**Methods of morbid histology and clinical pathology / by I. Walker Hall and G. Herxheimer.**

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# METHODS OF
# MORBID HISTOLOGY
AND
# CLINICAL PATHOLOGY


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# METHODS

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# MORBID HISTOLOGY

AND

# CLINICAL PATHOLOGY

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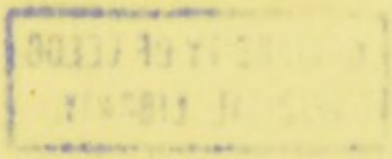

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1905

TO

THE MEMORY

OF

CARL WEIGERT

# PREFACE

THIS volume is designed to meet the requirements of students and pathologists working in hospitals and institutes. It is issued in response to enquiries for a laboratory handbook, containing the numerous, recently devised methods as well as those of earlier date, arranged in a form suitable for practical work.

Although we dare not hope to have fulfilled these conditions in their entirety, we trust that the book will prove useful to those for whom it has been prepared, and thus meet a demand in English medical literature.

It will be clearly understood that a book of this type makes no claim for originality. We have freely consulted well-known text-books, especially those of Gustav Mann, Sims Woodhead, Boyce, Mallory and Wright, Aschoff and Gaylord, Lee and Mayer, Schmorl, von Kahlden, Friedländer-Eberth, and Pollack, and the *Encyklopaedie der Mikroskopischen Technik*. To Schmorl's book, in particular, we are deeply indebted.

We have, however, except in a few cases, obtained the details of the methods from the original papers.

Our own work has thus consisted in selecting and grouping the methods, and in the addition of a few practical hints. We have also endeavoured to summarise roughly the chief theoretical considerations relative to each method. More than this seemed unnecessary, since there exist the excellent works of Gustav Mann, Fischer, Pappenheim,

Michaelis, and others, on the physical and chemical aspects of staining.

In the sections on bacteriology and clinical pathology, those methods only are included which a pathologist may require during his routine work.

In order to save time and space the references have been transferred to the end of the book, and there arranged in alphabetical order.

We are jointly responsible for the work, the personally written sections having been jointly criticised and reconstructed.

One of us owes much of his experience, especially in microscopical technique, to Weigert, whose assistant he was for several years. Partly for this reason, and partly because the name of Carl Weigert is so intimately associated with histological procedures, we intended to dedicate this volume to him as a token of the indebtedness we and many pathologists owe to this great master. Sudden death, however, removed Weigert from our midst before our task was completed. To his memory the book is now respectfully inscribed.

The other of us is also indebted to his former and present chiefs of department, Professors Sheridan Delépine and Lorrain Smith, for encouragement, guidance, and suggestive criticism.

We express our thanks to Mr. J. S. Webster and Mr. A. E. Quine for assistance in proof-reading and index revising.

WALKER HALL. G. HERXHEIMER.

*May* 1905.

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# CHAPTER I

## INTRODUCTION

### THE OBJECT OF MICROSCOPY; GENERAL CONSIDERATIONS; INSTRUMENTS

THE purpose of histological investigation is the study of objects too small to be visible to the naked eye or to the eye aided by a simple lens. Microscopical examinations of morbid tissues reveal conditions and permit diagnoses which cannot be made macroscopically, and may also confirm suppositions based upon naked eye observations. In addition to the actual diagnosis, facts of value in regard to prognosis, to ætiology, and to the correlation of pathological changes may be thus ascertained.

The objects of examination may be obtained from living or dead human tissues. In the former case, secretions and excretions as well as material gained by exploratory incisions or operations have to be dealt with; in the latter, the choice of proper material is facilitated by a general view of, and an access to, all the organs during the autopsy. In proof-excisions, etc., a diagnosis has frequently to be made from small and sometimes inappropriate fragments of tissue, and such a difficulty is often increased by the sense of responsibility and the rapidity of examination necessarily connected with a decision which may or may not indicate the need for operative interference.

When an entire organ or a large mass of tissue is available, much depends upon the selection of the part to be

examined. Although it is impossible to formulate general directions, as a standing rule, a wedge should be removed, preferably from the margin of the diseased tissue, that contains both healthy and diseased areas as well as the transition stages between the two conditions. Sometimes it is advisable, or even necessary, to take a series of wedges from the same or from various other areas.

For rapid recognition it is important that the direction of the section should be carefully considered when selecting the material; for instance, when removing a piece of kidney, both the cortex and medulla should be included, and the organ should be cut parallel to the ordinary longitudinal post-mortem section.

With small pieces of tissue removed during operations, etc., which are not easily recognised because of the absence of the usual landmarks, it is sometimes useful to make sections in two or more different directions, so as to ultimately examine the tissue in the correct plane.

Microscopical examinations may be made with fresh, untreated objects, or with objects which have been passed through certain manipulations, viz., hardening and imbedding in media, which facilitate the preparation of thin sections.

Histological investigations upon the fresh, untreated object are specially indicated for all fluids, for small fragments of torn or bruised material whose connection with their surroundings may be neglected, for the preparation of thick sections when such will suffice for the purpose in view (since only thick sections can be obtained from this unhardened material), and finally, for cases in which a apid diagnosis is desired.

A very practical method for such examinations is to freeze and cut the tissues upon an instrument called the freezing microtome, and then to treat the sections further. Although this method may be used for fresh objects, it has many drawbacks. These may be avoided by fixing and hardening the tissues in certain media before freezing; by such procedures thin sections can be obtained with but little loss of time.

When very thin sections are required, or cell changes

(such as, for instance, occur in freezing fresh tissues) must be minimised, or when very careful observations are necessary, or when serial sections are desired, or when the material is so soft or fragmented that it would be difficult to obtain satisfactory sections by the freezing method, then the material should be fixed, hardened, imbedded in either celloidin or paraffin, and cut upon their respective microtomes. By these means very thin sections may be prepared, and in a form which permits further treatment by all kinds of methods. Such procedures, however, occupy considerable time. When time is no object, the results are certainly unequalled; but it must be pointed out that with some rapid imbedding methods sufficiently thin sections can also be produced within a short time.

While fresh objects are generally examined in an unstained condition or after the addition of dilute staining solutions, sections obtained by simple freezing or after imbedding may be variously stained in order to make certain elements more prominent or distinct than others. In this regard histology has made great strides during the last decades.

Since the freezing method is easy of application, yields fairly thin sections, and permits considerable after-treatment of the tissues, it is very useful to the practical worker, and merits, therefore, a special chapter for its description.

For the pursuit of microscopical research it will be at once understood that numerous instruments and utensils are necessary, and these may now be shortly enumerated.

**The Microscope.**—Although there are numerous good microscopes on the market, personal taste and pocket must largely determine the choice of purchase.

Those made by Zeiss of Jena are generally supposed to be the best, but very good ones are also made by Leitz (Wetzlar), Reichert (Vienna), Ross, Powell and Lealand, Swift, Beck (London), Winkel (Göttingen), Hartnack (Potsdam), etc.[1] The beginner will be well advised to submit the instrument to expert opinion before concluding the purchase.

[1] A very cheap but quite reliable $\frac{1}{12}$th oil-immersion lens objective is now made by Gowlland, Selsey, Chichester, England.

Space does not here permit the consideration of the construction and theory of the microscope. The books of Frey and Behrens, Kossel and Schiefferdecker may be consulted by those specially interested. Practical tuition and experience in the actual use of the microscope is always preferable to written instructions, and the latter are accordingly here omitted.

Although a small plain stand may be used temporarily, a large one, which has a screw to move the large tube and a small micrometer screw for the exact focus, is much to be preferred.

The microscope should have two mirrors—plane and concave—to reflect the light on to the object of examination. For bacteria and for the finer histological details, an Abbe's condenser is also necessary. The plane mirror is intended for use with Abbe's condenser, the concave when no condenser is present. The reflected light is best obtained from a distance, preferably from a cloud, direct sunlight being always avoided; a room with windows looking north is therefore distinctly advantageous. No light is so good as the daylight; when this has disappeared, incandescent light is the best substitute. In this latter case the concave mirror should be used, the superfluous yellow rays of the artificial light being excluded by placing a blue glass between the light and the object. This is most easily achieved by placing a piece of blue glass under the condenser, an arrangement now generally attached to good microscopes; or, as formerly, a "Schuster ball" may be placed between the light and the microscope. (The "ball" is a globular glass vessel containing a solution of sulphate of copper and ammonia.) Working lamps specially designed for this purpose have been devised and constructed by Kochs-Wolz, Hartnack, Lassar, and others.

Two objectives are necessary, a $\frac{1}{4}$ and $\frac{1}{8}$ inch, or Zeiss A and D. For some purposes a **medium power** is useful, but not indispensable. For investigations upon nervous tissues a very low-power objective is required. In the examination of minute cell changes and of bacteria an oil-immersion lens is employed. For its application a condenser is used, such

being an accessory to every large microscope in the form of the so-called Abbe's lighting apparatus.

A nose-piece is very useful for easy and rapid changing of the objectives. Those are preferable which allow the objective to be directly screwed into the stand; when they are placed slantwise into the nose-piece, the dust can easily get into the objective from above, as this type are not closed completely. The microscopes made by Winkel are so constructed that no dust can enter, no matter the position in which the objective is placed.

An "iris" diaphragm is generally affixed to microscopes which have an Abbe condenser. It is much more convenient than the older method of changing the light apertures by inserting different sizes of diaphragms into a cylinder. The iris diaphragm permits a rapid changing of the apertures, and can be adjusted with the hand which moves the preparations. When the iris diaphragm is used with the oil immersion, it should be opened entirely, and the strongest available light be employed in order to study minute objects such as bacteria or other stained structures. For high magnifications the aperture is diminished in order to distinguish exact structures, although even here the colour picture is best determined with a fully opened diaphragm. In unstained preparations the iris is to be almost closed, since in this case it is the structures themselves, not stains, which are to be examined.

A No. 1 and a No. 2 ocular are required; rarely, a No. 4 may be used. As a rule, weak oculars are employed, since the strong ones tire the eyes and darken the picture.

Apochromatic lenses, although more expensive, possess certain advantages over the ordinary ones, and may be combined with strong oculars (compensation oculars) without the picture appearing too dark.

When using the oil-immersion lens, place a drop of cedar-wood oil on the cover-glass, lower the tube of the microscope with the large screw until the oil expands between the cover-glass and the lens, then use the micrometer screw until the picture is sharply defined.

To remove the oil from the immersion lens, wipe the

latter with a piece of soft wash-leather; in the same manner the oil may be removed from the cover-glass, or xylol may be added and the xylol and oil blown off by means of a capillary glass tube. If the oil has dried on the immersion lens, then it should be taken away with a drop of alcohol or a drop of chloroform, benzene, or xylol; the last reagent may be used for Canada balsam, but in this case the procedures must be rapidly carried out, so that the balsam which holds the lenses together is not thereby loosened.

Should spots be observed while examining the preparation, it can be easily determined by moving the oculars whether the speck is on the oculars, or by moving the preparation itself, whether the spot is on the slide or on the objective.

Specimens should be always first examined with the low power of the microscope in order to obtain a general impression, and then certain areas may be studied under the higher powers, their relations to surrounding structures being previously determined. The beginner will be often surprised to note how much an experienced worker uses the low-power objective, and how many details he is able to thus recognise. The former may be advised, when he has examined a part of the preparation with the high power, to again examine it with the low power. By such means he will learn to recognise details which the high power alone revealed to him before.

Also appertaining to a microscope, but only used for special purposes, are :—

I. A **movable stage**, which may be temporarily or permanently fixed. Although its general use is not advisable, since the preparation can be better controlled by hand, it is nevertheless valuable for thorough detailed examinations of the specimen for certain details and for enumeration of blood corpuscles, etc.

II. An **ocular micrometer**. This is a circular piece of glass which is placed in the ocular and upon which measured divisions are scratched.

III. A **warm stage** for the observation of living objects. Recently a simple electrical apparatus has been devised by Ross. It consists of a small disc of ebonite with a square gap in the centre for the cover-slip and objective of the microscope, and contains a coil of wire which, when connected with an electric light circuit, gives out heat sufficient to raise and maintain the temperature of the centre of the slide at 37° C. It can be slipped on and off the slide without altering the focus, and requires no attention.[1]

1 Sold by Drake and Gorham, 66 Victoria Street, London, W.

IV. A **drawing apparatus**, such as that of Edinger's, or the camera lucida of Abbe.

**Microtome**, *vide* Chapter II.

**Micro-photographic apparatus**, *vide* Chapter XVII.

**Various utensils** :—

*Razors* were more frequently used for cutting sections when fresh tissues were chiefly examined.

*Double knives* may be used for making sections of fresh or slightly hardened objects. They yield better sections than those obtained by the simple razor, especially in the hands of a novice.

*Scalpels* are required for various purposes.

*Scissors*, straight and curved.

*Forceps* of all sizes, including a pair of Cornet's forceps for "film" preparations.

*Spatulas* and *needles* are very necessary.

*Glass needles* are substituted for steel needles for all metallic solutions, and when testing for iron compounds.

*Leather strop* and a smooth stone, for sharpening razors and knives.

*Emery paper*, for sharpening needles, etc.

*Slides and cover-glasses* must always be kept very clean. Since, however, the silicates are being constantly decomposed, and both slides and cover-glasses are thus always coated with alkaline silicates, it is necessary to subject them to certain procedures before using them for histological purposes.

A simple and reliable method is to place both slides and cover-glasses in a mixture of equal parts of 1 per cent sulphuric and chromic acids ; the former should remain in the solution for an hour, the latter for 10 minutes. Both are then rinsed in distilled water to remove the acids, and then dried on a clean linen cloth, and kept dry in a dust-proof box or in absolute alcohol.

In order that used slides and cover-glasses may be again employed, a number of special methods have been recommended.

Dissolve the Canada balsam by dry heat, separate the slide and cover, and treat each separately.

I. **Knauer's Method.**—Boil the slides or covers in an enamelled pan containing 10 per cent lysol for about half an hour. Rinse in water until the water is quite clear. Dry on a clean cloth.

II. **Zettnow's Method.**—Prepare the following solution : Pour 2 litres of hot water over 200 gms. of chromate of potash ; add 200 c.c. of pure concentrated sulphuric acid, little by little, constantly stirring.

Place slides in the solution for 2-3 days ; wash in cold water ; dry. If stains are present, wipe them with a cloth dipped in alcohol.

Cover-glasses should be boiled in the solution for about 10 minutes, stirring constantly. If masses of resin appear on the surface, remove them with a piece of paper. Rinse with water, place in dilute caustic soda solution for 5 minutes, boil again in the special solution, wash in water, place in dilute caustic soda, then in several changes of alcohol ; dry.

III. **Funcke's Method.**—Heat the slides or covers, previously separated by the use of turpentine, in hydrochloric acid containing a few grains of potassium chlorate in each 30 c.c. Rinse in hot water. Heat in the following mixture for about half an hour, stirring repeatedly : equal parts of carbonate of soda, very small wood-shavings, and talc, made into a pulpy mass with a little water. Wash in hot water containing hydrochloric acid, then rinse in alcohol, ether, and dry.

IV. A more simple, easy, and useful method is as follows :—Separate the slides and covers by heating the slides over a Bunsen burner. Boil the slides and covers separately for 10 minutes in a strong solution of Hudson's dry soap. Rinse in water. Boil for 10 minutes in equal parts of 1 per cent chromic and sulphuric acids ; wash in water ; rinse thoroughly in alcohol ; dry.

*Test-tubes*, *glass rods*, *capillary tubes* (for fluids), *measures*, *funnels*, *bottles*, *watch-glasses*, and a number of *glass basins* are also necessary. Watch-glasses are not generally useful, as they are so easily upset and hold only small quantities of fluid. Small watch-glasses or porcelain basins are, however, useful when it becomes necessary to heat their contents over a flame. Hollowed glass blocks are sometimes used. Most convenient are deep basins without covers, which can be covered by a piece of plain glass. When treating a number of sections at a time, the "Siebdosen" of Steinach are specially advantageous. They are glass vessels whose bottoms are perforated like a sieve, and when in use are placed into larger glass holders containing the stain or other solutions. On removal of the inner vessel the stain runs out through the perforations, and leaves behind any sections or other contents, which may be then transferred to other fluids. (They are sold by Siebert, Alsenstrasse 19, Vienna.)

A *drop glass* or bottle *for the cedar-wood oil*, and a special *non-evaporating bottle for Canada balsam*, are essential.

Coloured *oil* or *wax pencils* and *labels* should be also near at hand.

