% alcohol.

3. Dehydrate in the usual way and embed in paraffin wax.

4. Cut sections at 4μ and place on dry slides previously covered with glycerin albumen fixative.

5. Remove paraffin wax as usual and carry the preparations through the usual graded alcohols to 70% alcohol.

6. Treat for the removal of mercuric deposits.

7. Wash well in tap water.

8. Stain in solution A for two minutes.

9. Wash in running tap water for five minutes.

10. Dip eight times in solution B.

11. Wash for five minutes in running tap water.

12. Immerse in solution D for one minute.

13. Wash in running tap water for ten minutes.

14. Dehydrate by dipping ten times in each of 50%, 70%, 80%, 90%, 95% and absolute alcohol.

15. Stain for eight minutes in solution F or G or H.

16. Wash thoroughly in distilled water.

17. Start the dehydration by rinsing briefly in 50%, 70%, 80% and 90% alcohol.

18. Rinse well in 95% alcohol.

19. Finish the dehydration by immersing for four minutes in each of two or three changes of absolute alcohol.

20. Dry carefully with filter paper.

21. Clear by immersion for four minutes each in two changes of xylol.

22. Mount in Canada in xylol or D.P.X.

Results:

Negri bodies: deep red, with the internal structure clearly delineated. Some of the Negri bodies showed inclusions like small granules encircled by a lighter halo, with basophilic granulations inside. Others had a dispersed fine granulation inside, intensified by their strong acidophilia and a large inclusion, also acidophilic, with a slightly basophilic centre. The Negri bodies, by reason of their characteristic internal structures, were clearly differentiated from erythrocytes. The nucleoli were shown as basophilic and could not possibly be mistaken for Negri bodies.

Notes:

(*a*) Of the three eosin solutions, the authors consider that No. 3 (i.e. solution H) gives the best results.

(*b*) The authors state that in view of their experience in using an alcoholic solution of acid eosin of (Schleicher, 1953) and another type (Kopsch, 1949–50) they are of opinion that the eosin used in their technique (Massignani and Malferrari, 1961) does not act solely as an acid eosin, but rather as a dye-mordant compound in which pH is not important.

(*c*) Copies of the original paper are obtainable from the authors, in English, Portuguese, Italian and French, as well as colour pictures and slides. Readers should refer to the original paper for further information.

Reference: Massignani, A. M. and Malferrari, R. (1961).

PHOSPHOTUNGSTIC HAEMATOXYLIN

An artificially ripened haematoxylin stain for formalin-fixed material

(After Ethel Lieb, 1948)

Solutions required:

A. Potassium permanganate, 0·5% aqueous
Note: This solution must be freshly prepared.

B. Oxalic acid, 2% aqueous

C. Iron alum, 4% aqueous

D. Phosphotungstic acid, 10% aqueous

E.

Haematoxylin	0·5 gm.
Red mercuric oxide	0·25 to 0·5 gm.
Hydrogen peroxide	2 ml.
Solution D	100 ml.
Distilled water	400 ml.

Place the distilled water in a 1 litre flask, add the haematoxylin and shake until dissolved. Add the phosphotungstic acid solution, then shake well. Heat to boiling point. Remove the flask from the source of heat; then cautiously add the mercuric oxide and shake to dissolve. Leave the mixture to cool to room temperature or thereabouts. Add the hydrogen peroxide and shake well. Plug the flask lightly with cotton wool, then allow it to stand for a few days to a week. Filter into the stock bottle, when the solution has assumed a deep brownish colour, which indicates that it is ready for use.

Technique:

1. Take paraffin section of formalin-fixed material down to water as usual.
2. Immerse in solution A (potassium permanganate) for five minutes.
3. Rinse in tap water.
4. Bleach by immersion in solution B (oxalic acid) for five minutes.
5. Wash in tap water.
6. Wash in distilled water.

7. Mordant in solution C (iron alum) for one hour.
8. Rinse well in tap water.
9. Wash in distilled water.
10. Immerse in the haematoxylin (solution E) for two to twenty-four hours.
11. Dehydrate in two changes of absolute isopropyl alcohol.
12. Clear in xylol.
13. Mount in D.P.X. or Emexel or Canada balsam in xylol.

Notes:

(*a*) The authoress states that despite the number of mordants proposed, it is difficult to make consistently good phosphotungstic acid haematoxylin preparations of formalin-fixed material. The difficulty may be due to inadequately oxidized staining solution, but the method described above has been found to give results that have been completely successful in every detail.

(*b*) Staining in the haematoxylin solution is usually complete in two or three hours, and should be checked at intervals, by microscopic examination.

(*c*) Bleaching in oxalic acid and potassium permanganate is stated to be necessary to give sharp contrast.

Reference: Lieb, E. (1948).

PHOSPHOMOLYBDIC ACID - EOSIN

For choline-containing lipids

Solutions required:

A. Acetone .. .. 1 volume
Ether .. .. 1 volume
B. Chloroform .. .. 1 volume
Absolute alcohol .. .. .. 1 volume
C. Phosphomolybdic acid.
1% in solution B.
D. Stannous chloride 1%.
in 3N hydrochloric acid.
E. Eosin 1% aqueous.

Technique:

1. Dip frozen sections into acetone-ether.

2. Immerse in the phosphomolybdic acid solution for fifteen minutes.

3. Rinse in solution B.

4. Dip into solution D.

5. Counterstain with the eosin solution for one to two minutes.

6. Mount in glycerine jelly.

Results:

Positive areas are stained blue, whilst negative areas are red.

Reference: Landing, B. H., Uzman, L. L. & Whipple, Ann (1952).

PICRO FUCHSIN (VAN GIESON) - HAEMATOXYLIN

For collagen and connective tissue

Solutions required:

A. Ehrlich haematoxylin

B. Van Gieson stain (picro fuchsin)

Technique:

1. Paraffin sections are mounted on slides and brought down to distilled water as usual.

2. Stain in Ehrlich haematoxylin for five to thirty minutes.

3. Blue in tap water in the usual manner; then examine while still wet, under the microscope: the nuclei should be dark blue, but if they are overstained or understained treat as described under Haematoxylin (Ehrlich) - Eosin (Stage 4).

4. Stain for three to five minutes with picro fuchsin; then rinse for a few seconds in water.

5. Examine while still wet, under the microscope, and continue the staining with picro fuchsin or continue the differentiation with water, whichever is necessary.

6. Dehydrate as usual; clear in xylol; mount in D.P.X. or in Cristalite, or Clearmount.

Results:

Nuclei of cells are stained dark brown to black. Collagen fibres: bright red. Erythrocytes, muscle, epithelia and other tissues are stained yellow.

Note:

If better colour contrast is desired in the nuclei it is recommended that Weigert haematoxylin be used in place of Ehrlich.

References:

Van Gieson, J. (1889).
Carleton & Leach (1947).

PICRO - GOMORI

(After D. W. Menzies, 1959)

A rapid and simple technique, being a modification of Mallory stain for the mass staining of pathological material for diagnostic and teaching purposes

Solutions required:

A. Haematoxylin (Weigert), A
B. Haematoxylin (Weigert), B
C. Alcohol, 95% .. .. .. 99 ml.
Glacial acetic acid .. .. 1 ml.
D. Picric acid, saturated, aqueous
E. Solution D .. .. .. 100 ml.
Orange G .. .. 1 gm.
F. Light green SF, yellowish, 10% aqueous
G. Phosphotungstic acid, 10% aqueous
H. Chromotrope 2R, 10% aqueous
I. Acetic acid, 10% aqueous ..
J. Solution F .. .. .. 3 ml.
Solution G .. .. .. 6 ml.
Solution H .. .. .. 6 ml.
Solution I .. .. .. 10 ml.
Water .. .. 75 ml.
K. Acetic acid, 1% aqueous

Technique:

1. Fix pieces of tissue in formol sublimate and embed in paraffin wax.

Note: The author states that formalin-fixation gives good results, but mercurial fixatives are superior, and a good general routine is to fix in 10% formol-saline. In the latter case the trimmed blocks should be immersed in a mercurial mixture for two hours before processing in the histokine or autotechnicon.

2. Carry paraffin sections through to water.
3. Treat for the removal of mercurial precipitate.
4. Stain in a mixture of equal volumes of solutions A and B for five to fifteen minutes.
5. Differentiate in acid alcohol (solution C).
6. Blue in tap water.
7. Immerse in solution E for one minute.
8. Wash in running water for five to ten minutes, controlling by microscopic examination at intervals until only the erythrocytes are coloured yellow.
9. Immerse in solution J for two minutes.
10. Immerse for one minute in each of three changes of 1% acetic acid.
11. Rinse in water.
12. Dehydrate through the usual graded alcohols.
13. Clear in xylol.
14. Mount in D.P.X. or Clearmount or Michrome mountant.

Results:

Erythrocytes: brilliant orange-yellow. Fibrin: orange-red or red. Cytoplasm of smooth muscle: deep purplish red. Epithelial cytoplasm varies from red to green. Collagen: clear green.

Notes:

(*a*) No special claim is made for this technique as a research tool. Its outstanding merits, according to the author, are speed and ease of operation and the fact that the need for microscopic control is reduced to the minimum.

(*b*) The important contrasts between smooth muscle and collagen, and between fibrin and erythrocytes are said to be consistently demonstrated, and it is the consistency of the staining

image which, according to the author, makes the technique a reliable one for routine work more particularly in laboratories where staining is carried out on a mass scale for teaching and diagnostic purposes.

(*c*) The author (Menzies) found that staining at the rate of seventy to eighty slides a day, the solutions used in dishes holding about 800 ml. each, required replacement every six to eight weeks. The slides were carried from dish to dish in stainless steel slotted racks.

(*d*) The method had been in routine use for diagnostic purposes in Menzies' laboratories for four years before the date of the paper referred to above.

(*e*) All the solutions listed above are stable.

Reference: Menzies, D. W. (1959).

PICRO ANILINE BLUE - EOSIN

For urinary casts

Solutions required:

A. Eosin yellowish 0·5% aqueous.

B. Aniline blue 1% aqueous .. 1 ml.
Picric acid saturated, aqueous .. 10 ml.
Glycerine .. .. 10 drops

Technique:

1. The urine is centrifuged and the supernatant liquid decanted as usual for microscopic examination.

2. One drop of eosin solution is added to the sediment and mixed by shaking from side to side for one to two minutes.

3. Two drops of Solution B are added and mixed. The colour of the sediment should now be distinctly blue-green; if it is reddish brown more of Solution B should be added till the blue-green colour is obtained, but too much should be avoided.

4. Some of the stained sediment is then transferred to a slide, covered with a coverglass and examined.

Note: The amounts of the two stains may be varied according to the amount and particular character of the sediment present. More eosin may be added if the cells have not been stained sufficiently red. Enough of the aniline blue should be added to stain the casts a distinct blue, but too much will stain them too dark.

More permanent slides may be made by adding more glycerine to the sediment and sealing the edges of the cover glass with Laktoseal.

This technique brings out the detailed structure of casts and cast-like bodies in a remarkable way. It does not furnish a differential stain since all the mucous material is also stained blue.

Results:

Hyaline casts are stained a clear blue of varying intensity. The more irregularly shaped bodies, sometimes classed as cylindroids, are similarly stained. An irregular distribution of material, a " mealy " structure, or a striated appearance sometimes becomes evident in bodies which appear perfectly homogeneous before staining. Mucous threads and amorphous material are also stained blue; they are clearly differentiated from the cast-like bodies by their structure. Granular material is usually stained darker. Mixed, fine granular casts present a striking picture of fine, dark granules powdered over the light blue, hyaline body. Some coarsely granulated casts are stained deep blue; the granules of the others are yellow, orange or dark reddish brown. Renal epithelial cells are usually red, sometimes orange or yellowish. Red blood cells are stained a brighter red. Pus cells are usually red, occasionally blue. Epithelial cells from the urinary passages are either red or blue.

Fat globules are unstained. In cells which are undergoing fatty degeneration, the fat cells are seen strikingly against reddish cellular material. The picric acid gives a light yellowish background.

Often the stain reveals the presence of lightly stained mucus-like envelope, apparently covering the cast material. This material is often clearly seen at the end of a cast which has apparently been squarely broken off.

Reference: Behre, J. A. & Muhlberg, W. (1936/7).

PINACYANOL - NEUTRAL RED

For supra-vital staining of blood

Note: Pinacyanol is superior to janus green in that it does not fade: it is very selective for mitochondria, and it does not inhibit the effect of neutral red. It has the disadvantage, however, of being extremely costly.

Solutions required:

Stock solutions:

A. Pinacyanol 0·1% in absolute alcohol.

B. Neutral Red 0·1% in absolute alcohol.

Staining solutions:

C. *for mitochondria only*

Solution A	1 ml.
Absolute alcohol	20 ml.

D. *for mitochondria, nuclei and other cell granules*

Solution A	1 ml.
Solution B	2 ml.
Absolute alcohol	20 ml.

Notes:

(*a*) Solutions C and D deteriorate after a few hours and should, therefore, be prepared only as and when they are required for immediate use.

(*b*) The proportions and dye concentrations of either of these two solutions may be varied to suit particular specimens.

Technique:

1. Scrupulously clean dry slides are flooded with solution C or D, whichever is required.

2. Drain and leave to dry, then use as follows (within a few hours at latest in the case of solution D as the dried dye combination does not keep well).

3. Place a drop of blood on each slide.

4. Cover with scrupulously clean coverslip and allow the blood to spread.

5. Seal the edges of the coverslips with soft paraffin wax (M.P. 38° C.), or Laktoseal, and examine under the microscope.

Results:

With Solution C (Pinacyanol only)

Mitochondria in still living and motile cells are stained deep blue to violet.

With Solution D

Mitochondria stained as above; nuclei and other cell granules are stained red.

Reference: Hetherington, D. C. (1936).

PICRO - NIGROSIN

For eleidin and keratin

Solutions required:

A. Picric acid, saturated, aqueous.

B. Nigrosin 1% aqueous.

C. Terpineol .. .. 1 volume
Origanum oil .. .. .. 1 volume

Technique:

1. Tissues should be fixed in 10% formalin, and frozen sections employed.
2. Sections are stained for five minutes in the picric acid solution.
3. Rinse in distilled water.
4. Stain in the nigrosin solution for one minute.
5. Wash in distilled water.
6. Rinse in 95% alcohol.
7. Clear in Solution C.
8. Mount in balsam or in Cristalite or D.P.X.

Results:

Eleidin: blue-black. Keratin: bright yellow.

Reference: Buzzi (1898).

PONTACYL BLUE BLACK SX - LUXOL FAST YELLOW

A general stain for animal tissues

Solutions required:

A.	Pontacyl blue black SX ..	1 gm.
	Tap water	100 ml.
B.	Potassium dichromate	2 gm.
	Distilled water	100 ml.
C.	Solution A	30 ml.
	Solution B	10 ml.
D.	Chrome alum, cryst. ($12H_2O$) ..	2 gm.
	Tap water	100 ml.
E.	Solution A	25 ml.
	Solution D	25 ml.
F.	Luxol fast yellow TN	2 gm.
	Alcohol, 95%	100 ml.

Technique:

1. Fix pieces of material in 10% formalin.
2. Dehydrate in graded alcohols.
3. Clear in xylol.
4. Embed in paraffin wax.
5. Fix sections to slides and remove paraffin wax with xylol as usual.
6. Pass through the usual graded alcohols into distilled water.
7. Stain in solution C or E for fifteen minutes.
8. Wash in tap water.
9. Differentiate in 70% alcohol until no more colour runs out of the section.
10. Counterstain in the luxol fast yellow (solution F) for five minutes.
11. Dehydrate with absolute alcohol.
12. Mount in Clearmount or Michrome mountant or proceed as follows:
13. Clear in xylol.
14. Mount in D.P.X. or Emexel or Clearmount or Michrome mountant.

Results with solution C (nuclear stain):

Nuclei: bluish green. Cytoplasm: pale yellowish green. Keratin: bright blue. Collagen: bright yellow. Muscle: pale green. Dark mitotic figures and nucleoli are quite well in evidence. After appropriate fixation, the secretion granules of the pancreatic acinar cells are well demonstrated. Elastic and hyaline cartilage are differentiated, the former staining green with the latter remaining clear.

Results with solution E (nuclear stain):

Nuclei: blue-black. Cytoplasm: deeper green than with solution C. In general the rest of the tissue-components are stained much the same as when solution C is used.

Notes:

(*a*) Good results were obtained with sections from a variety of organs from mice, monkeys and humans, using a number of fixatives which included 10% formalin, Bouin, Zenker, Susa–Heidenhain and strong Flemming's solution. Although pontacyl blue black SX is an acid dye, the authors found it to be an excellent nuclear stain even when used as a simple 1% aqueous solution, although it was found necessary to apply this solution to sections for an hour. The addition of a suitable mordant (chrome alum or potassium dichromate) was found to intensify the nuclear staining and reduce the time to fifteen minutes. Pontacyl blue black SX was used by Lillie (1945) as a stain for collagen.

(*b*) It is the belief of the authors that there are not many brilliant yellow stains among the known biological stains, and state that the results obtained with luxol fast yellow TN give a pleasing effect, which is so easy to achieve that it should become a very useful counterstain.

(*c*) See also pages 115 and 384.

Reference: Wood, M. L. & Green, J. A. (1958).

PONTACYL VIOLET - LUXOL FAST YELLOW TN

A general stain for animal tissues

Solutions required:

A.	Pontacyl violet	2 gm.
	Tap water	100 ml.

B.	Chrome alum	2 gm.
	Distilled water	100 ml.
C.	Solution A	25 ml.
	Solution B	25 ml.
D.	Ammonium chromate	2 gm.
	Distilled water	100 ml.
E.	Solution A	25 ml.
	Solution D	25 ml.
F.	Luxol fast yellow TN	2 gm.
	Alcohol, 95%	100 ml.

Technique:

1. Fix tissues in 10% formalin.
2. Dehydrate through the usual ascending grades of alcohol.
3. Clear in xylol.
4. Embed in paraffin wax.
5. Fix sections to slides and remove paraffin wax with xylol as usual.
6. Hydrate through the usual descending grades of alcohol.
7. Stain in solution C for fifteen minutes, or in solution E for one hour.
8. Wash in tap water.
9. Differentiate in 70% alcohol until no more colour runs out of the section.
10. Counterstain in luxol fast yellow (solution F) for five minutes.
11. Dehydrate with absolute alcohol.
12. Mount in Clearmount or in Michrome mountant, or proceed as follows:
13. Clear in xylol.
14. Mount in D.P.X. or Emexel or in Clearmount or Michrome mountant.

Results with solution C (nuclear stain):

Nuclei: deep purple to red. Collagen: brilliant yellow. Keratin: purple. Cartilage: yellow. Secretion granules of pancreas: red.

Results with solution E (nuclear stain):

Nuclei: pale violet. Cytoplasm: yellow to brown. Keratin: purple. Collagen and cartilage: yellow. Secretion granules of

pancreas: not clearly defined. Mitotic figures exhibit conspicuous metachromatic staining.

Note: The picture is improved if a green filter is used with the microscope lamp.

Notes:

(*a*) In the original paper, which also deals with the pontacyl blue black–luxol fast yellow technique, the authors remark that it is rather surprising that pontacyl blue black SX, which was used by Lillie (1945) as a stain for collagen has now been found to be a good nuclear stain, in simple aqueous solution, without a mordant.

(*b*) Both pontacyl blue black SX and pontacyl violet 6R are acid stains, but the first has one amino group and can, therefore, be regarded as amphoteric, whereas pontacyl violet is wholly acid. It is possible that here these two dyes are acting as hydrogen ion indicators. The addition of a suitable mordant to the aqueous solutions of either of these dyes reduces the staining time very considerably.

(*c*) The advantages of luxol fast yellow TN as a counterstain are mentioned on page 382, note (*b*).

Reference: Wood, M. L. & Green, J. A. (1958*a*).

PX STAIN

For keratin, vaginal smears, etc.

Technique:

1. Fix smears while still wet in a mixture consisting of equal parts absolute alcohol and ether, for two minutes.
2. Rinse in 70% alcohol.
3. Rinse in 50% alcohol.
4. Rinse in water.
5. Immerse in the staining solution for three minutes.
6. Immerse in dioxane for a few seconds.
7. Wash with absolute alcohol rapidly.
8. Rinse well in xylol and mount.

Results:

Nuclei: stained red. Cornified cells: red, pink or orange. Erythrocytes: orange. Basophile cells: blue or green.

Reference: Gurr, E. (1956, 1958*a*).

PROTARGOL - GALLOCYANIN

For nerve fibres, sheaths and cells

Solutions required:

A. Protargol (silver proteinate) .. 1% aqueous.
(Prepared by sprinkling the protargol powder on the surface of the water and leaving it to dissolve.)

B. Protargol 1% aqueous .. 50 ml.
Alcohol 95% .. .. .. 50 ml.
Pyridine pure .. .. .. 0·5 ml.

Note: The quantity of pyridine may be varied between 0·1 ml. and 2 ml. The higher concentrations facilitate the staining of thin fibres, whereas cell bodies and dentrites are better demonstrated with the lower proportions of pyridine.

C. Boric acid .. .. .. 1·4 gm.
Sodium sulphite anhydrous .. 2 gm.
Hydroquinone .. .. .. 0·3 gm.
Acetone .. .. 15 ml.
Distilled water .. .. .. 85 ml.
Dissolve each reagent in the above order in the water adding the next only after the previous one has been dissolved entirely.

D. Brown gold chloride .. .. 0·2 gm.
Distilled water .. .. .. 100 ml.
Glacial acetic acid .. .. 1 ml.

Note: Solutions A, B, C and D must be stored in dark bottles.

E. Oxalic acid 2% aqueous.

F. Sodium thiosulphite 5% aqueous.

G. Gallocyanin solution (Einarson).

H. Phosphotungstic acid 5% aqueous.

I.	Aniline Blue 1% aqueous	1 ml.
	Fast Green FCF	0·5 gm.
	Orange G	2 gm.
	Glacial acetic acid	8 ml.
	Distilled water	91 ml.

Note: This solution should be diluted 2 : 3 with distilled water before use.

Technique:

Note: Metallic instruments must not be used in the following procedures which should be carried out in a darkened room. Sections should be stained in black embryo dishes or in tubes covered all round with thick brown or black paper.

Fix in 10% formalin and embed in Celloidin or L.V.N. Sections are cut 15 to 25μ in thickness.

1. Immerse for twenty-four hours in ammoniated alcohol (conc. ammonia 1 ml., alcohol 50% 99 ml.).

2. Drain well and transfer to Solution A for six to eight hours at 37° C.

3. Drain well and transfer to Solution B in another staining dish, for twenty-four to forty-eight hours at 37° C.

4. Rinse for about five to ten seconds in 50% alcohol; then reduce with Solution C for about ten minutes.

5. Wash in several changes of distilled water; then tone in Solution D for ten minutes.

6. Wash in several changes of distilled water; then transfer for one to three minutes in Solution E, afterwards rinsing in distilled water.

7. Immerse for three to five minutes in Solution F; then wash thoroughly in distilled water.

8. Counterstain overnight in Solution G.

9. Wash thoroughly in distilled water then immerse in Solution H for thirty minutes.

10. Without washing transfer section to diluted Solution I for one hour; then wash with 70% alcohol and differentiate the counterstain in 95% alcohol.

11. Transfer to normal butyl alcohol; clear in cedarwood oil and mount.

Results:

Nerve fibres and neurofibrils: blue-black. Nissl bodies: pale blue. Nuclei: blue-black with silver and gold if a higher percentage of pyridine was used in solution. Myelin sheaths: bright yellow. Connective tissues: various shades of blue and green.

Reference: Foley, J. C. (1943).

PURPURIN

For calcium deposits in pathological tissues

Solutions required:

A. Purpurin, saturated in absolute alcohol (about 0·7%).

B. Sodium chloride, reagent grade, 0·75% aqueous.

Technique:

1. Fix material in 90% alcohol.
2. Dehydrate; clear; embed in paraffin wax.
3. Fix sections to slides; dewax and pass through the usual descending grades of alcohol to distilled water.
4. Stain in the purpurin solution for about ten minutes.
5. Immerse in sodium chloride solution for about five minutes.
6. Rinse with 70% alcohol until the stain ceases to come away in clouds.
7. Rinse with 90% alcohol and dehydrate with absolute alcohol.
8. Clear in xylol and mount in balsam.

Results:

Calcium deposits are stained red.

References:

Grandis, V. & Mainini, C. (1900).
Gurr, E. (1958*a*).
Schmorl, C. G. (1928).

PYRONIN - ALPHANAPHTHOL

(After Graham)

For oxidase granules in blood smears

Solutions required:

A. α-Naphthol, pure .. .. 1 gm.

Alcohol 40% .. .. .. 100 ml.
Hydrogen peroxide 20 vols. .. 0·2 ml.

Note: This solution deteriorates in four or five days when the hydrogen peroxide has been added, and it is, therefore, better not to add the hydrogen peroxide until the solution is required for immediate use.

B. Pyronin Y.. .. .. .. 0·1 gm.
Alcohol 40% .. .. .. 96 ml.
Aniline oil.. .. .. .. 4 ml.

C. Methylene blue 0·5% aqueous.

Technique:

1. Freshly spread air-dried blood smears are fixed in 10% formalin for two minutes; then washed well in distilled water.

2. Stain for five minutes in Solution A (as above); then wash for fifteen minutes in running water.

3. Stain for two minutes in Solution B (as above); then wash in distilled water.

4. Stain for one half to one minute in Solution C (as above); then wash with water; blot and dry thoroughly; mount in balsam.

Results:

Neutrophile granules, which give the oxidase reaction, are stained purple to red; while eosinophile granules are lighter red, larger and more refractile. Basophile granules are stained a deep purple; cell nuclei, blue; cytoplasm, pale blue; erythrocytes appear greenish yellow to pink.

Reference: Graham, G. S. (1916).

QUINCKE REACTION

For haemosiderin

Solutions required:

A. Ammonium sulphide solution,
concentrated .. .. .. 1 volume
Absolute alcohol .. .. 3 volumes

B. Basic fuchsin 0·5% in 50% alcohol.

Technique:

1. Tissues are fixed in neutral formalin 10%, or in absolute alcohol, and embedded in paraffin wax or Celloidin in the usual manner.

2. Bring sections down to distilled water, then immerse them from two to forty-eight hours in the ammonium sulphide solution.

3. Rinse thoroughly in distilled water.

4. Counterstain in the basic fuchsin solution for five to twenty minutes.

5. Wash in water; drain well; rinse in 80% alcohol.

6. Differentiate and dehydrate in absolute alcohol; clear in xylol and mount in Canada balsam or in Cristalite or D.P.X.

Results:

Haemosiderin, dark brown to black.

Reference: Quincke, H. J. (1880).

RAFFERTY'S STAIN
(Light Green-Haematoxylin)

A general purpose stain

Solutions required:

A. Haematoxylin (Ehrlich).

B. Light green (Michrome No. 240) .. 0·125 gm.
Water .. .. 100 ml.

Technique:

1. Stain sections in solution A for five to ten minutes.
2. Wash in tap water until blue.
3. Stain in solution B for five minutes.
4. Wash in water.
5. Dehydrate through the usual graded alcohols.
6. Clear in xylol and mount as usual.

Results:

Cellular elements are stained in various shades from purple to dark brown. Connective tissue fibres are stained, individually, a distinct green.

Notes:

(i) *For embryonic tissues it is advisable to use* 0·5 *per cent light green instead of* 0·125 *per cent if less mature fibres are to be displayed.*

(ii) In this technique there appears to be a series of colour compounds formed by the chemical union of light green with the complex haematoxylin-alum-tissue molecules.

(iii) This method, which is superior to the haemotoxylin-eosin procedure for displaying fibre structure in tissue sections was discovered by Mr. J. Rafferty, Senior Technician, Department of Anatomy, University College, Cork, during the course of work on the development of the Falg method.

Reference: Rafferty, J. (1960), unpublished.

RHODAMINE B - ANILINE METHYLENE BLUE

For splenic and lymphoid tissues

Solutions required:

A.	Methylene Blue, 2% alcoholic ..	10 ml.
	Aniline water	15 ml.
	Distilled water	30 ml.
B.	Rhodamine B 1% aqueous ..	2·5 ml.
	Distilled water	47·5 ml.
C.	Solution A (above)	3 volumes
	Solution B (above)	7 volumes

Technique:

Tissues are fixed in Zenker-Formol and embedded in paraffin wax.

1. After removal of mercurial precipitate by treatment with iodine in the usual manner (*see* pages 496–8) sections are stained two to three hours in Solution C; then washed rapidly with absolute alcohol.

2. Clear in xylol; mount.

Results:

Basophile protoplasm: blue. Chromatin: violet blue. Nucleoli: red. Connective tissue: faintly stained yellowish red. Muscle: yellowish red. Erythrocytes: bright red. Acidophile granules of leucocytes: bright red. Hyalin and granules of Russell: bright red. Nuclei of the small lymphocytes are faintly stained violet.

Reference: Houcke, E. (1928).

RESORCIN - FUCHSIN STAIN SIMPLIFIED

(After M. Krutsay, 1960)

For elastic fibres and as a substitute for Schiff's reagent in histology

Solutions required:

A. Basic fuchsin, 0·5% in 96% alcohol .. .. 100 ml.
Resorcinol.. .. .. .. 2 gm.
HCl, conc. .. .. .. 0·5 ml.
Distilled water .. .. .. 1·5 ml.

B. Formaldehyde, 40% .. ..

C. Solution A ⎫ equal
Solution B ⎭ volumes

Note: Solution A will remain stable for several months, but solution C should be prepared as and when required for immediate use.

D. Picric acid, saturated, aqueous

E. Periodic acid, 0·5% aqueous

TECHNIQUE I

For elastic tissues

1. Paraffin sections of formalin-fixed material are fixed to slides and dewaxed in the usual manner.
2. Wash in absolute alcohol.
3. Stain in solution C for thirty minutes.
4. Rinse in tap water.
5. Wash in 96% alcohol for one to two minutes to remove excess stain.

6. Rinse in distilled water.
7. Counterstain in the picric acid solution for two minutes.
8. Wash in 96% alcohol for one minute.
9. Dehydrate in alcohol.
10. Clear in xylol and mount in Canada balsam in xylol or D.P.X.

Results:

Elastic fibres: deep purple to violet. Nuclei: pale red. Cartilage: matrix: violet. Cytoplasm: yellow.

TECHNIQUE II

For use after periodic acid oxidation

1. Fix paraffin sections to slides and dewax in the usual manner.
2. Pass sections through to usual graded alcohols down to distilled water.
3. Oxidize in the periodic acid solution for twenty minutes.
4. Wash in tap water for five minutes.
5. Wash in 96% alcohol for one to two minutes.
6. Stain in solution A (basic fuchsin) for twenty minutes.
7. Rinse in tap water.
8. Wash in 96% alcohol for one to two minutes.
9. Dehydrate in absolute alcohol.
10. Clear in xylol and mount.

Results:

The following are strongly stained purplish red:

Thyroid and hypophyseal colloids, mucin, goblet cells, sarcolemma, basement membranes, glycogen, lipofuscin, hypophyseal basiphil cell granules, corpora amylacea, fungi, calcareous deposits, recent arterial hyalin and hyalin droplets and casts of renal tubules.

The following are stained moderately red:

Fibrin, connective tissue fibrinoid, amyloid, brush borders, and cartilage matrix.

The following are stained pink:

Collagen, reticular tissue, neuroglia, and myelin sheaths.

The following are unstained:

Cytoplasm, chromatin, muscle, elastic fibres, erythrocytes, and keratin.

TECHNIQUE III

For use after acid hydrolysis

1. Fix the sections to slides and remove the paraffin wax with xylol.
2. Pass through the usual descending grades of alcohol into water.
3. Hydrolyse in N/1 HCl at 58° C. for twelve minutes.
4. Rinse in tap water.
5. Stain in solution A (basic fuchsin) for twenty minutes.
6. Rinse in tap water.
7. Wash in 96% alcohol for one to two minutes.
8. Dehydrate in absolute alcohol and clear in xylol.
9. Mount in D.P.X. or Clearmount or Cristalite or if not available, in neutral Canada balsam in xylol.

Results:

Chromatin: purplish red. Other tissues: colourless.

Reference: Krutsay, M. (1960).

Notes:

The original author (Krutsay) used phenol-xylene and xylene in his dehydrating-clearing sequence, but if absolute alcohol is available, phenol-xylene should not be essential.

ROSE BENGAL - PHLOXIN

For staining and counting eosinophils in canine blood

(After C. Cueto & J. H. U. Brown, 1958)

Solutions required:

A.	Rose bengal 2B	10 gm.
	Tap water	100 ml.
B.	Phloxin B	10 gm.
	Tap water	100 ml.

C.	Solution A	0·2 ml.
	Solution B	0·8 ml.
	Tap water	49 ml.
	Propylene glycol	50 ml.

Note: The stock solutions A and B can each be prepared with only 1 gm. of the dyes, but in that case it will be necessary to add a few drops of chloroform (which must be renewed from time to time since it evaporates when the solutions have been kept in stock for a few weeks). If these two solutions are made to contain only 1%* of the dyes, then solution C will have to be modified as follows:

Solution A	2 ml.
Solution B	8 ml.
Water	40 ml.
Propylene glycol	50 ml.

Technique:

1. Dilute samples of the blood in white cell pipette and enumerate with a counting chamber as usual.

Results:

Eosinophils are stained brilliant red against a light red background.

Notes:

The authors state that workers, including Swingle (1955), have pointed out the difficulty in the staining of eosinophils of the dog by the methods that are in use for the blood of humans, rats and mice. The method described above was developed during the course of an investigation on the function of the adrenal cortex of the dog when it became necessary to make routine counts of these cells. It is claimed that with the rose Bengal–phloxin mixture more of the eosinophils can be seen, with a lower error of counting than usually given by single stains.

It is stated that errors in counting eosinophils stained with phloxine B can be reduced if the blood is collected without any anticoagulant such as heparin.

The original paper should be consulted for further information.

Reference: Cueto, C. & Brown, J. H. U. (1958).

* Without the addition of chloroform as a preservative, the weak solutions are liable to attack by air-borne micro-organisms with the destruction of the dyes. This does not occur with solutions containing 10% of the dyes.

SAFFRON - ERYTHROSIN

For connective tissue

Note: Saffron is the dried stigmata of crocus sativus, and should not be confused with safranin, which is an aniline dye.

Solutions required:

A. Saffron .. .. 2 gm.
Distilled water .. .. .. 100 ml.

Boil gently for an hour; allow to cool; then filter; add 1 ml. of 40% formaldehyde and 1 ml. of 5% tannic acid to the filtrate.

Note: Saffron solution deteriorates after a few weeks, and it is best to prepare the solution in small quantities, as required.

B. Delafield or Ehrlich haematoxylin.

C. Erythrosin, 1% aqueous.

Technique:

1. Fix small pieces of tissue in Bouin, Zenker-formaldehyde or in mercuric-formaldehyde.

2. Wash; dehydrate; embed.

3. Sections are stained for five to ten minutes with Delafield or Ehrlich haematoxylin; rinse in water.

4. Blue in tap water in the usual manner or in 1% sodium phosphate (Na_2HPO_4).

5. Stain for two to five minutes in 1% aqueous erythrosin.

6. Rinse quickly with water.

7. Differentiate with 70% alcohol for a few seconds, controlling under the microscope, until the collagen fibres are nearly colourless.

8. Rinse in water; stain for five minutes in saffron solution prepared as above; rinse with water.

9. Wash rapidly first with 70% alcohol then with absolute alcohol; clear in xylol and mount.

Results:

Nuclei are stained blue. Cytoplasm: varying shades of red. Muscle: pink. Elastic fibres: pink. Collagen: yellow.

Improved differentiation of most cells and tissues is obtained by employing this method in place of haematoxylin and eosin.