*Filter-paper*, of which a large quantity is requisite, should have a fairly smooth surface and be cleanly cut into small pieces.

The laboratory must be provided with *running water*, and *distilled water* should be kept in stoppered bottles.

The *working-table* should be covered with filter-paper, or, still better, with thick glass which can be easily cleaned.

As some objects are more easily recognised on a light background and others on a dark background (*e.g.* sections during clearing), the underground should be made of white and black glass plates, or the whole table may be covered with glass, under which pieces of black and white paper are arranged.

A *centrifuge* for fluids is frequently needed.

The necessary *stains*, *reagents*, *etc.*, are not now enumerated, as they will be described in the following chapters.

## CHAPTER II

### EXAMINATION OF FRESH OBJECTS

As already indicated, this method is rapid, and possesses the further advantage that the tissues are but little changed during its application. For the beginner, however, the recognition of unstained objects presents many difficulties, and the information obtained is frequently slight. Further, when a knowledge of the finer details or the relations of structures to one another is necessary, then the method is not satisfactory. But for the general structure of the cells and for certain pathological changes which are altered or lost during imbedding, etc., as for instance the so-called cloudy swelling, pigments, or fat, the method is very useful. Fat, however, can be also well hardened and stained in a special manner (*q.v.*).

It is at all events advisable to examine the fresh objects before hardening. Such examination will rarely make the hardening superfluous.

The most simple application of this method is in the investigation of pathological fluids. A drop of the material is placed upon a slide, covered with a cover-glass, and then examined: if rich in cellular constituents, it may be diluted with water, or, better, with normal saline solution; if poor in cells or in certain structures or formations, such as urinary casts, the fluid should be allowed to stand in a conical glass or centrifugalised (the better and surer method), and the sediment only be examined. For this purpose the ordinary hand centrifuges are sufficient, although the larger electric ones are more convenient. In both cases the sediment is removed from the bottom

of the glass with a pipette, and a drop, sufficiently small in size to just fill up the space between the cover and the slide, is examined. The cover-glass should not swim in the fluid, nor should it be pressed down upon the fluid.

It is sometimes useful to allow the drop of fluid to dry on the slide and be further treated with stains, etc., as in making a film preparation (see Blood and Bacteria).

For massive objects (generally organs), two methods of "fresh" examinations are available, viz. isolation of the cells by disturbing the relations of the structures, or the preparation of sections.

In the first method, the surface of the organ is scraped with a knife and the scrapings placed in a drop of fluid on the slide—(avoid the inclusion of much blood)—and a streak or film preparation is made therefrom; or a teased preparation is made by removing a small piece of the tissue with scissors, placing it in a drop of fluid, and tearing it into small fragments with two pointed needles. With very soft tissues, small portions may be crushed between two cover-glasses, but this procedure is rarely to be advised.

In other cases, small snips may be made with fine scissors, and the resultant piece spread out on a slide.

The film preparations are generally made from the looser tissues in order to rapidly identify abnormal cells, *e.g.* in certain tumours, or for the recognition of the so-called "granular bodies" in the central nervous system. The teased preparations are employed for the isolation of more closely bound cells, such as cardiac muscle cells, *e.g.* for albuminous or fatty granules, and sarcoma cells.

By placing small fragments of the tissues in *maceration or dissociation fluids* for a short time the subsequent teasing is much facilitated. Of the numerous solutions proposed only five will be here mentioned since, from a pathological standpoint, the method has not now the value it formerly possessed.

1. **Ranvier's 33 per cent Alcohol.**—Macerate 24 hours or more.
2. **Chromic Acid 1/5000 and Salts.**—Macerate 24 hours or more.

Potassium bichromate, 0·1-2 per cent solution. Macerate 2-4 days (nervous tissues).

3. **33 per cent Potassium Hydrate.**—Macerate ¼-1 hour. The tissues must be examined in the potash lye, since the cells are dissolved when water is added; or the potash must be neutralised with 50 per cent acetic acid. Specially used for muscles.
4. **Arnold's Iodine Solution.**—Ten parts of 10 per cent potassium iodide solution, to which are added 5-10 drops of a solution containing 5 gms. of iodine and 10 gms. of potassium iodide in 100 c.c. of water. If the solution becomes decolourised, add a further quantity of the latter mixture. Macerate 1 or more days.
5. **Digestion** of the tissues with gastric or pancreatic juice at 37° C. (Parasites, etc.). Macerate until required tissues are freed.

In the second method the tissues are cut into sections by razors or special knives.

When using *the simple razor* after fresh "setting" and sharpening, a section is cut as fine as possible and parallel to the selected surface. Only an expert will obtain good sections. This proceeding is used less now than formerly, other instruments having displaced the razor.

The *double knife* consists of two parallel blades arranged so that the space between them may be changed at will. Dip the instrument into water, so as to fill up the very narrow interspace. Make a perpendicular cut into the tissue: when the section enters, the water escapes. Then (without pressing to both sides) turn the double knife to both sides so as to loosen the lower edge of the section; pull back the knife; separate the two blades in water or normal saline solution; the section becomes free. With some practice, successful sections may be cut, but this procedure generally fails with soft tissues. Sections obtained by the razor or double knife are, as a rule, unsuited for the complicated manipulations of staining processes.

When examining specimens made from fresh objects, be they teased, film, or section preparations, it is advantageous to add certain fluids which render some structures more prominent than others.

Place a drop of the reagent at one side of the cover-glass and a small piece of filter-paper at the other; by this method the reagent enters the capillary space between the slide and the cover-glass and passes towards the blotting-paper. The changes induced in the tissue during its progress can be observed under the low or high power.

Amongst many others, the following chemicals deserve special mention :—

1. **Acetic Acid.**—This acid causes the nuclei to shrink slightly and to stand out more boldly.

   It is also useful in the demonstration of elastic fibres, which, being unaffected by acetic acid, are sharply defined from the connective tissue, which swells under its influence.

   Acetic acid differentiates albuminous and fatty granules, dissolving the former and not affecting the latter.

   A 1-5 per cent solution is generally employed, but in some cases the concentrated acid may be used. In the latter instance the acid is of course diluted in proportion to the amount of fluid already underneath the cover-glass. The addition of a few drops of a dye—as, for instance, fuchsin—to the acetic acid makes the nuclei still more distinct.
2. **Caustic Potash** (1-3 per cent).—Elastic fibres, pigments, fat, and bacteria are not acted upon, and so are more easily recognised ; the greater part of the other tissue structures become unrecognisable.
3. **Osmic Acid** (1 per cent) and the recently introduced Sudan III. and Scharlach R stain fat globules black and red respectively, thus rendering them distinctly visible.
4. **Mineral Acids**—HCl or $H_2SO_4$ (3-5 per cent).—The former liberates $CO_2$ from calcium carbonate, and effervescence occurs ; the latter produces calcium sulphate crystals.
5. **Iodine Solution** (Lugol's solution diluted with water).—This, in a manner similar to acetic acid, brings out the nuclei and cell contours, and is especially useful in that it yields a specific reaction with amyloid or lardacein. While it stains the tissues yellow, the amyloid material is coloured mahogany brown (see Amyloid). When the glycogen has not been washed out or dissolved by the solutions employed, it also is stained brown.
6. **Stains** (fuchsin, methylene blue, methyl green).—1 per cent solutions of these dyes in water, or preferably normal saline solution, are of great value in the examination of fresh tissues. They are drawn under the cover-slip by means of blotting-paper.

The above-described preparations are not permanent; if such are desired for further observation, it is necessary to surround the cover-glass with vaseline or wax in order to prevent evaporation, the edges of the cover-glass and surrounding area of the slide being first carefully dried. A hollow (hanging drop) slide may also be used, by placing a drop of the fluid to be examined on a cover-glass, inverting it over the cavity, and then painting it round with vaseline. Such a method cannot, of course, be used for sections.

For the study of living cells, and especially of their movements, warm stages and moist chambers are necessary. Recently, Deetjen's agar culture medium has been used. These, although mentioned here, belong to fields of special research.

# CHAPTER III

## THE FREEZING METHOD

For this method an apparatus is necessary which will serve for both the freezing of the object and for the production of fine sections. It is termed a "freezing microtome."

The freezing is induced by the use of ether or liquid carbonic acid gas. With the ether-freezing microtome an ether spray is produced by pressing a rubber ball connected with ether contained in a vessel and led to the under surface of a metal plate on whose upper surface is placed the object to be frozen. Instead of ether, ethyl chloride is also used; it is sprayed directly under the plate which carries the object. The method is simple, but very expensive.

The large ordinary microtomes may be also used for this purpose by affixing special freezing chambers, but the specially devised microtomes are much more convenient. Amongst these, Jung's so-called "student's freezing microtome" and "Cathcart's freezing microtome" will be found to give excellent results, considering their cheapness. They possess automatic adjustments for making sections of a definite thickness, and may be also employed for cutting paraffin blocks. Unfortunately, their ether-tube is somewhat easily blocked up, the object-holder is rather small, and since the knives are of a size which just fit into the apparatus, their cutting edge cannot be fully utilised.

With Becker's ether-freezing microtome (also to be obtained at a reasonable figure) these disadvantages are partly obviated; still it is necessary to use a considerable amount of ether, especially when the room temperature is somewhat high.

All these drawbacks may be avoided by the use of the fluid carbonic acid gas freezing microtome. The tissues are rapidly hardened, and fine sections may be obtained. The prices of these instruments are naturally higher than those of the ether-freezing microtomes, but the carbonic acid gas is relatively much cheaper than the pure ether which is necessary, and so the working expenses are small.

The liquid carbonic acid gas is supplied in large tubes, which stand the heat of our climate extremely well, although it is not advisable to place them too near to a fire or stove.

Different forms of these freezing microtomes have been devised. The older and somewhat dearer apparatus of Gaylord, the one recently modified by Aschoff (both made by Becker, Göttingen), and the Delépine freezing microtome (made by Beck, London) are all useful. The two latter types have been used by us for several years, and we can very highly recommend either of them.[1]

In all the instruments the tissue is placed on the freezing-plate, on the under surface of which the liquid carbonic acid is sprayed. The object is pressed slightly with the finger until the lower portion is frozen in order to obtain a flat cutting-surface. Some workers place the fresh tissues in the white of an egg, preserved for the purpose in a bottle containing a crystal of thymol; one of the writers always coats the object with a layer of thick gum.

When the lower part of the object is frozen, remove the upper soft layers with the microtome knife or with a sharp scalpel; the lower portion will, as a rule, be too hard for immediate cutting and it is necessary to wait until it is thawed a little, or to warm the surface by rubbing it with the fingers.

Before cutting, adjust the microtome for the thickness of section required; remove the sections from the knife with

[1] It was at one time intended to include a detailed description of these instruments, and to give the necessary instructions for their working. As, however, personal tuition and practical experience play so great a part in the acquisition of the necessary manipulations, the apparatus are here simply recommended and the purchaser advised to obtain the required instruction from laboratory workers.

a soft brush, or better with the finger in order to save the cutting edge, and place them in water, saline solution, or 70 per cent alcohol.

If the sections are curled or cannot be easily separated, place them in 50 per cent alcohol for some minutes, and then transfer them to water or to normal saline solution; they then straighten themselves out. The diffusion streams produced by the mixing of the alcohol and water are often of such intensity that the finer structures are damaged.

Sections obtained by this method can only be satisfactorily stained after further treatment. Successful results may be obtained by allowing the sections to remain in alcohol for a little time, or, according to Relly and Pick, by placing them in formol for four minutes before staining.

If it is desired to examine the fresh tissues in the most natural condition, sections may be transferred directly from the knife to the slide and examined in normal saline solution. The process of freezing is, however, an active one, and may have already altered the relation of the cellular structures.

This rapid method of freezing and staining fresh tissues is often employed for the diagnosis of operative or post-mortem material, but whenever more time is available, it is well to first fix the tissues in 10 per cent formol for a few hours, or if possible for 24 hours. The time may be shortened by placing the tissues in an oven or an incubator at 37° C. This additional treatment materially aids in the attainment of thin, well-staining sections.

When a piece of tissue has been fixed and hardened in formalin for a few hours it may be frozen directly, or after a slight rinsing in water, normal saline, or thin gum and syrup solution. The sections may be transferred to either water or alcohol prior to staining, but when alcohol is selected, sprinkling the razor or the frozen object should be carefully avoided. Sections which have been left in alcohol for a little time generally stain easily; should they not do so, they may be put into strong alcohol for a short time, when the desired effect will follow. Almost all general and special stains may be used, although with such methods

as that for fibrin (Chapter XI. section v.) certain manipulations are necessary.

The procedures of staining, etc., may be facilitated by using Wright's method of dealing with frozen sections. The section is spread upon a slide and dried thoroughly with filter-paper. A few drops of absolute alcohol are then allowed to run over the surface of the section, and are followed by a similar quantity of *very* dilute celloidin solution; the latter is allowed to dry in the air for a moment, and then placed in 70 per cent alcohol. The sections are now fast to the slide, and are not loosened by subsequent ordinary procedures.

Instead of formol, Orth's fluid (formol and Müller's fluid, Chapter IV.) may be used. 12-24 hours in the incubator are generally sufficient. The tissues, so hardened, must be washed before being frozen. Similarly, after fixing in pure Müller's fluid, corrosive sublimate solution, or Zenker's fluid, the pieces of tissue should be well washed in water. After these fixing and hardening reagents, objects can be frozen and cut quite easily, but this is not the case when they have been hardened in alcohol; they must then be left for 24 hours in running water, or they are better placed in formalin for some hours before freezing. It is also advantageous to put them into gum and syrup for 3-4 hours and then to coat them with thick gum just before freezing (Hamilton).

The freezing microtome method is also especially useful for the demonstration of certain substances which are altered or even dissolved during the passage of the tissues through the media necessary for imbedding, as, for instance, fat. In combination with the hardening of tissues in formol it permits, in comparison with the imbedding processes, an early diagnosis of operation and post-mortem material, and aids the selection of those tissues suitable for more detailed investigation. It is thus an important method for the practitioner, and quite merits the separate chapter here accorded.

For the finest cell structures this process is not so good as the imbedding methods, since the cells frequently appear somewhat changed and the sections are not so thin as those obtainable by the slower imbedding procedures.

# CHAPTER IV

## FIXATION—HARDENING

In the previous chapter certain fixing media were mentioned in connection with the freezing method for obtaining sections. The process of freezing rendered the tissues hard and enabled them to be cut. With material which is unsuitable for these procedures or when very thin sections are necessary, the pieces of tissue must be imbedded in a medium which can be cut on a microtome and which permits the attainment of the desired preparations. As a preliminary, the tissues are fixed and hardened.

By *fixation* is implied the preservation of the appearances present when the tissue is taken for examination; hence it is necessary to obtain the operative and post-mortem material as early as possible in order to avoid the occurrence of cell changes, and to place them in a sufficient quantity of fixing fluid to obviate any illusion as to their thorough fixation. The pieces of tissue should thus be of small size, be placed in at least ten times their own volume of the fixing solution, and allowed to remain in the medium for a period varying with each mixture. In order that the tissue may be in contact with the permeating fluid on all sides, it is well to place tow, cotton-wool, or filter-paper at the bottom of the vessel, or to suspend the blocks of tissue from the lid; portions of organs which float should be submerged by being weighted with filter-paper or glass rod.

*Hardening* is the term used to denote the process which gives to a tissue the consistency which is necessary for the attainment of thin sections. Some reagents fix and harden at the same time—*e.g.* alcohol, chromic acid salts, and for-

malin—when acting; others fix but harden so incompletely that a second hardening medium is necessary. For the second medium alcohol in increasing strengths is chiefly employed. It hardens by dehydration, and thus at the same time prepares the tissue for imbedding (*q.v.*) by removal of water. Before placing the tissue in the alcohol the majority of fixatives are removed by repeated rinsing in water, etc.

*Fixing and hardening media* are somewhat selective in their action; some preserve one constituent of a cell better than another, and others again act similarly in regard to other structures. While some fixing solutions stain the tissues at the same time as they fix them (*e.g.* fat is stained black during osmic acid fixation), others prepare the structures for the action of subsequent stains, acting as mordants for the latter; on the other hand, some staining methods fail only when the tissues have been hardened in certain media. There does not exist a general hardening solution which yields equally good results in all cases. It is thus often necessary to employ specific hardening fluids from the first moment of conservation, and for the reasons just stated, media which are composed of several fixing and hardening substances are frequently preferable to single fixing reagents.

*Fixatives* act by coagulating the cell albumins. According to v. Tellyesniczky, those substances are the best which, although they produce immediate death of the deeper layers of cells, yet only induce a slow coagulation and hence avoid marked shrinking. Such actions are fully exemplified by some of the fixing mixtures and especially by those containing acetic acid. The fixing solution acts better when it is slightly acid, a fact explained by Fischer as due to the alkaline reaction of most tissues and the consequent acceleration of coagulation in weakly acid media. v. Tellyesniczky remarks that good fixing media must also possess the property of rapid diffusion, in order rapidly to reach the deeper layers of cells.

For the theoretical considerations and grouping of fixing

media, reference may be made to the classical works of Fischer and Gustav Mann.

Of the large number of fixing fluids and mixtures that have been devised, we here include only those which, as a result of experience, have been more generally adopted.

### A. Formol

Formol or formalin is a 40 per cent watery solution of formaldehyde gas. It is generally put up in brown bottles to lessen its tendency to decomposition. Of this solution one part is mixed with nine parts of water to form a 10 per cent solution of formol, or, as some writers regard it, a 4 per cent solution of formaldehyde gas. The former term we adopt as the more correct one, inasmuch as the formaldehyde contents may vary slightly, and the 4 per cent solution of formaldehyde is commonly, but incorrectly, called 4 per cent formol. A 10 per cent solution of formalin is the best strength for fixation purposes.

Although formalin is here considered first, it is not because it is the oldest—on the contrary, it was only introduced into microscopical technics in 1893 by F. Blum; neither because of its widespread use, but rather because it may be recommended to the practical worker as a fixing fluid suitable for all general purposes. Each individual worker naturally falls into the habit of using one fixing medium for all except the most special purposes, even if a fluid does not exist which is suitable for all cases.

Formol has the following *advantages*:—(1) It is cheap; (2) the requisite strength may be quickly prepared, and it keeps well in brown bottles; (3) it facilitates rapid cutting and diagnosis; (4) pieces of tissue to be imbedded may directly or after rinsing in water be passed through 70 and 96 per cent and absolute alcohol (24 hours each) cleared and imbedded; (5) it permits the tissue to be otherwise after-hardened, as in some complicated methods for nervous tissues; (6) it hardens at the same time as it fixes, and this primary hardening, together with the subsequent alcohol hardening, gives the tissue a consistency

suitable for cutting, and there is but little shrinking; (7) it preserves fat well (freezing microtome sections); (8) it is a good fixative for blood constituents; (9) it allows the use of almost all stains, especially the principal ones (Schmorl recommends thorough washing before hardening with alcohol when carmine staining is employed); (10) it hardens even large pieces of tissue more rapidly than any other fixing medium, and if the formol be frequently changed, tissues may remain in it for some time without hurt, although finer structures suffer after an immersion of more than a month.

Against these advantages a few *disadvantages* may be cited:—(1) Alike with all other media, except alcohol, it dissolves glycogen and uric acid, and cannot therefore be used when these substances are under examination; (2) it sometimes diminishes the colour reactions of amyloid (lardacein) when the tissues are immersed for a long time (the same happens, however, with other fluids, even with alcohol, although not perhaps so quickly); (3) it does not fix finer cell structures, such as mitotic figures, so well as Flemming's or Hermann's mixtures; (4) it is frequently associated with the appearance of fine brown deposits. It sometimes changes the colour of biliary concrements—yellow to green,—and cartilage in the neighbourhood of blood clots are now and then altered so as to produce a pseudo-ochronosis (Heile). The recognition of these deposits may prevent wrong impressions.

### Method of Fixation and Hardening by Formol

1. Fix and harden in 10 per cent watery (or normal saline) solution, 4 hours to 4 or more days. Use 10 times as much fluid as the volume of the tissue. Heat (25-37° C.) hastens the process. Throw the used formol away; it cannot be again employed.
2. Wash in water (not necessary); cut on freezing microtome, or
3. Harden in 70 and 96 per cent alcohol, 24 hours each, and imbed.

Commercial formalin contains formic acid and yields an acid reaction. This does not seem a disadvantage for fixing, as v. Tellyesniczky advises the addition of 5 c.c. of acetic acid to each 100 c.c. of formol. Gustav Mann, however, recommends that for micro-chemical purposes the free

formic acid should be neutralised with magnesium or sodium carbonate and the aldehyde be freshly distilled; that when not used in full strength, formol should be neutralised and diluted with normal saline, not with water, which causes nervous structures and blood corpuscles to swell.

Formol has been also used in other **mixtures,** of which only the following are here given :—

1. **Orth's fluid,** an excellent general fixing medium.

| | |
|---|---|
| Formalin (40 per cent formaldehyde) | 10 c.c. |
| Müller's fluid . . . | 100 c.c. |

Prepare freshly; the mixture does not keep well. Fix the tissues 3-12 hours at 37° C. or for 24 hours at room temperature. Wash, freeze, and cut, or harden in alcohol and imbed.

2. **Formol alcohol.** Highly recommended by Fish.

| | |
|---|---|
| Formalin (40 per cent formaldehyde) | 10 c.c. |
| Alcohol (95 per cent) . . | 100 c.c. |

Fix tissues for 12-24 hours.

A similar mixture has been recommended by Parker and Floyd.

## B. Alcohol

Alcohol is a good general fixative, and also hardens at the same time; it is widely used for after-hardening purposes. For *fixing,* the alcohol must be strong, or it does not fix with sufficient rapidity.[1] 95-96 per cent alcohol is generally selected. The pieces must be small. Sufficient alcohol must be taken and changed frequently, absolute alcohol replacing the 95-96 per cent alcohol.

Fixation in alcohol has *disadvantages* which make it compare rather badly with formol and other fixatives. They are :—

(1) Excessive shrinking from sudden intense dehydration. The finer details are lost or distorted. Some workers will never use alcohol; one French writer terms it "the ideally bad fixing medium."

[1] When used for after-hardening alcohol is, on the other hand, used in increasing strengths, 70, 96 per cent, absolute alcohol.

(2) Excessive hardness and consequent difficulties in cutting.

On the other hand, alcohol has the following *advantages*, which account for its use in special cases :—

(1) Rapidity of preparation for imbedding; previous fixation or passage through increasing strength of alcohol being unnecessary. Hence it is specially useful for the diagnostic examination of tissues too small or too soft for the rapid freezing method. Such should be placed directly into absolute alcohol. This is the procedure in Lubarsch's rapid method (p. 39).
(2) For the staining of bacteria, alcohol fixation is largely adopted.
(3) Complete conservation of glycogen for subsequent staining.

**Alcohol mixtures** are numerous, but the one which chiefly minimises the disadvantages of alcohol fixation is the formol alcohol already mentioned.

To keep the alcohol absolute, and for the sake of economy, the following procedure may be employed. On the bottom of an exsiccator place pure dried white copper sulphate wrapped up in gauze, and fill up with alcohol. During dehydration, place the tissues on a draw-net; they then do not touch the copper; when the latter turns blue, replace it with the white sulphate or re-fuse it.

The alcohol may also be dehydrated by placing in it strips of gelatin which have been washed in 10 per cent salt solution and distilled water and then dried.

Sydney Young purifies alcohol by distilling it with benzine through a special form of condenser. The process is very rapid and the alcohol obtained is pure.

To determine whether the alcohol is sufficiently absolute for use, mix some drops of alcohol with pure water-free xylol; if no sediment appears (seen on a dark background), the alcohol is absolute or nearly so.

To test alcohol for its purity, P. Mayer adds to it

1 per cent of the following mixture:—Hæmatein 1 gm., aluminium chloride 1 gm., alcohol 100 c.c. With pure ethyl alcohol, the hæmatein does not precipitate after 24 hours.

## C. Chromic Acid Mixtures

Chromic acid itself is now rarely used as a fixing medium, and its salts only when combined with adjunctive substances.

1. **Müller's fluid** is the mixture more generally used:—

| | |
|---|---|
| Potassium bichromate . . | 2·5 gms. |
| Sodium sulphate . . . | 1·0 gm. |
| Water . . . . | 100 c.c. |

At one time this was the universal fixing medium; now it is almost only employed for the eye and nervous system, either in conjunction with or after fixation in formol.

Large pieces of tissue may be hardened in Müller's fluid, but several months are necessary, and the solution must be changed every day at first. Even small pieces require several weeks, but the process may be hastened by warming.

To avoid contamination by bacteria and moulds, add a small piece of camphor or a crystal of mercuric chloride.

When hardened, the tissues may be cut directly on the freezing microtome or passed through increasing strengths of alcohol and then imbedded. The dehydration is best performed in the dark, since chrome sediments appear on exposure to light. Such a procedure is important when treating Müller-fixed nervous tissues, since these may not be washed before transferring to alcohol.

2. **Erlitzky's fluid**:—

| | |
|---|---|
| Potassium bichromate . . | 2·5 gms. |
| Copper sulphate . . . | 0·5 gm. |
| Water . . . . | 100 c.c. |

Almost exclusively used for fixation of the central nervous system. Deceptive pigmentary deposits often arise (Tschisch); these may be removed by treating the tissue with hot or with acetified (Edinger) water.

## D. Mercuric Chloride

1. Concentrated watery solution of $HgCl_2$ . 100 c.c.
   Glacial acetic acid . . . . 5 c.c.

The acetic acid is not necessary, but its addition permits more thorough fixation.

2. **Zenker's solution** :—

| | |
|---|---|
| Mercuric chloride . . . | 5·0 gms. |
| Sodium sulphate . . . | 1·0 gm. |
| Potassium bichromate . . | 2·5 gms. |
| Water . . . . | 100 c.c. |
| Glacial acetic acid . . . | 5 c.c. |

Dissolve with heat; add acetic acid just before using (to avoid its evaporation).

Fix and harden small and thin pieces for 24 hours.

Helly advises the substitution of 5 per cent formalin for the 5 per cent glacial acetic acid. This fluid is specially useful for the stains employed for the demonstration of cell granules.

After mercuric chloride the tissues should be washed in running water for 24 hours and then, to avoid the presence of mercuric sediments, be transferred to 70 per cent alcohol, to which iodine has been added until a claret colour appears (Ravitz). The mercury combines with the iodine and the alcohol is gradually decolourised. When this occurs, change the alcohol, repeating the process until no decolorisation can be detected. Lugol's iodine solution (*q.v.*) is preferable, since the contained potassium iodide allows the combination of larger quantities of mercury. It is an advantage to replace the water in Lugol's solution by 90 per cent alcohol (Lee-Mayer, *Mikrosk. Technik.* 1901, p. 44). The tissues are then cut or imbedded in the ordinary manner. Should the sections contain mercuric sediments, place them in the iodine solutions for 30-60 minutes, washing them afterwards in alcohol. The sections then also stain better.[1]

3. **Klingmüller** and **Veiel** have recently recommended the use of a 5 per cent solution of **sublamin** in distilled water and after-hardening in alcohol. The solution is said to fix quickly (half-hour to several hours), to form no deposits, and to allow good staining effects. It is only necessary to stain for a short time.

For general work Zenker's fluid is not so useful as formalin. The necessary preparation, slow hardening, after-removal of mercury, and its deterrent action upon certain stains, constitute marked disadvantages.

Zenker's fluid is, however, specially useful for mitotic figures and for the finer details of cell structures. Tissues stained with Heidenhain's iron hæmatoxylin and the

[1] Because of the decomposition of the albuminates of mercury by iodine potassium iodide, Gustav Mann advises that this mixture should never be allowed to act upon tissues until they have been taken through the paraffin process.

Biondi-Heidenhain tricolour stain give the best results when fixed in Zenker's fluid, and it forms with other sublimate solutions the only possible type of fixative for Mallory's general staining method.

### E. Osmic Acid and Osmic Acid Mixtures

When osmic acid is used alone, a 1 per cent solution is generally employed. Better results are obtained by its combination with other substances.

1. **Flemming's mixture** :—

| | |
|---|---|
| Chromic acid 1 per cent . . | 15 c.c. |
| Osmic acid 1 per cent . . | 4 c.c. |
| Add glacial acetic acid up to . . | 1 c.c. |

Fix very small and thin pieces for about 24 hours; wash in running water 24-48 hours (or deposits of osmium may occasion wrong impressions); harden in increasing strengths of alcohol. The osmic acid penetrates badly, and there is often a marked difference between the fixation of the centre and edge of the flat pieces. Hence the necessary small size of the pieces of tissue. It is best to harden in brown bottles in the dark to prevent decomposition, and tightly stoppered to prevent evaporation.

Flemming's mixture is chiefly used for fat and mitotic figures, and for these purposes it is by far the best fixing solution extant.

Tissues fixed in Flemming's solution may be counterstained in safranin, aniline gentian violet, or carbol fuchsin (*q.v.*).

As a general fixing medium osmic acid cannot be recommended, because (1) it is expensive; (2) it penetrates very slightly, so that only small pieces can be taken; (3) the tissues take the stains very badly after fixation in osmic acid.

2. **Hermann's mixture** :—

| | |
|---|---|
| Platinic chloride 1 per cent . | 15 c.c. |
| Osmic acid 2 per cent . | 4 c.c. |
| Glacial acetic acid . . . . | 1 c.c. |

Merely substitutes platinic chloride for the chromic acid in Flemming's mixture. It is practically employed for the same purposes as Flemming's mixture; is dearer than Flemming's solution, but brings out finer plasma details better; the same counterstains may be used.

3. **Marchi's mixture** :—

| | |
|---|---|
| Müller's fluid mixture . . | 2 parts. |
| Osmic acid 1 per cent . . | 1 part. |

Specially used for nerve degeneration and sometimes for fat.

4. **Altmann's mixture** :—

| | |
|---|---|
| Potassium bichromate solution 5 per cent | Mix equal |
| Osmic acid 2 per cent . . | parts. |

Used for fixation of Altmann's granules.

### F. Picric Acid and Picric Acid Mixtures

Fix for 12-24 hours in cold saturated watery solutions of picric acid. Remove the picric acid with alcohol, not with water, and harden in increasing strengths of alcohol. Mitotic figures and other fine details are well fixed.

**Merkel's mixture** (chromic acid 1, platinic chloride 1, water 800 c.c.), **Carnoy's mixture** (glacial acetic acid 1, absolute alcohol 6, chloroform 3, are also useful for pathological work. Burchhardt's chrom-osmic-nitric mixture, Rabl's chrom-formic mixture, Flemming's chromic-acetic mixture, Rawitz's chromic-picric-nitric mixture, Pianese's mixture may here be just mentioned and the reader referred for further details to the handbooks of Gustav Mann and Lee and Mayer.

### G. Boiling Method

Place small pieces of tissue in water at 100° C. Boil for a few minutes: the albumins are coagulated. Harden in 96 per cent alcohol.

The method is useful for the fixation of œdematous tissues, viz. œdema of the lung, for renal casts and for the albuminous contents of cysts.

### H. Freezing and Drying Method (after Altmann)

Pieces of fresh tissue are frozen and dehydrated by being dried at a temperature of −20-30° C. in a vacuum over sulphuric acid; when quite dry they are imbedded in paraffin. Altmann believes that by this method the tissues are obtained in a condition free from any changes in volume, and only deprived of water.

Specimens fixed and hardened by the methods just described maintain their properties for a long period when kept in 80-90 per cent alcohol.

**Fixation and hardening of macroscopical specimens :—**

For the hardening of macroscopical specimens alcohol and, later, formol have been employed. Recently methods have been described by Melnikow-Raswedenkow, Kaiserling, Pick, Jores, and Westenhöffer, which preserve the natural colours of the specimens and harden them at the same time. Although the treatment of macroscopical objects is not strictly within the scope of this book, one of the methods is here given, since tissues so treated may be examined microscopically at any subsequent date. The organs or tissues are placed first in a formol mixture, then transferred to alcohol to regain their natural colours, and finally preserved in a solution containing potassium acetate, glycerin, and water.

Kaiserling's instructions are those more usually followed :—[1]

1. The organs are fixed in the formol mixture until they are just hardened. (Several days. Careful watching is required.) The best results are obtained by fixing in the dark.

| | |
|---|---|
| Formalin | 200 c.c. |
| Water | 1000 c.c. |
| Potassium nitrate | 15 gms. |
| Potassium acetate | 30 gms. |

2. Transfer to 80 per cent alcohol (1-6 hours), and then in 95 per cent alcohol until the colour fully returns (2-24 hours).
3. Mount in jars containing :—

| | |
|---|---|
| Glycerin | 400 c.c. |
| Potassium acetate | 200 gms. |
| Water | 2000 c.c. |

Westenhöffer uses a much simpler method of the same type ; for the demonstration of uric acid in tophi or in uric acid infarcts he found it essential to combine the formic acid contained in the formol with mercuric oxide.