References:

Foot, N. C. (1933).
Masson, P. (1929).

SAFRANIN - CRYSTAL VIOLET - FAST GREEN - ORANGE 2

(Kalter's quadruple stain)

Solutions required:

A. Safranin, O .. .. .. 0·2 gm.
Formalin (40% formaldehyde) .. 4 ml.
Sodium acetate .. .. .. 0·5 gm.
Alcohol 50% .. .. .. 100 ml.

B. Crystal violet 0·5% aqueous.

C. Fast green F.C.F. - Orange G saturated in clove oil

D. Orange 2 saturated in clove oil.

Technique:

1. Any fixative may be employed, but if Bouin is chosen, remove picric acid with a few drops of saturated lithium carbonate in the 70% alcohol of dehydration series. Should a fixative containing mercuric chloride be chosen, then it will, of course, be necessary to remove mercuric precipitate in the usual way.
2. After fixation, wash, dehydrate, clear and embed in paraffin wax.
3. Cut sections no thicker than 7·5μ and fix to slides, avoiding the use of glycerine albumin, which will cloud the stain.
4. De-wax with xylol; then pass through absolute, 90% and 70% alcohol to water.
5. Stain in the safranin solution for twenty-four hours.
6. Rinse with water.
7. Stain with the crystal violet for one to two minutes.

8. Wash with water.

9. Immerse in 50% alcohol for two minutes.

10. Immerse in 95% alcohol for two minutes.

11. Immerse in the fast green-orange 2 (Solution C) for five minutes.

12. Differentiate, examining under the microscope, until the connective tissue is stained to the desired depth of green.

13. Immerse in clove oil for ten minutes.

14. Transfer to Orange 2 in clove oil for ten minutes.

15. Differentiate, examining under the microscope at intervals, until the desired depth of staining has been achieved.

16. Immerse for ten minutes each in two changes of xylol.

12. Mount in balsam or D.P.X.

Results:

Nuclei: red. Nucleoli: purple or purplish red. Nuclear membrane: dark red. Cellular cytoplasm: pink to red, except in Henle's loop (light green). Connective tissue: green. Elastic fibres: yellow. Fibroblasts: green with purple nuclei. Muscle: reddish brown. Erythrocytes: orange. Polymorphonuclears show purple nuclei.

Notes:

The original author (Kalter) states that this technique, which is a development of Flemming's triple stain (Flemming, 1891), is particularly useful for histology students.

Reference: Kalter, S. S. (1943).

SAFRANIN - WATER BLUE

(After Unna)

For collagen fibres

Solutions required:

A. Safranin O 1% aqueous.

B. Water Blue 1% aqueous.. .. 10 ml.
Tannic acid 33% aqueous .. 10 ml.
This solution must be freshly prepared.

Technique:

1. Tissues should be fixed in 1% aqueous picric acid or in absolute alcohol, and Celloidin sections should be employed.
2. Stain for ten minutes in the safranin solution; then wash thoroughly in water.
3. Stain for ten to fifteen minutes in Solution B.
4. Wash thoroughly in distilled water.
5. Clear in Bergamot oil: then mount in Clearmount.

Results:

Collagen fibres are stained blue, while nuclei are red.

SCARLET R - ETHYLENE GLYCOL

An improved technique for staining fat, etc. in animal tissues, its chief advantages being: (*a*) Excellent differentiation without loss of stain out of the lipid particles. (*b*) A stable solution which does not dissolve lipid materials. (*c*) Sections are not shrunken but remain pliable. (*d*) More intense staining of fat.

Solutions required:

A. Ethylene glycol, pure, anhydrous.
B. Scarlet R .. .. 1 gm.
Ethylene Glycol, pure, anhydrous.. 100 ml.
Heat the ethylene glycol to 100–110° C. on a hot plate or in an oven, or over the bunsen flame, taking care that it does not catch fire; then add the stain and stir until all or most of it is dissolved. Filter when cold.
C. Ethylene glycol, pure, anhydrous.. 85 ml.
Distilled water .. .. .. 15 ml.
D. Ehrlich or Delafield haematoxylin.

Technique:

1. Fix material in 10% formalin and cut frozen sections.
2. Wash sections in water for two minutes or longer to remove the formalin.
3. Dehydrate the sections by agitating gently in pure anhydrous ethylene glycol for three to five minutes.

4. Immerse the sections in the stain (solution B) for two to three minutes, with gentle agitation.
5. Differentiate by agitating gently in 85% ethylene glycol.
6. Transfer to distilled water for three to five minutes.
7. Counterstain with Ehrlich or Delafield haematoxylin.
8. Wash well in tap water: mount in Aquamount.

Results:

Nuclei: blue. Fat: orange to red. Cholesterol: red. Normal myelin: unstained. Fatty acids: unstained.

Reference: Chiffelle, T. L. & Putt, F. A. (1951).

SCARLET R

For staining fat, etc. in animal tissues

Solutions required:

A. Scarlet Red, saturated in equal volumes of acetone and 70% alcohol.

B. Ehrlich or Delafield haematoxylin.

Technique:

1. Tissues are fixed in formalin and frozen sections are employed.
2. Sections are immersed for a second in 70% alcohol; then stained for two to five minutes, in the Scarlet R solution.
3. Wash quickly in 70% alcohol, and transfer to distilled water.
4. Counterstain with Ehrlich or Delafield haematoxylin.
5. Wash well in tap water; mount in glycerine or Aquamount.

Results:

Nuclei: blue. Fat: orange to red. Cholesterol: red. Normal myelin: unstained. Fatty acids: unstained.

Note: Acetone increases the solubility of the dye, but Chiffele & Putt's method (above) is to be preferred as acetone-alcohol is liable to dissolve out some lipid material from the sections.

Reference: Michaelis, L. (1901).

SCHIFF'S REAGENT (Feulgen reaction)

For deoxyribonucleic acid

Solutions required:

A. *Schiff's reagent**
Distilled water .. .. .. 100 ml.
Basic fuchsin (Michrome No. 412) 0·5 gm.

Boil the water; then allow it to cool to about 70° C. before adding the dye and dissolving by stirring. Allow the solution to cool to about 25° C., then add:
Potassium metabisulphite .. 1 gm.
HCl N/1 .. .. .. 10 ml.

Plug the neck of the flask with cotton wool; allow to stand several hours or overnight; then add:
Decolorizing charcoal .. 1 gm.

Shake well, then allow to stand for about half an hour before filtering.

Note: The finished solution should be colourless, and if kept in a well-closed bottle, free from dust and other extraneous matter, it will keep for many weeks or several months, even in a clear glass bottle exposed to daylight.

B. Potassium metabisulphite .. 0·5 gm.
HCl, conc. .. .. .. 5 ml.
Distilled water .. .. .. 100 ml.

C. Fast green FCF, 0·5% aqueous

Technique:

1. Sections of Zenker-fixed material are taken to water in the usual manner.

2. Treat for the removal of mercuric deposit by the standard technique.

3. Rinse in distilled water.

4. Rinse briefly in N/1 HCl.

5. Immerse in N/1 HCl for five minutes at 60° C.

* *Note:* This is sometimes referred to as Feulgen's Fuchsin.

6. Rinse briefly in cold N/1 HCl.

7. Rinse in distilled water.

8. Immerse in solution A for half to one hour.

9. Drain off excess fluid from slides; then rinse with three changes of solution B.

10. Wash well in running tap water.

11. Counterstain in the fast green solution for about thirty seconds.

12. Dehydrate through the usual graded alcohols.

13. Clear in xylol.

14. Mount in D.P.X.

Results:

DNA is seen in shades of reddish purple against a blue-green background.

Notes:

(*a*) Many other fixatives may be used instead of Zenker, but the time required for hydrolysis (step 5) varies with different fixatives. The following is a list of some of the fixatives stipulated by Bauer (1933), with times, expressed in minutes, for hydrolysis:

Bouin–Allen	22	Helly	8
Carnoy	8	Petrunkevitch	3
Carnoy–LeBrun	6	Regaud	14
Champy	25	Susa	18
Flemming	16	Zenker–Formol	5

Bouin is not recommended

(*b*) Feulgen's reaction is based on mild acid hydrolysis of deoxyribofuranose, which is the carbohydrate component of DNA, to release aldehyde groups, which then unite with Schiff's reagent to give a coloured product.

(*c*) Schiff reagent, following Feulgen hydrolysis, is generally believed to be specific for DNA.

(*d*) Basic fuchsin is a basic dye of the triphenylmethane group. It is the parent substance of a range of dyes varying in shade from red through purple to violet and blue, according to the number of methyl or ethyl groups added to the molecule of the original dye.

It is conceivable, therefore, that the purple and violet shades produced with Schiff's reagent may be due to molecular rearrangements involving methyl and/or ethyl groups.

References:

Bauer, H. (1933).
Dodson, E. O. (1946).
Feulgen, R. & Rossenbeck, H. (1924).
Gurr, E. (1958).
Lillie, R. D. (1951).
Overend, W. G. & Stacey, M. (1949).
Pearse, A. G. Everson (1953).
Stowell, R. E. (1945).
Tomasi, J. A. (1936).

SHORR'S STAIN

For vaginal smears, keratin, etc.

Staining solutions:

A. Harris's haematoxylin.

B. Biebrich Scarlet .. .. .. 1 gm.
Orange G .. .. .. 0·4 gm.
Distilled water .. .. .. 99 ml.
Glacial acetic acid .. .. 1 ml.

C. Phosphomolybdic acid .. .. 2·5 gm.
Phosphotungstic acid .. .. 2·5 gm.
Distilled water .. .. .. 100 ml.

D. Fast Green FCF .. .. .. 0·25 gm.
Acetic acid, 0·3% .. .. 100 ml.

Technique:

1. From the fixing solution, carry through alcohols to water; stain in Solution A for two minutes; then wash in running water for five minutes.

2. Stain for one minute in Solution B; then wash in water.

3. Mordant in Solution C for one minute; then wash in water.

4. Stain in Solution D for two minutes; then without washing:

5. Differentiate for one minute in 1% acetic acid.

6. Dehydrate in the usual manner; clear in xylol and mount in Clearmount.

Note: Solution A may be omitted in certain cases, as in the routine treatment of menopause with oestrogens. With this omission smears can be stained in five minutes.

Results:

Cornified cells: orange-red. Non-cornified cells: green.

Reference: Shorr, E. (1940).

SHORR'S STAIN
(Single solution)
For vaginal smears

Solutions required:

A. Harris Haematoxylin
B. Shorr's Stain (Single)

Technique:

1. Fix wet smears one to two minutes in equal parts of absolute alcohol and ether: then pass into two changes of absolute alcohol, followed by 80% and 66% alcohol.

2. Rinse with distilled water for ten seconds; then stain in solution A for two minutes.

3. Wash in running water for five minutes; then stain with solution B for two minutes.

4. Dehydrate with 66%, 80% and absolute alcohol; then clear in xylol and mount.

Results:

Cornified cells: orange-red. Non-cornified cells: green.

Reference: Shorr, E. (1941).

SILVER CARBONATE - ORCEIN - ANILINE BLUE - FAST GREEN

For demonstrating reticulin, elastin and collagen in the same tissue sections

Solutions required:

A. Celloidin 0·5% in equal vols. of ether and absolute alcohol.

B. Pot. Permanganate 0·25% aqueous.

C. Oxalic Acid 5% aqueous.

D. *Silver carbonate, Hortega (Foot's modification)*
Silver nitrate 10% aqueous .. 10 ml.
Lithium carbonate, saturated aqueous .. .. 10 ml.
Shake well; then allow to stand for ten minutes or so in a 25 ml. measuring cylinder.

Pour off the supernatent fluid, then transfer the precipitate to a 100 ml. measuring cylinder, and add about 75 ml. distilled water, shake well; allow to settle; then pour off the fluid and add a second lot of distilled water. This process should be repeated three or four times.

Finally add 25 ml. of distilled water to the precipitate and add 28% ammonia solution drop by drop until the precipitate is almost dissolved.

Make up to 100 ml. with 90% alcohol.

Filter and warm to 50° C. for 15 minutes before using.

E. Neutral Formalin 40% .. .. 20 ml.
Distilled water .. .. .. 80 ml.
Buffer to pH 7·0.

F. Gold chloride 0·2% aqueous.

G. Sodium Thiosulphate 5%.

H. Orcein 1% in 70% alcohol .. 100 ml.
Hydrochloric acid, conc. .. .. 1 ml.

Picro Aniline Blue

I. Aniline blue aqueous .. .. 0·1 gm.
Picric acid, saturated aqueous .. 100 ml.

or:

J. Picric acid, saturated aqueous .. 100 ml.
Fast Green FCF .. .. .. 0·2 gm.

Technique:

1. Material should be fixed in 10% formalin and embedded in paraffin wax by the standard technique.

2. Sections 4 to 5μ in thickness are fixed to slides, dewaxed and immersed in xylol for five minutes.

3. Wash and immerse in absolute alcohol for two minutes.

4. Immerse in solution A for five minutes (Celloidin).

5. Drain slides for one minute.

6. Immerse in 80% alcohol for five minutes.

7. Rinse in water.

8. Immerse in solution B (Pot. perman.) for five minutes.

9. Rinse in water.

10. Immerse in solution C (oxalic acid) for five minutes.

11. Rinse in tap water.

12. Wash in distilled water.

13. Immerse in the silver carbonate solution in an oven at 50° C. for ten to fifteen minutes (Soln. D).

14. Rinse in distilled water.

15. Immerse in solution E (20% neutral formalin) for five minutes.

16. Rinse in tap water.

17. Tone in solution F (gold chloride) for five minutes.

18. Rinse in tap water.

19. Immerse in solution G for two minutes.

20. Rinse in tap water.

21. Stain in solution H (Orcein) for ten to fifteen minutes in an oven at 37° C. or for one hour at room temperature.

22. Rinse in 70% alcohol, followed by tap water.

23. Stain in solution I (Aniline blue) for twenty to forty seconds or solution J (Fast green) for ten to twenty seconds.

24. Wash with 95% alcohol for six to eight seconds.

25. Rinse briefly with absolute alcohol.

26. Rinse with a mixture consisting of equal volumes of xylol and absolute alcohol until clear.

27. Rinse with several changes of xylol, and mount.

Results:

With Picro aniline blue:

Elastic fibres	reddish brown
Collagen	blue
Reticulum	black
Muscle	blue-green
Nuclei	tan to brown
Erythrocytes	yellowish tan
Cytoplasm	pale blue

With Picro fast green:

Elastic fibres	orange to reddish brown
Collagen	blue-green
Reticulum	black
Muscle	green
Nuclei	tan to brown
Erythrocytes	yellow to orange
Cytoplasm	light green

Reference: Lewis, A. L. & Jones, R. S. (1951).

SILVER NITRATE - GOLD CHLORIDE - PARACARMINE (Da Fano)

For Golgi apparatus

A. Cobalt nitrate 1% aqueous .. 100 ml.
Formalin .. .. 15 ml.

B. Silver nitrate 1·5% aqueous.
(*N.B.:* This should be stored in an amber or blue glass bottle.)

C. Cajal's Reducer.
Hydroquinone 2% aqueous .. 100 ml.
Neutral formalin .. .. .. 15 ml.
Sodium sulphite anhydrous .. 0·5 gm.
N.B.: This solution should be freshly prepared.

D. Gold chloride 0·2% aqueous.

E. Sodium thiosulphate 5% aqueous.

F. Paracarmine (Mayer).

Technique:

1. Pieces of tissue no thicker than 3 mm. are fixed from two to eighteen hours in the cobalt nitrate formalin solution, according to the size and nature of the material.
2. Wash the tissue quickly in a large volume of distilled water.
3. Immerse in the silver nitrate solution in the dark for thirty-six to forty-eight hours.
4. Wash quickly in a large volume of distilled water.
5. Trim the tissue to a thickness not exceeding 2 mm.
6. Immerse in Solution C (Cajal's Reducer) for two to twenty-four hours in the dark.

Note: For most soft tissues about four hours will suffice.

7. Wash in several changes of distilled water.
8. Dehydrate, clear and embed in paraffin wax in the usual way.
9. Cut sections up to 8μ in thickness.
10. Fix sections to slides; dewax and pass through descending grades of alcohol to distilled water.
11. Tone sections on slides by immersing in the gold chloride solution for five to ten minutes.
12. Wash quickly in distilled water.
13. Fix in 5% sodium thiosulphate (Solution E) for ten to fifteen minutes.
14. Wash thoroughly in distilled water.
15. Counterstain with paracarmine for about ten minutes.
16. Rinse with 90% alcohol, followed by absolute alcohol.
17. Clear in xylol and mount.

Results:

Golgi apparatus stained black while cells are pink or red.

Reference: Fano, C. Da (1921).

SILVER NITRATE - HYDROQUINONE

For the detection of gold in fixed tissues of experimental animals

Notes:

(*a*) The following technique must be carried out in the dark-room.

(*b*) Fixatives containing metals must be avoided.

(*c*) The use of metal instruments in handling the sections must be avoided.

Solutions required:

A. Gum acacia 10% aqueous, filtered 100 ml.
Silver nitrate, A.R. grade .. 2 gm.

Note: This solution should be prepared immediately before use.

B. Gum acacia 10% aqueous, filtered 100 ml.
Hydroquinone .. .. .. 1 gm.

Note: This solution should be prepared the day before it is required for use.

C. Solution A .. .. .. 10 ml.
Solution B .. .. .. 10 ml.
Citric acid 5% .. .. .. 0·5 ml.

Note: This solution should be prepared only when required for immediate use.

D. Sodium thiosulphate 5% aqueous.

Technique:

1. Small pieces of tissue are fixed in 20% formalin, and frozen sections are employed.

2. Rinse thoroughly in distilled water.

3. Immerse sections in Solution C and leave therein for five to ten minutes.

4. Plunge sections directly into the sodium thiosulphate solution, without prior washing, and leave therein for five minutes.

5. Wash thoroughly in several changes of distilled water.

6. Mount in Aquamount or in Farrant's medium.

Results:

The presence of gold is indicated by a black deposit in the cells.

Reference: Roberts, W. J. (1935).

SILVER NITRATE

For ascorbic acid

Reagents required:

A. Silver nitrate, 5% aqueous .. 100 ml.
Glacial acetic acid .. .. 5 ml.

B. Sodium thiosulphate, 1% aqueous

Technique:

1. Small, thin slices of fresh tissue are immersed directly in reagent A, *in the dark*, for about half an hour.

2. Wash thoroughly in repeated changes of reagent B.

3. Wash thoroughly in several changes of distilled water.

4. Dehydrate in dioxane (*see* p. 512) and embed in paraffin wax.

5. Press the wax sections on to slides and dewax with dioxane.

6. Mount the dewaxed sections in D.P.X., Clearmount, or Emexel.

Results:

Ascorbic acid is indicated by a black granular precipitate.

Notes:

(*a*) Localization is only approximate.

(*b*) Ascorbic acid is a sugar derivative which is soluble in water and in alcohol, and which is unstable to alkali, light and oxidation. It is a fairly strong aliphatic organic acid and possesses strong reducing properties. In biochemistry, silver nitrate, ferric chloride, ferricyanides, iodine, methylene blue and 2:6-dichlorophenol-indophenol have been used for the determination of the reduction capacity of ascorbic acid-containing substances. In histology, ascorbic acid is identified solely on its properties as a reducing agent: in the technique described above, ascorbic acid, if present, reduces the silver nitrate to metallic silver, which is observed as a black precipitate.

Reference, Girond, A., Leblond, C. P., Ratsimananga, R. & Rabowicz, M. (1936).

SILVER NITRATE

For controlled differentiation of cells and connective tissue fibres in silver staining

(After E. E. Lascano, 1960)

Solutions required:

A. Potassium permanganate, 1% aqueous

B. Oxalic acid, 5% aqueous

or:

Potassium metabisulphate, 2% aqueous

C. Iron alum, 2% aqueous

D. Silver nitrate, 5% aqueous

E. Methanamine, 3% aqueous

F. *Gomori's methenamine silver* (*Gomori*, 1946)

Solution D	5 ml.
Solution E	100 ml.

Shake until the heavy white precipitate disappears.

The solution will keep in the refrigerator for many months.

Note: When the solution is taken out of the refrigerator the precipitate will have reformed, but this will redissolve on shaking when the temperature of the solution rises to room temperature.

G.

Pyridine, pure, redistilled	1·5 ml.
Distilled water	98·5 ml.

H.

Gelatine powder	0·25 ml.
Warm water	100 ml.
Formaldehyde, 40%	2 ml.

Sprinkle the gelatine on the surface of the water; stir until dissolved; then add the formaldehyde.

I. (*Optional*) Gold chloride (brown or yellow), 0·2% aqueous

J. (*Optional*) Sodium thiosulphate, 5%

K. (*Optional*) Eosin or erythrosin, 1% aqueous

Technique:

1. Fix in 10% formalin for one to three days.
2. Dehydrate, clear and embed in paraffin wax as usual.
3. Cut sections as usual but do not mount them on slides.
4. Arrange a series of watch glasses containing xylol, the usual graded alcohols and distilled water in the order in which they are to be used for dewaxing and hydrating the sections.
5. Arrange a series of watch glasses containing the reagents in the order in which they will be used in the following technique.
6. Dewax and hydrate the sections by successively placing them in the watch glasses containing xylol, alcohols and water, handling the sections throughout as in the case of frozen sections.
7. Immerse in 1% potassium permanganate (solution A) for fifteen seconds to one minute.

Note: If the tissue has been fixed for a long time or if it contains a considerable quantity of blood, or if it is very cellular, the time in the potassium permanganate solution can be extended over one minute, sometimes as long as ten minutes, until a good impregnation of the connective tissue fibres is effected, but in such cases the staining of the cells might be suppressed.

8. Wash in distilled water for five to ten seconds.
9. Decolorize in 5% oxalic acid or 2% potassium metabisulphite for about ten to twenty seconds, until the brown colour disappears completely.
10. Immerse in a fresh lot of distilled water for one minute.
11. Immerse in 2% iron alum (solution C) for one minute.
12. Transfer to a fresh lot of distilled water.
13. Impregnate in Gomori's methenamine silver (solution F) for about two minutes.
14. Wash in solution G for about fifteen seconds.
15. Reduce in solution H for one minute.
16. Repeat steps 12 to 15 several times until the reticulum and collagenous fibres are completely stained, as the first staining is usually insufficient.

Note: If it is desired to stain the cells also, then the following procedure should be followed.

17. After the first insufficient staining of the reticulum and collagenous fibres, the sections are rinsed in distilled water.

18. Dip the section for one second in the silver solution (F).

19. Immediately reduce in the gelatine-formalin (solution H), omitting the pre-washing in solution G.

Notes:

(i) If necessary this step can be repeated.

(ii) Connective tissue fibres can be fully stained by this procedure but the cells and some tissues may remain unstained.

20. If the staining is too intense, decolorize in the iron alum (solution C).

Note: If the decolorization is carried too far, the desired depth of staining can be achieved again by repeating steps 12 to 15, but it is best to avoid prolonged soaking in the iron alum as this causes the staining to lose its precision.

*21. Tone in gold chloride (solution I) for five minutes or more.

*22. Fix in the sodium thiosulphate (solution J) for one to two minutes.

23. Counterstain in eosin or erythrosin (solution K), if desired.

Results:

Successful preparations show brilliant and complete staining: even the finest reticulum fibres are stained black, and the collagenous fibres light brown. When the staining of cells is achieved and the gold toning omitted, the cytoplasm is stained in various shades of yellow and the nuclei are black. In some cases only the cytoplasm is stained, with the nuclei barely visible. The background is transparent and without precipitates.

Notes:

(*a*) The original author (Lascano) states that this method, which is also applicable to frozen sections, can be applied to sections attached to slides but the results are less satisfactory, the structures possessing less brilliancy with little if any colour differentiation.

(*b*) The author states that there are no fixed rules or periods of time in his technique, described above, which may be varied with each kind of tissue. Sections must be stained one by one, and checked during the process by microscopic examination,

* Steps 21 and 22 are optional.

until a properly stained preparation is obtained. The complete staining of connective tissues, in particular, requires this control and adjustment to meet individual requirements.

(*c*) The technique was developed in Dr. Lascano's laboratories to overcome the problem of making satisfactory silver preparations of hospital laboratory cirrhosis liver specimens already embedded in paraffin wax.

(*d*) The original paper should be consulted for more detailed information and photomicrographs.

Reference: Lascano, E. F. (1960), *Stain Tech.*, 35, pp. 23–29.

SILVER STAINING

Enzymatic (pepsin or papain) removal of interfering connective tissue elements prior to silver staining of nerves

Solutions required:

A. Pepsin powder, 1% in N/10 hydrochloric acid (pH 1·8–2·0)

or

B. Papain, 1% in veronal acetate buffer (pH 6·0–6·5)

Technique:

1. Incubate celloidin sections in solution A or B at 37° C. for six to twelve hours.

Note: Pieces of unembedded tissues, after washing in water, may also be treated as above, but a longer incubation time will be necessary (from twelve to twenty-four hours).

2. Wash the sections or pieces of tissue thoroughly with water, then stain with silver nitrate as usual.

3. Embed pieces of tissue in L.V.N. or celloidin before staining with silver nitrate.

Results:

Collagenous elements, which would otherwise mask the appearance of nerves, are removed to permit a clearer and more accurate identification of the nerves.

Notes:

(*a*) The time required for hydrolysis by the enzyme (pepsin or papain) will depend upon the size and thickness of the sections or tissue blocks and their connective tissue characteristics. In those tissues where connective tissue is abundant, the time must be prolonged. However, care should be taken to avoid over-hydrolysis and consequent disintegration of the remaining tissue.

(*b*) The author (Bernick) observes that the dermis of the skin is composed of collagenous elements, of varying thickness, which interlace each other to form a network, and that these collagenous fibres are irregularly arranged and may become blackened with silver if such a section has not been pretreated with enzymes, as above.

(*c*) It is stressed that the enzymatic protein-hydrolysis described above does not remove the collagenous fibres completely. The action depends upon the density of the fibres present. The periodontal membrane, for example, contains a heavy concentration of collagenous elements and with enzymatic hydrolysis only partial loss of fibres results, but even this incomplete removal, the author states, permits the clear and accurate identification of the nerve supply to the membrane.

(*d*) For more detailed information the original paper should be consulted.

Reference: Bernick, S. (1955).

SILVER NITRATE - GOLD CHLORIDE

For demonstrating layers in human neurological lipofuscin granules

(After Nassar, Issidorides & Shanklin, 1960)

Solutions required:

A. Potassium permanganate, 5% aqueous
B. Sulphuric acid, 5% aqueous
C. Solution A .. .. .. 25 ml.
   Solution B .. .. .. 25 ml.
   Distilled water .. .. .. 450 ml.
D. Oxalic acid, 2% aqueous

E. Silver nitrate, 10% aqueous

F. Solution E .. .. .. 50 ml.
Pyridine, pure .. .. .. 3·75 ml.
Distilled water .. .. .. 200 ml.

G. Strong ammonia solution, spec. gravity 0·88 .. .. .. 7 ml.
Distilled water .. .. .. 18 ml.

H. Silver diaminohydroxide solution

Place 1 ml. of solution G in a scrupulously clean flask which has been washed out with three lots of distilled water. Add 7–8 ml. of solution E to the 1 ml. of dilute ammonia solution in the flask. Shake well. Cautiously add drop by drop more silver nitrate, shaking the flask between each addition, until the precipitate redissolves except for a faint turbidity which must be allowed to remain if the preparation is to be effective. A total of 9–10 ml. (including the the 7 or 8 ml. placed in the flask in the first place) of the 10% silver nitrate will be required.

I. Solution H .. .. .. 10 ml.
Pyridine .. .. 3 drops

J. Neutral formalin, 2% .. .. 1 volume
Absolute alcohol .. .. 1 volume
i.e. Formaldehyde, 40% solution, neutral .. .. 50 ml.
Distilled water, neutral .. 950 ml.

K. Gold chloride (brown or yellow), 0·2% aqueous ..

L. Sodium thiosulphate, 5% aqueous

M. Eosin or erythrosin, 1% in tap water

Technique:

1. Fix small pieces of fresh brain tissue in 10% neutral formalin for one week.
2. Wash in tap water.
3. Dehydrate through the usual graded alcohols.
4. Clear in xylol

5. Embed in paraffin wax.

Note: Pyridine may be used in place of alcohols and xylol.

6. Cut sections at 5μ and fix them to slides.
7. Dewax paired sections on slides labelled " A " and " B ".
8. Treat both sections with absolute alcohol.
9. Rinse both sections in 95% alcohol, then:
   Take section " A " through steps 10–18, below.
   Take sections " B " directly to step 18, below.
10. Rinse in 70% alcohol.
11. Wash in distilled water.
12. Oxidize in solution C for one to two minutes until the sections turn a brownish colour.

Note: This solution should be changed frequently.

13. Rinse well in distilled water.
14. Decolorize in solution D for two minutes.
15. Wash in running tap water for five minutes.
16. Rinse in 70% alcohol.
17. Rinse in 95% alcohol.
18. Place sections " A " and sections " B " in solution F for four to five hours at 50–55° C.
19. Rinse quickly in 95% alcohol.
20. Impregnate in solution I for five minutes at 50–55° C.
21. Rinse quickly in 95% alcohol.
22. Reduce in solution J for two minutes.
23. Wash in distilled water.
24. Tone in solution K until the sections turn greyish.
25. Fix in solution L (sodium thiosulphate) for two minutes.
26. Wash in tap water.
27. Counterstain lightly in 1% eosin or erythrosin.
28. Dehydrate and clear.
29. Mount in D.P.X. or Clearmount or Cristalite or neutral Canada balsam in xylol.

Results:

The lipofuscin granules appear as small distinct black dots, surrounded by a clear zone, in all sections impregnated without previous oxidation in silver diammine.

In all sections impregnated with silver diammine after initial oxidation, the granules appear as black rings with clear centres.

This is best seen in those parts of the cells where the granules are fewer and not overlapping. The diameter of the rings is approximately twice that of the dots.

Notes:

(*a*) The authors concluded from previous work of theirs, published in 1956,* that each lipofuscin granule in human neurons is built up of several concentric layers, which react differently to the several staining methods which they applied. The technique described above was developed for the purpose of revealing the layered structure of the lipofuscin granules.

(*b*) Histochemical work carried out by Shanklin & Issidorides (1959)† suggested the presence of free amino and carboxyl groups, cystine, unsaturated fats, protein-bound SH and SS groups and thereby supported the authors' view that these granules represent either a lipoprotein or a proteolipid complex.

(*c*) Reference should be made to the original paper (Nassar, Issidorides & Shanklin, 1960) for more detailed information and photomicrographs.

References:

Nassar, T. K., Issidorides, M. & Shanklin, W. M. (1960).
Nassar, T. K. & Shanklin, W. M. (1951).

* D'Angelo, C., Issidorides, M. & Shanklin, W. M. (1956).

† Shanklin, W. M. & Issidorides, M. (1959).

SILVER NITRATE - COPPER SULPHATE - GOLD CHLORIDE

For nerve fibres of Planarians

Solutions required:

A. Formalin, 40% formaldehyde .. 10 ml.
Alcohol, 95% .. .. .. 45 ml.
Glacial acetic acid .. .. .. 2 ml.
*B. Copper sulphate ($CuSO_4$, $5H_2O$), 10% aqueous
C. Silver nitrate, 1% aqueous
D. Pyrogallol, 1% aqueous

E. *Reducer* (*after A. Palmgren*, 1951)

Solution D	10 ml.
Nitric Acid, aqueous	1 ml.
Absolute alcohol	56 ml.
Distilled water	44 ml.

F. Gold chloride (brown or yellow), 0·5% aqueous

*G. Oxalic acid, 1% aqueous

H. Sodium thiosulphate, 5% aqueous

I. Copper sulphate ($CuSO_4$, $5H_2O$), 20% aqueous

J. Silver nitrate, 7% aqueous

K. *Bodian's developer* (*Bodian*, 1936)

Hydroquinone	1 gm.
Sodium sulphite (Na_2SO_3)	5 gm.
Distilled water	100 ml.

L. Oxalic acid, 2% aqueous

Note: Solutions B and G can be prepared by diluting solutions I and L respectively.

Technique I—Staining *in toto*

Technique:

1. Fix in solution A for twenty-four to forty-eight hours.
2. Without washing, transfer to 70% alcohol for three to ten days.
3. Wash for about an hour in distilled water.
4. Immerse for three hours in 10% copper sulphate (solution B) at 50° C.
5. Without washing, transfer to 1% silver nitrate (solution C) for one to one and a half hours at 50° C.
6. Wash for one minute in each of three lots of distilled water.
7. Reduce by immersing in solution E at 40–45° C. for one to five minutes.
8. Wash well in distilled water.
9. Tone in gold chloride 0·5% (solution F) for three to five minutes, until the nervous system becomes visible.
10. Wash in distilled water.
11. Reduce in 1% oxalic acid (solution G) for one to three minutes, until the nervous system is darkly stained.

12. Wash in distilled water.
13. Fix in 5% sodium thiosulphate (solution H) for about five minutes.
14. Wash in tap water.
15. Dehydrate and mount as usual.

Technique II—Staining Sections

1. Fix in the same fixative as used for *in toto* staining.
2. Dehydrate in graded alcohols as usual.
3. Clear in xylol and embed in paraffin wax.
4. Cut sections at 10μ and attach them to slides with glycerin albumen or Haupt's fixative.
5. Dewax and carry through the usual graded alcohols to water.
6. Immerse in 20% copper sulphate for 48 hours at 37° C.
7. Rinse briefly in distilled water; then immerse in solution J for twenty-four hours at 37° C.
8. Reduce in Bodian's developer (solution K) for three to five minutes.
9. Wash in distilled water.
10. Tone in 0·5% gold chloride (solution F) for two to four minutes until the sections become grey.
11. Wash in distilled water.
12. Reduce in 2% oxalic acid (solution L) for two to five minutes, controlling by microscopic examination, until the desired degree of staining is achieved.
13. Rinse in distilled water.
14. Fix in 5% sodium thiosulphate (solution H) for two minutes.
15. Wash well in tap water.
16. Dehydrate through graded alcohols as usual.
17. Clear in xylol.
18. Mount in Clearmount, D.P.X., or Emexel, or Canada balsam in xylol.

Notes:

(*a*) The author claimed that the technique gave simple and uniform results, whereas, investigators who were studying regeneration in planarians up to the time his paper was accepted for

publication had failed to stain the nervous system selectively, as far as he was aware.

(*b*) The planarian material stained *in toto* by the author was *Planaria vitta*, while that for sections was *Dugesia gonocephala*.

(*c*) The method is also applicable for tissues of higher animals if the concentrations of the copper sulphate and silver nitrate solutions are reduced. The optimum concentration for these two solutions must be determined by experiment in each case.

(*d*) For more detailed information, including photomicrographs, the original paper should be consulted.

Reference: Betchaku, T. (1960).

SODIUM VANILLIDENMALONATE

A simple and rapid method for staining neurological tissues

Solutions required:

A. Sodium vanillidenmalonate .. 1 gm.
Distilled water .. .. .. 100 ml.

B. Potassium permanganate .. 3 gm.
Distilled water .. .. .. 30 ml.

C. Tannic acid, 1% in 95% alcohol

D. Formic acid, 25%

Technique:

1. Fix material in 10% formalin; embed in paraffin wax, and cut sections 5 to 10μ in thickness.

2. Fix sections to slides; dewax with xylol, and pass through the usual descending grades of alcohol to distilled water.

3. Immerse in solution A for one minute.

4. Without rinsing, transfer the slides to solution B and leave therein for three minutes.

5. Rinse in distilled water.

6. Repeat 3, 4 and 5 until the sections turn brown.
7. Transfer the sections to solution C.
8. Differentiate quickly in solution D (formic acid 25%).
9. Wash well in distilled water.
10. Dehydrate in 95% and absolute alcohols.
11. Clear in xylol and mount.