Puppe has exactly demonstrated the theoretical basis of all these methods. In the formol solution, oxyhæmoglobin is changed into acid hæmatin, the latter being changed in the alcohol solution to alkali hæmatin, which in colour is very similar to that of oxyhæmoglobin, so that an approximately natural colour of the organ is produced.

[1] Some workers claim to obtain sharper colour-results by substituting the following for No. 1 solution :—

Sodium sulphate, 40 gms.
Magnesium sulphate, 40 gms.
Sodium chloride, 20 gms.
} Dissolve in 2000 c.c. of hot water ; add 200 c.c. of formalin.

For further details consult Shennan, *Scot. Med. and Surg. Journal*, 1899, p. 229.

# CHAPTER V

## DECALCIFICATION

Bones and tissues containing calcareous deposits cannot be easily cut until the calcium salts are removed. The necessary decalcification is best performed between the processes of fixation and after-hardening. The process may be summarised as follows :—

1. The tissues are fixed before being placed in the decalcifying medium, or a fixative is added to the latter, since the reagents employed, being very strong acids, materially alter unfixed cells.
2. The tissues, cut into very small pieces, are surrounded with a large excess of the reagent, the latter being frequently changed.
3. The tissues remain in the decalcifying fluid until all their calcium salts are removed, but no longer. This stage is best recognised by making sections with a sharp scalpel or testing with a needle. The exact moment for transference to the hardening fluid is not easy to determine. If the pieces are not entirely decalcified, notched knives result ; if they remain too long in the fluid after decalcification is complete, it is impossible to obtain good staining. Frequent testing is therefore very important. The time required varies considerably.
4. After decalcification the tissues are washed in running water for at least 24 hours—the addition of an alkali (lithium carbonate, ammonia, or sodium carbonate) to combine with the free acid is an advantage—and then hardened in alcohol.
5. The sections must be left for a considerable time in the staining fluid, as they only stain slowly ; the remaining acid being best removed by placing the sections in an alkaline solution, for instance, saturated watery solution of lithium carbonate, prior to staining.

Amongst the numerous decalcifying fluids, the following will be found useful :—

### A. Sulphurous Acid (concentrated) (Ziegler)

Fix the tissues in formol ; transfer them to this fluid. Decalcification is generally complete within 24 hours. Wash thoroughly in (alkaline) water. Harden in alcohol. Staining is generally very good. This is probably the best and most rapid decalcifying mixture extant. It has yielded excellent results in our hands.

### B. Trichloracetic Acid (Partsch)

| | |
|---|---|
| Trichloracetic acid 5 per cent | 90 c.c. |
| Formalin (40 per cent) | 10 c.c. |

This fluid decalcifies rapidly, alters the tissues but little, and does not interfere with the subsequent staining. Considerable experience with this mixture permits our strong recommendation. The pieces of tissues must be well fixed in formol before being transferred to the fluid, and the latter should be changed frequently.

### C. Nitric Acid

This is perhaps the most widely known decalcifying medium. Many formulæ have been devised ; the following is one of the best :—

1.

| | |
|---|---|
| Nitric acid | 10 c.c. |
| (Some workers take only 5 c.c.) | |
| Formalin 10 per cent | 100 c.c. |

2. Nitric acid is also used in **Haug's solution** :—

| | |
|---|---|
| Nitric acid, pure | 3-9 c.c. |
| Absolute alcohol | 70 c.c. |
| Water | 30 c.c. |
| Sodium chloride | 0·25 gm. |

This mixture is indicated for tissues fixed in mercuric chloride.

3. Nitric acid is also used in the **Phloroglucin method** :—

| | | |
|---|---|---|
| Phloroglucin | 1 gm. | Dissolve carefully over a flame in a stink cupboard. |
| Nitric acid, pure | 10 c.c. | |
| Add distilled water | 50 c.c. | |

The phloroglucin protects the tissues from the strong acid, and decalcification is very rapid.

4. Nitric acid alcohol mixtures. **Mayer** recommends :—

| | |
|---|---|
| Nitric acid . . . | 5 c.c. |
| Alcohol 90 per cent . . | 100 c.c. |

5. **Thoma's solution** contains :—

| | |
|---|---|
| Nitric acid, pure . . . | 20 c.c. |
| Alcohol 95 per cent to 96 per cent . | 100 c.c. |

The tissues are best hardened in alcohol and treated as follows :—After decalcification in several changes of fluid, they are washed in alcohol, transferred to 96 per cent alcohol (containing an excess of calcium carbonate to combine with the free acid). The chalk should be wrapped in filter-paper or lint so that it does not damage the tissues. Change the alcohol several times. When the alcohol no longer reddens litmus paper, allow the preparations to still remain there for several days (8-15 days in all), and then transfer them to absolute alcohol.

Schaffer, from numerous comparative experiments, recommends the following procedure :—Imbed the fixed and hardened tissue in celloidin, harden the celloidin preparations in 85 per cent alcohol, then decalcify in 3-5 per cent aqueous solution of pure nitric acid in Thoma's watery iodine, 12-24 hours (large pieces require a longer time). Transfer the celloidin block to a 5 per cent solution of lithium carbonate or sodium sulphate for 12-24 hours; change the solution once or twice; wash in running water 48 hours; dehydrate in increasing strengths of alcohol (up to 85 per cent) and cut.

### D. Hydrochloric Acid

This is most advantageously used in the form of

1. **Ebner's fluid** :—

| | |
|---|---|
| Hydrochloric acid . . | 2·5-5 c.c. |
| Sodium sulphate . . | 2·5-5 gms. |
| Alcohol . . . | 500 c.c. |
| Distilled water . . . | 1000 c.c. |

The stronger solution decalcifies more quickly than the weaker one or

2. **Haug's fluid** :—

| | |
|---|---|
| Hydrochloric acid . . . | 1 c.c. |
| Sodium sulphate . . . | 0·5 gm. |
| Alcohol . . . . | 70 c.c. |
| Water . . . . | 30 c.c. |

### E. Picric Acid

A saturated watery solution is used. The picric acid fixes and hardens at the same time; previous fixation is therefore unnecessary. The picric acid must be washed out with alcohol, *not* with water, since the latter would lead to maceration of the tissues.

Decalcification proceeds very slowly. This is also the case with

### F. Müller's Fluid and with Chromic Acid

The two latter methods are thus only applicable to pieces of tissue of small size and to those which contain a small percentage of calcium salts.

# CHAPTER VI

## IMBEDDING

THE previous chapters have dealt with the fixation, hardening, decalcifying, and partial and complete dehydration of the tissues. The latter are now supposed to be in absolute alcohol and ready for transference *via* an "intermedium" (Mayer) into the imbedding material, where they become permeated with a substance which preserves their component parts and their relative positions, and ultimately surrounds them with a protective coating. They can then be cut into thin sections on a microtome. Of the various substances suggested for the imbedding of tissues, two only are in general use:—

1. **Celloidin**
2. **Paraffin**

It is very difficult to advise the selection of either of these substances, but since each possesses certain advantages over the other, it is well to become efficient in the use of both. As a rule, individual workers use one of the methods more than the other, and consequently employ the former for general purposes, reserving the latter for certain special examinations. Thus in one laboratory the celloidin method is chiefly used; in another, the paraffin method. Frequent use of one method enables the worker to get good results and to avoid many pitfalls, and he is thus tempted warmly to recommend his own procedures. The choice of a method is therefore, at most, rather subjective, and on the whole it is best to use both methods with equal

facility. Some of the "objective" differences may be here stated:—

The paraffin method is the more rapid of the two, but the celloidin process demands less attention, since a lengthy immersion in celloidin is far less damaging to the tissues than a similar stay in hot paraffin.

For paraffin an exactly regulated oven is necessary, whereas celloidin imbedding may be accomplished without such apparatus, and is thus more useful to the student or practitioner when a laboratory is not available. It is a further advantage of the celloidin method that, hot solutions being unnecessary, there is no danger of the overheating which may occur in the paraffin method; tissues enclosed in the transparent celloidin can be more easily controlled than when they are being imbedded in paraffin.

Larger pieces of tissue can be manipulated and cut by the celloidin method than by the paraffin process.

The sections obtained from celloidin are usually thicker than those from a paraffin block, but this is not altogether a disadvantage. The thicker celloidin sections appear to yield a less changed and more complete representation of the tissues than is possible with the finer paraffin sections, and the latter often give the impression of a "burnt" condition and more or less shrinking of the structures.

With the harder forms of tissue, and the tissues which easily become brittle, such as muscle and skin, celloidin imbedding is distinctly preferable, since it is more difficult to obtain good sections when these are imbedded in paraffin.

Paraffin imbedding is most useful for the preparation of very thin sections, for the recognition of the finest structures of the cells, when it is necessary securely to fasten sections to a slide in order to avoid the separation of small particles, and most of all, when serial sections are to be made.

1. **Celloidin imbedding.**—Commercial celloidin (Schering) is a purified gun-cotton. It may be purchased in granules, in shavings, or in flat slabs. The latter require to be cut into small squares before using.

*To make a solution of the celloidin.*—Place the shreds, shavings, or squares in a wide-necked vessel or bottle which can

be well closed or stoppered ; cover them with absolute alcohol[1] and stir well ; after 24 hours, when they are quite soft and slippery, add an equal quantity of ether ; again stir freely ; allow to stand for 24 hours and again stir. The solution is now ready for use, but it must be shaken frequently in order to prevent the celloidin solution becoming thicker at the bottom than at the top. Three strengths should be made, thick, thin, and medium. The boxes of celloidin or pyroxylin sold in England contain the equivalent to 1 oz. of pure pyroxylin, and the contents of each box dissolved in 100 oz. (3000 c.c.) of ether alcohol yields a 1 per cent solution, 1 oz. in 50 oz. (1500 c.c.) a 2 per cent solution, 1 oz. in 25 oz. a 3 per cent solution. It is well to use a 2 per cent solution for a thin celloidin, and a 6-10 per cent for a thick celloidin ; a 3 per cent forming a medium strength.

Celloidin becomes very hard after six months or so, and is difficult to cut into pieces for dissolving. Large and old stocks should therefore be avoided.

For imbedding purposes, take small pieces of tissue (the beginner may be tempted to take large pieces) and transfer them from absolute alcohol to a mixture of equal parts of alcohol and ether for 24 hours. Then place them for 1-3 days in the thin celloidin, and for a similar period in the thick celloidin. The object is now to be mounted in a form in which it may be cut. The simplest way is to place the tissue on a cork or a block of wood (previously treated with alcohol ether for a long time in order to remove the tannin), or on the more expensive vulcanite blocks. Cork is now rarely used. Arrange the tissue on the block in the plane in which sections are desired, surround it on all sides with thick celloidin so that it is entirely imbedded, and allow it to dry in the air until a light finger-impression does not indent the celloidin ; then place the block with the tissue in 70-85 per cent alcohol (Busse and Lee recommend the latter), until it becomes quite hard (3-24 hours). Pure chloroform (water-free) may be used for hardening instead of the alcohol.

The preparations may be more easily affixed by wrapping a piece of paper around the cork or wooden block and securing it with pins. The tissue and celloidin are placed inside

[1] The absolute alcohol must be very pure. Mann recommends *pure* methyl alcohol (Merck) ; it dissolves the celloidin more rapidly and ensures more rapid penetration of the celloidin.

the paper, and the whole then hardened in alcohol. The pins and paper may be removed later.

It is preferable to allow the ether and alcohol to evaporate more slowly than is the case with air-dried specimens. This may be accomplished by placing the celloidin block under a glass cover in which a small tube containing chloroform is present; the ether alcohol evaporates more slowly and the celloidin block is also hardened more slowly. When sufficiently hard it is transferred to 70-85 per cent alcohol.

Sometimes better results are gained by the following slower procedure:—Pour thick celloidin into a small, open, shallow, dry glass vessel and place in it the celloidinised piece of tissue with the cutting surface downward. Allow the surface of the celloidin to just harden, then loosen the jelly-like mass from the sides of the vessel, and when the whole mass has a consistency upon which the finger leaves no impression, cover it with 70-80 per cent alcohol.

Lee-Mayer recommends the repetition of the process by pouring new celloidin over the preparations. When the celloidin is well hardened, remove the imbedded object from the vessel, and trim the mass containing the imbedded tissue into a square or oblong block. This may be attached directly to the microtome through the medium of a few drops of thick celloidin, or better, fastened to a cork or a piece of wood by coating the surface with thick celloidin solution, pressing the dried surface of the celloidin block into it, allowing it to dry for a few minutes, and then hardening in 80 per cent alcohol. The block may be also affixed to the freezing microtome in the following manner:—Place the hardened celloidin in water (to remove the alcohol) until it sinks, then coat it with a thick solution of gum, place it on the microtome, and freeze.

The imbedded blocks of tissue may be preserved indefinitely in 70 per cent alcohol, or kept in the dry condition by coating them with a layer of melted paraffin (Apathy).

The blocks may be marked for identification with a "negro" pencil, which is composed of a substance insoluble in alcohol (Weigert).

For the rapid examination of small pieces of material removed during operations, curettings, etc., the following shortened method has been used by one of us, and gives

satisfactory results. It allows the production of rather good preparations within a few days. A similar method has been devised by Kaufmann (see also p. 41.)

1. Place small pieces of the tissue in 96 per cent alcohol for 24 hours.
2. Transfer to absolute alcohol for 24 hours.
3. Place in alcohol ether, 2-3 hours.
4. Medium celloidin solution, 24 hours.
5. Attach to wooden block with thick celloidin. Dry in the air for a few minutes.
6. Harden in 70 per cent alcohol, 2-3 hours. The tissue is ready for sectioning.

Photoxylin may in all cases be used in place of the celloidin, the same precautions being observed.

2. **Paraffin imbedding.**—In celloidin imbedding the tissue is treated with a medium which becomes hard after the evaporation of its solvent; in paraffin imbedding heat is necessary to ensure the penetration of a substance which is hard when cold. To melt the paraffin an oven is therefore necessary, and this oven must be kept at a temperature which does not vary more than one degree Celsius. A slight draught or alteration in the pressure of gas may overheat the paraffin, and the tissue is materially damaged, or may underheat it, and the tissue is surrounded by unmelted, non-penetrating paraffin. An oven whose temperature is accurately regulated is thus an absolute necessity, and this constitutes a serious drawback to paraffin imbedding by those distant from a laboratory. Amongst the best forms of apparatus may be mentioned those of Hearson (London) and Lautenschläger (Berlin). Formerly the so-called Naples model was widely used. When ordering, it is necessary to state the range of temperatures desired. These vary with the melting points of the paraffins employed; as a rule, those melting at 51° C. are used, but during a hot summer harder paraffins of about 54° C. are necessary; intermediate grades are obtained by mixing paraffins with high and low melting points. The regulator should register one or two degrees above the melting point of the paraffin.

The tissue should be cut into small pieces, and be more thoroughly dehydrated than is necessary for celloidin imbedding. Transfer the piece of hardened tissue from

absolute alcohol to xylol: if a white opacity ensues, dehydration is incomplete, and it must be returned to absolute alcohol. Leave the piece of tissue in xylol until it is quite transparent (2-3 hours, examine with dark background). Put them into xylol containing as much paraffin as will dissolve on slight warming, and allow them to stand on the top of the oven or incubator for 2 hours. Then place them in a small tin, copper, or porcelain vessel full of pure paraffin freed from air or water by previous heating,[1] and allow them to remain in the oven for 1-2 hours. Then transfer them to a second vessel containing pure melted paraffin and leave them for a second 1-2 hours. It may be necessary to give them another paraffin bath of 1-2 hours.

Now the objects are ready for preservation, etc., in paraffin blocks, and these are made by placing the tissue in melted paraffin and allowing it to cool rapidly. For such purposes smear a small shallow patty-pan or deep watch-glass with fat or glycerin, in order to facilitate the later removal of the cooled paraffin. Small paper boxes, shaped somewhat like the cardboard boxes which contain cover-glasses, are sometimes used, or small or large iron chambers may be employed, the best type of the latter being that devised by Gaylord and made by Winkel of Göttingen. It consists of six or eight chambers in one frame, so that many pieces may be imbedded at the same time. These iron frames or chambers are then placed on a piece of flat glass. Into the patty-pans, watch-glasses, paper boxes or frames, melted paraffin is now poured; with a pair of forceps slightly warmed in the flame of a Bunsen burner the objects are removed from their paraffin bath and placed in the pans or frames with their cutting surface downwards. When a fine film appears on the surface of the paraffin, the objects being well covered with the fluid medium, plunge the vessel and paraffin suddenly into cold water and there leave it until the paraffin is thoroughly set. In order to hasten the appearance of the film, the procedure may be varied by holding the lower part of the vessel in water for a few moments. In a short time the cooled mass will rise to the surface of the water, or it may be easily freed from the tin. Then remove the surplus paraffin and trim the portion containing the tissue into square or oblong shape, making the sides perfectly parallel. The paraffin block is now ready for attachment to the microtome or to a wooden block by means of melted paraffin, in a manner similar to the preparation of the celloidin block.

[1] It is well to decant or to filter the paraffin before using, as it often contains adventitious particles. It filters quite easily through ordinary filter-paper. A "warm" funnel should be employed, or the procedure be conducted in the oven.

Instead of xylol, chloroform may be used; it permits a better infiltration of the paraffin, so that the objects need not stay so long in the hot paraffin, and it evaporates readily from the paraffin, so that the latter purifies itself. It is well, however, to change the tissue into a second bath of paraffin before finally imbedding.

Benzene also is a good clearing medium, especially for osmic acid preparations; xylol dissolves fatty substances.

Lee recommends cedar-wood oil for the same purpose; with this medium several changes of paraffin are necessary to get rid of the oil.[1]

Carbon bisulphide (recommended by Heidenhain) also is an excellent clearing medium; it dissolves more paraffin than xylol, and ensures better penetration. One of the writers employs this almost entirely for finer structures: its only objections are its smell and inflammability. The latter requires but little care, and the former only occurs when the liquid is agitated. Carbon bisulphide mixes well with alcohol, and it is best first to place the tissue in alcohol and carbon bisulphide (equal parts), then in carbon bisulphide, then in carbon bisulphide containing as much paraffin as it will dissolve, and finally in pure paraffin. With carbon bisulphide mixtures well-stoppered bottles must be used. The heat of the paraffin oven is quite sufficient to drive off the $CS_2$ from the tissues.

For a rapid examination of small objects the process of paraffin imbedding may be shortened so as seriously to compete with the freezing method, and even to constitute an improvement upon the latter when dealing with small pieces of tissue not particularly appropriate for the freezing method. It is a pity that such a shortened procedure is handicapped by the necessity for a paraffin oven.

**Lubarsch** first worked out this short paraffin method, and his modified and very practical procedure, to which he and his pupil Stein have recently again drawn attention, is as follows:—

[1] The oil may be removed from the paraffin, and the latter used again for imbedding, by putting a pledget of cotton-wool in the oily melted paraffin. The wool absorbs the oil almost entirely (Mair).

**Rapid paraffin imbedding method.**

1. Pieces up to ½ cm. in thickness are placed in a wide test-tube containing 10 per cent formalin for 10-15 minutes, the fluid being twice changed during such time.
2. Transfer to 90-95 per cent alcohol (change the fluid once) 5-10 minutes.
3. Absolute alcohol (change twice), 10 minutes.
4. Absolutely pure aniline oil until the pieces are completely transparent (10-30 minutes).
5. Xylol (change twice to three times, until the xylol is no longer tinged yellow), 10-20 minutes.
6. Imbed in paraffin, 10 minutes to 1 hour.

During all these stages the tissue is placed in the paraffin oven at 50° C., so that all the media act at this temperature. Hardening, imbedding, cutting, and staining may thus be completed in from 2-3 hours.

This method, as Lubarsch points out, is applicable for the rapid diagnosis of proof excisions and operative material, and then yields exceedingly good results, and it may be equally well employed for post-mortem tissues.

More recently **Henke** and **Zeller** recommend another rapid method for the preparation of sections.

They harden in acetone, which fixes the tissues like alcohol and at the same time prepares it for immersion in paraffin. The procedures are thus much simplified :—

(1) Place small pieces of tissue in pure acetone for ½-1½ hours. Larger pieces require a longer time. The acetone may be again used by placing fused copper sulphate at the bottom of the vessel.

(2) Transfer the tissues directly to fluid paraffin (52-56° C.) and place in the oven for ½-1½ hours. The acetone evaporates, and the paraffin permeates the tissue.

(3) Prepare the paraffin block ; cut ; stain as usual.

One of the writers finds it an advantage to fix the tissue in 10 per cent formalin for ½-4 hours prior to placing in acetone. Shrinking is thus almost entirely avoided.

Campbell recommends **pyridin** for formol-hardened tissues. Like acetone, it dehydrates, clarifies, and prepares for paraffin immersion. Fresh tissues may be placed directly into it. Time, about 48 hours. The staining properties of the tissues are unaltered.

A brief mention may be made of the soap-gelatin, glycerin-gelatin, and the celloidin-paraffin methods. With the latter, the tissue is first imbedded in celloidin, then transferred to origanum oil, then to xylol and to paraffin, or *vice versâ* ; or, according to Field and Martin, a solution containing both paraffin and celloidin is used for imbedding.

**Wright's formol-gelatin imbedding method**[1] for small fragments of tissue or curettings has yielded good results in our hands. The small fragments are placed close together in a watch-glass containing a 20 per cent warm solution of pure gelatin in water; the gelatin is allowed to solidify, and the block of gelatin is then placed in 4 per cent aqueous formalin for 24 hours. The formol acts upon the gelatin, renders it insoluble in water, and the imbedded tissue can then be placed on the microtome, and cut after freezing. The gelatin solidifies more rapidly when the watch-glass is placed in a refrigerator or in ice-cold water, and the action of the formaldehyde may be hastened by placing the gelatin mass in an oven at 50° C. after the formalin has already acted for an hour. Staining results are good.

Recently Bolton and Harris have advised a simultaneous hardening and imbedding method with formol agar. The reader is referred to the original paper for details. It has not yet been sufficiently used to permit a conclusion as to its practical utility.

**Rapid celloidin imbedding method.**—Scholz (*Deutsche Med. Woch.* 1905, p. 419).

(1) Place very small pieces of fresh tissue (or after formol or alcohol fixation) in acetone for ½-1 hour in an oven at 37° C.
(2) Transfer directly to thin celloidin (or first place in alcohol-ether for 15 minutes).
(3) Leave the objects in celloidin for 4-5 hours at 37-40° C.
(4) Add thick celloidin: allow to remain for 2-3 hours.
(5) Allow celloidin to dry slowly by chloroform method (p. 36). After about 12 hours transfer to alcohol for some hours, and then cut.

[1] This method we have also used for the preservation of thin slices of organs such as the liver, lung, kidney, or the several parts of the brain and spinal cord.

# CHAPTER VII

## SECTIONING

For this purpose a microtome is required, and the greater number of those available may be used for either celloidin or paraffin. The "slide" microtomes, which have replaced the old cylinder type, consist chiefly of a clamp or plate, movable in all planes, to which the object is affixed, and a sliding rod or bar which carries the knife. The clamp or plate is raised, lowered, or inclined by exact mechanical movements; the knife is directly moved by the hand, or the manual movements are transmitted to the knife by a crank or beam.

In Minot's and the Cambridge rocking microtomes the knife is fixed, and the object is moved to and from the cutting surface; in the majority of the other microtomes the object is fixed, and the knife is moved. The best microtomes are made by the Cambridge Scientific Co.; Schanze, Leipzig; Jung, Heidelberg; Becker, Göttingen. Those who have worked with the Cambridge rocking microtome value it highly; it is certainly very useful for serial sections. We find that made by Schanze (upon Weigert's designs) to be specially advantageous.

For ordinary work the smaller and simpler microtomes are useful; they cut more rapidly, and require less attention than the larger and more complicated ones.

Microtomes devised for special purposes are those of Cathcart, Delépine, Gudden (for large brain sections), and Minot (for serial sections).

The knife must be always carefully sharpened before using, and the cutting edge must not be touched by either needle, brush, or towel during the manipulations.

The best knives are made by Rogers of Sheffield and Walb of Heidelberg. A very useful razor for the Cambridge and similar microtomes is retailed by Hilliard, of Nicolson Street, Edinburgh.

**Paraffin sections.**—Before placing the wooden block on the holder of the microtome, trim the paraffin carefully so that its sides are parallel; leave only a small rim of paraffin around the tissue, or the sides of the section may be crinkled. If a wooden block is not used, as in Minot's microtome, fix the paraffin block to the holder by heating the latter in the flame until it will just melt the paraffin when the block is pressed against it. Put the holder and object in cold water for a few seconds.

Place the knife transversely and pull it through the block. Commence by cutting thick sections and gradually pass on to thin ones. If the cut surface of the block is ridged ever so slightly, or the under or inner edge of the knife scratches the paraffin on its return journey, choose another part of the knife, or re-strop.

Smooth the section on the knife with a camel's-hair brush while cutting.

Should the sections even then curl, re-sharpen the knife; if this does not avail, slightly warm (with a metal spatula) the upper and lower edges of the block, or cover the block with melted paraffin, and then remove the latter from these two sides only.

Transfer the sections directly to a slide with a brush, needle, or a pair of forceps, or float them on water at 45° C. or on 70 per cent alcohol. (See "Mounting.")

**Celloidin sections.**—These are generally cut in alcohol. Moisten the surface of the celloidin block with alcohol—a camel's-hair brush may be used, or a wash-bottle as introduced by Weigert.

Place the knife as much as possible in the longitudinal axis of the microtome, so that the knife and object are at a very slight angle. The knife must, however, cut the entire surface of the block. Transfer the section to 70 per cent alcohol with a brush or needle, or with a finger: the latter saves the knife edge the most. When the section curls,

at once smooth it out on the knife; when smooth, complete the sectioning.

From paraffin blocks, sections 5-7$\mu$ in thickness may be obtained; from celloidin blocks, sections 10-15$\mu$ in thickness are generally cut. For some purposes thick sections are preferable, *e.g.* for the examination of fat.

**Serial sections.**—These consist of a long row of single sections in unbroken succession. For their safe transfer to slides where they can be further treated numerous methods have been devised.

Paraffin imbedding yields the best serial sections. The slides must be thoroughly clean. They should be first washed with soap and brush, then in alcohol, and finally placed in alcohol and ether to remove any traces of fat. By the observance of such precautions the preparations keep better.

For the mounting of **paraffin serial sections,** the following methods give good results:—

1. **Capillary attraction method.**—Place the sections in water at 40-45° C. (viz. at a temperature just under the melting point of the paraffin). The partially rolled or crinkled sections at once straighten out. Float the sections on the slide by means of a spatula, or partly immerse a slide (previously dipped in alcohol and then in hot water) in the water just under the sections. Arrange the latter in order, withdraw the slide, and drain off the water. Or warm water may be placed on the slide and the sections placed directly on to it from the knife, and the water drained off. In both cases the glossy under-surface is to be attached to the slide. Put the slide in an incubator at 37-40° C. for 12 hours. The sections should be thoroughly adherent. If they contain small bubbles or are of a greyish-white colour, they should be heated over the flame until the paraffin just melts and then cooled before being further treated.

   Some workers smooth out the sections and remove the last traces of water by covering the slide and sections with a strip of paper moistened with alcohol and passing the thumb over it three or four times in one direction, gently pressing meanwhile. The paper is then raised slowly from one side and removed, the preparations being finally dried on the top of the paraffin oven (Shattock).

2. **The albumin-glycerin fixation** (Mayer).—Beat up the white of an egg, filter, and add an equal quantity of

glycerin and a crystal of carbolic acid or thymol. (Mayer adds 1 gm. of sodium salicylate to each 100 gm. of the mixture.)

Place a drop of this mixture on a slide, remove the surplus with the back of a finger, and then rub the remainder with the ball of the thumb so that only an invisible film is left. On this arrange the dry paraffin sections with the glossy side downwards, and flatten gently with a brush. Put the slides in an incubator for several hours: the albumin is coagulated and the sections are quite fast to the slide. Although less advisable, for rapid work the coagulation may be induced by passing the slides quickly through the flame several times.

The drawback to the method is, that the layer of albumin sometimes stains in the subsequent processes. The albumin-glycerin film should therefore be thinner than the beginner dares to make it.

3. **Combinations of the "capillary attraction" and "albumin fixation" methods.**—Of these the following will be found specially useful :—

Cover the cleaned glass slide with a thin film of the albumin-glycerin mixture ; coagulate the albumin by heat, and then place on it sections previously floated on warm water and incubate for 12 hours.

Personal experiences indicate the second method for thick, well-spread sections, or when a few wrinkles are harmless. For general purposes the third takes up a little more time, but gives excellent results. With the first method the sections often become loose during the further manipulations, especially when the tissues have been hardened in Müller's fluid or chromic acid mixtures.

While these procedures amply suffice for both single and serial paraffin sections, and for serial sections are most satisfactory, the following methods, amongst others, are specially devised for serial sections :—

**Strasser** prepares paper-gum-collodion plates. Strips of paper are first covered with a solution of gum arabic containing $\frac{1}{5}$ vol. glycerin, and then with collodion solution (about the consistence of glycerin) to which $\frac{1}{100}$ vol. of castor oil has been added. Sections are mounted on the plates in the following solution :—

| | |
|---|---|
| Celloidin . . . | 2 vols. |
| Ether . . . . | 2 vols. |
| Castor oil . . . | 3 vols. |

The plates with the sections are then put into benzene or turpentine for ½ hour, then into chloroform for 15 minutes, and finally into 95 per cent alcohol for several minutes. The collodion and the sections are now freed from the paper by dissolving the layer of gum in water, and are then ready for further treatment.

Strasser has more recently modified this proceeding.

**Schmorl** covers slides with an even layer of a solution containing

| | | |
|---|---|---|
| Cane sugar solution (1 : 1) | . | 300 c.c. |
| Alcohol (80 per cent) | . . | 200 c.c. |
| Yellow dextrin solution (1 : 1) | . | 100 c.c. |

Paraffin sections are carefully arranged on the wet layer, and the slide slightly heated to smooth out the sections; the slides with the sections are next placed in an incubator at 37° C. (3-4 hours), to harden and to allow the alcohol to evaporate; they are then transferred to xylol, chloroform, or turpentine for 10-15 minutes, afterwards being treated with absolute alcohol for 10-15 minutes; the alcohol is drained off, and the sections are then covered with a thin layer of celloidin (celloidin or photoxylin, 10; absolute alcohol, 100; ether, 100). When the layer becomes hard (1-2 minutes), detach the celloidin from the slide on three sides and place the whole in lukewarm water: the celloidin film with the sections affixed is only attached to the slide along one edge. The sections are then further treated as celloidin sections, avoiding the use of absolute alcohol (*vide* Chapter VIII.).

With Minot's or the Cambridge rocking microtome, long ribbons may be cut and fixed to the slide with the albumin-glycerin mixture.

**Celloidin serial section.**—With small sections and a little care, successful results may be obtained by the following simple method proposed by **Mallory** and **Wright**:—

When cutting the celloidin block, moisten it with 95 per cent alcohol, and as the sections are cut, arrange them in order on the knife, and draw them on to a clean, dry, and numbered slide. Pour over them ether vapour from a half-full bottle of ether, passing slowly down the edges of the celloidin in order to fasten down the frills. Transfer the slides to 80 per cent alcohol in order to harden the celloidin; preserve them in the same medium until required.

Celloidin serial sections are, however, generally prepared according to **Weigert's** method, which permits better and more exact results.

The slide is covered with a thin layer of collodion or celloidin. Closet paper, about twice as broad as the sections, is taken, and to this the sections are transferred from the knife edge in the order in which they are cut. It is well to place the first section close to the left hand of the operator, each successive section occupying a position nearer to the right hand. The piece of closet paper must be kept moist by being placed with the specimens uppermost on blotting-paper kept wet with 70 per cent alcohol and lying in a shallow dish; or the strip is placed on the knife itself (at the end which does not touch the celloidin block), and the alcohol on the knife holds it securely as well as penetrates the paper and keeps it moist. It is then always close at hand.

The strip with the sections is now laid upon the celloidinised slide; the sections, being underneath, stick to the slide, and the paper is carefully peeled off.

The preparation is now dried with filter-paper, a layer of thin celloidin is poured over it, and then the slide and sections, etc., are placed in 80 per cent alcohol to harden and be further treated; or, when the slides are placed in water or in a watery staining solution the celloidin peels off, taking the specimens with it, and they can be dealt with later as desired, following, of course, the usual procedure for the mounting of celloidin sections (see Chapter VIII.).