Results:

The method shows up the dendrites of the cells, the neuroglia cells, the course of the nerve fibres and of the blood vessels; the tigroid substance and sometimes the intracellular neurofibrils, and it gives excellent results for a general study of nerve centres and their connections, demonstrating the most delicate nerve extensions.

The different structures and the cellular elements assume a brown tint.

Notes:

(*a*) The author (Novelli) observes that although the methods for the microscopic study of nervous tissue are very numerous, for the most part they are modifications or variations of the few fundamental procedures, i.e. the Nissl method, Golgi silver impregnation, the so-called " photographic " methods of Cajal, the Bielschowsky ammoniacal silver impregnation method, and, finally, the Weigert iron haematoxylin method. Impregnation methods in particular are not easy to perform; they are lengthy and not always successful.

(*b*) Novelli's method, described above, however, differs from all other impregnation methods known at the present time in that a completely different reagent is employed, the procedure is rapid and easy to carry out. It is stated in the original paper that the method has proved to be successful on all occasions.

(*c*) It is stated that solution B (potassium permanganate) must be made up fresh each time; this, however, should not be necessary. Provided the solution is kept free from extraneous matter and in a well-closed bottle, stored in a cool place away from strong light, it should be good for a few days at the very least.

Reference: Novelli, A. (1952).

SOLOCHROME CYANIN R

A simple polychrome stain for neurological and general tissues

(E. Gurr & M. A. MacConaill, 1959)

Solution required:

Solochrome cyanin R (Michrome No. 750)	0·5 gm.
Glacial acetic acid	8 ml.
Water	92 ml.

Technique (for neurological tissues):

1. Fix pieces of brain or spinal cord in 10% formaldehyde.

Note: Do not use formaldehyde that has been neutralized with metallic compounds.

2. Demyelinate by treatment with successive baths of 96% alcohol, anhydrous cellosolve and anhydrous pyridine.
3. Embed in paraffin wax and cut sections at 8μ.
4. Fix sections to slides and carry through xylol to water in the usual way.
5. Immerse in the staining solution for five to eight minutes.
6. Wash in running water for five minutes.
7. Dehydrate through the usual graded alcohols.
8. Clear in xylol.
9. Mount in D.P.X. or Clearmount of neutral Canada balsam in xylol.

Results:

Erythrophile* (basic) tissue elements: pink to red. Acidic tissue elements are stained in shades of blue.

Notes:

(*a*) The picture of the tissue elements is not nearly so clearly marked as with the Falg or the Faviol techniques (pages 198–200, 203–6).

(*b*) For further information, reference should be made to pages 9–10 of this book.

References:

* MacConaill, M. A. (1949, 1951).
Gurr, E. & MacConaill, M. A. (1959).

SUDAN BLACK

For demonstrating the interstitial cells of the testis

Solutions required:

A. Potassium dichromate, 3% aqueous
B. Sudan black in 70% alcohol, unfiltered

Technique:

1. Fix material in Zenker-formol or in Regaud's fluid for several hours or overnight.
2. Immerse in solution A for three days at 37° C. changing the dichromate solution every twenty-four hours.
3. Wash in running water for twelve to twenty-four hours.
4. Dehydrate; clear; embed in paraffin wax.
5. Cut sections 5μ in thickness and fix them to slides.
6. Dewax with xylol; wash with absolute alcohol followed by 90% and 70% alcohols.
7. Immerse in the unfiltered Sudan black solution for ten to thirty minutes.
8. Wash briefly in 70% alcohol, but making sure that all excess dye is washed away from the slides.
9. Differentiate, if necessary, in 50% alcohol.
10. Wash in distilled water.
11. Mount in Farrants' medium or Aquamount or glycerine jelly.

Results:

Interstitial cells: cytoplasm blue and containing a variable number of darker coloured granules. Nucleus: unstained. Remainder of testis: light blue. Golgi apparatus of spermatocytes: often deep blue.

Notes:

(*a*) Sudan black in ethyl alcohol is liable to deteriorate after a few weeks, and I would, therefore, suggest either ethylene or propylene glycol (70% aqueous) be employed as the solvent instead, as these give stable solutions of Sudan black.

(*b*) The author (Threadgold) states that this method is to be preferred to his osmic acid method, details of which are given by

him in the same paper and which are described briefly on pages 336–7 of this book. It is stated that the Sudan black method shows cellular detail more clearly, and also all the stages in the cytomorphosis of the interstitial cell, but that it does not demonstrate the development of secretory granules: these, however, are well shown by the osmic acid method.

(*c*) The author states that the testis of a number of birds have been treated by his Sudan black technique, and that numerous dark blue granules in contact with small vacuoles (from which the lipid secretion droplets have been dissolved out during wax embedding) occur in the cytoplasm of the interstitial cells of birds. These granules are considered to be mitochondria.

(*d*) Counterstaining with Ehrlich haematoxylin or with eosin in 30% alcohol was found to obscure the results, although the eosin was found to give a fair contrast.

(*e*) It was found that sections took twenty-four hours to clear in Farrants' medium, and that it is advisable to hold the coverslip in place with a spring clip during that time.

(*f*) Readers should consult the original paper for more detailed information and photomicrographs.

Reference: Threadgold, L. T. (1957).

SUDAN BLACK

For lipids (especially those that are not well coloured by Sudan III or IV)

(J. R. Baker's technique)

Solutions required:

A. *Formaldehyde-saline.*

Formalin (Formaldehyde 40%) ..	10 ml.
Sodium chloride 10% aqueous ..	7 ml.
Distilled water	83 ml.

Note: Keep a few pieces of marble chips in the solution to maintain neutrality.

B. Formalin (Formaldehyde 40%) neutral.

Note : Keep a few pieces of marble chips in the bottle.

C. Potassium dichromate 2·5%
aqueous .. .. 88 ml.
Sodium chloride 10% aqueous .. 7 ml.
Note: Keep a few pieces of marble chips in the bottle.

D. *Dichromate-formaldehyde.*
Solution B .. .. 1 volume.
Solution C .. .. 19 volumes.

E. Potassium dichromate 5% aqueous

F. *Gelatine for embedding.*
Gelatine powder .. .. .. 25 gm.
Water .. .. 100 ml.
Sodium p-hydroxybenzoate .. 0·2 gm.

Sprinkle the gelatine on to the water and leave it to soak for an hour, afterwards warming in an incubator maintained at 37° C. until all the gelatine has dissolved, then strain through muslin while still warm.

Note: If Sodium p-hydroxybenzoate, which is added to prevent the growth of moulds and bacteria, is not available in the laboratory, then 0·25 to 0·5 gm. of Thymol should be used instead.

G. *Formalum* (*for hardening gelatine*)
Formalin (Formaldehyde 40%) .. 20 ml.
Potassium alum 5% aqueous .. 80 ml.
Keep marble chips in the bottle.

Note: Both gelatine blocks and gelatine sections may be preserved indefinitely in Formalum, which makes the gelatine very hard, thereby facilitating the cutting of thin sections which are non-sticky.

Important: Formalum must not be used in the acid haematein test for phospholipids (Baker 1946) as the alum would react with the haematein.

H. Sudan Black .. .. .. 0·5 gm.
Alcohol 70% .. .. .. 100 ml.
Boil for ten minutes under a reflux condenser; then cool and filter.

I. *Carmalum* (*Mayer*).

Technique:

1. Fix a piece of tissue not more than 3 mm. thick in the formaldehyde-saline for an hour.

2. Transfer, without washing, to the dichromate formaldehyde (Solution D) and leave for five hours.

3. Transfer, without washing, to 5% aqueous potassium dichromate and leave for about eighteen hours.

4. Leaving the tissue in the same solution, transfer to the paraffin oven at 60° C. for twenty-four hours.

5. Wash in running water for six hours.

6. Leave overnight in the melted gelatine in the oven at 37° C.

7. Cool the gelatine, preferably in a refrigerator.

8. Cut out a rectangular block containing the specimen.

9. Immerse the block overnight (or any conveniently longer time) in formalum, placing a marble chip in the capsule or tube.

10. Cut sections 8 to 10μ on the freezing microtome.

11. Transfer a section to 70% alcohol.

Note: It is best to transfer sections from fluid to fluid, up to stage 16 *in a Royal Worcester Porcelain thimble No. a.*4756, *size* 2.

12. Transfer to the Sudan black solution, and leave for $\frac{1}{2}$–4 minutes. (The best period is usually about $2\frac{1}{2}$ minutes.)

13. Wash in 70% alcohol for five seconds.

14. Wash in 50% alcohol for one minute.

15. Wash in water, sinking the section gently with a camel hair brush if it floats.

16. Transfer to Carmalum for two to three minutes. (The optimum time is usually three minutes.)

17. Rinse in distilled water.

18. Transfer the section to a fairly large dish, or a tongue jar of tap water, and leave for two minutes, or any conveniently longer time.

19. Wash again in another large bowl of water.

20. Transfer to a petri dish of water.

21. Float the section on to a slide.

22. Blot away excess water but do not allow the section to dry.

23. Mount in Farrants' medium, or in Aquamount.

24. Attach a clip to hold the coverslip to the slide: then leave overnight in the oven to harden the mounting media, before examining the preparation under the oil immersion objective.

Note: The slide may be examined after a quarter of an hour, if desired; then returned to the oven to complete the hardening.

Results:

Lipids, dark blue or blue-black. Cytoplasm: colourless or pale grey-blue. Chromatin: pink or red.

Note: If the results are not good, another section should be tried with variations of the staining times.

Never attempt to judge the colouring until the section is mounted and examined under the oil immersion objective.

It is recommended that the technique be learned on the intestine of the mouse, as it is scarcely possible to fail with this. Cut out a piece of empty intestine about 1 cm. long and immerse in formaldehyde-saline for five minutes, then open it by a longitudinal cut from one end to the other, taking care not to do any unnecessary damage to the villi.

The section should be left only one minute in the Sudan black and two and a half minutes in the carmalum.

References:
Baker, J. R. (1949).
Baker, J. R. (1955), personal communication.

SUDAN BLACK

For myelin and neutral fats

Solutions required:

A. Sudan black, saturated in 70% alcohol or 70% ethylene glycol

B. Carmalum (Mayer)

Technique:

Tissues are fixed at least three days in 10% formalin; then rinsed thoroughly in distilled water.

1. Frozen sections are immersed one minute in 50% alcohol; then one minute in 70% alcohol.

2. Stain for fifteen minutes to several hours in the Sudan black.

3. Rinse for a few minutes in 50% alcohol; then in distilled water.

4. Counterstain in carmalum for about three minutes; wash with distilled water; mount in glycerine jelly.

Results:

Neutral fat and myelin: blue-black to black; nuclei: red.

Reference: Carleton, H. M. & Leach, E. H. (1947), p. 259.

SUDAN BLACK - ETHYLENE GLYCOL

An improved technique for lipid staining, offering the advantage of a stable solution, excellent differentiation without loss of stain out of the lipid particles, and pliable unshrunken sections

Solutions required:

A. Ethylene glycol, pure, anhydrous.

B. Sudan black .. .. 1 gm.
Ethylene glycol, pure, anhydrous.. 100 ml.

Heat the ethylene glycol to 100–110° C. on a hot plate or in an oven, or over a bunsen flame, taking care that it does not catch fire; then add the stain and stir until all or most of it is dissolved. Filter when cold.

C. Ethylene glycol, pure, anhydrous.. 85 ml.
Distilled water .. .. .. 15 ml.

D. Carmalum (Mayer).

Technique:

1. Fix tissues for at least three days in 10% formalin.

2. Wash thoroughly in running water; cut frozen sections.

3. Dehydrate the sections by agitating gently in pure anhydrous ethylene glycol for three to five minutes.

4. Immerse the sections in the Sudan black solution from fifteen minutes to one hour, agitating gently at intervals.

5. Differentiate by agitating gently at intervals with 85% ethylene glycol (solution C) from one to ten minutes, controlling under the microscope while the section is still wet.

6. Transfer the sections to distilled water for three to five minutes.

7. Counterstain with carmalum for about three minutes.

8. Wash with distilled water.

9. Mount in glycerine jelly or Aquamount.

Results:

Lipid substances or particles are stained blue-black to black. Nuclei: red.

Reference: Chiffelle, T. L. & Putt, F. A. (1951).

SUDAN BLACK

For myelinated peripheral nerves

Solutions required:

A. *McManus fluid*

Cobalt sulphate or nitrate ..	1 gm.
Calcium chloride, 10%	10 ml.
Distilled water	80 ml.
Formalin (40% formaldehyde) ..	10 ml.

B. Formic acid, 50% .. .. 1 volume
Sodium citrate, 20% .. .. 1 volume

Note: Solution B is only required when decalcification is necessary.

C. Gelatine, 20%

D. Gelatine, 5%

E. Gelatine, 10%

F. Gelatine, 1%

G. Sudan black, saturated in 70% alcohol

Note: Solutions of Sudan black in alcohol deteriorate after a few weeks and I would, therefore, suggest that ethylene or propylene glycol be used instead as either of these two glycols give stable solutions of the dye (*see* page 428).

Technique:

1. Immerse pieces of fresh tissue in solution A for two days or longer: longer fixation, up to a month is apparently not harmful.
2. Wash thoroughly in running tap water.
3. Decalcify (if necessary) in solution B.
4. Wash thoroughly in running tap water.
5. Infiltrate with 2% gelatine for two to three days at 37° C.
6. Transfer to 5% gelatine and leave therein at 37° C. for the same length of time.
7. Immerse in 10% gelatine at 37° C. for the same length of time.
8. Transfer the dish of 10% gelatine containing the block to the refrigerator and leave it to chill for several hours.
9. Trim the block, by cutting away excess gelatine, then place it in cold McManus fluid (solution A) for twenty-four hours to harden the gelatine.

Note: Embedded specimens can be stored in this fluid at room temperature.

10. Cut thick serial sections, at an average thickness of 75μ, on a freezing microtome, and orient them on slides of suitable dimensions, on a warm stage, in a few drops of 1% gelatine.
11. Allow the excess fluid to evaporate, then place the slides in ice-cold McManus fluid in a grooved staining dish and transfer the whole to a refrigerator to harden the gelatine.
12. Wash the slides in cool, running tap water, then in distilled water, then transfer to 70% alcohol.
13. Immerse in freshly filtered Sudan black solution for fifteen to thirty minutes.
14. Drain the slides well.
15. Differentiate in two successive baths of 50% alcohol: this step takes anything from a few seconds to a few minutes and is best controlled by microscopic examination.
16. Wash well in water, then mount in glycerine jelly: allow the edges of the coverslips to dry thoroughly, seal with a non-aqueous cement such as Laktoseal. Alternatively, Aquamount may be used instead of glycerine jelly, in which case ringing of the coverslips is not necessary.

Results:

Myelin stains bright green to greyish green in thick sections as above. Large nerves and the smaller myelinated axons are well

stained, while nodes of Ranvier and the varicose character of the myelin sheath are clear. The author (Stilwell) claims that even some details of intrafusal endings and somatic motor terminations can be seen in sections over 50μ in thickness.

Notes:

(*a*) Myelin nerves have been demonstrated by Stilwell in this technique, in sections of bone, periosteum, ligament, skeletal muscle, the fibrous capsule of the diarthrodial joints and in fascia and other connective tissues, and readers are referred to the original paper for more detailed information and photomicrographs.

(*b*) It is claimed that as a method of studying peripheral nerves, practically all of which have myelinated motor or sensory fibres, this technique is of advantage because of its simplicity, and because it demonstrates the finest detail in myelin whilst obviating a background stain in tissues relatively free of adipose tissue.

Reference: Stilwell, D. L. (1957).

SUDAN BLACK
J. R. BAKER'S PYRIDIN EXTRACTION METHOD

For confirmation of lipids

Solutions required:

A.	Picric acid, saturated, aqueous ..	50 ml.
	Formaldehyde, 40%	10 ml.
	Glacial acetic acid	5 ml.
	Distilled water	35 ml.
B.	Potassium dichromate	5 gm.
	Calcium chloride, anhydrous ..	1 gm.
	Distilled water	100 ml.
C.	Sudan black in propylene glycol	
D.	Pyridine, pure, anhydrous	

Technique:

1. Fix pieces of tissue in reagent A for twenty-four hours.
7. Wash in 70% alcohol to remove excess picric acid.
3. Cut frozen sections and immerse some of them in pyridine for thirty minutes at 22° C.

4. Immerse in a fresh lot of pyridine for twenty-four hours at 60° C.

5. Wash in running water for two hours.

6. Immerse in solution B for one hour at 60° C.

7. Wash in distilled water.

8. Rinse in 70% alcohol.

9. Stain in the Sudan black solution for fifteen minutes.

10. Rinse in propylene glycol.

11. Mount in Aquamount or in glycerine jelly.

12. Take frozen sections from the same tissue block, omit stages 3 and 4, and proceed as at stages 5 to 11 above.

13. Compare the pyridine extracted sections with the unextracted sections.

Results:

Structures that are stained with Sudan black in the unextracted sections are confirmed as lipid-containing if they are unstained in the pyridine-extracted specimens.

Reference: Baker, J. R. (1946).

SUDAN BLUE

For demonstrating degenerated myelin

Solution required:

Sudan blue, saturated in 50%
alcohol or ethylene glycol

Technique:

1. Tissues should be fixed for at least three days in 10% neutral formalin.

2. Frozen sections are soaked one minute in 50% alcohol; then one minute in 70% alcohol; then stained from fifteen minutes to several hours in a saturated solution of Sudan blue in 50% alcohol.

3. Rinse for a few minutes in 50% alcohol; then in distilled water.

4. Counterstain in carmalum for three to five minutes.
5. Rinse in water; mount in glycerine jelly.

Result:

Degenerated myelin, blue.

Reference: Lillie, R. D. (1944).

SUDAN BROWN

For fat and for acute fatty degeneration not shown by Scarlet R

Solutions required:

A. Stock solution of Sudan brown saturated in isopropyl alcohol or ethylene glycol

B. Mayer's acid haemalum.

Technique:

1. Fix tissues in 10% formalin.

2. Frozen sections are stained for five minutes in a diluted solution of Sudan brown prepared by mixing 6 volumes of the stock solution with 4 volumes distilled water.

Note: This diluted solution keeps only for one day, and should, therefore, only be prepared immediately before use.

3. Float out in tap water.

4. Stain for two to five minutes in Mayer's acid haemalum; then rinse in tap water.

5. Immerse in tap water or in 1% sodium phosphate (Na_2HPO_4) until the section turns blue.

6. Rinse in distilled water.

7. Mount in Apathy's gum syrup or Aquamount.

Results:

Fat, brown; nuclei, bluish grey; protoplasm, colourless.

Reference: Lillie, R. D. (1948), p. 159.

SUDAN 2 - HAEMATOXYLIN

For degenerating and intact myelin and fat

Solutions required:

A. Haematoxylin 1% in absolute alcohol, at least one to five days old .. .. 50 ml.
4% iron alum aqueous .. .. 50 ml.
This solution should be prepared immediately before use.

B. Borax .. .. 1 gm.
Potassium ferricyanide .. .. 5 gm.
Distilled water .. .. .. 100 ml.

C. Iron alum .. .. .. 0·5% aqueous

D. Sudan 2, saturated in isopropyl alcohol or ethylene glycol .. 30 ml.
Distilled water .. .. .. 20 ml.
Mix well and allow to stand for ten minutes before use.

Note: This solution deteriorates within three to four hours.

Technique:

1. Formalin-fixed frozen sections are stained forty minutes with Solution A at 56° C. in a covered dish in an oven.

2. Rinse in water and differentiate for one hour with Solution B.

3. Rinse in distilled water and immerse for ten minutes in Solution C.

4. Stain ten to twenty minutes in Solution D; then float out in water.

5. Mount in Apathy's gum syrup or in Aquamount.

Results:

Degenerating myelin and fat: black; normal myelin, blue-black; nerve cells, grey; nuclei, deeper grey; red corpuscles, yellow to black; fats, orange yellow.

Reference: Lillie, R. D. (1948), p. 161.

SUDAN 3

For staining fat in faeces

Solution required:

Sudan 3 saturated in equal volumes of 70% alcohol and ether.

Technique:

1. A loopful of faeces is mixed with a drop of 50% alcohol.

2. Add one or two drops sudan 3 solution; then apply a cover-glass before examining.

Results:

Neutral fat appears as highly refractile droplets or yellowish flakes which stain orange to orange-red.

Note: In normal faeces there is no appreciable amount of neutral fat present.

Fatty acids appear as flakes, which stain faintly; or as fine needles which tend to collect in clusters and do not take the stain.

Soaps appear as yellowish flakes, rounded or gnarled bodies, everted like the pinna of the ear, or as coarse crystals, which do not take the stain.

On warming the preparation gently, fatty acids melt, whereas the soaps do not.

In the faeces of a healthy subject the only fat elements recognizable under the microscope are yellow calcium or colourless soaps.

SUDAN RED 7B - TOLUIDINE BLUE

A simple method for staining nuclei and lipids simultaneously

Solutions required:

A. Sudan red 7B, saturated in isopropyl alcohol (absolute)

B. Toluidine blue, 0·5% in isopropyl alcohol (absolute)

C.	Solution A	10 ml.
	Solution B	10 ml.
	Isopropyl alcohol (absolute) ..	5 ml.
D.	Distilled water	100 ml.
	HCl, conc.	0·2 ml.
E.	Ammonium molybdate	3 gm.
	Distilled water	100 ml.

Technique:

1. Fix material in 10% formalin or Bouin or calcium-formol.
2. Cut frozen sections, and drop them into solution C in a watch glass, or in a small petri dish.
3. Cover the watch glass or petri dish to prevent evaporation of the alcohol.
4. Allow the stain to act from one to ten minutes.
5. Differentiate, if necessary, in solution D.
6. Wash well in tap water.
7. Immerse the sections in solution E for about one minute if it is desired to retain the stain in permanent preparations.
8. Rinse well in distilled water.
9. Mount in Aquamount.
10. If desired, seal the edges of the coverslips with Laktoseal.

Notes:

(*a*) Any of the oil-soluble azo dyes (oil red O, oil red 4B, Sudan red 7B, Sudan black, Sudan blue, Sudan green, etc.) may be used.

(*b*) Crystal violet or a number of other basic dyes used as nuclear stains, or haemalum, can be used instead of toluidine blue.

(*c*) Lipids are distinctly coloured with the oil-soluble dye. Toluidine blue demonstrates labrocytes and other metachromatic elements; while crystal violet, used in place of toluidine blue in the above technique, is said to show amyloid degeneration very well.

(*d*) I would suggest that ethylene or propylene glycol might be used to advantage in place of isopropyl alcohol, in cases where more intense staining of lipids is desired as more concentrated solutions of the oil-soluble dyes can be obtained with this solvent, which like isopropyl alcohol, is miscible with water.

Reference: Arvy, Lucie (1958).

TETRACHROME STAIN

(After MacNeal)

For differentiation of types of leucocytes in smears

Staining solution:

Dissolve 0·3 gm. of the dry stain in 100 ml. of pure methyl alcohol by heating to 50° C. on a water bath. Shake well, and leave for four or five days with occasional shaking; then filter.

Technique:

1. Very thin, freshly prepared blood films are dried quickly at room temperature and stained immediately one to three minutes with 1 ml. of the staining solution prepared as above; then add 2 ml. buffer solution pH 6·8 and allow to stand for about five minutes.
2. Pour off the stain and wash with buffer solution pH 6·8 until the thin portions of the stained film are pink.
3. Blot carefully and examine.

Note: If it is desired to keep the films for any length of time before staining, they should be fixed immediately while still wet, with pure methyl alcohol for one minute.

Results:

Erythrocytes are stained yellowish red; polymorphonuclear neutrophiles: dark blue nuclei, reddish mauve granules, pale pink cytoplasm. Eosinophilic leucocytes: blue nuclei, red to orange-red granules, blue cytoplasm. Basophilic leucocytes: purple or dark blue nuclei, dark purple to black granules. Lymphocytes: dark purple nuclei, sky-blue cytoplasm. Platelets: violet to purple granules.

Reference: MacNeal, W. J. (1922).

THIONIN (Ehrlich)

For mucin

Solution required:

Thionin (Ehrlich), saturated, aqueous	0·5 ml.
Distilled water	10 ml.

Technique:

1. Tissues should be fixed in Zenker's fluid, washed in running water, dehydrated, cleared, embedded and sectioned in the usual manner.

2. Sections are mounted on slides and the mercuric precipitate from the fixative is removed by the standard technique.

3. Bring down to distilled water as usual; then stain from five to fifteen minutes in the thionin solution.

4. Dehydrate rapidly as otherwise the stain will be removed by the alcohol.

5. Clear in xylol and mount.

Results:

Mucin is metachromatically stained purple; while the basophil granules of the mast cells, Wharton's jelly of the umbilical cord, are purple; and the other tissue constituents are stained in varying shades of blue.

Reference: Hoyer, H. (1890).

THIONIN (Ehrlich)

For the differential staining of entamoeba in sections

Solutions required:

A. Thionin 0·25% aqueous.

B. Oxalic acid 2% aqueous.

Technique:

1. Pieces of tissue are fixed in absolute alcohol and embedded in Celloidin in the usual manner.

2. Immerse in the thionin solution for three to seven minutes.

3. Differentiate in the oxalic acid solution for thirty to ninety seconds, controlling by examination under the microscope.

4. Rinse in water.

5. Rinse in 70% alcohol.

6. Dehydrate by rinsing in two changes of 95% alcohol.

7. Clear in terpineol and mount in Clearmount.

Results:

Nuclei of amoebae are stained a rich brown colour, while the nuclei of all other cells are stained blue.

Reference: Mallory, F. B. (1938), pp. 2, 297.

THIONIN (Ehrlich)

For nerve cells and fibre tracts

Solution required:

Stain and fixative combined:

Thionin 0·3% in 10% formalin.

Technique:

1. Tissues are fixed and stained simultaneously by immersing in the above solution for a few days to three months.
2. Wash well in running water.
3. Dehydrate in ascending grades of alcohol in the usual manner.
4. Clear; embed in paraffin wax or in Celloidin.
5. Fix sections to slides and mount in Cristalite or D.P.X.

Results:

Cell bodies are stained blue, while the fibre tracts are red.

Reference: Chang, M. (1936).

THIONIN (Ehrlich)

For demonstrating malignant cells in biopsy material

Solution required:

Thionin 1% aqueous.

Technique:

1. Stain frozen sections for ten to sixty seconds in the thionin solution.

2. Rinse in water.

3. Mount in tap water or in Aquamount.

Results:

Nuclei: blue to purple. Collagen: red. Elastic tissues: green.

Reference: Mallory, F. B. (1938), p. 152.

TOLUIDINE BLUE

For acid mucopolysaccharides

Solutions required:

A.	Toluidine blue, 1% aqueous ..	1 ml.
	Distilled water	99 ml.
B.	Ammonium molybdate, 5% aqueous (freshly prepared)	
C.	Potassium ferricyanide, 1% aqueous (freshly prepared)	
D.	Reagent B	1 volume
	Reagent C..	1 volume

Technique:

1. Fix pieces of tissue in Zenker or in 10% formalin and embed in paraffin wax in the usual way.

2. Fix sections to slides; dewax and carry through to 70% alcohol.

3. If Zenker's fluid has been used, treat sections for the removal of mercurial precipitate in the usual way.

4. Wash in distilled water.

5. Stain in the dilute toluidine blue solution for five to fifteen minutes.

Note: This is to prevent loss of metachromasia which would otherwise occur when the sections are dehydrated in alcohol.

6. Immerse in solution D for 15 minutes.

7. Rinse well in distilled water.

8. Dehydrate rapidly.

9. Clear in xylol and mount in D.P.X. or Clearmount or Emexel.

Note: Instead of solution A, the following may be used:

Thionin, 1% aqueous ..	0·5 ml.
Distilled water	10 ml.

References:

Bensley, S. H. (1934).
Jones, Ruth M. (1950).

TOLUIDINE BLUE

For Nissl granules

Solution required:

Toluidine blue, 1% aqueous

Technique:

1. Human neurological material is fixed in formol saline for at least three days.
2. Dehydrate in alcohol as usual and clear in chloroform. Alternatively dehydrate and clear in pyridine (as page 502).
3. Stain in the toluidine blue solution for one and a half hours.
4. Rinse very rapidly in tap water.
5. Drain off excess water (but do not allow the sections to dry); then blot gently.
6. Differentiate and dehydrate rapidly with absolute alcohol.
7. Clear in xylol.
8. Mount in D.P.X.

Results:

Nissl granules: bluish purple.

Notes:

(*a*) The author states that it is not necessary to use Gothard's differentiator, which in any case was found to be too strong even when diluted to half strength with absolute alcohol.

(*b*) For more detailed information and photomicrograph, readers should consult the original paper.

Reference: Irugalbandara, Z. E. (1960).

TOLUIDINE BLUE

For mucus

Solution required:

Toluidine Blue 1·5% aqueous.

Technique:

1. Formalin-fixed material is embedded in paraffin wax in the usual manner.

2. Bring sections down to distilled water then stain for one or two minutes in the toluidine blue solution.

3. Wash with distilled water; drain well, then plunge the slide into two changes of 95% alcohol.

4. Dehydrate by immersing in two changes of acetone; then clear in xylol and mount.

Results:

Mucus, reddish violet; nuclei, blue; erythrocytes, yellow to greenish yellow.

TOLUIDINE BLUE

For the identification and counting of basophil leucocytes

(After A. W. A. Boseila, 1959)

Solutions required:

A. Toluidine blue .. .. .. 1 gm.
Alcohol, 60% .. .. .. 100 ml.

B. Toluidine blue .. .. .. 1 gm.
Alcohol, 40% .. .. .. 100 ml.

C. Sorensen phosphate buffer solution pH 7·7

Note: The most convenient way of preparing this is merely to dissolve a Michrome buffer tablet, pH 7·7, in 100 ml. of distilled water

D. Saponin .. .. 2·8 gm.
Alcohol, 50% .. .. .. 100 ml.

E.	Ammonium oxalate	1·2 gm.
	Potassium oxalate	0·8 gm.
	Distilled water	100 ml.
	Formalin (40% formaldehyde)	1 ml.
F.	Solution A	5 ml.
	Solution C	40 ml.
	Solution D	0·75 ml.
	Absolute alcohol	3 ml.
	Solution E	0·5 to 1·5 ml.

Technique I

For blood and bone marrow smears

1. Air-dried smears are fixed in absolute methyl alcohol for ten to twenty minutes.

Note: In the case of human blood, thick smears are recommended on account of the scarcity of basophil leucocytes.

2. Stain in solution A for thirty seconds to one minute.
3. Immerse for thirty seconds in 50% alcohol.
4. Drain off excess liquid and blot dry.

Technique II

For sections

1. Fix small pieces of tissue in absolute methyl alcohol for ten to twenty-four hours.
2. Clear in xylol.
3. Embed in paraffin wax.
4. Fix sections to slides and remove paraffin wax with xylol.
5. Wash in absolute alcohol (methyl or ethyl).
6. Wash in 90% alcohol (methyl or ethyl).
7. Wash in 70% alcohol.
8. Immerse in solution B (toluidine blue in 40% alcohol) for twenty minutes.
9. Wash in 70% alcohol.
10. Wash in 90% alcohol.
11. Dehydrate in absolute alcohol.
12. Clear in xylol.
13. Mount in Michrome mountant or proceed as follows:

14. Clear in xylol.
15. Mount in D.P.X. or Clearmount or Michrome mountant.

Technique III

For chamber-counting

1. A 1:10 mixture of the blood with the counting fluid (solution F) is made by measuring one volume of blood with a standard blood pipette and discharging into nine volumes of solution F, measured by a " Carlberg " micropipette, in an 8×70 mm. tube.
2. Shake the tube by hand until the diluted blood becomes clear, indicating complete haemolysis of the red cells.
3. Immediately fill counting chambers in the usual way.
4. Allow the counting chambers to stand in a damp container for at least five minutes to permit the white cells to settle.
5. To facilitate identification of the basophil, use a blue filter in the microscope lamp.

Results:

Basophil leucocytes are stained metachromatically, their granules being purplish red, while their nuclei as well as all other leucocytes and tissue components are stained faintly.

Notes:

(*a*) Human venous or capillary blood and rabbit venous blood as well as rabbit tissues were used by the authors.

(*b*) Jessen counting chambers with 0·4 mm. depth and 0·1 ml. volume were used for counting human basophils. In all other cases Fuchs–Rosenthal counting chambers with 0·2 mm. depth and 3·2 $mm.^3$ volume were used.

(*c*) In smears of human blood, the authors found that the average diameter of the basophil leucocytes was $10{\cdot}2 \pm 0{\cdot}2\mu$ (7–11μ), while that of the rabbit was $10{\cdot}8 \pm 0{\cdot}3\mu$ ($5{\cdot}4$–$8{\cdot}0\mu$).

(*d*) The technique is recommended by the author (Boseila) for routine use in haematological laboratories, in the belief that the simple methods described may help to throw more light on the unsettled problem of the role played by the basophil leucocyte in health and disease. The use of micropipettes instead of the commonly used standard leucocyte diluting pipettes offers the advantages of economy in the cost of apparatus and more accurate

dilution as well as providing adequate amounts of diluted blood for duplicate counts when required.

(*e*) The addition of the ammonium–potassium oxalate solution to the diluting fluid (solution F) had been found suitable, by the author (Boseila), for keeping the diluted blood in the fluid state.

(*f*) The diluting fluid (solution F) is a modification of Moore & James' (1953) diluting fluid (see page 446).

(*g*) For more detailed information and photomicrographs, the original paper should be consulted.

Reference: Boseila, A. W. A. (1959).

TOLUIDINE BLUE - EOSIN

A simple method for the identification and rapid counting of mast cells in tissue sections

Solutions required:

A.	Formaldehyde, 40%	10 ml.
	Alcohol, 95%	90 ml.
	Calcium acetate	1 gm.
B.	Toluidine blue, 0·25% in 70% alcohol	100 ml.
	Hydrochloric acid, conc. ..	0·5 ml.
C.	Alcohol, 95%	99·5 ml.
	Hydrochloric acid, conc. ..	0·5 ml.
D.	Eosin yellowish, 0·01% in 95% alcohol	

Technique:

1. Fix slices of tissue, not more than 3–5 mm. thick, in solution A for eighteen to twenty-four hours.

Note: Specimens may be left in this solution for at least a week without any apparent harm.

2. Wash for one hour in each of two changes of 95% alcohol.

3. Dehydrate; clear; embed in paraffin wax and cut sections at 8–12μ.

4. Mount sections on slides; dewax with xylol; wash in absolute alcohol followed by 95% alcohol.

5. Stain in solution B (toluidine blue) for one hour, in a closed staining dish to prevent evaporation of alcohol.

Note: Solution B should be filtered before use.

6. Rinse briefly in solution C to remove excess dye, until the sections appear colourless to the naked eye.

7. Counterstain lightly by immersing in solution D (eosin) for a few seconds.

8. Dehydrate rapidly in absolute alcohol.

9. Clear in xylol and mount in D.P.X., Cristalite, Clearmount, or Emexel, etc.

Results:

Mast cells are selectively stained deep blue against a pale pink background.

Reference: Smith, E. W. & Atkinson, W. B. (1956).

TOLUIDINE BLUE - SAPONIN METHOD

For enumeration of basophil leucocytes

(After J. E. Moore III & G. W. James III, 1953)

Solutions required:

A. Sodium chloride, A.R. .. .. 8·5 gm.
Distilled water .. .. .. 950 ml.

Dissolve, then make up the volume to 1 litre with distilled water.

B. Sodium hydroxide N/10

C. Solution A .. .. .. 200 ml.

Test and adjust, if necessary, to pH 7·0 by adding about 0·16 ml. of N/10 NaOH, or simply by shaking in two Michrome buffer tablets, pH 7·0, which should first be crushed to powder.

Toluidine blue .. .. .. 0·15 gm.
Alcohol, 95% .. .. .. 55 ml.

D. Saponin, saturated (30%) in 50% alcohol

Note: To prepare this solution shake the saponin, with the alcohol at intervals over several hours until it is all dissolved.

Note: The saponin solution should be made up freshly as and when required for use.

E. Solution C .. .. .. 51 ml.
Solution D .. .. .. 1 ml.

Filter before use

Note: Solution E should keep for four months at room temperature. Occasional filtration may be necessary to remove any sediment formed.

Technique:

1. Dilute fresh unclotted blood obtained by finger puncture, or citrated or oxalated venous blood, in the proportion of 1:10 with solution E in the standard leucocyte pipette.

2. Shake the pipette mechanically, or by hand, for three minutes.

3. Fill the four chambers of two Fuchs–Rosenthal (0·2 mm. deep) haemocytometers by capillary action in the usual manner.

4. Allow the counting chambers to stand for a few minutes in a moist-chamber.

5. Count one haemocytometer while the other remains in the moist chamber, using the 4 mm. objective.

6. After counting the number of basophils in all four chambers, total and average and multiply the number by the dilution factor, 3·13, which gives the absolute count in terms of basophils per centimetre.

Notes:

It is stated in the original paper that a total leucocyte count can be carried out simultaneously by counting two of the sixteen larger squares, averaging, and multiplying by fifty. The chamber should be filled immediately after shaking because if the pipette

is allowed to stand for longer than thirty minutes, a precipitate is liable to form around the leucocytes, making accurate counting impossible.

For more detailed information, the original paper should be consulted.

Reference: Moore, J. A. III & James, G. W. III (1953).

TRIBASIC STAIN (Barlow)

For mast-cell granules

Solutions required:

A. Iron alum, 5% aqueous .. .. 100 ml.
Celestin blue .. .. .. 0·5 gm.
Shake the iron alum solution with the dye in a flask; boil for three minutes, allow to cool; then filter, and add:
Sulphuric acid, conc. .. .. 2 ml.
Glycerine .. .. 14 ml.

B. Haemalum (Mayer)

C. Lithium carbonate, saturated, aqueous

D. Alcohol, 70% .. .. .. 99 ml.
HCl, conc. .. .. .. 1 ml.

E. Acridine red, 10% aqueous

F. Neutral red, 2% aqueous

G. Basic fuchsin, 1% aqueous

H. *Barlow's tribasic stain*
Solution E .. .. .. 10 ml.
Solution F .. .. .. 25 ml.
Solution G .. .. .. 5 ml.
Distilled water .. .. .. 20 ml.
Nitric acid, conc. .. .. 0·25 ml.
Boil the mixture vigorously for five to ten minutes; cool; then add:
Glycerine .. .. 50 ml.
Wash the glycerine out of the measuring cylinder with about 25 ml. of distilled water; add the washings

to the stain solution; then make up the final volume to 150 ml. with distilled water.

Note: Solution H deteriorates after repeated use, and it is recommended that the solution should be renewed after each batch of sections.

I. Lissamine fast yellow .. .. 0·1 gm.
Acetic acid, 1% aqueous .. 100 ml.

or

J. Light green, 0·25% aqueous

Technique:

1. Fix material in Bouin, or formol–saline or formol–sublimate, or Zenker (acetic or formol), or alcohol, or formol–acetic–alcohol.
2. If a mercury-containing fixative has been used, treat for the removal of mercuric precipitate.
3. Stain sections with the celestin blue solution for two minutes.
4. Rinse in tap water.
5. Stain in the haemalum solution for two minutes.
6. Rinse and blue in the lithium carbonate solution.
7. Differentiate in the acid alcohol (solution D), controlling by microscopic examination, until the cytoplasm is clear and the nuclei are delicately but firmly revealed.
8. Immerse in Barlow's tribasic stain (solution H) for five to fifteen minutes.
9. Wash quickly in running water.
10. Counterstain lightly with solutions I or J.
11. Rinse in water.
12. Dehydrate quickly with alcohol; clear in xylol; and mount in D.P.X., Cristalite, Clearmount, or Emexel.

Results:

Mast-cell granules are stained orange to red, the shade varying somewhat with the species of animal. Cartilage, keratin, and bacteria: pink or red. Mucin is occasionally stained a faint pink. Nuclei are blue, and other tissue elements, green or clear yellow, depending on which of the two counterstains (solutions I and J) have been employed.

The method gives satisfactory results on the tissues of man, ox, pig, dog, and rat.

Notes:

(*a*) The staining is progressive and should be controlled under the microscope as the density of colour and rapidity with which it is taken up varies with the species of animal and the state of the individual mast cells.

(*b*) The addition of nitric acid to the tribasic stain (solution H) reduces the affinity of the latter for nuclei and at the same time increases its selectivity for mast-cell granules, although other mucopolysaccharide-containing tissue elements also take up the stain to some degree. Of these, cartilage reacts most strongly, while gastric mucin, keratin, and elastic tissue may stain weakly, usually in a slightly different shade of red.

Reference: Barlow, R. M. (1957).

TRICHROME STAIN (Gomori)

Solutions required:

A. Delafield or Ehrlich Haematoxylin.

B. Lithium carbonate 1% aqueous.

C. Alcohol 70% .. .. .. 97 ml.
HCl, concentrated .. .. .. 3 ml.

D. Picric acid 1% in 50% alcohol.

E. Phosphotungstic Acid 3%.

F. Light green, or Fast green FCF, or
Aniline blue .. .. 0·5 gm.
Neoponceau (Michrome) .. .. 1·5 gm.

G. Solution F .. .. 1 volume
Acetic acid 2% .. .. 3 to 4 volumes

Technique:

1. Fix tissues in Bouin or 10% Formalin.
2. Stain sections in the Haematoxylin solution for ten minutes.
3. Blue in the lithium carbonate solution.
4. Differentiate, if necessary, with the HCl alcohol for predominance of the green or blue shades, or in picric alcohol for predominance of red shades, in the final picture.

5. Immerse the preparation in 3% phosphotungstic acid for ten to fifteen minutes.
6. Wash gently under the tap for one minute.
7. Stain in solution G for five to twenty minutes.

Note: The time is not critical although results will be slightly different.

8. Rinse in 2% acetic acid.
9. Dehydrate, clear and mount.

Results:

Nuclei: blue. Cytoplasm, muscle fibres, red cells, etc., in shades of red. Connective tissue: green or blue.

Note: Professor Gomori used Woodstain scarlet, which was not available under that name in Britain. The British equivalent at that time being Neoponceau. It is felt that this synonym is more suitable in this case, as the name Woodstain scarlet, indexed in literature on general biology, might suggest a botanical stain for woody tissues.

References:

Gomori, G. (1950), Personal communication.
Gurr, E. (1956).

TRICHROME STAIN (Masson), Modified

For epithelium, pituitary and thyroid glands, nerve (normal and tumour), etc.

Solutions required:

A. Regaud's haematoxylin.

B. Picric acid saturated in 95% alcohol 20 ml.
Alcohol 95% .. .. .. 10 ml.

C. *Violamine-acid fuchsin:*
Violamine, R .. .. .. 0·7 gm.
Acid fuchsin .. .. .. 0·3 gm.
Glacial acetic acid .. .. 1 ml.
Distilled water .. .. .. 100 ml.

D. *Acetic fast green*
Fast green, F C F .. .. 3 gm.
Glacial acetic acid .. .. 2 ml.
Distilled water .. .. .. 100 ml.

Technique:

1. Tissues are fixed in Bouin, Regaud, Zenker or formalin, and embedded in paraffin wax.

2. Mordant sections on slides with 5% iron alum previously heated to 45° C.

3. Wash with tap water; stain for five minutes in Regaud's haematoxylin; then rinse with 95% alcohol.

4. Differentiate with picric alcohol; then wash with running tap water.

5. Stain for five minutes in violamine R–acid fuchsin; then wash with distilled water.

6. Differentiate for five minutes in 1% phosphomolybdic acid; then without rinsing:

7. Flood the slide with solution D and leave for five to ten minutes.

8. Rinse with distilled water; then return to 1% phosphomolybdic acid for five minutes.

9. Leave in 1% acetic acid for five minutes.

10. Dehydrate in 95% alcohol, followed by absolute alcohol; clear in xylol and mount.

Results:

Nuclei: black. Argentaffin granules: black or red. Cytoplasm: vermilion. Collagen: green. Neuroglia fibrils: vermilion. Mucus: green. Keratin: vermilion. Intercellular fibrils: vermilion. Golgi apparatus: clear.

Reference: Lillie, R. D. (1945).

TRICHROME STAIN-PVA FIXATIVE

For confirmation of Amoebiasis

Solutions required:

A. *PVA-Fixative:*

Glacial acetic acid	5 ml.
Glycerin	1·5 ml.
Schaudinn's fixative	93·5 ml.
Polyvinyl alcohol (PVA)	5 gm.

Sprinkle the PVA on the surface of the mixed liquids, then stir for a few minutes with a glass rod. Heat, with constant stirring, to 75°–80° C. until the PVA has dissolved completely and the solution is clear.

Note: PVA-fixative should keep for several months without deterioration.

B. *Trichrome stain* (*Wheatley*, 1951)

Chromotrope 2R	0·6 gm.
Light green SF yellowish ..	0·15 gm.
Fast green FCF	0·15 gm.
Phosphotungstic acid	0·7 gm.
Glacial acetic acid	1 ml.
Distilled water	100 ml.

Weigh out the dry components of the stain and place them in a test tube. Add the acetic acid to the mixture in the test tube and agitate. Allow the tube to stand for fifteen to twenty minutes, then rinse it out with several lots of distilled water taken from the 100 ml. already measured out. Pour the rinsings into a 250 ml. flask and finally add the remainder of the distilled water. Shake well.

C. 90% alcohol	100 ml.
Glacial acetic acid	0·5 ml.

Technique:

Note: Fresh specimens, faeces or other materials suspected of containing protozoa are mixed with PVA-fixative. Immediately or months later, permanently stained films can be prepared from the preserved material. Microscope slide preparations are made as follows:

1. A drop of stool or other material (sigmoidoscocopic aspirates, culture sediments, etc.) is placed on a slide and mixed with three drops of PVA-fixative.

2. Spread the mixture with an applicator stick (do not make smears as in the case of blood) over about one-third of the area of the slide.

3. Allow the preparation to dry thoroughly overnight at room temperature, or for at least three hours at 37° C. in the incubator.

Note: Dried films remain satisfactory for staining for many months, if not indefinitely. During sigmoidscopy, the same aspirate or curetted material (the author, Brooke, 1960, states that samples should not be obtained with swabs), which is examined in wet mounts, can be preserved in PVA-fixative.

4. Immerse the preparation in 70% alcohol containing iodine for ten minutes.

5. Immerse for three to five minutes in each of two baths of 70% alcohol.

6. Immerse in solution B (Wheatley's trichrome stain) for six to eight minutes.

7. Rinse in solution C for ten to twenty seconds.

8. Immerse in 95% alcohol for five minutes.

9. Immerse in carbol-xylol for five to ten minutes.

10. Immerse in xylol for ten minutes.

11. Mount Canada balsam in xylol, D.P.X., Permount, or Cristalite or Clearmount.

Results:

The cytoplasm of thoroughly fixed and well stained *E. histolytica* trophozoites and cysts is blue-green, tinged with purple. *E. coli* cysts are slightly more purplish. Karyosomes of nuclei: brilliant ruby red. Background material usually stains green, resulting in a noticeable colour contrast with the protozoa. Organisms in the thick portions of the smears take the more neutral shades of red and green. In contrast with those stained with haematoxylin, trichrome smears have a transparency which enables identification of embedded protozoa. Helminth eggs and larvae can frequently be identified in the stained smears, usually stained red and contrasting strongly with the green background. Non-staining cysts and those staining predominantly red are most frequently associated with incomplete fixation. If unsatisfactory stained organisms are obtained it usually indicates incomplete fixation associated with poor emulsification. Thorough emulsification of preferably soft stools (or those first mixed with saline saline) will give critically stained cysts and trophozoites. Degenerate forms stain pale green. Owing to the contrast between the organisms and the background, larger protozoa can be seen with the one-sixth-inch objective, but it is preferable to use the oil immersion objective.

Reference: Brooke, M. M. (1960).

Notes:

(i) The author (Brooke) states that the greatest advantage of the PVA-fixative is that it makes possible the performance of satisfactory examinations for amoebiasis without the patient being present. It appears that one of the major uses of the PVA-fixative has been by public health laboratories which are generally dependent upon the delivery of specimens by mail. By using a two-vial collection kit, described in the original paper, such a laboratory can perform satisfactory examinations for amoebae and other intestinal parasites. Brooke also states that in attempting to establish a diagnosis, the patient can be directed to collect and preserve specimens passed at home, particularly those during the hours of diarrhoea or dysentery. The specimens preserved in PVA-fixative can be mailed to the clinic or delivered at the convenience of the patient.

(ii) The fixative makes possible the successful staining of organisms occurring in fluid stool specimens. The solution which serves as an adhesive as well as a preservative and prevents loss of organisms during the staining procedure, may be found to be of wider application in microtechnique for collecting and preserving other kinds of biological specimens.

TWEEN METHOD (Gomori)

For lipases

Solutions required:

A. Tween 80 (or Crill 7)

Note: Tween 80 is a U.S.A. product; Crill 7 is a British product.

B. Veronal acetate buffer M/5, pH 7·4

C. Calcium chloride, anhydrous, 10%

D. Solution A .. .. .. 1 ml.
Solution B .. .. .. 5 ml.
Solution C .. .. .. 3 ml.
Distilled water .. .. .. 45 ml.

Filter; then add a crystal of thymol as a preservative and store in a refrigerator where the solution keeps reasonably well.

E. Lead nitrate, 2% aqueous

F. Ammonium sulphide, conc. sol. 1 volume
Distilled water .. .. .. 5 volumes

Technique:

Note: Gomori states that celloidin protection is useful but only permissible in the case of saturated substrates; the unsaturated ones penetrate the celloidin membrane very poorly.

1. Material should be fixed in 10% formalin at 4° C., and the frozen sections cut as thin as possible. Alternatively, material may be fixed in acetone at 4° C. and embedded in celloidin–paraffin wax.
2. Fix frozen sections to slides, without the use of glycerine albumen, and leave to dry thoroughly in the atmosphere at room temperature. If sections of double embedded material are used, fix to slides with glycerine albumen and allow to dry thoroughly in the air at room temperature.
3. Incubate the slides at 37° C. in reagent D.
4. Rinse the slides in distilled water.
5. Immerse in reagent E for ten minutes at 45° C.
6. Wash in repeated changes of distilled water for five to ten minutes.
7. Immerse in reagent F for about five minutes.
8. Wash in running water for five to ten minutes.
9. Counterstain as desired.
10. Wash thoroughly in water and mount in Aquamount or in glycerine jelly; or dehydrate, clear, and mount in D.P.X. or Clearmount.

Results:

Lipases are indicated by a dark brown deposit.

Reference: Gomori, G. (1952), pp. 203–206.

UREA SILVER NITRATE

For nerve fibres and nerve endings

Note: In this technique, nerve fibres and nerve endings of the peripheral and central nervous system are preferentially stained.

Applied to paraffin sections on slides, the technique gives rapid and constant results, and eliminates the necessity of gold toning.

The following fixatives are recommended:

(I).	Chloral hydrate	25 gm.
	Alcohol 50%	100 ml.
(II).	Formalin, undiluted (i.e. Formaldehyde 40%)	20 ml.
	Alcohol 95%	80 ml.
(III).	Ammonium bromide	2 gm.
	Formalin, undiluted (i.e. Formaldehyde 40%)	15 ml.
(IV).	95% or Absolute Alcohol	
(V).	Bouin's fluid.	

Solutions, I, II, III and IV are satisfactory for Central Nervous System and nerve trunks.

Solution I has been used with satisfaction for striated muscle tissue.

Solution II is suitable for gland and smooth muscle tissue.

Solution V for gland and smooth muscle tissues and for embryos.

Tissues may also be fixed in 10% formalin with good results, but an excessive staining of connective tissue has been observed when this fixative has been employed.

Solutions required:

A.	Picric acid, saturated aqueous ..	50 ml.
	Mercuric cyanide	0·5 gm.
B.	Silver nitrate 1% aqueous	100 ml.
	Urea	25 gm.
	Solution A	3 drops
C.	Hydroquinone	2 gm.
	Urea	25 gm.
	Distilled water	100 ml.
	Sodium sulphite, anhydrous ..	10 gm.

Technique:

1. Fix the material in one of the above fixatives (I, II, III, IV or V) and embed in paraffin wax.
2. Fix sections to slides and remove paraffin wax with xylol.
3. Rinse with two changes of absolute alcohol.
4. Wash with 90% followed by 80% alcohol.

5. Immerse slides directly into solution B for one to one and a half hours at 50 to 60° C. in an oven.

6. Rinse quickly in two changes of distilled water.

7. Reduce by immersing in solution C for three minutes at 25 to 30° C., agitating the slides gently for the first two minutes.

8. Wash thoroughly in four or five changes of distilled water.

9. Wash with 50% followed by 70% and 80% alcohols.

10. Examine under the microscope while the preparation is still wet and if it is found that the staining is not complete, repeat step 5 using the original urea-silver nitrate solution and reduce the time to ten to fifteen minutes; then repeat steps 6, 7, 8 and 9.

11. Rinse with 95% alcohol.

12. Dehydrate with two changes of absolute alcohol.

13. Clear in xylol and mount.

Results:

Nerve fibres are stained from brown to black, while nerve endings are usually black, and nerve cells from yellow to brown. The background is usually yellow, but its appearance depends upon the kind of tissue and the fixative employed.

Reference: Ungewitter, L. H. (1951).

VERHOEFF'S STAIN

For elastic fibres, nuclei and collagen

Solutions required:

A. Haematoxylin 5% in absolute alcohol .. .. 20 ml.
Ferric chloride (hydrated) 10% aqueous .. .. .. 8 ml.
Iodine solution (1 gm. iodine, 2 gm. KI, 50 ml. water) .. 8 ml.

Note: Solution A deteriorates after twenty-four hours.

B. Ferric chloride hydrated 2% aqueous.

C. Van Gieson stain (picro fuchsin).

Tissues should be fixed in Zenker or in 10% formalin: if the former is used mercurial precipitates are removed by the iodine in the staining solution and it is not, therefore, necessary to treat the sections or tissues with iodine before staining.

Technique:

Paraffin wax, Celloidin or L.V.N. may be used for embedding.

1. Sections are brought down to distilled water; then immersed in Solution A for one quarter to one hour until quite black.
2. Differentiate for a few minutes in Solution B, controlling by examination in water under the low-power objective.
3. Wash with tap water; then immerse in 95% alcohol to remove iodine.
4. Wash in tap water for five minutes; then counterstain in Van Gieson for three to five minutes.
5. Differentiate in 95% alcohol; then dehydrate.
6. Paraffin sections are cleared in xylol; Celloidin or L.V.N. in terpineol (after 95% alcohol).
7. Mount in Cristalite or in balsam or D.P.X.

Results:

Elastic fibres: intense blue-black to black. Nuclei: blue to black. Collagen: red. Other tissue elements: yellow.

Reference: Verhoeff, F. H. (1908).

WATER BLUE - ORCEIN - SAFRANIN

For demonstrating epithelial fibres

Solutions required:

A. Water blue .. .. .. 1 gm.
Orcein .. .. 0·75 gm.
Glycerine .. .. 20 ml.
Absolute alcohol .. .. 50 ml.
Acetic acid 5% .. .. .. 100 ml.

B. Eosin 1·25% alcoholic.

C. Hydroquinone 1% aqueous.

D. Safranin O, aqueous 1%.

E. Potassium dichromate 0·5%.

Technique:

1. Specimens of skin are fixed in 10% formalin and embedded either in paraffin wax or in Celloidin.

2. Bring sections down to distilled water; then stain for ten minutes in a mixture consisting of:

Solution A	10 ml.
Solution B	3 ml.
Solution C	3 ml.

3. Wash well in distilled water.
4. Stain for ten minutes in the safranin solution.
5. Wash thoroughly in distilled water.
6. Immerse in 0·5% potassium dichromate solution from ten to thirty minutes.
7. Wash in distilled water; dehydrate in absolute alcohol; then clear in oil of bergamot.
8. Examine under the microscope; then if necessary differentiate alternatively with absolute alcohol and oil of bergamot until the depth of the safranin stain has been reduced.
9. Mount in balsam or in Cristalite or D.P.X.

Results:

Epithelial fibres are stained red, while the nuclei are pale violet; plasmasomes, red; cytoplasm, blue to violet; granules of the neutrophil leucocytes, sky blue; elastic fibres, red; collagen fibres, blue.

References:

Carleton, H. M. & Leach, E. H. (1947), p. 285.
Unna, P. G. (1910*a*).

WEIGERT - FRENCH ELASTIN STAIN

(Moore's modification)

Preparation of the dry stain:

A. Ferric chloride, anhydrous, A.R. grade .. .. 30 gm.
Distilled water .. .. .. 65 ml.
Dissolve; then make up the volume to 100 ml. with distilled water.

Note: This solution must be freshly prepared.

B. Crystal violet .. .. .. 2·5 gm.
Basic fuchsin .. .. .. 2·5 gm.
Dextrin .. .. 1·0 gm.
Resorcin, pure .. .. .. 10·0 gm.
Distilled water .. .. .. 500 ml.

Note: The water must be heated to about 95° C. in a large evaporating basin, the dyes and dextrin added and stirred until dissolved. The resorcin is then added and the solution raised to boiling point with constant stirring.

Procedure:

1. When Solution B begins to boil, add 62 ml. of Solution A slowly, in small portions at a time over a period of five minutes, with constant stirring.
2. Continue with the boiling and stirring for a further period of two or three minutes until a coarse precipitate is obtained.
3. After cooling filter by means of a Buchner funnel and flask attached to a suction pump.
4. Wash the precipitate with distilled water until the runnings are colourless and the filtrate a clear azure blue; this usually requires 8 to 10 litres distilled water.
5. The preparation is then dried overnight in an incubator, after removing the filter paper.

Preparation of the staining solution:

1. The dried elastin stain is now dissolved in 550 ml. absolute alcohol plus 1 ml. HCl in a 1-litre flask, the neck of which is plugged lightly with cotton-wool. Solution is effected by boiling gently for about thirty minutes on a water bath or electric hot-plate.

2. Cool; filter; add 19 ml. concentrated HCl; then shake well and allow to stand for at least twenty-four hours.

Staining technique:

1. Sections are brought down to distilled water; then treated with 0·5% aqueous potass. permanganate for five minutes.

2. Rinse and bleach with 5% aqueous oxalic acid; then wash in running water.

3. Transfer to elastin stain for at least two hours at 37° C. or for one half to one hour in an oven at 60° C.

4. Blot and treat with absolute alcohol for three to five minutes.

5. Rinse with water and counterstain for three minutes with neutral red (Jensen).

6. Rinse and differentiate the neutral red for a few seconds in absolute alcohol.

7. Rinse in distilled water, then pour on 0·5% picric acid aqueous and wash off immediately with running water.

8. Blot; dehydrate in absolute alcohol; clear in xylol and mount.

Results:

Elastic fibres: blue-black. Nuclei: red. Erythrocytes and muscle: yellow.

Notes: Best results are obtained after the stain has been kept in stock for several weeks, when it becomes perfectly selective and remains so indefinitely.

The use of Coplin's jars, which can be "topped-up" occasionally to make good loss by evaporation, is to be recommended.

Picric acid gives a beautiful contrast to the neutral red and enhances the appearance of the elastic fibres by causing them to stand out against a neutral background. Care must be taken when using it, however, as overstaining tends to give the red nuclei an unpleasant brownish tinge. It is perhaps advisable, until the technique has been mastered, to use neutral red only, ensuring that it is properly differentiated in absolute alcohol.

It is claimed that this modification gives greater selectivity than either Sheridan or Weigert elastin stains, and consistently excellent results are obtained provided the stain is properly prepared.

Solutions of Moore's elastin stain will keep for several years without deterioration.

The dry stain requires time and great care for its preparation; the majority of workers will, no doubt, wish to purchase the stain ready for use, but for those who have the time and prefer to prepare the stain themselves, the method is given above.

References:

Moore, G. W. (1943).
Gurr, E. (1950*b*, 1956).

WEIGERT - PAL TECHNIQUE

For myelin sheaths in brain and spinal cord and for peripheral nerves and ganglia

Solutions required:

A. *Weigert's rapid fixative:*
Potass. dichromate .. .. 5 gm.
Fluorchrome powder (chromium fluoride) .. .. .. 2 gm.
Distilled water .. .. .. 100 ml.
Dissolve by heat; cool and filter.

B. Haematoxylin 10% in absolute alcohol .. .. 10 ml.
Absolute alcohol .. .. 90 ml.

C. Saturated lithium carbonate, aqueous .. .. .. 7 ml.
Distilled water .. .. .. 93 ml.

D. Potass. permanganate .. .. 0·25%

E. Oxalic acid 1% .. .. .. 50 ml.
Potass. sulphite, anhydrous 1% .. 50 ml.

Technique:

1. Slices of the material 2 to 5 mm. thick are fixed in 10% formalin; then transferred to Solution A for four to seven days.

2. Wash in running water for several hours; then dehydrate and embed in Celloidin, L.V.N. or in paraffin wax, or cut frozen sections (in which case 2% ammon. bromide should be added to the formalin fixing solution).

3. Sections 10 to 20μ in thickness are stained twenty-four to forty-eight hours in a freshly prepared mixture consisting of 1 volume Solution B and 9 volumes Solution C.

4. Immerse for one half to three minutes in Solution D; then rinse in distilled water.

5. Differentiate in Solution E for one half to three minutes or until the white matter is blue-black, and the grey matter almost colourless.

6. Counterstain with safranin 1% aqueous, if desired, for one half to two hours according to thickness of sections.

7. Wash thoroughly in water: dehydrate; clear and mount.

Results:

Myelin sheaths: blue-black. Myelinated fibres: black or blue-black. Grey matter: white or slightly yellow. Other structures: unstained (unless a counterstain has been used).

Note: See page 199, note (*b*).

References:

Pal, J. (1886, 1887*a*, 1887*b*, 1888).
Weigert, C. (1885).

WOOL GREEN - HAEMATOXYLIN - BIEBRICH SCARLET

For connective tissue and muscle

Solutions required:

A. Picric acid, saturated in 70% alcohol.

B. Weigert's haematoxylin A.

C. Biebrich scarlet 1% in 1% acetic acid aqueous.

D. Weigert's haematoxylin B.

E. Wool Green S 1% in 1% aqueous acetic acid.

F. Acetone and xylol, equal volumes of each.

Technique:

1. Immerse sections in the picric acid solution for two minutes.

2. Wash thoroughly in running tap water.

3. Stain for five to seven minutes in Weigert's haematoxylin A.

4. Rinse in water and stain for three to five minutes in the Ponceau S solution.

5. Wash in water.

6. Immerse in Weigert's haematoxylin B.

7. Wash thoroughly in water.

8. Stain for three to five minutes in the wool green solution.

9. Decolorize for two minutes with 1% acetic acid.

10. Pour off excess acid; rinse well in distilled water; drain and blot carefully.

11. Rinse well with acetone.

12. Rinse with two washings of Solution F.

13. Clear in xylol and mount.

Results:

Muscle and cytoplasm: red. Connective tissue and basement membranes: green to dark blue.

Reference: Lillie, R. D. (1945).

WRIGHT'S STAIN

For general differentiation of blood corpuscles; for malarial parasites; trypanosomes, etc. in sections.

This stain is extensively used in America instead of Leishman stain which appears to be preferred by many British workers.

Solutions required:

Formol-saline, neutral, buffered:

A. Formalin (40% formaldehyde) .. 100 ml.
Sodium chloride, A.R. .. .. 8·5 gm.
Distilled water .. .. .. 1 litre
Acid sodium phosphate, monohydrate .. .. 4 gm.
Anhydrous disodium phosphate 6·5 gm.

B. Wright's stain.

C. Acetic acid, 0·08% aqueous.

Technique:

1. Fix pieces of tissue in Solution A for sixteen to forty-eight hours.

2. Dehydrate in the usual ascending grades of alcohol; clear and embed in paraffin wax.

3. Fix sections, not exceeding 5μ in thickness to slides; remove wax with xylol; pass through descending grades of alcohol down to neutral distilled water.

4. Stain for three to five minutes in a freshly prepared mixture consisting of one volume of Wright's stain and two volumes of neutral distilled water, in a stoppered staining jar.

5. Rinse with neutral distilled water.

6. Differentiate with the acetic acid solution, controlling by examination under the microscope, until the protoplasm of the cells is pink, and only nuclei are blue.

7. Wash with neutral distilled water.

8. Dehydrate quickly with absolute alcohol; clear in xylol; mount in Cristalite.

Results:

Erythrocytes: yellowish red. Polymorphonuclears: dark purple nuclei, reddish violet granules, pale pink cytoplasm. Eosinophiles: blue nuclei, red to orange-red granules, blue cytoplasm. Basophiles: purple to dark blue nuclei, dark purple to black granules. Lymphocytes: dark purple nuclei, sky blue cytoplasm. Platelets: violet to purple granules. Malarial parasites and Leishmania: chromatin, red; cytoplasm, blue. Trypanosomes: chromatin, red.

Note: The timing of the staining either before or after dilution may be altered to suit individual requirements.

Staining effects similar to Giemsa are obtained by staining for ten minutes in Wright's stain diluted with four times its volume of distilled water buffered to pH 6·5.

Reference: Wright, J. H. (1902).

WRIGHT'S STAIN

For general differentiation of blood corpuscles; for malaria parasites, trypanosomes, etc., in smears

This stain is extensively used in America instead of Leishman stain, which appears to be generally preferred by British workers.

Best results are obtained with very thin films, and the distilled water used should be buffered to pH 6·5–7·0.

Technique:

Fixation is unnecessary unless the films are to be kept for any length of time before staining, in which case they should be fixed

for five minutes with pure methyl alcohol; then blotted and dried at room temperature.

1. Place 1 ml. of the stain on a dried blood film and leave it to act for one minute; then add 2 ml. distilled water and rock the slide gently to mix.

2. Allow this diluted stain to act for three to five minutes; then pour off and wash with distilled water until the thin portion of the films appears pink to the naked eye.

3. Pour off excess stain; blot and dry at room temperature.

Results:

Erythrocytes: yellowish red. Polymorphonuclears: dark purple nuclei, reddish violet granules, pale pink cytoplasm. Eosinophiles: blue nuclei, red to orange-red granules, blue cytoplasm. Basophiles: purple to dark blue nuclei, dark purple to black granules. Lymphocytes: dark purple nuclei, sky-blue cytoplasm. Platelets: violet to purple granules. Malarial parasites and Leishmania: chromatin, red; cytoplasm, blue. Trypanosomes: chromatin, red.

Note: The timing of the staining either before or after dilution may be altered to suit individual requirements.

Staining effects similar to Giemsa are obtained by staining for ten minutes in Wright's stain diluted with four times its volume of distilled water buffered to pH 6·5.

Reference: Wright, J. H. (1902).

SECTION 3

APPENDIX

FIXATION

The main objects of fixation are:

(*a*) To kill the cells suddenly and uniformly so that they retain, as near as possible, the same appearance which they possessed in life.

(*b*) To preserve the tissues, cells, etc., by the inhibition of putrifactive and autolytic changes.

(*c*) To set and hold intra-cellular bodies, cells, etc., by precipitation in the positions which they occupied in life, thereby facilitating the closest possible study of the histology and cytology of the cells.

(*d*) To facilitate differentiation in the refractive indices of certain cell elements which would otherwise be invisible owing to the exceedingly narrow margin between the refractive index of one type of cell and that of other types.

(*e*) To protect the cells and tissue constituents from the subsequent processes such as dehydration, clearing, embedding, staining, etc., prior to their examination under the microscope.

(*f*) To facilitate proper staining of tissues. Here it should be mentioned that some fixatives act as mordants while others act as inhibitors for certain stains, and it is, therefore, of considerable importance that a suitable fixative should be employed for a particular staining technique or a particular staining technique should be chosen to suit material which has already been treated with a particular fixative. Recommendations as to suitable fixatives are given for most, if not all, of the staining techniques described in this book.

It is essential, if good results are to be achieved, that tissues should be removed from the body with the least possible delay and fixed immediately. Bacteria, protozoa and other unicellular

organisms should be living at the instant of fixation. The following points must be observed in order to avoid failure, waste of time, effort and materials:

1. Pieces of tissue should, whenever possible, be cut into slices 2 to 6 mm. in thickness to permit penetration of the fixative throughout within a reasonable time.

2. The container in which the material is to be fixed should be of sufficient size to take the pieces of tissue without their folding or bending.

3. If large organs are to be fixed, large incisions should be made to allow thorough penetration of the fixing fluid.

4. A volume of the fixing fluid roughly about twenty to fifty times as great as that of the material to be fixed is necessary.

5. Material must not be left in the fixing fluid beyond the necessary time, but should be taken out, washed, dehydrated, cleared and embedded or stored in a fluid, suitable for the particular material, until it is required for embedding.

6. After fixation, and before proceeding to dehydration, careful washing out of the excess fixative is necessary, except in the case of alcohol which requires no washing out. In most cases running water is employed for this purpose, but for some tissue cells and cell constituents, and for some fixatives, alcohol must be used. In all cases, however, it is necessary to use liberal quantities of the liquids for washing out.

7. A fixative suitable to the material to be examined and compatible with the stains to be employed should be chosen, as disregard of this factor will, as previously stated, lead to failure and disappointment as well as waste of time, effort and materials: the importance of this rule cannot be over-emphasized.

FIXATIVES

The number of these from which to choose is legion, although the number in everyday use is comparatively small. Details of some of the more commonly used of these are given below: recipes of others are given elsewhere in this book, see pages 532–9 and Index.

Acetic Acid, Glacial

Recommended for:

Rapid fixation of strongly contracting organisms.

Technique:

Fix in the warm acid for a maximum period of fifteen minutes; then remove excess acid by washing in 30–50% alcohol.

Remarks:

Acetic acid glacial is rarely used alone: it causes the swelling of cell constituents, etc., and it is of most value when mixed with such substances as formalin, alcohol, mercuric chloride, etc., to counteract their shrinking effect.

Acetone

Recommended for:

Rapid fixation of brain tissue for rabies diagnosis (R. D. Lillie, 1948). It is also employed for fixing tissue enzymes, particularly phosphatases and lipases.

Technique:

Thin slices of tissue are fixed in pure acetone for twelve to twenty-four hours at 0° C.; they are then dehydrated by immersion in two changes of pure acetone for two hours in each at room temperature, and afterwards cleared by immersing for half an hour in each of two changes of benzene before infiltration with paraffin wax.

Alcohol, Absolute

Recommended for:

Glycogen, Amyloid, Fibrin, Hyaline, Haemofuscin, Phosphatase.

Technique:

Fix from two hours to several days according to the nature of the material; dehydrate; clear.

Remarks:

Unsuitable for fats and lipines as these are dissolved by the higher concentrations of alcohol.

Allen's Fixative (B.15)

Recommended for:

Chromosomes.

Recipe:

Picric acid, saturated, aqueous	75 ml.
Formalin (formaldehyde 40%)	20 ml.
Acetic acid, glacial	5 ml.
Chromic acid	1·5 gm.
Urea	2 gm.

Technique:

Fix tissues from four to sixteen hours then immerse in frequent changes of 70% alcohol over a period of forty-eight hours or until the yellow coloration due to picric acid ceases to come out.

Remarks:

The solution deteriorates very rapidly and it is, therefore, essential that this fixative should be prepared only when it is required for immediate use.

Bouin's Fluid

Recommended for:

Embryological specimens, for elementary bodies, Purkinje cells, Argentaffin reaction, and for animal tissues in general.