**Dimmer** has modified the preceding method, so that the sections are only coated with celloidin on one side, and staining is thus facilitated. Slides are covered with a gelatin solution (made by dissolving 16 gms. of gelatin in 300 c.c. of warm water), and are then dried. The sections are placed on the gelatinised slide and covered with thin collodion or phloroglucin (after Weigert); they are then transferred to water at 50° C., when the gelatin dissolves and the collodion film, which covers the sections on one side only, is liberated.

**Obregia** produces a similar effect by coating the slides with a layer of

| | |
|---|---|
| Cane sugar (sat. watery solution) . . . | 30 c.c. |
| Alcohol (95 per cent) . . . . | 20 c.c. |
| Dextrin syrup . . . . . | 20 c.c. |

and covering the affixed sections with a solution of photoxylin (6 gms. in equal parts of absolute alcohol and ether 100 c.c.). Obregia employs the same method for paraffin sections.

**Jordan** fixes celloidin sections with the albumin-glycerin fixative, and Lee advises a similar procedure.

**Fr. Müller** recommends a modification of Auburtin's method. He covers the slide with a thin film of albumin glycerin as for paraffin sections, and heats it in the flame until the steam rises. The sections are then arranged in order on the slide with 95 per cent alcohol, stretched and gently pressed down with blotting-paper. When the alcohol has evaporated and the sections begin to turn white, a few drops of alcohol ether are allowed to drop from a pipette at the edges of the sections; after the alcohol ether has evaporated, 10-15 minutes, transfer the slide with its sections first into 70 per cent alcohol and then into water. The sections are now quite fast to the slide. Phenol xylol is used for clearing instead of xylol (Chapter VIII.).

v. Tellyesniczky, instead of albumin glycerin, recommends diluted egg-white solution. He shakes up the white of an egg in 100 c.c. of distilled water and filters, claiming with this liquid to make a much thinner film than is possible with the albumin-glycerin mixture.

Large glass plates are preferable to ordinary slides for serial sections. They should be about as broad as three ordinary slides and slightly longer. Mica covers are very suitable for the large plates.

Care should be taken to mark the successive celloidin films with a brush dipped in watery methylene blue directly they are completed. It is difficult accurately to mark them at a later stage. It is also well to become accustomed to a certain order of succession in arranging the sections on the slides.

When serial sections are desired from celloidin blocks which have been stained in bulk, **Langhans'** method is advised.

During the actual cutting of the sections, wet the knife with origanum oil, and place the sections on slides covered with a thin layer of the same oil. The preparations at first appear milky, owing to the mixing of oil and 70 per cent alcohol, but soon become clear. After blotting, they are mounted in Canada balsam.

To avoid the milky clouds **Schmorl** employs a mixture of 1 part of absolute alcohol and 3 parts of origanum oil during the cutting process.

# CHAPTER VIII

## FURTHER TREATMENT OF SECTIONS—CLEARING, MOUNTING

THE sections, whether from imbedded or unimbedded tissues, must still be further treated. Except when sections are to remain unstained, the general purpose of the further proceedings is to make some structures more prominent than others by allowing them to combine with one or more dyes. The following chapters will treat of these processes.

The stained or unstained specimens are now to be dehydrated in alcohol, cleared in xylol or in certain oils, and then mounted in Canada balsam, the procedures differing slightly for celloidin and paraffin sections respectively.

In some cases alcohol and xylol cannot be employed, as for instance when tissues are stained for amyloid (or lardacein) and when particular stains are used to demonstrate fat; the sections are then examined and preserved in glycerin or lævulose, and made permanent by edging the junction of the cover-glass and slide with gold size, wax, or paraffin by means of a brush or the stump end of a match. When dry, zinc white or shellac may be superimposed.

Glycerin gelatin,[1] however, forms a better mounting medium.

Place the section on the slide; dry it between folds of blotting-paper; pour on a drop of warm (fluid) glycerin gelatin [1] and cover. The drop spreads evenly between the slide and cover-glass and solidifies rapidly while cooling.

[1] **Glycerin gelatin.**—A useful formula is the following:—
Water, 200 c.c.
Gelatin, 20 gms.
Powdered white shellac, 2 gms.
Farrant's solution, 25 c.c.
Dissolve. Filter while warm.

**Paraffin sections.**—Three methods for further treatment may be followed, but they are all based on the same principle.

1. Single and serial sections are mounted after the method described in the previous chapter, and are treated in the following manner:—

> When the sections are arranged on the slides (either singly or in series) they are placed in the incubator (see Chapter VII.), and when all the water has evaporated the slides and sections are placed in xylol for 10-15 minutes to remove the paraffin, then transferred to absolute alcohol for 10 minutes to remove the xylol, and then put into 80 per cent alcohol for a short time. Staining, decolourising, etc., are now proceeded with, and at the end of these stages the preparations are either in water or alcohol. In order to obtain complete dehydration they are placed in absolute alcohol and then cleared in xylol; when all traces of water have been removed no white sediment appears on transference of the slide to xylol. The process of clearing is best observed by placing the preparation on a dark background; when quite transparent the section is carefully dried between several folds of blotting- or filter-paper, a drop of thin xylol balsam (Canada balsam dissolved in xylol) is placed upon it and a cover-glass affixed. The balsam should only just fill up the space between the cover-glass and the slide. No direct pressure with needles, etc., is permissible, but to obtain an even spreading of the balsam, and to remove air bubbles, small lead weights about the same size as the cover-glass may be left on the preparation for several days. Small cartridge cases filled with lead are useful for this purpose.
>
> If the Canada balsam is too thick it spreads unevenly. It may be thinned by the addition of a few drops of xylol; but it must not be too thin, or the cover-glass will float on the liquid, drying will be very slow, and excessive shrinkage may lead to denudation of the specimen and the entrance of air.

2. The single unaffixed paraffin sections are stained without first removing the paraffin, being transferred from the knife or razor directly into 80 per cent alcohol, then stained, dehydrated in absolute alcohol, cleared in xylol (the paraffin is now removed), and mounted in Canada balsam. The sections may also be placed directly into staining fluids at 40° C. when first cut: they smooth themselves

out in the heat and are afterwards dehydrated and cleared.

Two points deserve special remark :—(*a*) Staining before the paraffin is removed requires much longer time ; (*b*) xylol dissolves paraffin, therefore the section which otherwise might break up into small particles must be treated with xylol on the slide. Transference from the alcohol to the slide may be performed by means of a lifter or spatula, or better, by dipping the slide in the alcohol, drawing the section into position with a needle or brush, and then draining the alcohol off.

Remove the superfluous alcohol, add xylol and allow it to remain until the section is transparent, then dry with filter-paper, in order to make the section quite flat, and mount in Canada balsam. Do not blot after absolute alcohol ; the sections then easily stick to the filter-paper.

When alcohol must be avoided for the sake of special staining, the section may be mounted from water after the staining and dried in the incubator at 37° C., or drawn rapidly through the flame several times, then cleared in xylol and mounted in balsam.

This method is chiefly used for the staining of bacteria in tissues and for other delicate structures, and is not applicable to all staining processes.

3. With thicker sections and those prepared from dense organs, such as the liver or kidney, the paraffin may be removed by placing them directly into xylol. The preparations may then be transferred to absolute alcohol, to 80 per cent alcohol, and to the staining solutions, washed and then dehydrated in absolute alcohol, cleared in xylol, and therefrom drawn on to a slide and mounted in Canada balsam.

**Celloidin sections.**—The further treatment of celloidin sections is more simple than that of paraffin sections, since it is unnecessary to remove the celloidin, which but rarely affects the processes of staining and is seldom stained itself. Still, some precautions must be taken, if good preparations are to be obtained. Absolute alcohol must be avoided, since it may dissolve, or at least soften, the celloidin. Thus, as only completely dehydrated sections can be passed through xylol, another clearing reagent must be substituted for xylol. Hence other substances, such as bergamot, cedar and origanum oils (not clove oil, which dissolves celloidin), were formerly used for celloidin sections.

The best, simplest, and most thoroughly worked out method is that of Weigert.

The stained section is placed in 96 per cent alcohol and then transferred to **phenol xylol** (xylol, 3 parts; melted cryst. carbolic acid, 1 part).[1] On account of the ingredients of this mixture the sections need not be entirely dehydrated; 96 per cent alcohol is sufficient for the necessary treatment, and the use of absolute alcohol is thus avoided. During the stay in the phenol xylol the sections lose their last traces of water, and they can be transferred from the phenol xylol to the slide. It is well now to place a drop of xylol on the section to see if it is quite transparent; when so, it should be dried and mounted in Canada balsam.

Celloidin serial sections and celloidinised paraffin sections are, of course, all similarly treated, in order to avoid the use of absolute alcohol.

Phenol xylol cannot be used for sections stained with aniline dyes, since the carbolic acid removes such stains. Weigert advised the following treatment in these cases:—

After 96 per cent alcohol, place the section on the slide, add a drop of xylol, dry between folds of blotting-paper, again add xylol and dry, and repeat the procedure until the section is quite transparent. Then mount in balsam.

When it is necessary to stain the section on the slide, press the section on the slide with blotting-paper and proceed as usual (see fibrin method). It is sometimes desirable to remove the celloidin from the section, particularly when it also has become stained. This may be performed by passing the sections from 96 per cent to absolute alcohol, then transferring them to alcohol ether for 10 minutes, afterwards returning them to absolute alcohol, clearing in xylol and mounting in balsam.

Sections should not remain long in xylol; they become hard after a time. Instead of xylol Canada balsam, Weigert used in his neuroglia and in some other staining methods Lee-Mayer's already published colophonium, dissolved in turpentine and shellac; the colours keep better and longer than they do in xylol balsam.

**Frozen sections** and those obtained by the use of razors or double knives may be treated by the methods followed for either paraffin or celloidin sections. No imbedding medium being present, no dependent precautions are necessary. One of the writers, however, prefers to treat

[1] When preparing this medium, pour the xylol in the measuring glass first, then add the melted carbolic acid, otherwise the carbolic acid easily crystallises out. The vessel should be previously well warmed.

such sections with 96 per cent alcohol and phenol xylol (like celloidin sections), since frozen and fresh sections shrink considerably when dehydrated in absolute alcohol.

A short tabular account of the procedures already given may now be appended.

### Summary of the Stages in Celloidin Imbedding and Mounting

1. Fix the tissue in formol, Müller-formol, Zenker's fluid, etc.
2. Wash in water 24 hours, when necessary.
3. Harden and dehydrate in 70 per cent alcohol, 24 hours.
4. Continue hardening and dehydration in 90-96 per cent alcohol, 24 hours.
5. Finally complete in absolute alcohol, 1 to several days.
6. Alcohol-ether, 24 hours.
7. Thin celloidin solution, 1 to several days.
8. Thick celloidin solution, 1 to several days.
9. Make the celloidin block.
10. Harden the celloidin block in 85 per cent alcohol.
11. Cut; preserve the sections in 70 per cent alcohol.
12. Stain—remove surplus stain, etc.
13. Dehydrate in 96 per cent alcohol.
14. Clear and finally dehydrate in phenol xylol.
15. Place on slide.
16. Clear in xylol.
17. Mount in Canada balsam.

### Summary of the Stages in Paraffin Imbedding and Mounting

1-5. Proceed as in the celloidin method.
6. Clear in xylol (chloroform, cedar-wood oil, or carbon bisulphide at 37° C.), until the tissue becomes transparent (2-5 hours).
7. Xylol (chloroform or carbon bisulphide) paraffin on the oven (2 hours).
8. First paraffin bath in incubator or oven, 1-2 hours.
9. Second paraffin bath in incubator or oven, 1-2 hours.
10. Imbed in paraffin.
11. Suddenly immerse in cold water.
12. Cut; mount sections on slide.
13. Remove paraffin in xylol (10 minutes).
14. Remove xylol in absolute alcohol (10 minutes).
15. 70-80 per cent alcohol, several minutes.
16. Stain—remove surplus stain, etc.
17. Dehydrate in absolute alcohol.
18. Clear in xylol.
19. Mount in Canada balsam.

## Mounting for Comparison of Normal with Abnormal Tissues beneath the Microscope

Shattock recommends the method of superposition and the method of composite blocks :—

**Superposition.**—Sections (about 2 $\mu$) are prepared from paraffin blocks : one is mounted on the slide, another on the cover-glass. Both are dehydrated, cleared and mounted with xylol balsam in such a way that the section on the lower side of the cover-glass is directly superposed upon the section on the slide. There is a distinct microscopic interval between them, owing to the intervention of a thin layer of the mounting medium, and both sections can be readily studied even beneath a $\frac{1}{12}$ oil-immersion lens.

**Composite blocks.**—The result obtained by mounting two different sections side by side, cut from separate blocks, though each block has been trimmed so as to present one free straight edge uncovered by paraffin, is unsatisfactory, since it is practically impossible to adjust the two sections so that their edges are microscopically in precise apposition.

Two blocks having been prepared in the usual manner, a perfectly flat face is obtained by cutting on a microtome which cuts in an exact vertical plane. The block is then pared by hand at right angles to the face until the tissue is exposed. Two blocks similarly prepared are lightly clamped together and warmed until the exterior of the paraffin softens. The composite block is then placed (with the cutting surface downwards) in a mould into which paraffin is poured. When set, sections are prepared and stained.

# CHAPTER IX

## STAINS AND STAINING

THE advantages gained by the staining of tissues are the easier recognition of the constituent elements and the demonstration of structures otherwise almost invisible. In addition, the dyes sometimes satisfy the affinities of certain simple or compound substances present in the tissues, and thus yield pure micro-chemical reactions. Hence the endeavours now being made to discover staining reactions which will indicate the presence and distribution of such complex organic substances as nuclein, protamines, carbohydrates, etc. Micro-chemistry must not be viewed as alone applicable to metallic elements such as iron, mercury, or phosphorus.

It is only within the latter half of the last century that dyes have been used for histological purposes, although thousands of years ago primitive races employed stains in the preparation of coloured fabrics. In 1849 carmine was first applied in microscopical work, and about 1860 the advent of the synthetical coal-tar colours—aniline dyes—gave a great impulse to the staining of animal tissues. First introduced into histological technics by Weigert, their use has been widely extended by the practical methods of Weigert and Ehrlich, while more recently the theoretical aspects of colour-chemistry has had its pioneer in the latter of these two great masters.

Concerning the precise processes which occur during the staining of tissue elements nothing definite is yet known. Formerly two theories held the field. One supposed that a distinct chemical change occurred—a dye,

insoluble in water, being formed from the combination of tissue and stain; the other, that the staining was due to physical forces alone, and consequently "mechanical" in character.[1]

Of late the "solution" theory advanced by Witt in 1890 has gained many supporters. He takes an intermediate position: the process is neither purely physical nor purely chemical. Dyes may dissolve in solid tissues just as they do in liquid media; just as it is possible to shake out dyes from watery substances by means of amyl alcohol or ether, so the tissues may take up the dye from its watery solution. The stained fibre is a "starre lösung" of the dye in the substance of the fibre. The chemical reaction does not alone follow the molecular weights of the substances, but is also dependent on the physical conditions which prevail, as is the case with solutions in general.

Whatever theory or theories ultimately explain the interaction of stains and tissue elements, it is undeniable that the tissues possess certain affinities for dyes and especially for aniline dyes, although both may interact with varying intensities and under dissimilar conditions.

In textile dyeing as well as in histology, the various stains have been classified in two main groups, the "substantive" or "direct," and the "adjective" or "indirect." The former (through physical, chemical, or physico-chemical processes) permits a direct interchange between the fibre and the dye; the latter requires the interaction of a third substance before the fibres are stained. Such a substance is known as a *mordant*, and the resultant colour compound (mordant + dye) is termed a *lake*. The mordant, to be useful in histological work, must however form a "lake" which will ensure a "fast" colour combination with the tissues. The mordant may be added to the adjective or indirect stain, or the tissue may be treated with it prior to the use of the stain.

[1] A detailed account and consideration of the theoretical aspects of staining is here purposely omitted. The worker is referred to the admirable and comprehensive treatise on *Physiological Histology* by Gustav Mann (Oxford, 1902), which is widely recognised as the standard English publication on this subject, and to Pappenheim's *Grundriss der Farbchemie*.

In England, the term "lake" is often restricted to the union of basic mordants and colour acids; in Germany it is extended to include compounds formed between acid mordants and colour bases.

Some of the "fixing" fluids already mentioned act as mordants. Heidenhain attributes this in part to the fact that the more generally used mordants are albumin precipitants, and that such precipitation is essential for fixation.

Chromic acid and chromate fixing fluids act as mordants. Certain metals make useful mordants, such as iron for hæmatoxylin; certain aniline dyes also serve the same purpose. Broadly speaking, an acid mordant is selected to produce a fast combination with a basic dye, and a basic mordant with an acid dye.

In histological work, dyes are employed for diffuse or differential staining; the latter frequently represents a somewhat loose example of micro-chemical reaction, and the stain is then termed "specific." Only a few dyes are "elective" for single constituents of the tissues, and so permit precise micro-chemical reactions; the majority stain more than one of the tissue elements, but may exhibit special affinities for certain of these.

Two methods, based upon these characteristics, are available for the "elective" or "specific" staining of tissues:—

(1) The *progressive* method—in which the dye is allowed to act until the affinities of certain constituents have been satisfied, and the action is then interrupted;

(2) The *regressive* method—in which the tissues are over-stained, and then the dye is washed out from all structures except those for which it possesses the strongest affinities. This procedure is termed "differentiation." As a general inference, substances which absorb the stain slowly, retain it strongly, a point of importance in connection with the staining of the tubercle bacillus. The chief differentiating media for aniline dyes are alcohol, acid alcohol (both being in general use for basic

stains), acid stains, aniline oil, and a mixture of aniline oil and xylol. The regressive methods give less constant pictures than the progressive methods, since the results depend largely upon the thickness of the sections, the concentration of the differentiating fluid and its capacity and duration of penetration, as well as the "personal equation" of the worker. These conditions are increased when the complicated procedures of certain methods render it difficult to determine the time when differentiation should be interrupted. Still, it is a distinct advantage to be able to modify the depth of the stain at the wish of the stainer, particularly when such modification can be easily controlled by repeated microscopic examinations.

When two or more structures are to be demonstrated by different dyes, as, for instance, nuclei and plasma, the stainings may be simultaneously carried out by the use of "mixtures." As a rule, it is better to employ two separate stains, using one after the other. A combination of both methods is exemplified in van Gieson's stain (*q.v.*), in which a mixture containing two colours is used after a single dye.

Simultaneous staining with mixtures is chiefly used in the examination of blood preparations, and those employed consist of certain combinations of acid and basic dyes, which have resulted from Ehrlich's classical researches. The varying affinities of different cells and cell structures to dyes of different constitutions have permitted certain important chemical conclusions. A mixture of acid and basic dyes may result in the formation of neutral stains, for which some cell constituents evince special affinities (*vide* Blood).

If, when a single dye is used, certain tissue elements yield a colour-result differing from those of the other structures, the staining is termed *metachromatic* (Ehrlich): lardacein, mucin, and mast cell granules exhibit these properties. Generally speaking, the metachromasia arises from the chemical constitution of the dye, but sometimes it is due to the formation of new compounds during the preparation of the mixture (for instance, polychrome methylene

blue), so that, as a matter of fact, stain mixtures are formed, and the metachromasia is more apparent than real.

Tissues may be stained in bulk or in sections. In the former the staining is completed before imbedding, the method being rarely used in pathological work, since it is generally difficult to determine what stains shall be used before the examination of the tissue is made. The staining of single or serial sections is the usual method.

Tissues may be also stained while present in the body or immediately after removal therefrom. These procedures are termed "vital" or "survival" staining, and sometimes are used for nervous tissues and blood.

Observance of the following precautions will be found to facilitate the processes of staining:—

1. The staining solutions should be carefully filtered; as a rule, just before being used. They should contain neither putrefactive organisms nor decomposition products; such may be avoided by the addition of an antiseptic, such as a crystal of thymol, etc.
2. In preparing the staining solutions, the instructions should be minutely followed.
3. It is well to remember that certain solutions lose their staining properties after a certain interval, while others need an interval of "ripening" before they may be used, for example, hæmatoxylin solutions.
4. The actual staining may be intensified:—
   (*a*) by long action of the stain.
   (*b*) by increasing the concentration of the stain.
   (*c*) by staining in the warm (30-50° C.).
   (*d*) by the addition of a substance, such as aniline oil.
5. The quantity of the stain should be sufficient to allow the section to spread itself evenly. When the sections are not stained on the slide, large basins should be used. If the section is wrinkled or curled, transference to dilute alcohol for a few moments when using a watery stain, or to stronger alcohol when using a dilute alcoholic stain, will cause it to straighten out when returned to water or to a watery stain. These transfers should be avoided if possible, or only made into dilute alcohol, since the diffusion currents created may injure the finer structures of the tissues.

The chemistry of the stains which are more generally used in pathological investigations may now be briefly considered.

### 1. Natural Colours

Hæmatoxylin, Carmine, Brazilin, Alcannin, Litmus, Orcein, Carminic acid, Purpurin, Alizarin, etc. Hæmatoxylin and Carmine are chiefly used for pathological work.

**Hæmatoxylin** ($C_{16}H_{14}O_6$) is obtained from the wood of *Hæmatoxylon campechianum* (growing in Domingo, Hayti, Jamaica, etc.), by extraction with ether. Its constitution has been determined by Perkins and Yates; it is composed of a pyrogallol radical joined to pyrocatechin. It is not a dye, and only becomes one when oxidised to Hæmatein ($C_{16}H_{12}O_6$). With such mordants as chrome alum, vanadium, copper, and iron, "lakes" are formed. Without these the tissues would not be stained, the dye thus belonging to the "adjective" group of stains.

**Carmine** is obtained from the female *coccus*. It contains proteid, calcium, and alum in addition to the dye, *Carminic* acid. The constitution of the latter is not yet clear. Lieberman has given it the formula $C_2H_{22}O_{13}$, and considers it to be related to the oxyquinones.

### 2. Aniline Colours

#### A. Acid

**Acid fuchsin** is the sodium salt of Rosanilin-disulphonic acid.

**Eosin** is the potassium salt of tetrabrom-fluorescein.

**Erythrosin** is the sodium salt of tetriodo-fluorescein.

**Orange G** is the sodium salt of the disulphonic acid of benzene-azo-$\beta$-naphthol.

**Sudan III.** is azo-benzene-azo-$\beta$-naphthol.

**Fett ponceau or Scarlet R** is azo-orthotolnol-azo-$\beta$-naphthol (contains two $CH_3$ groups).

**Picric acid** is tri-nitro-phenol.

#### B. Basic

**Methylene blue, Thionin, Toluidin blue, Methylene violet** (the latter being contained in the so-called **polychrome methylene blue**) are related to the **thio-diphenyl-amines** or thiazins; they all contain sulphur. The simplest of these bodies is **thionin** or **Lauth's violet**, which is diamido-thio-diphenyl-amine or amido-diphenthiazin.

**Basic fuchsin**, or Rosanilin, is methyl-tri-amido-triphenyl-carbinol. Rosanilin hydrochloride is generally used.

**Methyl violet** belongs to the same group as fuchsin, and is a mixture of tetra-penta- and hexa-methyl-para-rosanilin.

**Crystal violet** and **gentian violet** are also para-rosanilin compounds; the former is hexa-methyl-para-rosanilin chloride;

the latter is a mixture of the chlorides of penta- and hexa-methyl para-rosanilin.

**Dahlia** is a compound of methyl violet and fuchsin.

**Aniline blue** is triphenyl-rosanilin.

**Safranins** are amido derivatives of azonium bases.

**Bismarck brown** and **Vesuvin** are related to the azo bodies, being triamido-azo-benzene; the commercial salt is generally the hydrochloride of the disazo body.

# CHAPTER X

## STAINING OF NUCLEAR AND PLASMA STRUCTURES

EHRLICH'S observation—that basic dyes stain the nucleus most intensively, while acid dyes are selective for the plasma—may be considered as the starting-point or basis of the applications of theoretical conclusions to histological staining methods. It has been therefrom inferred, that nuclear structures are acid in reaction (nucleinic), while protoplasmic elements are basic in character. A combination of the two groups of dyes, or successive staining first with one and then with the other, will thus result in the staining of the several cellular constituents.

### A. GENERAL NUCLEAR STAINS[1]

(1) Natural dyes { (*a*) Hæmatoxylin. (*b*) Carmine.

(2) Basic aniline dyes—Methylene blue, methyl green, etc.

The aniline dyes possess affinities for bacteria as well as for nuclei, hence methylene blue and fuchsin are used as general bacterial stains; when they are employed for staining bacteria in tissues, the nuclei are also dyed, and diagnosis is thus materially assisted. Methyl green, however, does not possess this affinity for bacteria, but stains the chromatin of the cell very intensely. Methylene blue is now chiefly used in blood pathology for staining nuclear

[1] In the following chapters phenol xylol is often mentioned; instead of this medium some of the ethereal oils may be used, and in the case of paraffin sections pure xylol is generally employed. In the directions for the preparations of staining solutions, as a rule, "water" means "distilled water."

structures. Formerly, it was recommended for general nuclear staining; its colour combinations are, however, not very permanent, and there are other disadvantages which make it less preferable than hæmatoxylin.

The same may be said of the other basic aniline dyes: they are chiefly used for special purposes, and appear, therefore, in later chapters. Safranin and fuchsin are often employed for mitotic figures.

We may now briefly consider the two dyes most frequently selected for general nuclear staining, viz. hæmatoxylin and carmine.

### A. Hæmatoxylin

Hæmatoxylin solutions must always "ripen" before they can be used, because oxidisation is necessary for the production of the blue stain, hæmatein. The time for staining and the intensity obtained are dependent, therefore, upon the age of the solution. The relation between these conditions is somewhat inconstant, and the time for staining must be ascertained for each solution; those that stain very rapidly usually stain diffusely (often the celloidin as well) and yield poor results; the addition of dilute alum solution may, however, render the solution usable.

Frequent filtration is necessary to remove the sediment which is constantly being formed during the oxidisation.

Hæmatoxylin is not used by itself for pathological work; as one of the "adjective" stains, it should be combined with a mordant, and, accordingly, alum or iron are generally added to its solutions, or are used as mordanting fluids prior to staining.

The only fixing medium which diminishes its staining intensity is osmic acid.

Hæmatoxylin stains nuclei blue (it is perhaps the most permanent nuclear stain), mucus, calcareous deposits dark blue, and large aggregations of bacteria dirty light blue. The plasma is also tinged light blue. Pure nuclear staining may be obtained by the progressive method of interruption, or by the regressive method of overstaining and differen-

tiation. The latter has generally given the best effects in our hands, although the results depend upon the composition of the solution and upon the "personal equation" of the operator. Alum solutions ($\frac{1}{2}$-1 per cent) or 1-2 per cent hydrochloric acid in 70 per cent alcohol have been employed for differentiating purposes. After-washing is of great importance in hæmatoxylin staining, several hours being often necessary to obtain a bright colour. Washing is specially necessary after acid differentiation: traces of acid left in the preparation lead to further decolorisation.

The "ripening" and "after-washing" are considerably minimised by Weigert's latest hæmatoxylin mixture, and with this stain differentiation is not necessary.[1]

The most useful solutions are:—

## I. Combinations of Hæmatoxylin or Hæmatein with Alum

1. **Böhmer's alum-hæmatoxylin** (1865-68).

Solution (1). Dissolve 1 gm. of hæmatoxylin in 10 c.c. of absolute alcohol.

Solution (2). Dissolve 20 gms. of potash alum in 200 c.c. of warm water. Filter when cold.

Allow the two solutions to stand for 24 hours, then mix and expose to air and light in an open vessel for 8 days. Filter.

2. **Delafield's alum-hæmatoxylin** (1885).

Solution (1). 400 c.c. of a saturated solution of ammonia alum.

Solution (2). 4 gms. of hæmatoxylin dissolved in 25 c.c. absolute alcohol.

Mix. Expose for 3-4 days. Filter. Add 100 c.c. of glycerin and 100 c.c. of 96 per cent alcohol, and filter. The mixture may be diluted with distilled water.

3. **Ehrlich's acid-hæmatoxylin** (1886).

| | |
|---|---|
| Hæmatoxylin | 2 gms. |
| Absolute alcohol | 100 c.c. |
| Glycerin | 100 c.c. |
| Water | 100 c.c. |
| Potash alum | in excess. |
| Glacial acetic acid | 10 c.c. |

[1] For an account of the methods for the oxidisation of hæmatoxylin with mercuric chloride, etc., consult Gustav Mann, *Physiological Histology*, p. 240.

Allow to stand for a week. Expose to air and light; then filter: after six months the best staining results are obtained. If kept in well-stoppered brown bottles, the solution remains clear and no further precipitate is deposited.

Gustav Mann in 1892 substituted 0·5 to 2 gms. of hæmatein (which had just been introduced into histological work by Mayer in 1891) for the hæmatoxylin in Ehrlich's solution; he adds the glacial acetic to the hæmatein and dissolves the latter either in the acid or after the addition of 25 c.c. of alcohol; the glycerin is next added, the mixture thoroughly shaken, and the alum then dissolved in hot water, and while hot, poured into the mixture. The solution is kept in a well-stoppered bottle.

With hæmatein, as Mayer pointed out, no "ripening" time is necessary, the solution being at once ready for use.

4. **Mayer's hæmalum** (1891).

Solution (1). Dissolve 1 gm. of hæmatein in 50 c.c. of 90 per cent alcohol; warm.

Solution (2). Dissolve 50 gms. of potash alum in 1000 c.c. of distilled water.

Mix; cool; filter. (2 per cent acetic acid may be added if acid-hæmatein is desired.)

The mixture is at once ready for staining purposes.

Mayer recently recommends the following mode of preparation:—

Dissolve 1 gm. of hæmatoxylin in boiling water; add water up to 1 litre.

Add sodium iodate, 0·2 gm.; alum, 50 gms. Dissolve at room temperature.

If chloral hydrate 50 gms. and citric acid 1 gm. are added, the solutions will keep better.

## II. Other Hæmatoxylin or Hæmatein Combinations

These consist in the addition of chrome, copper, or iron salts, molybdic or phosphotungstic acids to the hæmatoxylin solution for mordanting purposes. They are chiefly used in Weigert's stains for nervous tissues and in Heidenhain's iron-hæmatoxylin method.

**Weigert's** last hæmatoxylin mixture may be placed among the general stains; the other combinations appear in connection with special methods:—

5. **Weigert's iron hæmatoxylin.**—In this staining mixture the iron is contained in the staining solution; no previous mordanting is necessary.

| | | |
|---|---|---|
| Solution (1). | Hæmatoxylin . . | 1 gm. |
| | Alcohol (96 per cent) . | 100 c.c. |
| Solution (2). | Liq. ferri sesquichlor . | 4 c.c. |
| | (Pharmak. German. IV. sp. gr. 1·124) | |
| | Hydrochloric acid conc. . | 1 c.c. |
| | Water . . . | 100 c.c. |

Both solutions keep well; the first must be allowed to ripen for some days, and should not be kept longer than six months, as it then stains rather diffusely, possibly from over-oxidisation. Mix equal parts of each solution just before staining. The mixture stains at once. A quantity sufficient for 4 days only should be made, since the best effects are then obtained. It may, however, continue to stain for 8-14 days. The hydrochloric acid is used to avoid overstaining. Differentiation in acid alcohol is therefore not necessary (Weigert), although sometimes advisable. The iron solution may be used without the acid. Differentiation is then always necessary.

**Summary of staining sections with hæmatoxylin.** — This is more or less the same with all the five hæmatoxylin solutions.

1. Stain in hæmatoxylin mixture, 3-15 minutes.
2. Differentiate in $\frac{1}{2}$-1 per cent potash-alum solution, or better, in acid alcohol (1 c.c., HCl 100 c.c. 70 per cent alcohol).
3. Wash for several hours in tap water, generally overnight;

or To obtain the blue colour rapidly, expose the section to ammonia vapour or place in lithium-carbonate solution for a few seconds. Wash thoroughly.

(Stain with a plasma stain eosin, orange G, van Gieson.)

| | |
|---|---|
| 4. Dehydrate in 90 per cent alcohol. | 4. Dehydrate in absolute alcohol. |
| 5. Clear in phenol xylol. | 5. Clear in xylol. |
| 6. Mount in Canada balsam. | 6. Mount in balsam. |
| (For celloidin sections.) | (For paraffin sections.) |

Ehrlich's acid hæmatoxylin keeps well and does not overstain, and is more generally useful than Böhmer's or Delafield's mixtures; the alum hæmateins have the advantage of being immediately ready for use.

**Weigert's solution** has the following special advantages, and gives most satisfactory results:—

1. Easy and rapid preparation of well-keeping stock solutions. (It is well to have a good supply made, so that the percentage of iron is not subject to much variation.)

2. The nuclei are stained deeply and quickly (1-2 minutes).
3. No overstaining when hydrochloric acid is included.
4. Long washing is unnecessary.
5. The secondary plasma stain is accentuated (see van Gieson's stain). For combined nuclear-plasma staining Weigert's iron hæmatoxylin is probably without an equal.