Recipe:

Picric acid, saturated aqueous	75 ml.
Formalin	25 ml.
Glacial acetic acid	5 ml.

Technique:

Fix from eighteen hours to two days; then wash in 50% followed by 70% alcohol until most of the yellow coloration, due to

picric acid, is extracted. Alternatively the picric acid can be washed out with the alcohols after the tissues have been embedded and sectioned.

It is not essential that all the picric acid, which serves as a mordant enhancing many staining effects, should be entirely extracted from the fixed tissues.

Remarks:

This fixative, which keeps indefinitely and causes only slight shrinkage of tissues, is compatible with almost every staining technique: it is considered to be a valuable fixative for most purposes, although it is unsuitable for kidney and mucin. Its penetration power is great, and delicate material should be left in contact with this fixative only for the minimum time, to avoid over-fixation: this applies to cytological work in particular. There are many modifications of Bouin, of which Allen's Fixative, B.15, has proved to be the most satisfactory for chromosomes in mammalian tissues.

Bouin - Duboscq (Duboscq-Brasil, or Alcoholic Bouin)

Recommended for:

Arthropods containing parasites and protozoan cysts, and for chitinous tissue.

Recipe:

Absolute alcohol	48 ml.
Formalin (formaldehyde 40%) ..	30 ml.
Glacial acetic acid	7·5 ml.
Picric acid, saturated aqueous ..	12·5 ml.
Distilled water	15 ml.

Technique:

Fix from eighteen hours to two days; then wash in 70% alcohol.

Remarks:

This is stated to be more penetrating than aqueous Bouin and for this reason it is employed for hard tissues.

Carnoy's Fluid

Recommended for:

Glycogen; for animal tissues in general, and for plant cytology.

Recipe:

Absolute alcohol	60 ml.
Chloroform	30 ml.
Glacial acetic acid	10 ml.

Technique:

Fix for one and a half to two hours; then transfer to two changes of absolute alcohol before clearing and embedding.

Remarks:

A rapid fixative with great penetrating power.

Carnoy - Le Brun Fluid

Recommended for:

Insects and ticks, and as a rapid and penetrating fixative for plant tissues.

Recipe:

Absolute alcohol	30 ml.
Glacial acetic acid	30 ml.
Chloroform	30 ml.
Mercuric chloride	to saturation (about 10 gm.)

Technique:

Fix from half to one minute; then wash in 95% alcohol.

Champy's Fluid

Recommended for:

Plant and animal tissues in general; mitochondria and other cytological detail.

Recipe:

Potass. dichromate 3% aqueous	.. 35 ml.
Chromium trioxide 1% aqueous	.. 35 ml.
Osmic acid 2%	.. 20 ml.

Technique:

Fix for six to twenty-four hours; then wash in running water for the same length of time.

Flemming's Fluid (Strong)

Recommended for:

Cytology; fat.

Recipe:

A.	Chromium trioxide 1%	.. 30 ml.
	Acetic acid, glacial	2 ml.
B.	Osmic acid 2%	8 ml.

Mix A and B immediately before use.

Technique:

Fix for one to twenty-four hours according to the material; for chromosomes an hour is sufficient. Wash with running water for twenty-four hours.

Remarks:

The penetration power of this fixative is poor; and that of the weak solution (*see* below) is poorer still; however, either solution gives good results with basic stains, particularly safranin, and iron haematoxylin. Quite apart from their high cost, Flemming fixatives should definitely not be employed as general fixatives, but only in special cases for very small pieces of tissue where fixation extending through a layer of about four or five cells in thickness is sufficient, as this is the limit of their penetrating power even in loose-celled tissues.

Flemming's Solution (weak)

Recommended for:

All purposes for which Flemming's strong solution is used.

Recipe:

A.	Chromic acid 1%	25 ml.
	Acetic acid 1%	10 ml.
B.	Osmic acid 1%	10 ml.
	Distilled water	50 ml.

Technique:

Used in the same way as the strong solution (*see* above), except that the volume of the fixative required is about eight or ten times that of the material to be fixed.

Remarks:

See under Flemming's Fluid (strong).

Formalin Neutral

Recommended for:

Animal tissues in general, particularly neurological tissue.

Formula:

Formalin (formaldehyde 40%) ..	100 ml.
Tap water	900 ml.
Magnesium carbonate	to excess

Shake well and allow to stand several hours at least; then decant off the volume of the clear fluid required for fixation.

Technique:

Fix at least twenty-four hours at room temperature, or six to eight hours at 50–60° C. Washing out is unnecessary.

Remarks:

Formalin penetrates well, tissues may be kept in it for long periods without undue hardening, although there is a gradual decrease in basophilia of cytoplasm and nuclei, and certain cytoplasmic structures are not hardened by it sufficiently to permit paraffin embedding. For this reason the proportion of formalin (formaldehyde 40%) to water is often increased to 1 : 3.

Formalin Buffered

Recipe:

Neutral formalin, as above	1 litre
Sodium dihydrogen phosphate, monohydrate, A.R.	4 gm.
Disodium phosphate anhydrous, A.R.	6·5 gm.

Technique:

As for neutral formalin.

Remarks:

Neutral formalin turns acid on keeping owing to the production of formic acid, whereas the buffered fixative remains neutral.

Recommended for:

All purposes for which neutral formalin is employed where a neutral fixative is required.

Formol - Alcohol

Recommended for:

Glycogen in animal tissues. Fibrin. Peroxidase. Plant tissues, particularly pollen tubes in styles.

Recipe:

Formalin (40% formaldehyde) ..	100 ml.
Alcohol 70%	900 ml.

Technique:

Fix for three to six hours; then dehydrate, clear and embed. Alternatively, if it is not convenient to dehydrate, clear and embed at once, the tissues may be stored for long periods without deleterious effects in 70% alcohol.

Remarks:

This fixative, which penetrates quickly, while compatible with most stains, is particularly suitable for indigo carmine.

Helly's Fluid

Recommended for:

Animal tissues in general, but particularly for blood-forming organs.

Recipe:

Potassium dichromate	5 gm.
Mercuric chloride	10 gm.
Sodium sulphate crystals	2 gm.
Neutral formalin (40% formaldehyde)	10 ml.
Distilled water	200 ml.

Technique:

Fix for twelve to twenty-four hours. Wash in running water for the same time. Transfer to 80% alcohol; dehydrate; clear and embed.

Remarks:

Not recommended for bacteria, cytoplasm degeneration, necrosis or regeneration.

Hermann's Fluid

Recommended for:

Cytological work.

Recipe:

Platinic chloride 10%	6 ml.
Osmic acid 1%	32 ml.
Glacial acetic acid	4 ml.
Distilled water	38 ml.

N.B.—The solution should be freshly prepared.

Technique:

Fix from twelve to sixteen hours, wash in running water for three to six hours; then treat as for Flemming fixed-material.

Remarks:

While this fixative mordants chromatin for staining with basic stains, it inhibits staining with acid stains. Good plasma staining is difficult if not impossible after this fixative.

Kleinenberg's Fluid

Recommended for:

Embryos; marine organisms, arthropods, chitinous material.

Recipe:

Sulphuric acid 1%	100 ml.
Picric acid, saturated aqueous ..	49 ml.

Technique:

Wash out the picric acid with warm 70% alcohol, followed by increasing strengths of alcohol.

Remarks:

This fixative is a powerful penetrant of chitin.

Lewitsky's Fluid

Recommended for:

Plant cytology.

Recipe:

Formalin (formaldehyde 40%) ..	100 ml.
Distilled water	100 ml.
Chromic acid 5% aqueous	100 ml.

Technique:

Fix tissue for twelve to twenty-four hours; then wash for six to sixteen hours in running water.

Marchi's Fluid

Recommended for:

Animal and plant tissues generally.

Recipe:

Potassium dichromate 2·5% aqueous	100 ml.
Sodium sulphate crystals	1 gm.
Osmic acid 1% aqueous	50 ml.

Note.—The last item should be added immediately before use.

Technique:

Immerse thin pieces of tissues, not more than 2 mm. thick in the fixative for four to eight days; then wash in running water for twelve to sixteen hours. Transfer to 70% alcohol, afterwards dehydrating, clearing and embedding in the usual way.

Remarks:

This fixative is also employed to blacken nerve fibres.

Navashin's Fluid

Recommended for:

Cytological study (usually of plant tissues).

Recipe:

Chromic acid	1·5 gm.
Acetic acid 10% aqueous	100 ml.
Distilled water	60 ml.
Formalin (formaldehyde 40%) ..	40 ml.

Note.—The formaldehyde should not be added until the solution is required for immediate use.

Technique:

Fix material for one to two days; then wash twelve to sixteen hours in running water. Dehydrate; clear and embed.

Orth's Fluid

Recommended for:

Demonstration of acute degenerative processes to be stained with Giemsa, Wright, or Leishman Stain, and for Intestine.

Recipe:

Potassium dichromate	2·5 gm.
Sodium sulphate crystals	1 gm.
Distilled water	100 ml.
Formalin (formaldehyde 40%) ..	10 ml.

The last item should not be added until the fixative is required for immediate use.

Technique:

Fix pieces of tissue up to 1 cm. in thickness for two to four days; then wash in running water from twelve to twenty-four hours. Transfer to 80% alcohol; dehydrate; clear and embed.

Remarks:

This fixative may be employed as a general fixative for animal tissues. It is useful where a firm consistency of tissue is required, but it is not recommended for sharp histological detail. The value

of the sodium sulphate in the above recipe is extremely doubtful and it appears that this constituent may be left out without any noticeable effect.

Petrunkevitch's Cupric Paranitrophenol Fixative (Petrunkevitch, 1933)

Recommended for:

Tissues in general.

Recipe:

Alcohol 60%	100 ml.
Nitric acid, pure (sp. gr. 1·41—1·42)	3 ml.
Ether	6 ml.
Cupric nitrate $Cu(NO_3)$ 2, $3H_2O$ ..	2 gm.
Paranitrophenol pure, cryst. ..	5 gm.

Technique:

Fix material for twelve to twenty-four hours. Wash in 70% alcohol; dehydrate; clear and embed.

Remarks:

All stains commonly in use may be employed after this fixative, which causes less hardening than most other fixatives.

Regaud's Fluid

Recommended for:

Mitochondria and rickettsia in animal tissues.

Recipe:

Potassium dichromate	3 gm.
Distilled water	100 ml.
Formalin (formaldehyde 40%) ..	25 ml.

Technique:

Fix material for three days changing the fluid every day. Immerse in 3% potassium dichromate for six to eight days; then wash in running water for twenty-four hours. Dehydrate; clear and embed.

Remarks:

Suitable for Giemsa stain and for Masson's trichrome stain.

Schaudinn's Fluid

Recommended for:

Animal tissues in general. Protozoa.

Recipe:

Mercuric chloride saturated aqueous	66 ml.
Absolute alcohol	33 ml.
Glacial acetic acid	1 ml.

N.B.—The acid should be added immediately before use.

Technique:

Fix tissues for six to sixteen hours. Wash in several changes of 70% alcohol.

Remarks:

The fixative may be used at about 65° C. when less time is required for its penetration.

Susa Fluid (Heidenhain)

Recommended for:

Animal tissues in general.

Recipe:

Mercuric chloride, saturated aqueous	50 ml.
Trichloracetic acid	2 gm.
Formalin (formaldehyde 40%) ..	20 ml.
Distilled water	30 ml.
Glacial acetic acid	4 ml.

Technique:

Fix for five to twelve hours; then wash out with 95% alcohol.

Remarks:

Compatible with most stains, but not with Weigert's elastin stain. Susa offers the advantage over most other fixatives in that it causes less shrinkage and less hardening, thereby rendering tissues easier to cut.

Zenker's Fluid

Recommended for:

Perfect histological detail in animal tissues in general.

Recipe:

Potassium dichromate 2·5% aqueous	100 ml.
Mercuric chloride	5 gm.
Glacial acetic acid	5 ml.

Technique:

Immerse slices of tissue in the fluid for six to twenty-four hours according to the nature of the material and the thickness of the slices. Wash in running water for twelve to twenty-four hours; then transfer to 80% alcohol.

Remarks:

Unsuitable for Mitochondria. Suitable for Mallory's connective tissue stain; for demonstration of Muscle, Fibrin, Haemofuscin, Purkinje cells, etc.

DEHYDRATION, CLEARING, EMBEDDING, SECTIONING, ETC.

CELLOIDIN METHOD FOR EMBEDDING TISSUES

For preserving the relations of cell layers of different consistency, as are contained in the eye; for large objects; for pieces of central nervous system; and for hard tissues such as decalcified bone.

Solutions required:

A. *Celloidin* 8%

Celloidin flakes	25 gm. damped with absolute alcohol*
Absolute alcohol	150 ml.
Ether	163 ml.

**Note:* This may be obtained in 25-gm. bottles, ready damped with absolute alcohol.

Pour the alcohol and ether into a clean, absolutely dry, wide-mouth bottle of about 32 ounce capacity, and mix by inverting the bottle several times, releasing the stopper at intervals, to release the pressure of ether vapour; then add the Celloidin flakes and invert the bottle as before. Leave for about 12 hours to dissolve; inverting or shaking the bottle gently at intervals.

B. *Celloidin* 4%

Celloidin solution 8%	2 volumes
Absolute alcohol	1 volume
Ether	1 volume

Mix as described above, in a large-stoppered wide-mouth bottle.

C. *Celloidin* 2%

Celloidin 4%	2 volumes
Absolute alcohol	1 volume
Ether	1 volume

D. Cedarwood oil, for clearing .. 1 volume
Chloroform .. .. .. 1 volume

Technique:

(*a*) Wet Method

1. Pieces of tissue not thicker than 5 mm. are fixed in the usual manner.

2. Wash in running water for the prescribed time for the particular fixative. If a fixative containing mercury has been used, remove mercurial precipitate by the standard technique.

3. Immerse the pieces of tissue for two hours in each of the following: 50%, 70% and 90% alcohol.

4. Immerse in absolute alcohol from two to sixteen hours, according to the nature and thickness of the tissue.

5. Immerse for twenty-four hours in a mixture consisting of equal volumes of absolute alcohol and ether, in a stoppered wide-mouth bottle, which must be absolutely dry.

6. Impregnate with 2% Celloidin solution from five to seven days.

7. Transfer to 4% Celloidin for five to seven days.

8. Impregnate with 8% Celloidin for three or four days.

9. The tissue is then taken out of the Celloidin and put into a mould made by folding a piece of writing paper, and the whole is then placed in a desiccator and left for several days, lifting the desiccator lid for a few seconds each day to accelerate the hardening of the Celloidin. If, through shrinkage of the Celloidin during this process, the tissue becomes exposed, pour on more Celloidin solution to cover it. Hardening of the block may be hastened by placing 1–2 ml. of chloroform in the bottom of the desiccator.

The block is hard enough for sectioning when no impression is left after pressing with the ball of the thumb.

10. The base of the hardened Celloidin block is dipped into 8% Celloidin then fixed to a roughened wooden or a vulcanite block by pressing firmly, afterwards leaving for at least half an hour with a weight on top.

11. Expose to chloroform vapour for half an hour; then attach the wooden or vulcanite block to the microtome holder, or store the Celloidin block mounted on the wooden or vulcanite block in 80% alcohol until required for sectioning.

12. The microtome knife and the Celloidin block must be kept moist with 70% alcohol and each section as it is cut must be transferred by means of a camel-hair brush, moistened with 70% alcohol, into a suitable vessel containing 70% alcohol in which the sections can be stored indefinitely until required for staining.

13. When required for staining the sections should be removed from the 70% alcohol by means of a small camel-hair brush, or a piece of thin glass rod bent at one end, and transferred to a series of watch glasses containing the reagents and stains, arranged on the bench in the order in which they are to be used. For instance, if it is desired to stain the sections with Haematoxylin and Eosin, the steps are as follows:

14. Immerse sections in 50% alcohol for a few minutes; then transfer to water.

15. Stain with Ehrlich Haematoxylin by the standard technique.

16. Blue in tap water; then stain in Eosin (aqueous solution).

17. Transfer to 70% alcohol; then immerse for five minutes in each of two lots of 96% alcohol.

Note: Absolute alcohol must be avoided as Celloidin is dissolved by it.

18. Immerse for five minutes each in two lots of Carbol-Xylol.

19. Pass into two changes of xylol.

20. Mount in balsam or D.P.X.

(*b*) **Dry Method**

1. Proceed exactly as described above up to and including step No. 8; then take the tissue out of the Celloidin and put it into a paper mould as described in step No. 9 (above).

2. Place the block in a desiccator for a day, lifting the lid for a few seconds every hour or so; then leave in the desiccator overnight.

3. Next morning place the block in a mixture consisting of equal volumes of cedarwood oil and chloroform and add another 8 volumes of cedarwood oil, one volume at a time, every hour for small objects, or every day in the case of large objects. The Celloidin should now be wholly transparent.

4. Fix the Celloidin block to the wooden or vulcanite block as described in step 10 (above).

5. Sections may now be cut without the necessity of moistening the knife or the block.

Note: Blocks prepared by this method are stored in a dry wide-mouth stoppered bottle.

CELLOIDIN - PARAFFIN WAX (Double Embedding)

For serial sections embedded in Celloidin

Solution required:

Celloidin 1% in Methyl Benzoate

This is prepared by adding the appropriate weight of air-dried Celloidin flakes to a quantity of methyl benzoate in a clean dry corked flask or bottle. Shake well; allow the flask or bottle to stand upright for an hour or so; then leave it inverted for an hour, afterwards leave it lying horizontally for a time; then stand it upright again, and repeat the process at intervals throughout the day and leave the bottle lying on its side overnight: next morning, solution should be complete and it is only necessary to shake the bottle well to ensure a homogeneous solution.

Technique:

1. Tissues are fixed and washed in running water, and any mercurial precipitate removed in the usual manner.

2. Immerse for two hours in each of the following: 50%, 70% and 90% alcohol.

3. Transfer to absolute alcohol for two to sixteen hours.

4. Immerse in Methyl Benzoate - Celloidin solution for twenty-four hours, at the end of which time pour off the solution and replace with a fresh lot in which the tissue should remain for a further forty-eight hours: if the tissue is not now clear, transfer it to a fresh lot of Methyl Benzoate - Celloidin solution for a further period of seventy-two hours.

5. Immerse in three changes of pure benzene, for four hours in the first lot, eight hours in the second, and twelve in the third.

6. Transfer to a mixture consisting of equal parts of paraffin wax and pure benzene in the embedding oven for an hour.

7. Immerse in two changes of pure paraffin wax from a quarter of an hour to six hours in each, depending upon the thickness and the nature of the tissue.

Note: It is of utmost importance that tissues should be kept in the embedding oven just long enough for the paraffin wax to penetrate fully. Prolonged heating in the oven causes shrinking and hardening of the tissues rendering sections difficult to cut. If, on the other hand, any of the Methyl Benzoate remains in the tissue and sufficient time has not been allowed for proper penetration of the paraffin wax satisfactory sections cannot be cut. It is best, whenever possible, to cut thin slices of tissue for embedding, preferably not more than 5 mm. thick so that the total time for impregnating in the pure paraffin wax need be no longer than three hours. Large objects such as whole embryos need a total time of twelve hours in pure paraffin wax. Pieces of brain and spinal cord about 5 to 10 mm. thick, skin, and large objects such as whole embryos need at least three changes of pure paraffin wax for a total time of about 12 hours, whereas organs such as spleen, containing a large amount of blood, muscle, fibrous tissue, require no longer than a total of three hours in the paraffin baths. It is, however, only by experience that the technique of embedding can be mastered.

8. Cast the tissue in an embedding mould and proceed as in the case of paraffin sections (see page 500).

CELLOIDIN - PYRIDIN

A rapid method of dehydrating, clearing and embedding, which obviates the use of alcohols and the consequent hardening of tissues

Reagents required:

A. Pyridin, extra pure, redistilled .. 1 volume
Celloidin 4% in equal volumes of absolute alcohol and ether .. 1 volume

B. Celloidin 8% in equal volumes of absolute alcohol and ether.

Technique:

1. Pieces of tissue are fixed in the usual manner.

2. Wash in running water for the prescribed time for the particular fixative employed. If a fixative containing mercury has been used, remove mercurial precipitate by the standard technique.

3. Immerse the tissue in two changes of Pyridin, from two to eight hours in each, according to the nature and the thickness of the tissue.

4. Immerse for twenty-four hours each in two changes of a mixture consisting of equal volumes of Pyridin and 4% Celloidin (formula as above).

5. Immerse in 8% Celloidin for twelve hours.

6. The tissue is then removed from the Celloidin bath, blocked and cut into sections by the standard technique described on pages 487-9.

FROZEN SECTIONS

For the identification of fat in tissues; for certain impregnation methods for the central nervous system; and for the rapid examination of pathological material, such as pieces of tumour, which may be sectioned, stained and diagnosed within a few minutes of their removal by the surgeon in the operating theatre

Sections as thin as 5μ may be cut, and an advantage of this method is that there is a lesser degree of shrinkage than in the case of paraffin-embedded material. It is not, however, possible to cut serial sections by this method, and sections cannot be stored before staining as in the case of paraffin-embedded material. Frozen sections should be employed only for the specific purposes mentioned above and not as an alternative to paraffin embedding. It should be noted that frozen sections are manipulated in the same way as Celloidin sections, but greater care must be exercised on account of the absence of any embedding mass.

Tissues should not be frozen too hard or the sections will curl up and split.

A special microtome is required for cutting frozen sections.

Rapid Method for Staining Fats

Solutions required:

A. Sodium chloride A.R. grade 0·9% in distilled water .. .. 95 ml.
Formalin .. .. 5 ml.

B. Sudan Black B, saturated in 70% alcohol (this should be freshly filtered).

Note: Ethylene or Propylene Glycol may be employed as the solvent (see page 428).

C. Apathy's mountant.

Technique:

1. Thin slices of tissue are fixed for ten minutes at 37–40° C. in Solution A.
2. Transfer the material directly from the fixative to the freezing microtome and cut sections about 5μ in thickness.
3. By means of a camel-hair brush, moistened with 50% alcohol, transfer sections from the microtome knife directly to the first of a series of dishes previously arranged as follows, in order of their use: 70% alcohol, Sudan Black solution (as above), 50% alcohol; distilled water.
4. After immersion in 70% alcohol (in the first dish) for two minutes, stain in the Sudan Black for ten minutes, or longer if time permits.
5. Rinse in the 50% alcohol.
6. Transfer to the distilled water: mount in Apathy's medium, or, if there is sufficient time, counterstain for about three minutes in Carmalum; then rinse in distilled water before mounting.

Results:

Neutral fat and myelin: blue-black to black; nuclei: red.

GELATINE EMBEDDING

This method of embedding is employed when sections of loose friable tissues are required. Dehydration is entirely eliminated since the embedding takes place directly from water. The gelatine which is retained during the staining processes holds the tissues together without absorbing the stain itself.

Solutions required:

A. *Gelatine* 5%

Gelatine	5 gm.
Phenol 1% aqueous	95 ml.

Warm in an oven to about 45° C. then stir until the gelatine has dissolved; raise to about 60° C. before filtering through fine calico.

B. *Gelatine* 10%

Phenol 1% aqueous	90 ml.
Gelatine	10 gm.

Prepare exactly as for Solution A.

C. *Gelatine* 15%

Phenol 1% aqueous	85 ml.
Gelatine	15 gm.

Prepare exactly as for Solution A, except that temperature should be raised to about 75° C. before filtering.

D. *Gelatine* 20%

Gelatine	20 gm.
Phenol 1% aqueous	80 ml.

Prepare as for Solution A, but raise temperature to 95° C before filtering.

E. *Gelatine* 1%

Gelatine	1 gm.
Phenol 1% aqueous	99 ml.

Dissolve by warming.

Technique:

1. Small pieces of tissue, not more than 3 mm. in thickness are fixed for twenty-four hours in 5% formalin in physiological saline.

2. Wash in running water.

3. Immerse in 5% gelatine in an incubator at 37° C. for twenty-four hours.

4. Immerse in 10% gelatine overnight in an incubator at 37° C.

5. Immerse in 15% gelatine in an incubator for several hours at 37° C.

6. Embed in 20% gelatine and leave to set.

7. Cut out blocks of tissue and immerse them in formalin for twenty-four hours.

Note: The blocks may be stored indefinitely in this formalin solution if desired.

8. Rinse blocks in water and trim.

9. Freeze blocks thoroughly until they are uniformly white.

10. Allow the block to thaw somewhat until the knife cuts easily.

11. Cut sections up to 5μ in thickness and float onto slides with distilled water.

12. Drain off excess water and float sections on slides with two or three drops of 1% gelatine.

13. Drain off excess 1% gelatine and leave the slides in an oven at 37° C. until the sections are dry.

14. Immerse slide in 10% formalin for ten minutes to fix the gelatine; then stain in the usual manner with Sudan 3, or Scarlet R, Nile blue or osmic acid, or store the slides in the 10% formalin until required.

LOW VISCOSITY NITROCELLULOSE (L.V.N.)

For embedding tissues

Chesterman and Leach's technique using Low Viscosity Nitrocellulose (L.V.N.) offers advantages over the older method of embedding in Celloidin, in that penetration is quicker, considerably thinner sections can be cut, it is easier to use and considerably cheaper than Celloidin. With L.V.N. technique large blocks, such as half a cat's brain, can be cut at 15μ; small blocks 5 × 5 mm., can be cut at 5 to 7μ on a paraffin microtome without any special modification or attachment.

L.V.N. is supplied damped with normal butyl alcohol; it is more explosive than Celloidin and it should be handled with care. When dry it will explode if hit. Exposure to sunlight should be avoided.

Solutions required:

Note: Solutions A and B each contain 20% of Nitrocellulose:

A. Absolute alcohol .. .. 210 ml.
Ether .. .. 250 ml.
Dibutyl phthalate .. .. 5 ml.*

Mix well and add 140 gm. L.V.N. (as supplied damped with N. butyl alcohol).

B. Absolute alcohol .. .. 210 ml.
Ether .. .. 250 ml.

Mix well and add 140 gm. L.V.N. (damped with N. butyl alcohol).

From Solution B prepare also 5% and 10% solutions by diluting with a mixture consisting of equal parts of absolute alcohol and ether.

C. Xylol .. .. 2 parts
Toluol .. .. 1 part
Beechwood creosote .. .. 1 part

Procedure for embedding tissues:

1. Fix and dehydrate tissues as usual; then immerse in ether-absolute alcohol (equal parts) for one day.

2. Immerse in 5% L.V.N. for three to five days.

3. Transfer to 10% L.V.N. for one to two days.

4. Transfer to 20% L.V.N. (Solution B above) for one to five days.

5. Embed in Solution A.

* *Note:* In the original paper (Chesterman & Leach, 1949) and in E. Gurr (1950*a*, 1953), tricresyl phosphate was stipulated: this has now been replaced by dibutyl phthalate, and thanks are due to Professor F. Bergel of The Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, for calling attention to the potential danger of handling tricresyl phosphate. Professor Bergel states that tricresyl-o-phosphate is highly toxic, more than 7–30 mg./kg. producing severe paralysis, and while the corresponding meta- and para-compounds show little sign of having this toxicity, the tricresyl phosphates on the market, up to the present time, always contain some of the ortho compound.

References:

Aldridge, W. N. (1954).
Thompson, R. H. S. (1954).
Martindale's Extra Pharmac. (1941), vol. 1, 200–201.

6. Allow to harden slowly in a desiccator. In one to five days the block should be adequately hard. At this stage it should be a stiff but easily deformable gel not altered in shape or size by shrinkage; it should be considerably less hard than a Celloidin block is usually made.

7. Plunge the block into 75% alcohol. Change the alcohol twice over a period of one to three days.

8. Trim the block, removing the hard outer rim of the L.V.N. Use 20% L.V.N. to mount it on the wood or fibre block. Hardening is complete in a few minutes. Dip into 75% alcohol for a few more minutes.

Cutting sections:

Cut the sections " dry ". If a Celloidin microtome is used the tilt of knife should be the same as that used for cutting Celloidin, but the angle the knife makes with the direction of travel should be between 25° and 45° instead of the usual 75° used for Celloidin sections; this prevents the rolling of sections.

Procedure for handling sections:

1. Collect the sections in 75% alcohol; handle and stain as usual. Dyes tend to stain L.V.N. less than Celloidin.

2. Mount sections on to a slide from a bowl of 96% alcohol. Flatten with tissue paper moistened with 96% alcohol; press the paper with a glass rod and then remove it. Repeat this several times.

3. Treat similarly several times with equal parts of absolute alcohol and chloroform.

4. Treat similarly several times with the solution C.

5. Treat similarly several times with xylol.

6. Mount in balsam or D.P.X.

Reference: Chesterman, W. & Leach, E. H. (1949).

MERCURIC CHLORIDE PRECIPITATES IN SECTIONS: METHODS FOR REMOVAL

Before proceeding to the staining of sections of tissues which have been fixed in fluids containing mercuric chloride it is necessary to carry out the following procedure, which is essential for

the removal of the deposits of mercuric chloride which would obscure the picture.

Method I

Solutions required:

A.	Iodine	0·5 gm.
	Alcohol 70%	100 ml.
B.	Sodium hyposulphite	7·5 gm.
	Alcohol 96%	100 ml.
	Distilled water	900 ml.

A crystal of thymol should be added to the stock bottle.

Technique:

1. Sections are mounted on slides and the paraffin wax removed with xylol. If there is any doubt as to the nature of the fixative which has been used, examine a section under the microscope: mercuric chloride, if present, will be seen as a fine brown granular deposit, more abundant in the centre of the section than on the outer edges.
2. Wash with absolute, followed by 90% alcohol.
3. Immerse for half to two minutes in Solution A.
4. Wash well with water.
5. Immerse in Solution B for half to two minutes or until the natural colour of the sections has been restored.
6. Rinse well with water.
7. Stain, dehydrate and clear in the usual manner.

Method II (Cellosolve Method)

Simultaneous dehydration of tissue blocks and removal of mercuric precipitates of fixatives

1. Fix for the required time.
2. Wash for a short time in water or immerse directly in cellosolve.
3. Immerse in three changes of cellosolve for a total time of twenty-four to forty-eight hours.

4. Clear in two changes of toluene or benzene for a total time of one to two hours.

5. Embed in paraffin wax as usual.

Results:

The mercuric precipitates are completely eliminated from the tissues.

Notes:

This method not only saves time in the embedding process but when the tissues have been fixed in fluids containing mercury compounds, it also obviates the use of iodine and sodium thiosulphate (page 497), and in consequence, the mordant action of iodine also. It would appear that the needle crystals or spots deposited in tissues which have been fixed in fluids containing mercury compounds, which according to Baker (1950) represent a reaction product between mercuric chloride and tissue phosphates, are soluble in cellosolve and are thus dissolved and washed away.

Reference: Gonzalez, G. (1959).

PARAFFIN WAX EMBEDDING AND SECTIONING

(*a*) Dehydration

Technique:

1. Pieces of tissue are fixed and washed by any of the standard methods.

2. Immerse in 50% alcohol from twelve to twenty-four hours.

3. Transfer to 70% alcohol for the same length of time as stage 2.

4. Transfer to 90% alcohol for the same length of time.

5. Transfer to 96% alcohol for the same length of time.

6. Immerse in absolute alcohol for the same length of time.

Note: Where possible use a series of corked specimen tubes for the above procedures. By occasionally shaking the tube containing the specimen the process of penetration of the alcohol is accelerated. Twelve hours in each change of alcohol is sufficient for small pieces, but larger pieces of tissue usually require eighteen or

twenty-four hours. Hard tissues may be softened by Lendrum's technique which consists of immersing the tissues in 4% aqueous phenol for one to three days, after washing out the fixative; dehydration is then carried out in the manner described above.

Rapid dehydration of small slices of tissue:

Thin slices not more than 5 mm. in thickness are immersed for half an hour in each of 50%, 70%, 90%, 96% and absolute alcohol.

(*b*) Clearing

Xylol, cedarwood oil, benzene, toluene or chloroform are the reagents most frequently used for this purpose.

Xylol is the most rapid of these in displacing the absolute alcohol, but it has the disadvantage of rendering tissues brittle; therefore, if xylol is used as the clearing agent, tissues must be subjected to it only for the minimum time necessary to displace the absolute alcohol.

Cedarwood oil is slow in its action but it has the advantage of not hardening tissues even after prolonged immersion.

Benzene is the best clearing agent and may be employed for the most delicate tissues: it causes the minimum shrinkage, penetrates tissues fairly rapidly and subsequently evaporates from them in the paraffin embedding bath.

Toluene is also a very satisfactory clearing agent in that tissues can be subjected to it for at least twenty-four hours without risk of their undergoing shrinkage.

Chloroform hardens tissues to a lesser degree than xylol but requires two or three times as long to penetrate and clear the tissue. It rapidly evaporates from the paraffin embedding bath, and it is particularly suitable for large pieces of pathological tissue.

Technique:

1. Small pieces of material not more than 5 mm. thick may be cleared by immersing for fifteen to thirty minutes in each of two changes of xylol or cedarwood oil or benzene or toluene. Larger pieces up to 1 cm. thick require one-and-a-half to three hours in each of two changes of the clearing agent, while bulky specimens such as whole embryos require up to six hours in each of the two changes. If at the end of the times prescribed above the specimens

are not translucent or transparent they should be left in the clearing agent until they have reached that stage.

(*c*) Embedding

Technique:

1. Transfer the object from the clearing agent to a mixture consisting of approximately equal parts of paraffin wax and the clearing agent in a tube and place the whole in the oven set at a temperature from about 50 to 60° C. for one half to sixteen hours, depending upon the size and nature of the object; half an hour is sufficient for objects up to 3 mm. thick; an hour for 5 mm., two hours for 1 cm., and from eight to sixteen hours for bulky specimens such as whole embryos.

2. Transfer to pure paraffin wax in the oven from a quarter to eight hours.

3. Transfer to another bath of pure paraffin wax for the same length of time.

Note: Specimens up to 3 mm. thick usually require half an hour in each of the two baths of pure paraffin wax, while specimens 5 mm. in thickness require about an hour; and 1 cm. about four hours; very bulky objects about eight hours in each of the two baths of wax.

Pathological material containing thrombi, emboli, etc., striated and non-striated muscle, organs containing a large amount of blood (spleen, etc.), and fibrous tissue should be subjected to immersion in the embedding baths for the minimum time necessary for the wax to penetrate thoroughly, as they are particularly liable to hardening and shrinkage when exposed to heat for prolonged periods.

Casting the Paraffin Block

1. Smear the inside of the embedding angles and the embedding base-plate very thinly with liquid paraffin; then adjust the angles on the plate to form a mould of a suitable size.

2. Fill the mould with molten paraffin wax; then place the object in the wax and arrange it so that it is set in the right plane for sectioning.

3. When the wax block so formed is partially set, immerse it gently, while still in the mould, in cold water to ensure rapid

cooling and thereby obviating crystallization of the wax and consequent crumbling of the block when it is mounted on the microtome and sections are cut.

Section Cutting

This can only be learnt by practical experience under skilled guidance in the laboratory, and it is not proposed to make any attempt to deal with the subject here. However, readers are referred to the specialist books on section cutting.

Mounting Sections on Slides and Hydrating

1. Wet the tip of the finger slightly with glycerine albumen (Mayer) and make a smear over an area large enough to take the section in the centre of the slide.

2. Pick a section up with a needle or forceps and place it over the smear of albumen.

3. With the thumb, gently press the section on to the smear so that it is quite flat and without folds or creases, taking care not to damage the section in the process.

Note: If the sections are curled up or folded, first place a drop of 1% potassium dichromate on the slide and float the section on this; then heat very gently until the section floats out flat. Blot round the edges of the section to remove excess solution; then carefully but thoroughly blot until all traces of liquid are removed. Leave the slide on a warm surface for a few minutes to drive away the last traces of water; then proceed as follows:

4. Gently warm the slide until the paraffin wax just melts; then wash away all traces of wax with two or three changes of xylol.

5. Remove the xylol by washing the preparation thoroughly with absolute alcohol.

6. Wash with two changes of 90% alcohol.

7. Wash with 70% alcohol.

8. Wash with two changes of distilled water if an aqueous stain is to be used; but if an alcoholic stain will be used staining may commence immediately after washing with 70% alcohol.

9. Proceed to stain in accordance with the staining technique it is desired to employ.

Note: If the section appears opalescent when the xylol or when the absolute or the 90% alcohol is added the presence of water is indicated and it is necessary to retrace each step until the preparation no longer appears opalescent when taken down to alcohol.

PARAFFIN WAX - PYRIDIN

A rapid method of dehydrating and embedding

1. Material is fixed and washed by the standard method.

2. If a fixative containing mercury has been used, remove mercurial precipitate by the standard technique.

3. Immerse the tissue in two changes of Pyridin for two to eight hours in each, according to the nature and thickness of the tissue.

4. Transfer to a mixture of equal parts of molten paraffin wax and Pyridin in the embedding oven for one half to sixteen hours, depending upon the size and nature of the object.

5. Transfer to pure paraffin wax in the oven for a quarter to eight hours, depending upon the thickness and nature of the material.

6. Cast into block and cut sections in the usual manner.

WATERWAX D23

(Edward Gurr)

A very rapid and simple method of embedding tissues, obviating the use of dehydrating and clearing agents.

Waterwax D23 is an amorphous water-soluble wax which sets at 56° C to form translucent blocks similar in appearance to paraffin wax but with the complete absence of any trace of crystallization. Fresh or fixed material may be used.

Technique:

1. Shake off excess water before immersing the pieces of tissue directly into a bath of waterwax in the embedding oven at 55–6° C. and leave for an hour.

2. Transfer to a second bath of waterwax and leave for an hour.
3. Transfer to a third bath of wax for an hour, or longer.
4. Cast the block and leave it to cool in the atmosphere or in a refrigerator. Care must be taken not to allow the block to come into contact with water.
5. After cutting sections, float them out on water, which dissolves away the wax, and take them up on slides.
6. Stain, dehydrate and mount immediately in the usual way.

Notes:

(*a*) Fat, if present, should be dissolved out with several changes of acetone, before the tissues are immersed in the wax.

(*b*) Blocks should be kept dry and stored in airtight containers as the wax is liable to take up moisture from the atmosphere.

(*c*) Sections can be attached to slides by means of Haupt's adhesive (see pages 529–30).

POLYVINYL ALCOHOL

A simple method of embedding fat-containing tissues that are incompatible with Carbowax, Waterwax, and other water-soluble waxes

Solution required:

Polyvinyl alcohol powder	20 gm.
Tap water	80 ml.
Glycerine	20 ml.

Technique:

1. Fix pieces of tissue as usual.
2. Wash well in running water.
3. Place 80 ml. of tap water in a 250 ml. beaker and heat to about 80–90° C.
4. Sprinkle the 20 gm. of polyvinyl alcohol powder, a little at a time, with constant stirring, on the surface of the hot water.
5. Still stirring, raise the temperature to boiling until all the powder has gone into solution, then turn off the heat.
6. While the solution is still warm, stir in the 20 ml. of glycerine.

7. Infiltrate tissue blocks in the medium in a vacuum embedding bath for one hour at 56° C.; then transfer to a covered dish, in the incubator at 56° C. for two hours per day for about seven to ten days until the block has solidified.

8. Trim and cut as in the case of celloidin blocks.

ESTER WAX (1960), STANDARD

(H. F. Steedman, 1960*a*)

Diethylene glycol distearate, neutral	300 gm.
Glyceryl monostearate	150 gm.
Polyethylene glycol (300) distearate	50 gm.

Melt the diethylene glycol distearate first and heat it until it clears. Add the glyceryl monostearate, and when this is dissolved add the remaining ingredient. Stir well while the mixture is still hot, then filter through a ring of fine calico.

Notes:

The author (Steedman) states that this modification of his earlier (1947) ester wax allows for variations in commercial samples of stearate esters and produces an ester wax of physical stability which gives consistent results.

Ester wax (1960) melts at 48° C. and sections, flat, may be cut from 1μ to 12μ at temperatures between 17° and 27° C., and ribbons may be obtained.

The wax is soluble in absolute alcohol and 95% ethyl alcohol, cellosolve, cedarwood oil, chloroform, xylol and a number of other solvents both of the aliphatic and the aromatic series. Among the clearing agents recommended are xylol, normal butyl alcohol and absolute ethyl alcohol.

A block measuring 20 × 10 × 10 mm. is ready for sectioning, at a room temperature of 20° C., in about an hour after pouring. Cutting speed recommended is 30 to 50 sections a minute. After an appropriate length of time in the clearing agent the tissue slices should be transferred to a bath consisting of approximately equal parts of the wax and clearing agent. Time of infiltration in the pure wax will depend, of course, on the size and nature of the specimen, but a piece of liver about 4 to 5 mm. in thickness will take about three hours with stirring, or about four hours

without. Ester wax, which is an opaque white or cream coloured substance, is brittle when cut in thick slices, and it is recommended, therefore, that only thin slices should be cut away during the trimming of the block. The microtome knife bevel angle should be between 20° and 30° (but not greater than 30°).

For further information the original paper should be consulted.

ESTER WAX (1960) TROPICAL

(H. F. Steedman, 1960*b*)

Recipe:

Diethylene glycol distearate, neutral	300 gm.
Glyceryl monostearate	150 gm.
Triethylene glycol monostearate ..	50 gm.

This is prepared in the same way as the standard ester wax (see page 504).

Notes:

The author (Steedman) states that once the wax is melted and its temperature allowed to fall to 50° C., it will stay liquid at that temperature for about four days. The liquid wax will become cloudy if kept at that temperature, but it may be heated to 80° C. and filtered. The filtrate will remain clear for at least another four days, after which it should be heated and refiltered. Specimens may be infiltrated at 50° C. if desired for four days or even longer.

Sections and ribbons may be cut at 3 to 10μ when the room temperature is as high as 37·5° C., after the block has been at that temperature for a week. Good sections and ribbons may be obtained at this temperature provided the block and knife are kept in a cool place (about 15° C.).

Steedman also calls attention to the difference between the melting point and setting point of embedding waxes. This is something which has not been generally realized; there is usually a difference of several degrees between the two. It should be mentioned here that in the case of paraffin waxes, the temperatures given as the melting points are really the setting points. That is,

the temperature at which the molten paraffin wax sets, or the temperature at which the solid wax just begins to melt. Therefore it may happen that such a wax with a stated melting point of, say, 58° C. will not be completely melted until the temperature is taken up several degrees beyond this point.

POLYESTER WAX

A ribboning embedding medium

(H. F. Steedman, 1957)

Reagents required:

A. *Polyester wax*
Polyethylene glycol (400) distearate .. .. 990 gm.
Cetyl alcohol .. .. .. 10 gm.
Warm gently in a pan over an electric hotplate till melted; then stir well and put the preparation aside to cool in the atmosphere.

B. Distilled water .. .. .. 1 litre
Nipa ester No. 82121* .. .. 1·5 gm.
Boil the water first, then while the water is still boiling add the Nipa ester and stir in until dissolved.

C. Solution B (cold) .. .. 200 ml.
Amylopectin .. .. .. 2 gm.

D. *Amylopectin adhesive*
Solution B .. .. .. 800 ml.
Solution C .. .. .. 200 ml.
First raise the temperature of solution B to boiling, then add slowly, with stirring, solution C. On cooling this reagent is ready for use.

Note: Solution D should not be filtered, and it should always be shaken before use.

Technique:

1. Take pieces of fixed material into 96% alcohol through to absolute alcohol and xylol. Alternatively the material can be taken from 96% alcohol into cellosolve, if desired.

* Made by Nipa Laboratories Ltd., Pontypridd, Glam., S. Wales.

2. Transfer the tissue to a mixture of any of the above solvents and polyester wax, or directly into the pure wax.

Note: The infiltration time recommended by the author (Steedman) is approximately one third longer than that for paraffin wax.

3. Cast the block, as with paraffin wax: do not immerse the wax in water, but leave it to cool in the atmosphere. It is not necessary to surround the block with cold water.

4. Cut sections at the speed of paraffin section (about 80–100 per minute). Do not handle the block.

5. Float the sections or ribbons on to the amylopectin adhesive, either on a microscope slide or in a dish.

6. Flatten the sections by leaving them on the amylopectin solution for five to ten minutes at room temperature (18° C.), or by placing the slide with the amylopectin and the section on it, on a warm plate for about thirty seconds.

7. Carefully draw off the excess amylopectin solution from the slide, then leave the section to dry at room temperature overnight.

Note: It is essential that the excess amylopectin solution is well drained off the slide before drying, as otherwise amylopectin will show as fine black dots under the microscope.

8. Carry the sections through to water, as in the case of paraffin sections, then stain, dehydrate, clear and mount as usual.

Notes:

(*a*) The wax has a melting point of 37° C.

(*b*) The author (Steedman) claims that the main advantage of his polyester wax over paraffin wax, his earlier ester wax (Steedman, 1947), and Chesterman & Leach's (1956) modification of the latter, is its low melting point which reduces hardening and shrinkage of tissues as well as obviating other heat-induced artefacts.

(*c*) Heavy, not readily volatile solvents such as cedarwood oil are not recommended for use with polyester wax. However, alcohols, ethers, and esters are recommended, as well as aromatic hydrocarbons such as xylol and benzene, for those who prefer them. The author states that 96% alcohol is excellent.

(*d*) All the known adhesives for tissue sections failed to prevent the loosening of polyester wax sections from slides, and of more

than eighty new reagents investigated, amylopectin adhesive (solution D, above) proved to be the most successful. This adhesive sticks the sections to glass more firmly than any other medium, but because it breaks down and loses its adhesive properties after eight days, a preservative (Nipa ester No. 8221) is added to prevent this.

Reference: Steedman, H. F. (1957).

CELLOIDIN PROTECTION OF ENZYMES, Etc., IN PIECES OF TISSUE AND SECTIONS

To obviate the loss of water-soluble substances during processing

Solutions required:

1% and 0·5% Celloidin in equal volumes of ether and absolute alcohol.

Technique:

1. Material to be examined for enzymes should be chilled for about twenty minutes in a refrigerator, then cut into slices, not more than 3 mm. in thickness, and fixed in acetone at 0° C. to 4° C. for twenty-four hours.

2. Dehydrate for about twelve hours in each of three changes of absolute acetone.

3. Immerse in the celloidin solution for twelve to twenty-four hours.

4. Drain rapidly; then immerse for about forty-five minutes in each of two changes of chloroform.

5. Embed in paraffin wax at a temperature not exceeding 56° C. for a maximum time of one and a half hours.

6. Cut sections at 3 to 8μ, floating them out on lukewarm water.

7. Fix sections to slides with albumen.

8. Place slides on a warm plate or on a box-type microscope lamp to dry the adhesive thoroughly.

9. Place the slides with the dry sections in the oven for a few minutes until the wax melts to form a protective coating to the sections.

Note: Sections treated in this way remain unchanged for many years.

10. For use, dewax the sections with xylol.

11. Wash well with absolute alcohol.

12. Wash with 95% alcohol, draining and drawing off the excess with a piece of filter paper.

13. Flood slides with 0·5% celloidin.

14. Shake off excess celloidin solution.

15. Harden the celloidin coat by exposure to chloroform vapour, or by immersion in 80% alcohol for a few minutes.

References:

Gomori, G. (1946).
Gomori, G. (1952).

MISCELLANEOUS DEHYDRATING AND CLEARING REAGENTS

ANILINE OIL

For clearing, etc.

This reagent, which is used in the preparation of a number of staining solutions, such as aniline gentian violet, aniline fuchsin, etc., will absorb a fair amount of water (more than will cajeput oil) without clouding, and it is, therefore, particularly useful in wet climates as a clearing agent in place of xylol. However, after clearing, the aniline oil must be removed from tissues with two or three changes of xylol, benzene or chloroform. It is not very miscible with paraffin wax, and unless it is similarly washed out of sections it will impart a brownish coloration, which will become darker with age. A good quality aniline oil, which should be anhydrous, will clear tissues from 95% or even 90% alcohol, and sections from 70% alcohol, but it should not be used after osmic acid fixation or after any fixatives containing oxidizing agents such as chromium trioxide, dichromates, etc., as even traces of these are liable to cause oxidation of the aniline with the

formation of a blackish blue or brownish compound that will be taken up by the tissue to obscure the picture. Gatenby & Beams (1950, p. 69) state that in recent years the use of aniline oil for clearing delicate tissues (e.g. mammalian testicular material) has become widespread because the higher alcohols can be avoided. The same authors state that dehydration is started with 50% or 70% alcohol, and that the alcohol is gradually replaced with aniline oil.

Aniline oil should be stored in tightly closed dark bottles, as it will absorb water from the atmosphere and darken in colour.

BUTYL ALCOHOL TERTIARY

For dehydrating and clearing tissues, for paraffin embedding, in place of ethyl alcohol and xylol. The reagent is miscible with water and with paraffin wax, and causes less shrinkage and hardening of tissue than does ethyl alcohol and xylol. It is also a useful substitute for ethyl alcohol for dehydrating material stained with methylene blue and other dyes which are easily extracted by ethyl alcohol.

Technique:

After fixing and washing tissues in the usual manner pass into:

1. Tertiary butyl alcohol (T.B.A.) 50% aqueous for 1–2 hours.
2. 70% Aqueous T.B.A. 2 hours to several days.
3. 85% Aqueous T.B.A. for 1–2 hours.
4. 95% Aqueous T.B.A. for 1–3 hours.
5. Pure T.B.A. for 3 changes of 4 hours in each.
6. Equal parts of liquid paraffin and T.B.A. for 1–2 hours.
7. Infiltrate in paraffin wax.

CAJEPUT OIL

For clearing

This reagent will absorb small amounts of water without clouding, and it is, therefore, particularly useful in wet climates as a clearing agent in place of xylol. Cajeput oil is considerably more expensive than xylol, however.

CARBOWAX 400

This is an organic solvent recommended by Zugibe, Fink & Brown (1959) for oil red O and Sudan 4 for staining carbowax-embedded and frozen sections.

The authors state that Carbowax 400 fulfils all the requirements of an efficient solvent for lipid staining postulated by Chiffelle & Putt (1951), and has, in addition, several other advantages making it more practical than propylene or ethylene glycol. These advantages are (i) Carbowax (or Waterwax) sections are stained three times as fast; (ii) frozen sections are stained five times as fast; (iii) the staining solution is more rapidly prepared; and (iv) the intensity of staining is greater.

CEDARWOOD OILS

Cedarwood oil for clearing is the refined natural cedarwood oil which is employed for clearing pieces of tissue prior to infiltration with, and embedding in, paraffin wax. It is a thin oil, of variable refractive index depending on the source and the particular batch: the average refractive index of the oil being 1·510. Cedarwood oil for clearing is unsuitable for use with oil immersion lenses, and the latter type of oil would be quite unsuitable for clearing tissues.

CELLOSOLVE

(Ethylene glycol monethyl ether)

For dehydrating thin slices of tissue and sections

This reagent is a colourless, inflammable liquid, miscible with water, alcohol, xylol, cedarwood oil, clove oil, and various other oils and solvents. It is a good solvent for many stains, and is coming into increasing use both for animal and plant histology, in place of ethyl alcohol. Many biologists employ cellosolve in preference to the ethyl alcohol for dehydration. However, although some workers believe cellosolve to be superior to all other dehydrating agents as it obviates hardening and distortion of

most tissues, it is unsuitable for bulk material as it tends to cause distortion of protoplasmic cells owing to the rapidity of its dehydrating action.

Technique:

1. Wash pieces of tissue, not more than 5 mm. thick and immerse directly into cellosolve for half an hour.
2. Immerse in a fresh bath of cellosolve for half to one hour.
3. Immerse in a third bath of cellosolve for the same time.
4. Complete the dehydration in a fourth bath of cellosolve for an hour-and-a-half.
5. Clear in xylol for an hour.
6. Immerse in a bath of molten paraffin wax for half an hour.
7. Transfer to a second bath of paraffin wax for an hour.
8. Complete the infiltration by immersing in a third bath of wax for an hour; then cast the block and cut sections.
9. Fix sections to slides; then remove paraffin wax with xylol.
10. Rinse in two changes of cellosolve.
11. Apply the stain; then wash with water, or alcohol.
12. Immerse for one to three minutes in each of three changes of cellosolve.
13. Clear in xylol, benzol, clove oil or cedarwood oil.
14. Mount in balsam, Cristalite or D.P.X. (Lendrum and Kirkpatrick).

DIOXANE

For the dehydration and clearing of tissues

This reagent is a colourless inflammable liquid, solidifying at 10° C., miscible with water and paraffin wax, alcohol and xylol as well as many other organic solvents of the aliphatic and of the aromatic series. It is used and preferred by many workers in place of the orthodox alcohol-xylol-cedarwood oil method for dehydrating and clearing tissues, as the technique is simpler and quicker. It also has the advantage of eliminating brittleness and shrinkage of tissues. Very recently I have found that certain

triarylmethane dyes (e.g. light green SF) are slightly soluble in dioxane to produce colourless solutions. That is to say, dioxane appears in some cases to act as a decolorizing agent by combining with such dyes.

Warning.—Care should be taken as Dioxane vapour is toxic.

Technique:

1. Transfer tissues directly from the fixative to a well-stoppered specimen jar containing Dioxane with a thin layer of anhydrous calcium chloride over which is placed a circle of surgical or zinc gauze to separate the tissue from the dehydrating agent. Allow the Dioxane to act from three to twenty-four hours, depending upon the size and thickness of the tissue.

Note: Tissues which have been treated with a fixative such as Müller or Zenker, containing potass. dichromate, must be washed from two to twelve hours in running water, depending upon the size and the nature of the tissue, before being transferred to Dioxane.

2. Transfer to a mixture of equal parts of paraffin wax and Dioxane for half to one hour in a paraffin embedding oven.

3. Transfer to pure paraffin wax, allowing a somewhat longer time for impregnation than for tissues cleared by the orthodox method.

Note: The Dioxane can be used several times provided the calcium chloride is changed.

ETHYLENE GLYCOL

A solvent, inflammable when heated, burning with an intense, almost invisible flame; which may also be employed as a dehydrating and differentiating agent for the Sudan colours, in place of acetone-alcohol, 70% or 50% alcohol; giving stable solutions, without loss of stain from the lipid particles.

Technique:

1. Prepare the staining solution by heating and stirring about 0·75 gm. of the dye with 100 ml. pure anhydrous ethylene glycol

to 100° C. Filter the solution while it is still hot, and again after it has been allowed to cool.

2. Cut frozen sections, from formalin fixed material, and wash them in water for about five minutes to remove excess formalin.

3. Dehydrate the sections by agitating them gently with a camel hair brush for three minutes in each of two changes of pure anhydrous ethylene glycol.

4. Transfer the sections to the staining solution for five to seven minutes, agitating them gently at intervals.

5. Differentiate by agitating the sections gently in 85% ethylene glycol in water for two to five minutes, controlling by examining under the microscope at intervals.

6. Transfer to a large dish of distilled water for three to five minutes.

7. Float sections onto slides; drain and carefully blot away excess water.

8. Mount in glycerine jelly, Farrant or Aquamount.

Note: Either ethylene or propylene glycol may be used; however, the former usually costs less than the latter.

Reference: Chiffelle, T. L. & Putt, F. A. (1951).

PROPYL ALCOHOL, NORMAL (OR ISO)

For dehydrating and clearing tissues prior to embedding

Technique:

1. Pieces of fixed tissue are placed directly into normal propyl alcohol and left therein overnight.

2. Transfer directly into a bath of paraffin wax M.P. 40° C.

3. After infiltration of the 40° C. wax, transfer to a bath of 52–54° C. paraffin wax for a few minutes; then cast the block.

Note: This method prevents hardening and distortion of tissue: it is particularly recommended for scirrhous carcinoma, connective tissue, tumours, etc.

TERPINEOL

For dehydrating sections

Technique:

1. After staining and before dehydrating, wipe the slides and blot sections carefully, without allowing them to dry completely.
2. Transfer to terpineol and agitate for a few seconds.
3. Immerse in a second lot of terpineol for a few seconds.
4. Drain and wipe the slides carefully.
5. Clear with xylol, and mount as usual.

Note: The destaining action of alcohol is avoided with this method, which is harmless to the vast majority of stains, Neutral Red being an exception. If desired, Cajeput Oil may be used for clearing.

AQUEOUS MOUNTING MEDIA

A.C.S. MOUNTANT

(Edward Gurr)

(R.I. 1·418)

A new fluid mountant for spiders, mites, ticks, etc., especially suitable for whole mounts. Specimens are preserved by A.C.S. without shrinkage and distortion, and the mountant does not crystallize or darken with age. A.C.S. is miscible both with water and with alcohol, and stains may be incorporated with this mountant which also clear specimens.

The mountant is unsuitable for Lepidoptera.

APATHY GUM SYRUP MOUNTANT

A fairly quick-drying aqueous mountant which sets very hard and may be used in place of Farrant for fat preparations; it is, however, usually definitely acid in reaction. Hardening of the mountant may be hastened if the slides are left on warm plate. Apathy may also be used for ringing glycerine mounts.

AQUAMOUNT

(Edward Gurr)

(R.I. 1·435)

A moderately quick-drying, transparent and colourless, aqueous mountant which, unlike Apathy and Farrant, is neutral in reaction. It takes somewhat longer to harden than does Apathy but, unlike the latter, it does not tend to crystallize or become excessively brittle. Aquamount is preferable to Apathy or Farrant for fat preparations.

BERLEZE'S FLUID

A mountant and killing fluid used in Entomology. Living specimens of Acarina and Insecta are killed by placing them directly into a drop of this fluid on the slide, but specimens which have been stored in alcohol should be washed with 10% aqueous

acetic acid before mounting. The fluid takes from one to two weeks or even longer to set, after which time the coverslips should be ringed with a waterproof cement and ringed with a layer of Canada balsam in benzene, or Laktoseal can be applied immediately.

DOETSCHMAN'S GUM CHLORAL MOUNTANT AND STAIN

This is a modification, containing basic fuchsin, of Berleze's fluid.

This valuable reagent is used in entomology for killing, fixing, dehydrating, clearing, staining and mounting specimens in one operation. Highly chitinized specimens should be treated with 10% potassium hydroxide from sixteen to twenty-four hours, then washed well in water before mounting directly into a drop of Doetschman's fluid on a slide. For permanent mounts the preparations should be placed in the oven at 37–50° C. from six to twenty-four hours. Ring the slides with a waterproof cement.

Reference: Gatenby & Beams (1950), p. 207.

FARRANTS' MEDIUM

Refractive index 1·432

This consists of a viscous solution of gum arabic in a mixture of glycerine and water, together with a preservative, usually arsenic or phenol, although neither of the latter can be relied upon to prevent the growth of some species of air-borne micro-organisms that bring about the decomposition of this mountant. It is a very old mounting medium, having been devised by Farrants about eighty years ago, and which was, until a generation or so ago, extensively used for preparations stained with Sudan 3 and other fat stains of the same group. At the present time, however, it seems to have fallen out of favour.

GLYCERINE

Used as an aqueous mounting medium for temporary preparations. Only the purest neutral glycerine should be used.

GLYCERIN JELLY

Refractive index 1·414

This is a very old mounting medium, first used nearly a century ago, and still used to some extent today for fat preparations, frozen sections, gelatine sections, and whole mounts. However, Dade (1960) states that glycerin jelly is not a good medium for " fluid " mounts; that its refractive index is too low, and that it has no advantages over glycerin or lactophenol which he favours for " fluid " mounts.

For frozen sections of fat preparations, the more modern medium, Aquamount, is to be preferred, as, unlike glycerin jelly, this is a liquid which sets hard. Moreover, glycerin jelly has to be melted before it can be applied.

Preparations mounted in glycerin jelly may be preserved for some time by painting the edges of the coverslips with molten glycerin jelly, and when this has set, two coats of Laktoseal should be applied with a fine, soft brush, allowing the first coat to dry thoroughly (a few minutes) before the second coat is applied. Gatenby & Beams (1950) state that gold size may be used for sealing glycerin jelly mounts on slides. However, it would appear that Laktoseal is preferable on account of the rapidity with which it dries and seals.

GLYCHROGEL MOUNTANT

For gelatine sections, teased preparations, nematodes etc.

A.	Chrome alum	0·2 gm.
	Distilled water	30 ml.
	Dissolve by warming.	
B.	Gelatine granules	3 gm.
	Distilled water	50 ml.
	Glycerine	20 ml.

Warm the water to 45° C. then shake in the gelatine a little at a time until dissolved and add the glycerine.

Add solution A to solution B; shake thoroughly; then filter and add about 0·1 gm. camphor as a preservative.

Keep in a well-closed bottle to prevent evaporation.

Reference: Wotton, R. M. & Zwemer, R. L. (1935).

LACTOPHENOL

Refractive index 1·460

This is a non-drying medium used for making fluid mounts of objects that are difficult or impossible to mount in Canada balsam in xylol or other solidifying mountants. Lactophenol is used for small whole mounts, such as insects, mites, mosses, algae, microfungi, and small coelenterates. It is in universal use by mycologists.

Dade (1960) states that a particular virtue of the lactophenol method is that, in its simplest form, manipulation is completed in a single operation. It is, therefore, especially suited to fragile objects that cannot withstand repeated transfers from one fluid to another without disintegration. Various stains can be incorporated in lactophenol, which then acts as a combined fixative, staining agent and mountant. The lactic acid, which is an ingredient of lactophenol, prevents delicate material from collapsing, and it will, in fact, cause dried, shrunken cells to expand and to appear to regain the natural forms they possessed when living.

For further information, reference should be made to the original paper by Dade (1960).

REYNE'S MOUNTANT

For the preservation and mounting of small insects

Chloral hydrate	50 gm.
Water	50 ml.
Glycerin	12·5 ml.
Gum arabic, white, powdered	30 gm.

Dissolve the chloral hydrate in the water, then sprinkle the powdered gum on the surface of the solution and stir occasionally. Leave overnight, then when all the gum has gone into solution, stir in the glycerin and allow the preparation to stand overnight, to get rid of air bubbles, before it is used.

Note:

It is claimed that preparations mounted in Reyne's fluid are exceptionally clear and require no sealing or heating. This medium, which is said to harden within a few days, has been used for mounting dissected crustaceans such as copepodes.

Reference: Reyne, A. (1950).

UVAK

(Edward Gurr)

An aqueous mountant for fluorescence microscopy. Uvak is moderately quick-drying and it sets hard without crystallizing. The mountant is of particular use for making permanent preparations of specimens that have been stained with acridine orange and other fluorescence dyes which are removed from tissues by dehydrating and clearing fluids.

NON-AQUEOUS MOUNTING MEDIA

CANADA BALSAM

Strictly speaking " Canada balsam " is natural Canada balsam, a pale-yellow viscous fluid obtained from the balsam fir, indigenous to Canada: this fluid is useless as a histological mounting medium. "Canada balsam, dried " is the natural Canada balsam which has been dried by heat until it has become a brittle solid.

" Canada balsam in xylol " (or in benzene or other suitable solvents) is prepared by dissolving the dried natural balsam in xylol, etc. When an histologist sees the words " Mount in Canada balsam " (or simply " balsam ") he takes it to mean, and correctly so, that the preparation is to be mounted in Canada balsam in xylol. However, the term " Canada balsam " can be misleading to those who are new to the language and customs of histology, and such workers have been known to mount in true Canada balsam: i.e. natural Canada balsam, and with most unsatisfactory results. It is for this reason that this definition of " Canada balsam " is given here.

Although Canada balsam in xylol is still used extensively it has a serious disadvantage in that it is prepared from a natural resin which is uncertain and variable in chemical composition, usually somewhat acid in reaction, and while it may be neutralized, will revert to an acid reaction after a time, however careful one is in handling and storing the mountant. For this reason it is better to employ a synthetic mountant of definite and unvarying chemical composition. Short descriptions of mountants of this kind are given below: they also have the advantage of being considerably less costly than Canada balsam.

CLEARMOUNT

(Edward Gurr)

Refractive index 1·515

A colourless, neutral, synthetic mountant, with a drying time approximately the same as Canada balsam in xylol. This mountant, which remains neutral indefinitely and does not cause the fading of even the most delicate stains, is miscible with xylol, absolute alcohol, benzene, toluol, dioxane and many other solvents.

CRISTALITE

Refractive index 1·515

This synthetic mountant has all the properties of Clearmount except that it dries much more rapidly and is not miscible with alcohol.

DAMAR XYLOL

This is an antiquated mountant now apparently little used, not that antiquity in itself is necessarily a fault: in fact a number of mountants, stains and reagents of greater antiquity than damar xylol are still used today and it would be difficult to replace them with satisfactory substitutes. But damar xylol and any other mountant containing gum damar, is unreliable and not to be recommended, particularly today when the microscopist has a variety of excellent and reliable modern synthetic mounting media, such as D.P.X. (Kirkpatrick & Lendrum), Cristalite, Clearmount, Emexel, Michrome mountant and others, from which to choose. No matter how carefully gum damar-containing mounting media are prepared to ensure clear bright solutions free from traces of water, on being kept in stock for any length of time, even in tightly closed bottles, the solutions are liable to become cloudy and unusable, due to adulterants in the raw gum, which defy removal. As long ago as 1913, Lee stated that after ample experience he was convinced that not one of the gum damar solutions proposed by various authors could be depended on for permanent preservation; that " sooner or later, sometimes after a few weeks or days, or it may be only after months or years, granules make their appearance in mounts."

D.P.X.

(Kirkpatrick & Lendrum, 1939, 1941)

Refractive index 1·515

This excellent synthetic mountant, which is colourless and neutral and does not cause fading of stains and on which Cristalite and probably other proprietary synthetic mountants are based, has the added advantage of being one of the least costly of the

synthetic resinous mountants: however, there is a considerable degree of shrinkage of the mountant on drying and it should therefore be applied liberally to allow for this.

EMEXEL MOUNTANT

(Edward Gurr)

Refractive index 1·495

A clear, colourless synthetic, xylol miscible, slightly acid mounting medium. This mountant has a drying time intermediate between Clearmount and Cristalite, and it does not darken with age.

FLUORMOUNT

(Edward Gurr, 1951*b*)

Refractive index 1·515

A synthetic, colourless, neutral, xylol miscible, non-fluorescent mountant for fluorescence microscopy.

HYMOUNT

Refractive index 1·666

A pale-straw coloured synthetic quick-drying mountant of high refractive index, which is miscible with a number of aromatic and aliphatic solvents such as xylol, benzene, chloroform, acetone and absolute alcohol.

LPM MOUNTANT

(W. S. Ollett, 1947, 1951)

Refractive index 1·515

A synthetic mountant similar in composition to D.P.X., but the solvent is monochlorobenzene and not xylol. Ollett found that xylol leads to fading of Gram's stained preparations, and accordingly devised LPM with the object of avoiding the use of a xylol-containing mountant.

MATAMOUNT

(Edward Gurr)

This mountant was designed for mounting cellulose acetate strips in electrophoresis. Its use is described by Bodman (1960). Cellulose strips processed by Bodman's technique are rendered completely transparent and can be used as lantern slides for projection purposes.

MEEDOL BALSAM

(Edward Gurr)

Refractive index 1·515

A xylol miscible mountant, pale amber in colour and hardly distinguishable in appearance from Canada balsam in xylol. This is one of the least costly of all resinous synthetic mounting media. Unlike Canada balsam, but like the other synthetic mountants described in this paragraph, Meedol balsam has the advantage of remaining neutral indefinitely under normal conditions of storage and handling.

MICHROME MOUNTANT

Refractive index 1·515

A synthetic neutral mountant miscible with alcohol and with xylol. Sections may be mounted directly from 95% alcohol if desired. It does not cause the fading of stains even after several years. However, Clearmount, which is odourless, is perhaps to be preferred.

S.Q.D. BALSAM

(Edward Gurr)

Refractive index 1·515

An exceptionally quick-drying, synthetic, xylol miscible, synthetic mountant, similar to Meedol balsam.

VENETIAN TURPENTINE

For mounting filamentous algae and other delicate material

Solutions required:

A. Glycerin 5%.
B. Venetian turpentine 10% in absolute alcohol.

Technique:

1. Stain the material in suitable aqueous stains.

2. Transfer to Solution A, and leave therein for several days in an open dish.

3. Wash with several changes of 95% alcohol.

4. Wash with two or three changes of absolute alcohol.

5. Transfer the material quickly from absolute alcohol to Solution B (10% Venetian turpentine in absolute alcohol), in an open dish.

6. Place the dish with contents over soda lime in a desiccator for several days until the fluid becomes viscous.

7. Mount on slides.

ADHESIVES, CEMENTS, IMMERSION OILS AND MISCELLANEOUS REAGENTS

CARBOCHROME INK

This consists of a stable colloidal suspension of finely divided carbon in distilled water, and is employed for injection, for which purpose the ink is usually diluted to about one-fifth its standard strength. Unlike Indian ink, it does not contain gum and other substances necessary to preserve gummy solutions which are subject to attack and decomposition by air-borne micro-organisms. Carbochrome ink, diluted to suitable proportions for the particular class of animal, is non-toxic to laboratory animals, except the guinea pig, which is adversely affected by carbon injections. It is not only more suitable for injection than Indian

ink, but also as a negative stain for spirochaetes, etc., on account of the absence of granules, which occur in Indian ink. It should be noted, however, that for the marking and labelling of laboratory apparatus and specimens, it is not waterproof, and, therefore unsuitable. For such purposes waterproof Indian ink is more satisfactory. Carbochrome ink contains approximately 11% of carbon, the average particle size of which is ½ to 1μ, although ther emay be a few aggregates present up to but not exceeding, 10μ.

CEDARWOOD OIL

For microscopic immersion lenses

" Cedarwood oil for microscope immersion lenses " is not the same as " cedarwood oil for clearing."

Occasionally one sees " cedarwood oil for microscopy " stipulated; such phraseology is vague and confusing as it could imply either type of cedarwood oil, since both are used for microscopy. When specifying cedarwood oil, therefore, it should be made quite clear which type is required by stipulating either " for immersion lenses " or " for clearing " as neither type can be used for the same purpose as the other.

Cedarwood oil for microscope immersion lenses is prepared by dissolving gums or resins in cedarwood oil " for clearing " to bring it to the required viscosity and refractive index. There are a number of different brands of cedarwood oils for immersion on the market under various proprietary names, which appear to differ slightly from each other in refractive indices, viscosity and depth of colour, usually pale yellowish or pale amber. Although immersion oils with a refractive index of 1·520 is recommended by some microscope manufactures, oils possessing a refractive index of 1·515 are used by a large number of microscopists.

In my own laboratories, Optoil, which is a synthetic immersion oil (*see* page 531) is normally used, and only occasionally cedarwood oil for immersion lenses.

A good make of cedarwood immersion oil, such as Cedronol, is excellent for mounting Gram-stained preparations, which often fade when mounted in xylol balsam and most synthetic mountant (but see LPM mountant, page 523 for this purpose).

Cedarwood oils for immersion can be thinned, if desired, by stirring in a little cedarwood oil for clearing.

COVERLAC

A clear, colourless, synthetic neutral lacquer, refractive index 1·518, which may be used instead of mounting media and coverglasses for smears and sections. It is not claimed that Coverlac ensures a uniformly flat optical surface; indeed this is impossible without the usual mounting medium and coverglass, but except for very critical work, Coverlac offers an economical means of covering smears and sections. Preparations are stained, dehydrated and cleared as usual; excess liquid is removed, and the back of the slides dried thoroughly: Coverlac is then dropped on to the slides; which should be placed on a flat surface, to form a protective film. The slides are then placed in a drawer, or covered in a similar manner, to protect them from dust overnight, after which the coating of Coverlac may be hardened off in an oven overnight and during the next day.

FLUOROIL

(Edward Gurr)

Refractive index 1·515

This is a clear, colourless, non-sticky, non-drying, synthetic immersion oil, which does not fluoresce in ultra-violet light, a property which renders it suitable for fluorescence microscopy, for which this oil is intended.

The oil is miscible with xylol and benzene.

GELATIN ALCOHOL ADHESIVE

(M. A. Albrecht)

For attaching frozen sections to slides

Sprinkle 1·5 gm. of powdered gelatin on to the surface of 120 ml. of warm water (about 50–55° C.) in a beaker, then after

about ten minutes stir until the gelatin is completely dissolved. Add and stir in 80 ml. of absolute alcohol.

Method of use:

1. Immerse frozen sections, 15–50μ in thickness in the adhesive for five minutes or longer.

Note: The depth of the liquid should be at least 2·5 cm.

2. Transfer sections to slides by means of a fine brush.
3. Allow excess fluid on the slides to evaporate.
4. Blot sections on slides with filter paper.

Note: It may be necessary to damp the filter paper with 95% alcohol.

5. Immerse the slides, with the sections on them, in 95% alcohol.

Result:

The remaining gelatin on the slides will congeal, holding the sections firmly attached to the slides. If necessary, the sections can be subsequently coated with celloidin solution (0·5% celloidin in equal volumes of absolute alcohol and ether).

Notes:

The method was developed to overcome the difficulty of teasing frozen sections, particularly large ones, on to slides without their becoming dry and perhaps damaged in the process.

The method is of particular value for large frozen sections of brain tissue.

Reference: Albrecht, M. A. (1954).

GLYCERIN - ALCOHOL

For softening brittle or crumbly material already embedded in paraffin wax

Solution required:

Absolute alcohol .. .. .. 54 ml.

Water	36 ml.
Glycerine	10 ml.

Technique:

1. Cut the block at one side so that the surface of the tissue is exposed.

2. Soak the block in glycerin–alcohol for several hours or overnight or longer if necessary.

Results:

The glycerin–alcohol penetrates and softens the tissue, despite the presence of the paraffin wax, and makes section cutting easier.

Notes:

Soaking in water has a similar effect, but glycerin–alcohol penetrates more quickly and makes the tissue softer: also maceration is avoided and glycogen and water-soluble proteins are not extracted.

References:
Baker, J. R. (1941).
Carleton, H. M. & Leach, E. H. (1937).

HAUPT'S ADHESIVE

Gelatine powder	1 gm.
Water	60 ml.
Phenol, 5% aqueous	40 ml.
Glycerine	15 ml.

Warm 50 ml. of the water to 50–60° C. in a beaker. Sprinkle the gelatine powder on the surface of the water. Leave for about fifteen to thirty minutes for the gelatine to dissolve, then stir well and add the glycerine. With the remaining 10 ml. of water rinse out the measuring cylinder that has been used for the glycerine. Add the rinsings to gelatine–glycerine mixture. Add the phenol solution; stir well; then filter.

Notes:

Johansen (1940) states that he considers Haupt's fluid by far the best adhesive yet devised for attaching sections to slides, and that he finds it may be used for affixing unicellular and many colonial algae. He also directs that a drop of the adhesive should be smeared evenly on the slide to leave a barely perceptible layer. A few drops of 3 to 4% formalin are then added and the section floated on this, on a warm-plate at 40 to 43° C. (for sections embedded in paraffin wax melting point 58 to 60° C.) until the section has flattened out. The slide is then allowed to cool, when the excess formalin is drained off, and the section positioned as desired. If the specimen, fixed to the still wet slide, is placed in an oven together with a dish of full-strength formalin, the formaldehyde fumes will reinforce coagulation of the gelatine.

Reference: Haupt, A. W. (1930).

KOLLOFAST

An excellent fluid, superior to Kaiserling, for preserving either anatomical or botanical museum specimens permanently in their natural colours: the preservative is equally satisfactory for both normal and pathological specimens. Brightly coloured specimens such as lizards and snakes, and leaves and fruits are preserved permanently in Kollofast, showing their natural colours and with the areas of infection, if any, clearly differentiated as in life. This technique is a two solution method: specimens are fixed in solution A, the time of fixation varying according to the size and nature of the specimen. After fixation and washing in water, the specimens are preserved permanently in solution B.

LAKTOSEAL

(Edward Gurr)

A quick-drying, transparent, colourless cement for sealing lactophenol, glycerine and other aqueous mounts.

Bodman (1960) uses Laktoseal in conjunction with Aquamount for preparing permanent preparations of starch gels in electrophoresis.

MACCONAILL'S GLUCOSE SYRUP

This may be used in certain cases as an aqueous mountant in general histology. It is an extremely useful product with many applications.

MacConaill's glucose syrup is recommended for clearing and mounting, without previous dehydration, certain tissues and tissue sections, museum specimens, etc. Clearing of sections takes about one minute; slices of tissue are cleared from half an hour to several hours, depending on their thickness; while a four-month foetus usually takes about ten to twelve hours. The syrup acts as a preservative against bacterial and fungal destruction of tissue. Tissues immersed in the syrup rapidly become hard; they can be taken out, drained of the surplus syrup, and then preserved in the candied state; when required, tissues preserved in this way can simply be immersed again in the clearing syrup. If desired, these syrup preparations can be softened in water, then cut up, when and as required, and recleared.

Reference: MacConaill, M. A. (1937).

OPTOIL

(Edward Gurr)

Refractive index 1·515

Optoil is a clear, colourless, odourless, synthetic immersion oil, which is non-sticky, and non-drying. This oil, which is xylol miscible, is particularly suitable for prolonged observations, as it does not thicken or dry even when left on slides for several months. Although, unlike Cedronol A and B, and possibly other cedarwood oils for immersion, Optoil is unsuitable as a mountant for Gram-stained preparations; as an immersion oil it is preferred by many workers to thickened cedarwood oils, all of which possess the disadvantage of drying out and becoming too thick on prolonged exposure to the atmosphere.

RECIPES

SUNDRY FIXATIVES

ALTMANN'S FLUID

Osmic acid 2%	1 volume
Potass. dichromate 5%	1 volume

5-AMINOACRIDINE HYDROCHLORIDE FIXATIVE

For acid mucopolysaccharides
(After G. Williams & D. S. Jackson, 1956)

5-Aminoacridine hydrochloride	2 gm.
Absolute alcohol	50 ml.
Distilled water	50 ml.

Thin slices of tissue are fixed for forty-eight hours; then washed thoroughly with 50% alcohol to remove excess 5-aminoacridine hydrochloride before dehydration and embedding in paraffin wax.

Note: The authors tested this fixative on thin slices of human and guinea pig tissues, comprising costal cartilage, aorta, stomach and kidney.

AOYAMA'S FIXATIVE

Cadmium chloride 1%	200 ml.
Formalin (40% formaldehyde)	80 ml.
Distilled water	220 ml.

APATHY'S FIXATIVE

Mercuric chloride 5% aqueous	200 ml.
Acetic acid 10% aqueous	25 ml.
Distilled water	75 ml.
Absolute alcohol	200 ml.

BENDA'S FLUID

Osmic acid 2%	20 ml.
Chromic acid 1%	75 ml.
Glacial acetic acid	1 ml.

Note: The solution should be freshly prepared.

BENSLEY'S FIXATIVE

Potassium dichromate 5% aqueous	250 ml.
Mercuric chloride	25 gm.
Distilled water	200 ml.
Formalin (40% formaldehyde)	50 ml.

BOWIE'S FIXATIVE

Mercuric chloride 5%	500 ml.
Glacial acetic acid	10 ml.
Potassium dichromate	12·5 mg.

BRANCA'S FIXATIVE

Picric acid, saturated, aqueous	420 ml.
Mercuric chloride	30 ml.
Formalin (40% formaldehyde)	80 ml.
Glacial acetic acid	7·5 ml.

CETYL PYRIDINIUM CHLORIDE - FORMALDEHYDE FIXATIVE

(After G. Williams & D. S. Jackson, 1956)

For acid mucopolysaccharides

Cetyl pyridinium chloride	2·5 gm.
Formaldehyde 40%	50 ml.
Distilled water	450 ml.

Thin slices of fresh tissue are fixed for forty-eight hours.

Note: The authors tested this fixative on thin slices of human and guinea pig tissues, comprising costal cartilage, aorta, stomach and kidney.

CHROMIC ACID FIXATIVE

Chromium trioxide	10 gm.
Distilled water	100 ml.

CIACCIO'S FIXATIVE

Potassium dichromate 10%	200 ml.
Formic acid 1% aqueous	200 ml.
Formalin (40% formaldehyde)	80 ml.
Distilled water	20 ml.

CRETIN'S FIXATIVE

Picric acid, saturated, aqueous	300 ml.
Mercuric chloride	13 gm.
Distilled water	150 ml.
Formalin (40% formaldehyde)	50 ml.

DEL RIO - HORTEGA'S FIXATIVE

Uranium nitrate 5%	75 ml.
Formalin (40% formaldehyde)	25 ml.
Distilled water	150 ml.

DREW'S FIXATIVE

Sodium chloride 1%	400 ml.
Cobalt nitrate	10 gm.
Formalin (40% formaldehyde)	100 ml.

DURIG'S FIXATIVE

Potassium dichromate 10%	150 ml.
Formalin (40% formaldehyde)	50 ml.
Distilled water	350 ml.

EMIG'S FIXATIVE

Copper acetate 1% aqueous	75 ml.
Glacial acetic acid	7·5 ml.
Formalin (40% formaldehyde)	75 ml.
Methyl alcohol, absolute	75 ml.

Note: The normal use of this fixative is in botany (for algae).

GILSON'S ACETO ALCOHOL CHLOROFORM FIXATIVE

Absolute alcohol	100 ml.
Glacial acetic acid	100 ml.
Chloroform	100 ml.

GILSON'S FLUID

Mercuric chloride	5 gm.
Glacial acetic acid	1 ml.
Nitric acid, conc.	3·75 ml.
Alcohol 95%	15 ml.
Distilled water	230 ml.

GULLAND'S FIXATIVE

Alcohol 95%	450 ml.
Formalin (40% formaldehyde)	50 ml.

HARTZ FIXATIVE

Mercuric chloride 6% aqueous	400 ml.
Formalin (40% formaldehyde)	100 ml.
Trichloracetic acid	0·5 gm.

HELD'S FIXATIVE

Potassium dichromate	5 gm.
Distilled water	500 ml.
Formalin (40% formaldehyde)	18 ml.
Glacial acetic acid	8 ml.

HENNING'S FIXATIVE

Picric acid, saturated, aqueous	60 ml.
Mercuric chloride	16 gm.
Distilled water	112 ml.
Absolute alcohol	250 ml.
Chromium trioxide	0·4 gm.
Nitric acid, conc.	72 ml.

HOLLANDE'S FIXATIVE

Distilled water	250 ml.
Copper acetate	6·25 gm.
Formalin (40% formaldehyde)	25 ml.
Glacial acetic acid	2·5 ml.
Picric acid	to saturation*

HOYER'S FIXATIVE

Mercuric chloride 5%	80 ml.
Potassium dichromate 5%	64 ml.
Distilled water	100 ml.

JACKSON'S FIXATIVE

Alcohol 90%	100 ml.
Formalin (40% formaldehyde)	12·5 ml.
Glacial acetic acid	12·5 ml.

* Add about 5 gm. of picric acid and shake at intervals over several hours; allow to stand overnight; then filter or decant.

KAHLE'S FLUID (DIETRICH'S FLUID)

Alcohol 95%	30 ml.
Formalin	12 ml.
Glacial acetic acid	2 ml.
Distilled water	60 ml.

KAISER'S FIXATIVE

Mercuric chloride 5% aqueous	166 ml.
Acetic acid, glacial	7·5 ml.
Distilled water	84 ml.

KENYON'S FIXATIVE

Potassium dichromate	9·5 gm.
Copper sulphate crystals	5 gm.
Distilled water	200 ml.
Formalin (40% formaldehyde)	50 ml.

KINGSBURY'S FIXATIVE

Mercuric chloride 5% aqueous	150 ml.
Copper sulphate (crystals) 10%	25 ml.
Distilled water	50 ml.
Copper dichromate	6·25 gm.
Formalin (40% formaldehyde)	25 ml.

KINGSLEY'S FIXATIVE

Picric acid, saturated, aqueous	50 ml.
Distilled water	180 ml.
Sodium chloride	1·75 gm.
Formalin (40% formaldehyde)	20 ml.

KOHN'S FIXATIVE

Mercuric chloride 5%	64 ml.
Potassium dichromate 5%	116 ml.
Glacial acetic acid	12·5 ml.
Distilled water	70 ml.

KOLLMANN'S FIXATIVE

Chromium trioxide 5%	100 ml.
Potassium dichromate 10%	125 ml.
Distilled water	25 ml.
Nitric acid	5 ml.

MANN'S FIXATIVE

Osmic acid 1% aqueous	100 ml.
Sodium chloride, pure	0·75 gm.

MAYER'S FIXATIVE

Distilled water	250 ml.
Nitric acid, conc.	6·25 ml.
Picric acid	4 gm.

Shake at intervals over a period of several hours; then filter.

MÜLLER'S FLUID

Potass. dichromate	2·5 gm.
Sodium sulphate	1 gm.
Distilled water	100 ml.

NOVAK'S FIXATIVE

Mercuric chloride 5%	106 ml.
Chromium trioxide 1%	75 ml.
Glacial acetic acid	7·5 ml.
Distilled water	44 ml.
Formalin (40% formaldehyde)	25 ml.

OHLMACHER'S FLUID

Absolute alcohol	64 ml.
Chloroform	12 ml.
Acetic acid glacial	4 ml.

Immediately before use add mercuric chloride, 16 gm.

ORR'S FIXATIVE

Osmic acid 2%	80 ml.
Acetic acid 1% aqueous	20 ml.

PICRIC NITRIC ACID

Picric acid, aqueous, sat.	95 ml.
Nitric acid, conc.	5 ml.

PICRO-SUBLIMATE-ACETIC ACID (RATH'S FLUID)

Picric acid, saturated, aqueous	50 ml.
Mercuric chloride, hot saturated aqueous	50 ml.
Glacial acetic acid	40 ml.

PICRO-SUBLIMATE (RAHL'S FLUID)

Picric acid, saturated aqueous	50 ml.
Mercuric chloride, saturated aqueous	50 ml.
Distilled water	100 ml.

RAWITZ FIXATIVE

Osmic acid 1%	70 ml.
Distilled water	180 ml.
Nitric acid, conc.	12·5 ml.
Picric acid	3 gm.

ROMEIS' FIXATIVE

Alcohol 95%	56 ml.
Distilled water	54 ml.
Formalin (40% formaldehyde)	12·5 ml.
Glacial acetic acid	2·5 ml.

ROSKIN'S FIXATIVE

Distilled water	235 ml.
Mercuric chloride	14 gm.
Glacial acetic acid	15 ml.

RUFFINI'S FIXATIVE

Distilled water	235 ml.
Formic acid	15 ml.
Mercuric chloride	6·25 gm.

SCHAFFNER'S FIXATIVE

Chromium trioxide 1% aqueous	75 ml.
Distilled water	175 ml.
Acetic acid, glacial	1·75 ml.

SPULER'S FIXATIVE

Osmic acid 1%	12·5 ml.
Acetic acid 1% aqueous	150 ml.
Distilled water	87·5 gm.
Picric acid	3 gm.

SWANK & DAVENPORT'S FIXATIVE (1934)

A. Formalin 10% ..

B.	Osmic acid 1%	82 ml.
	Potassium chlorate 1%	62·5 ml.
	Acetic acid 10%	25 ml.
	Distilled water	80·5 ml.

Immerse fresh tissue in solution A for twenty-four hours; then transfer, without washing, to solution B and leave therein for one week.

Reference: *Stain Tech.* (1934), **9**, 11.

SWANK & DAVENPORT'S FIXATIVE (1935)

Osmic acid 1%	2·5 ml.
Potassium chlorate 1%	150 ml.
Acetic acid 10% aqueous	25 ml.
Formalin (40% formaldehyde)	30 ml.
Distilled water	22·5 ml.

Reference: *Stain Tech.* (1935), **10**, 88.

TAKAHASHI'S FIXATIVE

Osmic acid 1%	125 ml.
Chromium trioxide 1%	20 ml.
Hydrochloric acid, conc.	1 drop
Distilled water	105 ml.

TELLYESNICZKY'S FIXATIVE

Potassium dichromate 10%	75 ml.
Distilled water	175 ml.
Glacial acetic acid	12·5 ml.

TURCHINI'S FIXATIVE

Picric acid, saturated, aqueous	200 ml.
Ammonium molybdate	17 gm.
Distilled water	50 ml.
Formalin (40% formaldehyde)	50 ml.

WINGE'S FIXATIVE

Chromium trioxide 1%	190 ml.
Distilled water	15 ml.
Formalin (40% formaldehyde)	25 ml.
Glacial acetic acid	18·5 ml.

ZENKER-FORMOL

See Helly's fluid.

HYDROGEN ION INDICATORS

ALIZARIN

pH 4·0–6·8 and 11·0–13·0

Alizarin	0·5 gm.
Alcohol 70%	100 ml.

ALIZARIN RED

pH 4·0–6·8 and 11·0–13·0

Alizarin red	0·5 gm.
Distilled water	80 ml.
Absolute alcohol	20 ml.

ALIZARIN YELLOW G

pH 10·1–12·1

Alizarin yellow G	0·1 gm.
Alcohol 50%	100 ml.

ALIZARIN YELLOW GG

pH 10·0–12·0

Alizarin yellow GG	0·1 gm.
Alcohol 50%	100 ml.

BROMOCRESOL GREEN

pH 3·6–5·2

Bromocresol green	0·04 gm.
N/100 NaOH	5·72 ml.
Distilled water	74·28 ml.
Absolute alcohol	20 ml.

BROMOCRESOL PURPLE

pH 5·2–6·8

Bromocresol purple	0·04 gm.
N/100 NaOH	7·4 ml.
Distilled water	72·6 ml.
Absolute alcohol	20 ml.

BROMOPHENOL BLUE

pH 3·0–4·6

Bromophenol blue	0·04 gm.

N/100 NaOH	5·92 ml.
Distilled water	74·08 ml.
Absolute alcohol	20 ml.

BROMOTHYMOL BLUE

pH 6·0–7·6

Bromothymol blue	0·04 gm.
N/100 NaOH	6·4 ml.
Distilled water	73·6 ml.
Absolute alcohol	20 ml.

CHLOROPHENOL RED

pH 5·0–6·6

Chlorophenol red	0·04 gm.
N/100 NaOH	9·44 ml.
20% Alcohol	90·56 ml.

CONGO RED

pH 3·0–5·2

Congo red	0·1 gm.
Distilled water	80 ml.
Absolute alcohol	20 ml.

o-CRESOLPHTHALEIN

pH 8·2–10·4

o-Cresolphthalein	0·04 gm.
Alcohol 50%	100 ml.

m-CRESOL PURPLE

pH 0·5–2·5 and 7·0–9·0

m-Cresol purple	0·04 gm.
N/100 NaOH	10·88 ml.
Distilled water	69·12 ml.
Absolute alcohol	20 ml.

CRESOL RED

pH 7·2–8·8

Cresol red	0·04 gm.

N/100 NaOH	10·48 ml.
Distilled water	69·52 ml.
Absolute alcohol	20 ml.

DIMETHYL YELLOW INDICATOR

pH 2·9–4·0

Dimethyl yellow	0·1 gm.
Alcohol 70%	100 ml.

GALLEIN

pH 3·8–6·6

Gallein	0·1 gm.
Alcohol 50%	100 ml.

HAEMATOXYLIN

pH 0·0–1·0 and 5·0–6·0

Haematoxylin	0·5 gm.
Distilled water	80 ml.
Absolute alcohol	20 ml.

INDIGO CARMINE

pH 11·5–14·0

Indigo carmine	0·25 gm.
Distilled water	80 ml.
Absolute alcohol	20 ml.

LACMOID

pH 4·4–6·4

Lacmoid	0·25 gm.
Alcohol 70%	100 ml.

LITMUS

pH 4·5–8·3

Litmus	0·5 gm.
Distilled water	100 ml.

METHYL ORANGE

pH 2·9–4·1

Methyl orange	0·1 gm.
Distilled water	80 ml.
Absolute alcohol	20 ml.

METHYL RED

pH 4·2–6·2

Methyl red	0·04 gm.
Alcohol 50%	100 ml.

METHYL VIOLET

pH 0·1–3·2

Methyl violet indicator	0·25 gm.
Distilled water	80 ml.
Absolute alcohol	20 ml.

α-NAPHTHOLBENZEIN

(p-naphtholbenzein) pH 8·5–9·8

α-Naphtholbenzein	1 gm.
Alcohol 70%	100 ml.

α-NAPHTHOLPHTHALEIN

pH 7·3–8·7

α-Naphtholphthalein	0·1 gm.
Alcohol 50%	100 ml.

NEUTRAL RED

pH 6·8–8·0

Neutral red	0·1 gm.
Distilled water	80 ml.
Absolute alcohol	20 ml.

p-NITROPHENOL

pH 5·0–7·0

p-Nitrophenol	0·25 gm.
Distilled water	80 ml.
Absolute alcohol	20 ml.

PHENOLPHTHALEIN

pH 8·2–10·0

Phenolphthalein	1 gm.
Alcohol 70%	100 ml.

PHENOL RED

pH 6·6–8·4

Phenol red	0·04 gm.
N/100 NaOH	11·36 ml.
Distilled water	68·64 ml.
Absolute alcohol	20 ml.

ROSOLIC ACID

pH 6·8–8·2

Rosolic acid	0·5 gm.
Alcohol 50%	100 ml.

THYMOL BLUE

pH 1·2–2·8 and 8·0–9·6

Thymol blue	0·04 gm.
N/100 NaOH	8·6 ml.
Distilled water	71·4 ml.
Absolute alcohol	20 ml.

THYMOLPHTHALEIN

pH 9·3–10·5

Thymolphthalein	0·1 gm.
Alcohol 50%	100 ml.

UNIVERSAL INDICATOR

pH 1·0–10·0

Universal indicator powder	0·1 gm.
Alcohol 70%	100 ml.

or

1 Universal indicator tablet dissolved in 10 ml. of 70% alcohol

Note: A comparator is not required with Michrome universal indicator, as it gives very definite colour changes as follows:

pH 1 cherry red	pH 2 rose	pH 3 red-orange
pH 4 orange red	pH 5 orange	pH 6 yellow
pH 7 yellow-green	pH 8 green	pH 9 blue-green
pH 10 blue		

REAGENTS, SUNDRY

ACETONE - XYLOL

Acetone, absolute	100 ml.
Xylol (water-free)	400 ml.

This reagent clears tissues and sections from 90% alcohol.

ALKALINE ALCOHOL

Alcohol 90%	99·5 ml.
Ammonium hydroxide (sp. gravity 0·88)	0·5 ml.

CARBOL XYLOL (Phenol Xylol)

Xylol, anhydrous	300 ml.
Phenol crystals	100 gm.

Dissolve by shaking.

EAU DE JAVELLE

A. Bleaching powder	50 gm.
Water	250 ml.

Shake well; then allow to stand for several hours in a closed bottle.

B. Potassium carbonate	37·5 gm.
Water	250 ml.

Mix solution A with solution B; allow the mixture to stand for several hours; then decant or filter through glass wool.

FARRANTS' MEDIUM

Gum acacia powder	150 gm.
Water	150 ml.

Warm the water to about 40° C.; sprinkle the gum on the surface of the water a little at a time and stir in with a glass rod: then stir in:

Arsenious oxide	0·5 gm.

Leave overnight; then stir well before adding:

Glycerine	150 ml.

Stir well; filter through surgical lint, or decant after standing for several days or weeks, if necessary.

GLYCERINE ALBUMEN (Mayer)

*Egg albumen	100 ml.
Glycerine	100 ml.
Sodium salicylate	2 gm.

Shake well; allow to stand in a closed glass vessel for several hours; then filter if necessary.

* Four or five fresh eggs are required to produce 100 ml. of albumen.

GLYCERINE JELLY

Gelatine powder	100 gm.
Water	650 ml.

Warm the water, in a 2-litre beaker or an enamel bowl, to about 40–50° C.; then sprinkle the gelatine on the surface of the water a little at a time, stirring in each lot with a glass rod; then add and stir in:

Glycerine	700 gm.

Pour the mixture into screw cap storage jars, and add a crystal of thymol to each.

Keep the jars tightly closed.

LACTOPHENOL (Amann)

Lactic acid	16 ml.
Glycerine	31 ml.
Distilled water	20 ml.
Phenol, cryst.	20 gm.

Warm on a water bath until the phenol has gone into solution; then store in a dark glass bottle.

SCOTT'S TAP WATER SUBSTITUTE

Sodium bicarbonate 0·7% aqueous	1 volume
Magnesium sulphate, cryst., 4% aqueous	1 volume

STAINS AND REAGENTS

ACETIC THIONIN

Thionin (Ehrlich)	0·1 gm.
Distilled water	120 ml.
Glacial acetic acid	6 ml.

ACETO CARMINE (SCHNEIDER)

Carmine	0·4 gm.
Distilled water	55 ml.
Heat to boiling, then add:	
Glacial acetic acid	45 ml.

Raise to boiling point again; then cool and filter.

ACID FUCHSIN (ALTMANN)

Aniline water	100 ml.
Acid fuchsin	20 gm.

ACID FUCHSIN (MASSON)

Acid Fuchsin	0·5 gm.
1% Acetic acid, aqueous	100 ml.

ALBERT'S STAIN (for Diphtheria)

A.

Toluidine blue 0·3% aqueous	75 ml.
Methyl green 0·4% aqueous	75 ml.
Glacial acetic acid	1·5 ml.
Absolute alcohol	4 ml.

Shake well, heat to about 80° C. then allow to stand for twenty-four hours before filtering.

B.

Iodine, resublimed	1 gm.
Potassium iodide	1·5 gm.
Distilled water	150 ml.

Dissolve the potassium iodide in about 3 to 5 ml. of the water; then add the iodine and shake until dissolved before adding the remainder of the water.

ALIZARIN RED (SCHROTTER)

Alizarin red S	5 gm.
Distilled water	100 ml.
Distilled water	99 ml.

ALUM CARMINE (MAYER)

Distilled water	50 ml.
Alum carmine powder	3·5 gm.

Shake well; then boil for a minute; cool and filter.

AMMONIA CARMINE

Ammonia carmine powder (Michrome No. 364)	0·5 gm.
Distilled water	100 ml.

ANILINE BLUE, ACETIC (MASSON)

Aniline blue 1% aqueous	100 ml.
Glacial acetic acid	2 ml.

ANILINE BLUE, ALCOHOLIC

Aniline blue, alcohol soluble	1 gm.
Absolute alcohol	70 ml.
Distilled water	30 ml.

ANILINE BLUE, AQUEOUS

Aniline blue, water soluble	1 gm.
Distilled water	100 ml.

Store in a well-closed bottle to which a few drops of Chloroform or a crystal of thymol is added to inhibit the growth of air-borne organisms and consequent decomposition of the stain.

ANILINE CHLORIDE (ANILINE HYDROCHLORIDE)

Aniline Hydrochloride	10 gm.
Distilled water	100 ml.

ANILINE CHLORIDE (ANILINE HYDROCHLORIDE), ACIDIFIED

Aniline chloride	5 gm.
Sulphuric acid 25%	100 ml.

ANILINE - BLUE - ORANGE G (MALLORY)

Aniline blue-Orange G (Michrome No. 422)	2·5 gm.
Distilled water	55 ml.

Dissolve, and filter.

ANILINE GENTIAN VIOLET (STIRLING)

Gentian violet	5 gm.
Aniline oil	2 ml.
Water	88 ml.
Alcohol 95%	10 ml.

Dissolve by shaking thoroughly; allow to stand twenty-four hours then filter.

ANILINE SULPHATE

Aniline sulphate	5 gm.
Distilled water	100 ml.

ANILINE SULPHATE, ACID

Aniline sulphate	1 gm.
Distilled water	27 ml.
Absolute alcohol	63 ml.
Sulphuric acid N/10	10 ml.

ANILINE WATER

Aniline oil	5 ml.
Distilled water	100 ml.

Shake vigorously: then allow to stand. Filter immediately before use.

ANILINE XYLOL

Aniline oil	1 volume
Xylol	1 volume

BEST'S CARMINE (STOCK SOLUTION)

Potassium carbonate	1 gm.
Potassium chloride	5 gm.
Carmine	2 gm.
Distilled water	60 ml.

Heat gently for five to ten minutes until dissolved: then cool and add:

Ammonia solution (sp. gr. 0·880)	20 ml.

or:

Best's Carmine powder (Michrome No. 387)	10 gm.
Distilled water	100 ml.

Heat to boiling point; cool and add:

Strong ammonia solution (sp. gr. 0·880)	20 ml.

BIEBRICH SCARLET

Biebrich scarlet	1 gm.
Alcohol 70% (or distilled water)	100 ml.

BORAX CARMINE (GRENACHER)

Borax	4 gm.
Carmine	3 gm.
Distilled water	100 ml.
Alcohol 70%	100 ml.

Shake the borax and carmine with the water: then boil gently for thirty minutes; allow to cool; make up to the original volume with distilled water; then add the 100 ml. 70% alcohol. Mix well and allow to stand several days before filtering.

BORAX CARMINE (MAYER)

Borax	1 gm.
Carmine	1 gm.
Distilled water	50 ml.
Absolute alcohol	50 ml.

Shake the borax and carmine with the water: then boil gently for thirty minutes; cool; make the volume up to 50 ml. with distilled water; add the alcohol; then filter.

BORREL'S BLUE

Borrel's blue powder (Michrome No. 376)	2 gm.
Distilled water	50 ml.

Boil for five minutes: then cool and filter and make the volume up to 50 ml. with distilled water.

BRAZILIN (HICKSON)

A.	Iron alum 10% aqueous	10 ml.
	Distilled water	20 ml.
	Absolute alcohol	70 ml.
B.	Brazilin 0·5% in 70% alcohol	

CARBOL FUCHSIN (ZIEHL NEELSEN)

Basic fuchsin 10% in absolute alcohol	10 ml.
Phenol 5% aqueous	100 ml.

Mix well and allow to stand overnight before filtering.

CARBOL GENTIAN VIOLET

Gentian violet 10% in absolute alcohol	10 ml.
Phenol 2% aqueous	100 ml.
Alcohol 95%	20 ml.

Mix well; then filter.

CARBOL METHYLENE BLUE

Phenol 5% aqueous	100 ml.
Methylene blue	1 gm.
Absolute alcohol	10 gm.

CARBOL THIONIN

Thionin (Ehrlich)	0·125 gm.
Phenol crystals	1 gm.
Alcohol 10%	100 ml.

Heat to dissolve, or triturate in a mortar; filter when dissolved. The solution deteriorates after a few weeks.

CARBOL TOLUIDINE BLUE

Toluidine blue	1 gm.
Phenol (cryst.)	5·0 gm.
Distilled water	100 ml.

CARMALUM (MAYER)

Carminic acid	1 gm.
Potash alum 5%	200 ml.

Boil for half an hour. Cool and filter. Add a crystal of thymol or a few drops of chloroform, to the stock bottle, as preservative.

CARMINE (DE GROOT)

Iron alum 10% aqueous	0·5 ml.
Potash alum 10% aqueous	25 ml.
Carminic acid	1 gm.
Distilled water	74·5 ml.
Hydrochloric acid, conc.	1 drop

Boil the carminic acid with the water; then shake in the other ingredients and filter.

CARMINE (Meriwether)

Potassium carbonate 10% aqueous	19 ml.
Potassium chloride 10% aqueous	62·5 ml.
Carmine	2·5 gm.
Distilled water	18·5 ml.

Boil for two minutes; cool and filter; then add:

Ammonium hydroxide (sp. gravity 0·88)	25 ml.

CHROME HAEMATOXYLIN (GOMORI)

Haematoxylin 1% aqueous	50 ml.
Chrome alum 10% aqueous	15 ml.
Potassium dichromate 10% aqueous	1 ml.
Sulphuric acid, conc.	0·1 ml.

CHROME HAEMATOXYLIN (HANSEN)

Haematoxylin 1% aqueous	30 ml.
Chrome alum 10% aqueous	30 ml.
Potassium dichromate 10% aqueous	2 ml.
Sulphuric acid, conc.	0·2 ml.

CHROME HAEMATOXYLIN (SCHWEITZER)

Haematoxylin 1% aqueous	50 ml.
Chrome alum 10% aqueous	50 ml.
Potassium dichromate 10% aqueous	2·75 ml.
Distilled water	22·25 ml.
Sulphuric acid, conc.	4 ml.

COTTON BLUE - LACTOPHENOL

Cotton blue	1 gm.
Lactophenol	100 ml.

COTTON BLUE - MAGENTA LACTOPHENOL

Cotton blue 1% in lactophenol	8 ml.
Magenta 1% in lactophenol	100 ml.
Lactophenol	100 ml.

DORNER'S NEGATIVE STAIN

Nigrosin	10 gm.
Distilled water	100 ml.
Formalin	9·5 ml.

EOSIN, ALCOHOLIC

Eosin, alcoholic	1 gm.
Tap water	30 ml.
Absolute alcohol	70 ml.

EOSIN, AQUEOUS

Eosin, yellowish (or bluish)	1 gm.
Tap water	100 ml.

FAST-GREEN FCF IN CELLOSOLVE

Cellosolve	100 ml.
Fast-green FCF	3 gm.

Heat in a flask on a water bath for about half an hour with occasional stirring; then cool and filter.

FAST GREEN FCF, AQUEOUS

Fast green FCF	0·5 to 1 gm.
Distilled water	100 ml.

FLAGELLA STAIN MORDANT (LOEFFLER)

Tannic acid 20% aqueous	100 ml.
Ferrous sulphate, cryst.	20 gm.
Distilled water	40 ml.
Basic fuchsin 10% in absolute alcohol	10 ml.

Dissolve the ferrous sulphate by shaking vigorously with the cold distilled water (do not heat) then add the tannic acid and basic fuchsin.

GALLOCYANIN (EINARSON)

Gallocyanin	0·3 gm.
Chrome alum	10 gm.
Distilled water	200 ml.

Boil gently for five minutes; then cool; filter, and make up to the original volume with distilled water.

GIEMSA STAIN

Giemsa stain	3·8 gm.
Pure methyl alcohol	250 ml.
Pure glycerine	250 ml.

Triturate with a mortar and pestle, adding the alcohol and glycerine in small portions at a time during a period of fifteen minutes. Transfer to a stoppered flask and allow to stand for twenty-four hours with occasional shaking; then filter.

GOODPASTURE'S ACID POLYCHROME METHYLENE BLUE

Methylene Blue	0·25 gm.
Potassium carbonate 0·25% aqueous	100 ml.

Boil for half an hour, then cool under the tap and add 0·75 ml. glacial acetic acid.

Shake well then boil gently for five to ten minutes; cool and make up the volume to 100 ml. with distilled water.

GRAM'S IODINE

Iodine pure	1 gm.
Potass. iodide	2 gm.
Water	300 ml.

Dissolve the potass. iodide in about 10 ml. of the water; then add the iodine, shake till dissolved; add the remainder of the water.

or:

Gram's Iodine powder (Michrome No. 435)	1 gm.
Water	88 ml.
Hydrochloric acid, conc.	0.5 ml.

HAEMALUM (HARRIS)

Haematoxylin 0·5% aqueous	100 ml.
Potash alum	5 gm.
Mercuric oxide powder	0·25 gm.

Heat to boiling point, then allow to cool, and filter.

HAEMALUM (MAYER)

Haematein	0·1 gm.
Absolute alcohol	5 ml.
Potash alum 5% aqueous	100 ml.

HAEMATEIN (RAWITZ)

Haematein	0·25 gm.
Distilled water	35 ml.
Glycerine	50 ml.
Ammonia alum 10% aqueous	35 ml.

HAEMATOXYLIN (ALZHEIMER)

Haematoxylin 10% in absolute alcohol	10 ml.
Lithium carbonate 1% aqueous	3 ml.
Distilled water	87 ml.

HAEMATOXYLIN (ALUM), AQUEOUS

Haematoxylin 0·25% aqueous	400 ml.
Ammonia or potash alum	20 gm.
Thymol	1 gm.

Note: The solution deteriorates after two or three months.

HAEMATOXYLIN (BÖHMER)

A. Haematoxylin 3·5% in absolute alcohol

B. Potash alum 0·3% aqueous

For use mix 1 part of solution A with 9 parts of solution B.

HAEMATOXYLIN (COLE)

Haematoxylin 10% in absolute alcohol	6 ml.
Ammonia alum 10% aqueous	6 ml.
Glacial acetic acid	7·5 ml.
Glycerine	29 ml.
Absolute alcohol	20 ml.
Distilled water	28 ml.

HAEMATOXYLIN (DELAFIELD)

Haematoxylin 3·5% in absolute alcohol	100 ml.
Ammonia alum 6·25% aqueous	320 ml.
Glycerine	80 ml.

Mix thoroughly and allow to ripen for at least three months; then filter before use.

HAEMATOXYLIN (EHRLICH)

Haematoxylin 2% in absolute alcohol	100 ml.
Potash alum 2·5% aqueous	100 ml.
Glycerine	100 ml.
Glacial acetic acid	10 ml.

Mix well; allow to ripen in a 500-ml. closed bottle for three months

or:

Dissolve 1 gm. Ehrlich haematoxylin powder (Michrome No. 370) in a mixture of 4 ml. glacial acetic acid, 80 ml. 50% alcohol and 40 ml. glycerine; then filter. The solution requires no ripening and is ready for immediate use.

HAEMATOXYLIN (HANSEN)

Iron alum 10% aqueous	45 ml.
Haematoxylin 10% in absolute alcohol	7·5 ml.
Distilled water	47·5 ml.

Mix well; boil; then filter and make the volume up to 100 ml. with distilled water.

HAEMATOXYLIN (HARRIS)

Haematoxylin 10% in absolute alcohol	5 ml.
Mercuric oxide	0·25 gm.
Potash alum 10% aqueous	100 ml.
Glacial acetic acid	4 ml.

Mix the haematoxylin and alum solutions: raise to boiling point, then add the mercuric oxide and when the solution turns deep purple turn off the heat: then cool and add the acetic acid.

HAEMATOXYLIN (HEIDENHAIN)

No. 1

Iron alum	4 gm.
Distilled water	100 ml.

Dissolve by shaking. Do not heat.

No. 2

Haematoxylin 10% in absolute alcohol (ripened three to six months)	5 ml.
Distilled water	95 ml.

HAEMATOXYLIN (IRON) (ANDERSON)

No. 1

Haematoxylin 10% in absolute alcohol	5 ml.
Absolute alcohol	45 ml.
Distilled water	50 ml.
Calcium hypochlorite 2% aqueous	3 ml.

No. 2

Iron alum 4% aqueous	100 ml.
Sulphuric acid conc.	3 ml.

For use, mix two volumes of solution No. 1 with one volume of No. 2; this mixture keeps for a few hours only, and should therefore be prepared only when needed for immediate use.

HAEMATOXYLIN (JANSSEN)

Iron alum 10% aqueous	50 ml.
Haematoxylin 10% in absolute alcohol	10 ml.
Methyl alcohol, absolute	15 ml.
Glycerine	15 ml.
Distilled water	20 ml.

Allow the mixture to stand for a week before use.

HAEMATOXYLIN KULTSCHITZKY

Haematoxylin 10% alcoholic	10 ml.
Acetic acid 2% aqueous	100 ml.

Dissolve the haematoxylin in the alcohol then shake thoroughly with the acetic acid solution.

HAEMATOXYLIN (MARTINOTTI)

Ammonia alum 10% aqueous	15 ml.
Methyl alcohol, absolute	15 ml.

Glycerine	15 ml.
Haematein	0·2 gm.
Distilled water	55 ml.

Dissolve the haematein in the distilled water; then mix well with the other ingredients.

HAEMATOXYLIN (PAQUIN & GODDARD)

Iron alum 10% aqueous	50 ml.
Haematoxylin 10% in absolute alcohol	8 ml.
Glycerine	13 ml.
Ammonium sulphate 5%	14 ml.
Absolute alcohol	16 ml.
Distilled water	11 ml.

HAEMATOXYLIN PHOSPHOMOLYBDIC (MALLORY)

Haematoxylin 1% aqueous	100 ml.
Chloral hydrate	6 gm.
Phosphomolybdic acid 10%	1 ml.

HAEMATOXYLIN PHOSPHOTUNGSTIC (MALLORY)

Haematoxylin 10% alcoholic	1 ml.
Phosphotungstic acid 10%	20 ml.
Distilled water	80 ml.

Ripen by adding 4 drops hydrogen peroxide (20 volumes) or 5 ml. of 1% Potassium permanganate.

HAEMATOXYLIN (RAWITZ)

Haematoxylin	1 gm.
Distilled water	55 ml.
Ammonia alum 10% aqueous	10 ml.
Glycerine	35 ml.

HAEMATOXYLIN REGAUD

Haematoxylin 10% in absolute alcohol	10 ml.
Glycerine	10 ml.
Distilled water	80 ml.

HAEMATOXYLIN (SASS)

Ammonia alum, saturated, aqueous (about 13%)	100 ml.
Haematoxylin	1 gm.

Glacial acetic acid	3 ml.
Sodium or potassium iodate	1 gm.

HAEMATOXYLIN (SCHRÖDER)

Lithium carbonate 1% aqueous	4 ml.
Haematoxylin 1% aqueous	30 ml.
Distilled water	66 ml.

HAEMATOXYLIN (SQUIRE)

Haematoxylin 10% in absolute alcohol	10 ml.
Ammonia alum	1·2 gm.
Calcium chloride	8 gm.
Distilled water	10·5 ml.
Absolute alcohol	96 ml.

Dissolve the calcium chloride in the water; then shake in the ammonia alum and mix the solution with the haematoxylin and the alcohol.

HAEMATOXYLIN (THOMAS)

Dioxane	40 ml.
Haematoxylin	2·5 gm.
Hydrogen peroxide 3%	2 ml.
Distilled water	44 ml.
Ethylene glycol	16·5 ml.
Phosphomolybdic acid	16·5 gm.

Dissolve the haematoxylin in the dioxane; dissolve the phosphomolybdic acid in the water and the ethylene glycol; mix the two solutions; then add the hydrogen peroxide and mix thoroughly.

HAEMATOXYLIN (WEIGERT)

A. Haematoxylin 10% in absolute alcohol (ripened three to six months)	10 ml.
Absolute alcohol	90 ml.
B. Ferric chloride (hydrated) 30% aqueous	4 ml.
Hydrochloric acid	1 ml.
Distilled water	95 ml.

HITCHCOCK & EHRICH STAIN

Malachite Green 2% aqueous	15 ml.
Acridin red 2% aqueous	45 ml.

JENNER STAIN

Jenner stain powder	0·5 gm.
Pure methyl alcohol	100 ml.

Dissolve by heating in a flask, lightly plugged with cotton-wool, on a water bath. Allow to cool; then filter.

LEISHMAN STAIN

Leishman stain powder	0·15 gm.
Pure methyl alcohol	100 ml.

Dissolve by heating in a flask, lightly plugged with cotton-wool, on a water bath. Allow to cool; then filter.

LIGHT GREEN, AQUEOUS

Light green SF	0·5 to 1 gm.
Distilled water	100 ml.

LIGHT GREEN IN CELLOSOLVE

Cellosolve	100 ml.
Light Green	3 gm.

Heat in a flask on a water bath for about half an hour, with occasional shaking or stirring; then cool and filter.

LITHIUM CARMINE (Orth)

Lithium carbonate, saturated aqueous	100 ml.
Carmine	5 gm.

Boil for fifteen minutes then cool and filter.

or:

Lithium Carmine (Michrome No. 366)	2 gm.
Distilled water	50 ml.

Heat to boiling point; allow to cool; then filter.

LUGOL'S IODINE

Iodine pure	1 gm.
Potassium iodide	2 gm.
Water	100 ml.

MAGENTA-COTTON BLUE-LACTOPHENOL

Cotton blue-lactophenol	4 ml.
Magenta 1% aqueous	2 ml.
Lactophenol	50 ml.

MAY-GRUNWALD STAIN

May-Grunwald stain powder	0·25 gm.
Pure methyl alcohol	100 ml.

Dissolve by heating in a flask, lightly plugged with cotton-wool, on a water bath. Allow to cool, then filter.

METHYL GREEN - PYRONIN (PAPPENHEIM - UNNA)

Methyl green	0·15 gm.
Pyronin (Y or G)	0·25 gm.
Phenol 0·5% aqueous solution	80 ml.
Glycerine	20 ml.
Absolute alcohol	2·5 ml.

Dissolve the powder in the phenol solution; then shake vigorously with the glycerine and alcohol: warm to dissolve; then filter.

METHYL VIOLET (JENSEN)

Methyl violet 6B	0·5 gm.
Distilled water	100 ml.

METHYLENE BLUE, AQUEOUS

Methylene blue	1 gm.
Distilled water	100 ml.

METHYLENE BLUE (BORAX) (MANSON)

Methylene blue	2 gm.
Borax 5% aqueous	100 ml.

Note: Do not heat above 60° C.

METHYLENE BLUE (BORAX) (UNNA)

Methylene blue	1 gm.
Borax 1% aqueous	100 ml.

Note: Do not heat borax methylene blue solutions over 60° C.

METHYLENE BLUE (LOEFFLER)

Methylene blue	3 gm.
Absolute alcohol	30 ml.
Potass. hydroxide 0·01% aqueous	100 ml.

METHYLENE BLUE, POLYCHROME (UNNA)

Methylene blue	1 gm.

Potass. carbonate	1 gm.
Absolute alcohol	20 ml.
Water	100 ml.

Heat to 60° C. for fifteen minutes. Cool and filter.

or:

Polychrome methylene blue (Michrome No. 1024)	3 gm.
Distilled water	200 gm.
Absolute alcohol	10 ml.

Dissolve by heating to 60°C. and maintain at that temperature for fifteen minutes. Cool and filter; then add the alcohol and mix thoroughly.

METHYLENE BLUE SULPHURIC (GABBET)

Distilled water	75 ml.
Sulphuric acid, conc.	25 ml.
Methylene blue	2 gm.

Pour the sulphuric acid into the water carefully, in a flask, then add the methylene blue and shake or stir to dissolve.

MUCICARMINE (MAYER)

Stock solution:

Carmine	1 gm.
Aluminium chloride, anhydrous	0·5 gm.

Mix thoroughly by grinding to a homogeneous powder in a mortar; then place the mixture in a flask with 100 ml. of 50% alcohol and boil for two and a half to five minutes on a water bath, shaking the flask at intervals. Cool under the tap at once and filter when cold.

For use, dilute one volume of this solution with nine volumes distilled water;

or:

Shake 5 gm. of Mucicarmine powder (Michrome No. 356) with 200 ml. of 50% alcohol; then heat to boiling point and continue boiling for 2½ minutes. Cool; filter.

MUCI HAEMATEIN, ALCOHOLIC

Haematein 0·2% in 70% alcohol	100 ml.
Nitric acid	0·1 ml.
Aluminium chloride	0·1 gm.

MUCI HAEMATEIN, AQUEOUS

Haematein 0·5% aqueous	40 ml.
Aluminium chloride 0·5%	20 ml.
Glycerine	40 ml.

Dissolve by warming and shaking; then cool and filter.

NEISSER'S STAIN

A. Methylene blue	0·2 gm.
Absolute alcohol	4 ml.
Glacial acetic acid	10 ml.
Distilled water	190 ml.
B. Bismarck brown	0·2 gm.
Distilled water	100 ml.

NEUTRAL RED (JENSEN)

Neutral red	0·1 gm.
1% acetic acid	0·2 ml.
Distilled water	100 ml.

NISSL'S METHYLENE BLUE

Venetian soap	0·175 gm.
Distilled water	80 ml.

Dissolve; then add:

Methylene Blue 2% aqueous	20 ml.

Shake vigorously then store in a well-closed bottle for at least four months, before use, shaking once or twice a week during this period.

ORANGE FUCHSIN (SQUIRE)

Acid fuchsin 10% aqueous	10 ml.
Orange G 3% aqueous	200 ml.
Distilled water	30 ml.
Absolute alcohol	60 ml.

ORANGE G, AQUEOUS

Orange G	1 gm.
Distilled water	100 ml.

ORCEIN (UNNA - TÄNZER)

Orcein	1 gm.
70% Alcohol	100 ml.
Hydrochloric acid, conc.	1 ml.

PARACARMINE (MAYER)

Carminic acid	1 gm.
Aluminium chloride, hydrated	0·5 gm.
Calcium chloride, anhydrous	4 gm.
Alcohol 70%	100 ml.

PERIODIC ACID - SCHIFF

(After Ritter & Oleson, 1950)

A. Periodic acid 4 gm.
Distilled water 100 ml.
M/5 sodium acetate* 50 ml.

B. Reducing rinse

Potassium iodide	1 gm.
Sodium thiosulphate, pentahydrate	1 gm.
Distilled water	20 ml.
Dissolve, then add:	
Absolute alcohol	30 ml.
followed by:	
Hydrochloric acid 2N	0·5 ml.

C. Fuchsin - Sulphite

Basic fuchsin	1 gm.
Distilled water	200 ml.
Hydrochloric acid 2N	5 ml.
Potassium metabisulphite	2 gm.
Decolorizing charcoal	about 1 gm.

First boil the distilled water; then cool to about 50° C. and dissolve the basic fuchsin in it, followed by the potassium metabisulphite. Add the hydrochloric acid; plug the neck of the flask with cotton wool before shaking. Leave the flask to stand overnight; then add the charcoal, shake well, then filter.

D. Sulphite rinse water

Distilled water	100 ml.
Potassium metabisulphite	0·4 gm.
Hydrochloric acid, conc.	1 ml.

* Prepared by dissolving 0·193 gm. crystalline sodium acetate in 50 ml. of absolute (ethyl) alcohol.

PHLOXIN - PROPYLENE GLYCOL

(For eosinophile counts)

Phloxin 0·1% in distilled water	50 ml.
Propylene glycol	50 ml.

This reagent is stated to give more consistent results with no destruction of eosinophiles.

Reference: Henneman, Wexler & Westenhaver (1949).

PICRO ANILINE BLUE, ALCOHOLIC

Aniline blue alcohol soluble	1 gm.
70% alcohol..	50 ml.
Picric acid, 1% in 70% alcohol	50 ml.

PICRO ANILINE BLUE, AQUEOUS

Aniline blue water soluble	0·1 gm.
Picric acid saturated, aqueous	100 ml.

PICROCARMINE

Picrocarmine powder (Michrome No. 1087)	1 gm.
Distilled water	50 ml.

Boil gently for five minutes: allow to cool; then filter and make up the volume to 50 ml. with distilled water.

PICRO INDIGO CARMINE

Picric acid, saturated aqueous	100 ml.
Indigo carmine	0·25 gm.

RANDOLPH'S BLOOD COUNTING FLUID

Solution A

Methylene blue	0·1 gm.
Propylene glycol	100 ml.

Solution B

Eosin, bluish	0·1 gm.
Distilled water	100 ml.

Mix equal volumes of the two solutions and use as a diluting fluid in the white cell pipette. The mixture of the blood and the diluting fluid should remain in the pipette for fifteen minutes before filling the counting chamber.

Reference: Randolph, T. G. (1943).

RED CELL COUNTING FLUID (O. T. George, 1952)

Sodium citrate 3% aqueous	100 ml.
Formalin (Formaldehyde 40%)	1 ml.
Eosin, bluish	0·6 gm.

This solution stains the erythrocytes and causes the cells to lie flat in a single focal plane, thereby facilitating enumeration.

SAFRANIN

Safranin	1 gm.
Distilled water or 70% alcohol	100 ml.

SCARLET R, ALKALINE (HERXHEIMER)

Absolute alcohol	35 ml.
Distilled water	5 ml.
Sodium hydroxide 10% aqueous	10 ml.
Scarlet R	1 gm.

Place in a flask and plug the neck lightly with cotton wool. Heat on a hot water bath until a considerable portion of the dye has gone into solution; then allow to cool before filtering.

SHORR'S STAIN

A. Harris haematoxylin.

B. Biebrich scarlet	1 gm.
Orange G	0·4 gm.
Distilled water	99 ml.
Glacial acetic acid	1 ml.

Heat the water: add the stains; stir until dissolved; allow to cool; then add acetic acid; shake well and filter.

C. Phosphotungstic acid 10%	25 ml.
Phosphomolybdic acid 10%	25 ml.
Distilled water	100 ml.
D. Fast green FCF aqueous 5%	5 ml.
Acetic acid 1%	32 ml.
Distilled water	64 ml.

SHORR'S STAIN
(Single solution)

Biebrich scarlet, aqueous	0·5 gm.
Orange G	0·25 gm.
Fast green FCF	0·075 gm.
Phosphotungstic acid	0·5 gm.

Phosphomolybdic acid	0·5 gm.
Glacial acetic acid	1 ml.
Alcohol 50%	100 ml.

Dissolve by warming; then allow to cool and filter.

or:

Shorr's Stain powder (Michrome No. 521)	1 gm.
Alcohol 50%	55 ml.
Glacial acetic acid	0·5 ml.

Dissolve the stain by warming with the alcohol; allow to cool; then add the acetic acid, shake well and filter.

TOLUIDINE BLUE (PONDER'S STAIN) FOR DIPHTHERIA

Toluidine blue 0·02% aqueous	100 ml.
Glacial acetic acid	1 ml.
Alcohol	5 ml.

TOLUIDINE BLUE (PUGH)

Toluidine blue	0·2 gm.
Glacial acetic acid	5 ml.
Absolute alcohol	2 ml.
Distilled water	43 ml.

WRIGHT'S STAIN

Wright's stain powder	0·3 gm.
Methyl alcohol, pure	100 ml.

Dissolve by heating in a flask, lightly plugged with cotton wool, on a water bath. Allow to cool; then filter

or:

Wright's stain (Michrome)	1 tablet
Methyl alcohol, pure	33 ml.

Crush the tablet; then dissolve by shaking or heating on a water bath.

REFRACTIVE INDICES

Absolute alcohol (ethyl)	1·361
Acetic acid, glacial	1·371
Acetone	1·359
Aniline oil	1·586
Aniseed oil	1·557
Aquamount	1·435
Balsam tolu	1·640

Refractive Indices (cont.)

Benzene (benzol)	1·501
Benzyl benzoate	1·568
Bergamot oil	1·464
Butyl alcohol (normal)	1·399
Cajeput oil	1·457
Canada balsam, natural, dried	1·535
Canada balsam, natural	1·521
Canada balsam, synthetic, in xylol	1·515
Canada balsam, neutral, in xylol	1·515–1·520
Carbon bisulphide	1·630
Carbon tetrachloride	1·460
Castor oil	1·490
Cedarwood oil, natural	1·504–1·510
Cedarwood oil for immersion lenses	1·506–1·518
Cellosolve	1·405
Chloroform	1·447
Cinnamon oil	1·567
Clove oil	1·533
Colophonium, dried	1·545
Colophonium turpentine	1·510
Creosote, beechwood	1·538
Cristalite	1·515
Crown glass	1·518
Dammar, dried	1·520
Dammar xylol	1·520
Dimethylaniline	1·558
Dimethylphthalate	1·480
Dioxane	1·423
D.P.X.	1·515
Ethyl acetate	1·372
Ethylene glycol	1·432
Eucalyptol	1·450
Farrants' medium	1·432
Glycerine	1·460
Glycerine albumen	1·405
Glycerine jelly	1·414
Gum chloral (Berleze)	1·440
Gum chloral (Defaur)	1·415
Isobutyl alcohol	1·397
Methyl alcohol (absolute)	1·328
Methyl benzoate	1·515
Methyl salicylate	1·537

Refractive Indices (cont.)

Monobromnaphthalene-alpha	1·660
Olive oil	1·473
Optoil	1·515
Origanum oil	1·458
Paraffin, liquid	1·471
Pyridine	1·509
S.Q.D. balsam	1·515
Turpentine oil	1·473
Water (distilled)	1·333
Xylol	1·492

SATURATED SOLUTIONS OF REAGENTS

To prepare 100 ml. dissolve:

	grammes in mls. water
Ammonia alum	13 ,, 92
Aluminium chloride, hydrated	75 ,, 60
Potash alum	13·3 ,, 92
Ammon. molybdate	39 ,, 88
Ammon. oxalate	5·5 ,, 97
Aniline chloride	54 ,, 56
Aniline sulphate	6 ,, 96
Barium chloride	33 ,, 91·5
Borax	5·9 ,, 98
Boric acid	5·1 ,, 97
Chloral hydrate	120 ,, 31
Chrome alum	22 ,, 90
Chromic acid	103·5 ,, 62·5
Citric acid	87 ,, 44·5
Cobalt nitrate	125 ,, 31·5
Ferric ammon. sulphate	62 ,, 64
Ferric chloride	125 ,, 29
Ferrous ammon. sulphate	31·5 ,, 85
Ferrous sulphate	51 ,, 73
Hydroquinone	7 ,, 95
Lactose	17 ,, 90
Lithium chloride	66 ,, 62
,, citrate	47 ,, 74
,, sulphate	33 ,, 88·5
Magnesium chloride	79 ,, 47·5
Magnesium sulphate	72 ,, 58·5

Saturated Solutions of Reagents (cont.)

	grammes in mls. water
Mercuric chloride	7·5 ,, 99
Mercurous nitrate	7·7 ,, 99
Oxalic acid	13 ,, 91
Phosphomolybdic acid	135 ,, 46
Phosphotungstic acid	160 ,, 64
Potass. acetate	94 ,, 44·5
,, bromate	7·7 ,, 98
,, bromide	55 ,, 82
,, carbonate	78 ,, 74·5
,, chlorate	6 ,, 98
,, chloride	31 ,, 87
,, dichromate	14·5 ,, 95
,, ferricyanide	37 ,, 82·5
,, ferrocyanide	30·5 ,, 86
,, hydroxide	78 ,, 68
,, iodide	99 ,, 70
,, nitrate	35 ,, 85
,, oxalate	34 ,, 86
,, permanganate	7 ,, 97
,, sulphate	11·5 ,, 97
Resorcinol	68·5 ,, 45
Rochelle salts	68·5 ,, 63
Silver nitrate	164 ,, 65·5
Sodium acetate	65 ,, 53
,, bicarbonate	8·5 ,, 98
,, bisulphate	87 ,, 60
,, carbonate, anhydrous	27·5 ,, 96
,, chlorate	72 ,, 73
,, chloride	31·5 ,, 89
,, hydroxide	77 ,, 74
,, nitrate	65·5 ,, 73
,, nitrite	56·5 ,, 76
,, salicylate	65 ,, 60
,, sulphate	45·5 ,, 72·5
,, sulphite, anhydrous	28·5 ,, 95·5
,, thiosulphate	93 ,, 46
,, tungstate	73 ,, 82
Tartaric acid	76·5 ,, 54
Trichloracetic acid	148 ,, 10·7
Uranium nitrate	120 ,, 54·5
Zinc chloride	128 ,, 61

SPECIFIC GRAVITIES (at 4° C.)

Acetic acid glacial	1·049	Ethyl acetate	0·898
Acetone	0·788	Ethyl alcohol (abs. alc.)	0·816
Aniline oil	1·022	Eucalyptol	0·921
Benzene (benzol)	0·876	Isobutyl alcohol	0·806
Benzyl alcohol	1·050	Mercury	13·500
Benzyl benzoate	1·114	Methyl alcohol	0·790
Carbon bisulphide	1·260	Methyl salicylate	1·184
Carbon tetrachloride	1·589	Octyl alcohol	0·834
Chloroform	1·475	Pyridine	0·988
Dimethylaniline	0·956	Toluol	0·867
Dioxane	1·030	Xylol	0·860
Ether	0·752		

STAIN SOLUBILITIES AND MOLECULAR WEIGHTS

Notes:

The letters A, B and N indicate acid and basic neutral dyes.

Many stains in aqueous solutions, particularly dilute aqueous solutions, are attacked and decomposed by airborne micro-organisms when kept in stock for any length of time, e.g. eosin, aniline blue. In such cases it is best to add a small crystal of thymol or a few drops of chloroform as preservative. In the case of alcoholic solutions such precautions are unnecessary, since the alcohol itself acts as a preservative.

Solutions of auramine decompose when heated above 35–40° C. Aqueous solutions of eosin should be prepared by using water of a slightly alkaline reaction as traces of acid cause precipitation of the acid radical of the dye and consequent loss of staining affinity and colour. Water buffered pH 7·2–8·0 gives good results with eosin. Alkaline solutions of methylene blue decompose when heated above 60–65° C.

Note:

The structural formulae, solubilities, and molecular weights of other dyes will be found on pages 36–76 of this book. More complete information on these and many other dyes is given in *Encyclopaedia of Microscopic Stains* (E. Gurr, 1960).

	Stain	*Mol. Wt.*	*Approximate percentage solubilities at 15° C. In water*	*In alcohol*
A	Acid violet B	483·39	3·0	nil
A	Alizarin	240·20	0·25	1·75
B	Amethyst violet	435·00	7·00	12·5

	Stain	Mol. Wt.	Approximate percentage solubilities at 15° C. In water	In alcohol
A ..	Azoeosin	380·35	1·0	0·05
A ..	Azophloxin	509·43	11·0	nil
A ..	Benzoazurin	758·69	1·50	nil
A ..	Benzopurpurin 4B ..	724·72	1·0	0·25
B ..	Bismarck brown R.. ..	461·39	1·5	3·0
A ..	Bordeaux B or R	502·43	4·75	2·25
B ..	Brilliant green	482·62	5·0	8·0
A ..	Carmoisin A	502·43	2·75	1·0
B ..	Chrysoidin R	262·73	0·1	1·5
A ..	Coeruline	554·42	1·00	0·02
A ..	Congo corinth	697·65	0·5	0·1
A ..	Corollin, alcoholic	290·30	nil	1·25
A ..	Crocein scarlet	556·49	1·50	0·50
A ..	Eosin, yellowish	691·9	44·0	2·0
A ..	Eosin, ethyl	714·07	nil	1·0
A ..	Eosin, scarlet	624·09	10·0	3·0
A ..	Erio green	560·61	4·50	4·50
B ..	Ethyl violet..	492·12	6·0	5·0
A ..	Fast acid violet 2R.. ..	648·63	4·00	3·15
A ..	Fast light yellow	380·35	2·5	0·05
A ..	Fluorescein (Uranin) ..	376·27	50·0	7·0
A ..	Guinea green, B	662·74	5·00	5·00
B ..	Hofmann's violet	421·99	6·0	7·5
A ..	Hydrazine yellow	380·35	5·00	0·07
A ..	Indigo carmine	466·36	1·3	nil
A ..	Isamine blue	767·79	5·50	1·50
B ..	Janus black B	453·96	1·0	1·5
B ..	Janus red	459·96	1·0	2·0
A ..	Manchester yellow ..	256·15	1·0	nil
B ..	Meldola blue	338·82	2·00	0·1
A ..	Metanil yellow	375·37	5·0	1·5
A ..	Methyl blue	711·77	50·0	nil
B ..	Methyl violet 6B	484·06	4·7	9·5
B ..	Methylene green	364·85	1·5	0·1
A ..	Naphthalene brown ..	400·38	1·20	0·03
B ..	New blue R	310·77	1·85	0·07
B ..	Night blue	576·19	2·25	2·25
A ..	Orseillin BB	584·54	1·50	2·00
A ..	Oxydianil yellow	737·8	2·25	nil

	Stain	Mol. Wt.	Approximate percentage solubilities at 15° C. In water	In alcohol
B ..	Phenosafranin	322·78	6·5	5·25
A ..	Ponceau 2R	480·4	5·0	0·1
A ..	Ponceau de xylidine ..	556·49	5·0	0·1
A ..	Pontacyl carmine 6B ..	566·52	2·25	0·5
A ..	Pontacyl green B	690·79	5·00	5·00
A ..	Pontamine sky blue 5BX ..	992·83	6·0	0·5
A ..	Purpurin	256·20	nil	0·7
B ..	Pyronin, B	358·89	10·0	0·5
A ..	Quinalizarin	272·20	nil	0·5
A ..	Quinoline yellow	555·49	0·5	nil
B ..	Rhodamine B	479·00	2·0	1·75
B ..	Rhodamine 6G	450·94	1·5	6·5
B ..	Rhoduline violet	378·89	3·00	20·00
A ..	Rose bengale 2B	1049·88	30·0	7·0
A ..	Rosolic acid	290·30	nil	1·2
B ..	Setoglaucine	399·35	5·50	5·50
A ..	Soluble blue 8B	799·80	40·0	nil
A ..	Solway purple	431·39	0·80	nil
A ..	Sudan brown	298·32	nil	2·0
A ..	Sudan 1	248·27	nil	0·25
A ..	Sudan 4	380·43	nil	0·5
B ..	Thioflavine, T	318·86	2·0	1·0
A ..	Titan yellow	695·73	1·5	nil
B ..	Toluylene blue	290·79	8·0	2·5
A ..	Tropoeolin O	316·27	3·0	3·0
A ..	Tropoeolin, OO	375·37	0·2	0·2
A ..	Tropoeolin, OOO No. 1 ..	350·60	2·4	0·5
A ..	Tropoeolin, OOO No. 2 ..	350·32	3·0	0·15
B ..	Turquoise blue	423·93	7·00	15·00
B ..	Victoria blue R	458·02	0·6	4·25
A ..	Vital new red	986·87	1·3	nil
A ..	Water blue	728·77	50·0	nil
A ..	Wool violet 4BN	761·91	5·0	0·75

CONVERSION TABLES

1 milligramme = 0·001 gramme = 0·015432 grains.
1 grain = 0·0648 grammes = 64·8 milligrammes.
1 gramme = 15·432 grains.

1 ounce (avoirdupois) = 28·3495 grammes.
1 ounce (troy) = 31·1035 grammes.
1 pound (avoirdupois) = 453·5924 grammes.
11 pounds = 5 kilogrammes.
1 ml. = 0·001 litre = 1 cc. = 16·23 minims.
10 minims = 0·616 ml.
1 fluid drachm = 3·697 ml.
1 fluid ounce = 29·673 ml.
8 fluid ounces = 236·583 ml.
1 pint (English Imperial) = 567·94 ml.
1 litre = 1,000 ml. = 33·81 fluid ounces.
1 gallon (English Imperial) = 4·5435 litres.
1 inch = 2·54 centimetres = 25·4 millimetres.
1 foot = 30·48 centimetres = 0·3048 metres.
1 yard = 0·9144 metres.
1 metre = 1·09361 yards = 39 inches (approximately).

To convert Centigrade degrees to Fahrenheit, multiply by $\frac{9}{5}$ then add 32.

To convert Fahrenheit to Centigrade degrees, deduct 32; then multiply by $\frac{5}{9}$.

DILUTION OF PERCENTAGE SOLUTIONS TO REQUIRED LOWER CONCENTRATIONS

It is often desirable to keep concentrated stock solutions of stains and reagents and dilute these to lower percentages as and when required. A simple rule, which may not be known to all readers for carrying out such dilutions, is as follows:

It is desired to prepare a dilute solution of X% from a more concentrated solution of Y%.

This is done by taking X ml. of solution Y and making up the volume to Y ml.

Examples:

1. To prepare a 0·25% solution of say, acid fuchsin from a 10% solution of the dye.

Here X = 0·25 and Y = 10.

Therefore 0·25 ml. of the 10% solution is diluted to 10 ml.

2. To prepare 100 ml. of 0·5% solution of safranin in 70% alcohol from a 5% aqueous solution of the dye.

As regards the dye, X = 0·5 ml. and Y = 5 ml. Therefore, to make 100 ml., a quantity of 5 ml. of the 5% safranin is needed. But the final solution has to be in 70% alcohol.

As regards the alcohol, which has to be diluted from absolute alcohol, X = 70 and Y = 100.

Therefore 5 ml. of 5% safranin plus 70 ml. of absolute alcohol and 25 ml. of water are required to prepare 100 ml. of 0·5% of the dye in 70% alcohol.

3. To prepare 0·03% sodium carbonate from a 10% solution.

Here X = 0·03 and Y = 10, so 0·3 ml. of the 10% solution has to be diluted to 100 ml. with water.

ATOMIC WEIGHTS

Element	*Symbol*	*Atomic Weight*	*Element*	*Symbol*	*Atomic Weight*
Aluminium	Al	26·98	Helium	He	4·00
Antimony	Sb	121·76	Holmium	Ho	163·50
Argon	A	39·95	Hydrogen	H	1·01
Arsenic	As	74·91	Indium	In	114·76
Barium	Ba	137·36	Iodine	I	126·92
Beryllium	Be	9·02	Iridium	Ir	193·10
Bismuth	Bi	209·00	Iron	Fe	55·84
Boron	B	10·82	Krypton	Kr	83·7
Bromine	Br	79·91	Lanthanum	La	138·92
Cadmium	Cd	112·41	Lead	Pb	207·21
Calcium	Ca	40·08	Lithium	Li	6·94
Carbon	C	12·01	Lutecium	Lu	175·0
Cerium	Ce	140·13	Magnesium	Mg	24·32
Cesium	Cs	132·91	Manganese	Mn	54·93
Chlorine	Cl	35·45	Mercury	Hg	200·61
Chromium	Cr	52·01	Molybdenum	Mo	96·0
Cobalt	Co	58·94	Neodymium	Nd	144·27
Copper	Cu	63·57	Neon	Ne	20·18
Dysprosium	Dy	162·46	Nickel	Ni	58·69
Erbium	Er	167·64	Niobium	Nb	92·91
Europium	Eu	152·00	Nitrogen	N	14·0
Fluorine	F	19·00	Osmium	Os	191·5
Gadolinium	Gd	156·90	Oxygen	O	16·0
Gallium	Ga	69·72	Palladium	Pd	106·7
Germanium	Ge	72·60	Phosphorus	P	31·02
Gold	Au	197·20	Platinum	Pt	195·23
Hafnium	Hf	178·60	Potassium	K	39·09

Atomic Weights (cont.)

Element	*Symbol*	*Atomic Weight*	*Element*	*Symbol*	*Atomic Weight*
Praseodymium	Pr	140·92	Tantalum	Ta	180·88
Protactinium	Pa	231·0	Tellurium	Te	127·61
Radium	Ra	226·05	Terbium	Tb	159·2
Radon	Rn	222·0	Thallium	Tl	204·39
Rhenium	Re	186·31	Thorium	Th	232·12
Rhodium	Rh	102·91	Thulium	Tm	169·4
Rubidium	Rb	85·48	Tin	Sn	118·7
Ruthenium	Ru	101·7	Titanium	Ti	47·9
Samarium	Sm	150·43	Tungsten	W	184·0
Scandium	Sc	45·1	Uranium	U	238·07
Selenium	Se	78·96	Vanadium	V	50·95
Silicon	Si	28·06	Xenon	Xe	131·3
Silver	Ag	107·88	Ytterbium	Yb	73·04
Sodium	Na	22·99	Yttrium	Y	88·92
Strontium	Sr	87·63	Zinc	Zn	65·38
Sulphur	S	32·06	Zirconium	Zr	91·22

SECTION 4

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