When, however, a light nuclear stain is desired, as, for instance, a contrast stain to Sudan III. or Scharlach R (for fat) or fuchsin (tubercle bacilli), Delafield's or Ehrlich's hæmatoxylin solutions yield the best results, since after washing in ammonia or exposure to ammonia vapour the nuclei are a bright blue.

## B. Carmine or Carminic Acid

Carmine contains carminic acid, proteid, alum, and calcium (Lieberman, Mayer). The latter substance, as well as the alum, appears to play some part in the staining process.

Space permits the inclusion of only a few formulæ. Carmine is not now so frequently used by the pathologist as it was in former days.

1. **Alum carmine** (Grenacher, 1879).

| | |
|---|---|
| Carmine . . . | ½-1 gm. |
| Alum solution 1-5 per cent . | 100 c.c. |

Boil for 20 minutes; cool; filter.

2. **Carm-alum** (Mayer, 1891).

| | |
|---|---|
| Carminic acid . . . | 1 gm. |
| Potash alum . . . | 10 gms. |
| Distilled water . . . | 200 c.c. |

Warm; dissolve; filter.

A few drops of formol, or 5 per cent sodium salicylate, or a crystal of thymol may be added for keeping purposes.

With both dyes:—

(1) Stain 15 minutes to several hours.
(2) Wash well in water.
(3) Dehydrate; clear; mount in balsam.

When a plasma dye is also used, it is interpolated between stages 2 and 3.

Nuclei red. Carm-alum tinges the plasma slightly; if

pure nuclear staining is desired, differentiate in 1 per cent potash alum, or $\frac{1}{1000}$ hydrochloric acid alcohol, washing thoroughly afterwards. Carm-alum cannot be used with tissues reacting distinctly alkaline.

3. **Lithium carmine** (Orth, 1883).

| | |
|---|---|
| Carmine . . . | 2½-5 gms. |
| Cold saturated watery lithium carbonate . . . | 100 c.c. |

(1) Stain 1-3 minutes; (2) acid alcohol (¼ to several hours); (3) wash in water; (4) dehydrate; clear; mount in balsam.

A plasma stain may be used after stage 3. Between 1 and 2, do not wash in water.

This is the best carmine stain for pathological work. In our experience, excellent results are obtained by differentiation in acid alcohol for 12-24 hours; the nuclei are then alone stained.

4. **Watery borax carmine** (Grenacher, 1879).

| | | |
|---|---|---|
| Carmine | 0·5-0·75 gm. | Dissolve by boiling. |
| Borax . | 1-2 gms. | |
| Water . | 100 c.c. | |

To the hot solution add 5 c.c. of 0·5 per cent acetic acid until the solution is deep red. Allow to stand for 24 hours; filter, add a crystal of thymol.

Strain for about 15 minutes, and decolourise in acid alcohol.

5. **Acid alcohol carmine** (Mayer, 1883).

| | | |
|---|---|---|
| Carmine . . | 4 gms. | Dissolve by boiling. |
| Water . . | 15 c.c. | |
| Hydrochloric acid | 30 drops. | |

Add 95 c.c. of 85 per cent alcohol, filter hot; neutralise with ammonia; filter when cold.

(1) Stain 1 to several minutes; (2) differentiate in acid alcohol; (3) dehydrate in alcohol; (4) 96 per cent or absolute alcohol; (5) phenol xylol or xylol; (6) Canada balsam.

This method may be used when for other purposes the sections may not be passed through water.

Although carmine gives good nuclear staining, especially the lithium-carmine solution, it is in no sense comparable to that obtained with hæmatoxylin. The colour, however,

is permanent. Carmine stains are useful in the following conditions :—

(1) For tissues containing certain pigments, as in anthracosis and brown atrophy, the contrast being better than when the blue nuclear dyes are employed.
(2) When structures other than nuclei are stained blue, *e.g.* elastic fibres, fibrin, and some bacteria.

Lithium carmine gives constant and reliable results.

### B. General Plasma Stains

Eosin and orange G are the best counterstains for hæmatoxylin or methylene blue, and picric acid is generally used as a contrast to carmine. These dyes will be considered under "combined" staining, as they are but rarely used alone.

**Ammonia carmine** is useful in some cases, and Honegger's formula deserves mention here :—

> Rub carmine into a thick paste with the smallest possible quantity of strong liquid ammonia ; spread it in thin layers on a dish or glass plate and allow it to dry ; pulverise it ; leave it in the open air for 24 hours ; dissolve it in distilled water.
>
> When using, add a few drops of this mixture to distilled water until the latter assumes a reddish colour. Stain $\frac{1}{2}$-24 hours, or until the sections are decidedly red ; wash thoroughly. The nuclei stain slightly (they may be subsequently stained with hæmatoxylin), but the connective tissues and muscle fibres are stained a deep red.

### C. Combined Nuclear and Plasma Stains

#### I. The Staining is Simultaneous

#### Picro-carmine

**Ranvier,** who introduced this combination, considered it to be an ammonio-picric carminate ; **Mayer** has since shown it to be an indefinite mixture of ammonium picrate, ammonium carminate, free ammonia, aluminium, and lime.

1. **Ranvier's picro-carmine.**—Add a saturated solution of ammonia carmine to a saturated watery solution of picric acid; evaporate the mixture on a water-bath to about one-fifth of the original volume; when cool, filter; dissolve the yellow-red powder in distilled water, making a 1 per cent solution.

   **Neumann** stains sections in Ranvier's picro-carmine, and differentiates them under a low-power objective in 10 c.c. of glycerin, containing 4 drops of hydrochloric acid. Nuclei, red; connective tissues, fibrin, and protoplasm, yellow.

2. **Weigert's picro-carmine.**—Place 2 gms. of carmine and 4 c.c. of ammonia in a well-stoppered bottle; allow to stand for 24 hours; add 200 c.c. of cold watery saturated picric acid solution; allow to stand for 24 hours, shaking occasionally, until solution is complete; add acetic acid until a slight permanent precipitate remains after thorough shaking; filter; after 24 hours add traces of ammonia until the precipitate is dissolved. Stain for 5-10 minutes.

3. **Orth's picro-lithium carmine :—**

| | |
|---|---|
| Lithium-carmine solution (containing $2\frac{1}{2}$ per cent carmine) | 1 part. |
| Saturated watery picric acid solution | 2 parts. |

Picro-carmine mixtures are useful for fresh tissues. A small amount of the stain left in the preparation intensifies the staining and does no harm. Sections must not be transferred to water. The surplus stain is removed by blotting-paper and the preparation mounted in glycerin or Farrant's medium.

Although useful for fresh tissues, picro-carmine mixtures are now but rarely used by the pathologist.

## II. Successive Staining

This procedure yields more precise results than the method of combined staining permits. The nuclei are generally stained first, the protoplasmic structures later.

**Carmine and picric acid.**—**Lithium carmine** may be used for staining the nuclei, and, after differentiation, the protoplasm may be stained with **picric acid**. A useful procedure is to add a few drops of saturated alcoholic picric acid solution to the alcohol used for dehydration, any excess of picric acid being removed by absolute alcohol. This is valuable for keratin and muscles.

**Hæmatoxylin and eosin.**—After staining in hæmatoxylin and washing, treat the preparation with eosin (1 gm. in 100 c.c., 90 per cent alcohol), removing any excess with absolute alcohol.

Eosin gives good results after fixation in formol or in Orth's (formol—Müller) fluid, red blood corpuscles being specially brilliant.

Hæmatoxylin may also be combined with picric acid, as for keratin, etc.; but the sections must be overstained, since picric acid partly decolourises the hæmatoxylin.

**Hæmatoxylin and orange G.**—A 1 per cent watery solution of **orange G** is generally used. Stain $\frac{1}{4}$ to several hours.

A large number of basic nuclear-staining aniline dyes are often combined with acid plasma stains, *e.g.* safranin, gentian aniline blue, safranin aniline blue, methyl green eosin, and methylene blue eosin. The majority of these are described under "Blood" (*q.v.*).

It will there be shown how many combinations of methylene blue and eosin are possible. Of these, **von Willebrand's** solution is useful for staining sections.

## III. Staining with Combined Plasma Stains

The use of more than one stain for after-nuclear staining permits a better differentiation of the intercellular structures, and often demonstrates their varying chemical constitutions. One of the best examples is

**van Gieson's staining method.**—After preliminary staining with hæmatoxylin the sections are transferred to a mixture of picric acid and acid fuchsin. van Gieson did not at first give exact instructions for the preparation of his solutions; these have been worked out later.

Prepare the following stock solution :—

| | | |
|---|---|---|
| Acid fuchsin (patent rubin S is better) | . 1·5 gm. | Keeps |
| Saturated watery picric acid (0·6 per cent) | . 150 c.c. | well. |

For general use, add 10 c.c. of the saturated watery picric acid solution to 1 c.c. of the stock solution. The mixture keeps well.

Stain for 10 seconds; wash rapidly; dehydrate in alcohol, xylol (or phenol xylol for celloidin) balsam.

van Gieson introduced this stain in 1889 for neuroglia; Ernst applied it to general staining.

Eosin is a diffuse rather than a differential stain; when combined with hæmatoxylin, nuclei are blue and the remaining cell structures are red. Picric acid fuchsin is differential, if not elective. When combined with hæmatoxylin, the nuclei are brown; protoplasm, yellow; connective tissue fibres, light red; muscle fibres, voluntary and involuntary, bright yellow; hyaline substances, orange to yellow (but inconstant); lardacein and fibrin, yellow.

Each worker, as a rule, adopts one nuclear-plasma method of staining for general impressions and prefers it before all others. Van Gieson's method may be thoroughly recommended for such a purpose; it is not only a well-defined differential stain for nuclei and general protoplasmic structures, but it indicates clearly any slight departure from usual staining, and often suggests early pathological changes and the need for elective stains. The solution is easily made, keeps well, and stains rapidly, the necessary procedures being quite simple. Its advantages are now almost universally recognised.

The presence of picric acid in the mixture demands deep previous staining with hæmatoxylin; if this is not obtained, the nuclei may be somewhat indistinct. For this reason, van Gieson's stain is allowed to act for a few seconds only. When using Weigert's iron hæmatoxylin, decolorisation need not be feared; van Gieson's mixture may penetrate for more than 10 seconds, yet sharp, instructive pictures are produced.

The method may be summarised as follows:—

1. Stain in iron hæmatoxylin, 5-10 minutes. Decolourise in acid alcohol, if necessary.
2. Wash in water.
3. Stain in van Gieson's mixture, 10-30 seconds.
4. Wash rapidly in water.

| | | |
|---|---|---|
| 5. 96 per cent alcohol | or | 5. Absolute alcohol. |
| 6. Phenol xylol | ,, | 6. Xylol. |
| 7. Canada balsam | ,, | 7. Canada balsam. |
| (For celloidin sections.) | | (For paraffin sections.) |

Hansen prepares van Gieson's staining mixtures as follows:—

| | |
|---|---|
| Cold saturated picric acid . . . | 100 c.c. |
| Acid fuchsin solution (2 per cent) . . | 5 c.c. |

Add 12 drops of 2 per cent acetic acid.

Sections are placed in this mixture for 20 minutes, then washed in water containing 2 drops of the stain to each 3 c.c., and finally transferred to absolute alcohol.

This procedure is more complicated than the original method, and does not yield any better results.

**Rubin and orange.**—Delépine recommends the following proportions of rubin and orange. The staining results are not so multicoloured and brilliant as those obtained by van Gieson's staining solution, but they are especially helpful in examining the finer details.

**Solutions**:—

| | | | |
|---|---|---|---|
| I. Rubin | 2 gms. | II. Orange | 5 gms. |
| Alcohol | 50 c.c. | Alcohol | 50 c.c. |
| Water | 50 c.c. | Water | 50 c.c. |

For use, take

| | |
|---|---|
| Rubin solution I. | 10 parts. |
| Orange solution II. | 20 ,, |
| Water | 500 ,, |

Protoplasm, orange; connective tissues, red; muscle fibres, orange; fibrin, orange; colloid and hyaline, orange-red.

**Erythrosin and picric acid.**—Powell White advises the following stain for muscular and fibrous tissues:—

| | |
|---|---|
| Saturated alcoholic solution of erythrosin | 4 c.c. |
| Saturated watery solution of picric acid | 50 c.c. |
| Water, up to | 100 c.c. |

Add excess of calcium carbonate; allow to stand for 15-30 minutes, shaking occasionally; filter. Stain for 15-30 minutes. If the mixture loses its staining properties, add a few drops of dilute acetic acid and reneutralise with calcium carbonate. Muscle fibres, yellow to yellow-brown; fibrous tissue, pink; red blood corpuscles and protoplasm, yellow.

The stain is used after hæmatoxylin or hæmalum.

## D. Methods for the Demonstration of Special Nuclear Structures

The **mitotic figures** which constitute indirect nuclear division demand our primary consideration. It is customary, when studying mitotic changes, to fix the tissues as rapidly as possible after their removal from the body.

Operative material is therefore selected for such investigations, since by the time post-mortem material is available the mitotic figures are said to have run through their ordinary stages (*e.g.* after Wentscher). Wolff, however, states that many mitotic figures may still be observed some days after death, and doubts that the mitotic changes thus continue. It is safer, however, to fix and to harden the tissues as early as possible.

Alcohol, or better, formol may be used as fixatives, and hæmatoxylin, or Gram's or Weigert's methods, be employed for staining. If present, the mitoses may then be easily observed. It is well, however, for special purposes, to fix in sublimate solutions, or, best of all, in Flemming's mixture. With the former, Biondi-Heidenhain's stain may be combined; with the latter, safranin is probably the most useful. With these procedures the mitoses are much more evident. The following are the various stages of the process:—

1. **Fixation in Flemming's solution; staining with safranin**:—

Small pieces of tissue are fixed and hardened in Flemming's solution (Chapter III.) for 24 hours in a brown glass bottle, washed 24 hours, hardened in increasing strengths of alcohol, imbedded and cut. They are then stained in

(1) 1 per cent watery safranin solution, 12-24 hours.
(2) Rapid washing in water.
(3) Rapid differentiation in 0·5 - $\frac{1}{1000}$ HCl in 70 per cent alcohol.
(4) Differentiate in absolute alcohol (best controlled under low objective), until the colour ceases to come away in clouds, and the nuclei are weak red in colour.
(5) Xylol; balsam.

The mitotic figures stand out clearly by reason of their intense staining. They should not be confused with deeply stained masses of degenerated nuclear material.

In addition to the mitoses, fat is stained black by this method. Tubercle bacilli may also be stained; sometimes they are black because of the fatty material with which they are associated; at other times they are red from the long action of the safranin and the slight differentiation; this is generally the case when the safranin is dissolved in aniline water instead of water, as for instance in Babes' solution—saturated solution of safranin in aniline 2 c.c., water 100 c.c., dissolved at 80° C.; or, as Lee recommends,

equal parts of safranin in aniline water, and safranin in absolute alcohol. This conjoined staining of tubercle bacilli with nuclear structures is, however, too inconstant for general diagnostic purposes.

Instead of safranin, the tissues may be stained with carbol fuchsin (*vide* "Bacteriology") for one hour, or with 1 per cent watery methyl violet, 12-24 hours, and differentiated as before. With carbol fuchsin the elastic fibres are sometimes stained violet.

To satisfactorily stain bacteria and at the same time to demonstrate mitoses, Gram's method (*vide* "Bacteriology") is useful, the differentiation being concluded when the mitotic figures are well defined.

Hermann's mixture (Chapter IV.) may be used when very accurate fixation of the centrosome, spindle threads, and chromosomes is necessary.

Osmic acid fixation (Flemming, Hermann), combined with safranin staining, is by far the best method for mitoses.

2. **Fixation in mercuric chloride mixtures.**—After the fixation harden in alcohol, add iodine solutions to remove mercurial deposits, imbed, cut, and **stain by Ehrlich-Biondi-Heidenhain's method** :—

| | | |
|---|---|---|
| Saturated watery solution of orange | . | 100 c.c. |
| ,, ,, acid fuchsin | . | 20 c.c. |
| ,, ,, methyl green | | 50 c.c. |

To obtain saturated solutions, excess of the stain should be added, and allowed to stand for several days.

For staining, the mixture must be diluted by the addition of 60 to 100 parts of distilled water to 1 c.c. of the stain. The mixture may be used when the addition of acetic acid causes it to turn a deeper red ; a drop placed on filter-paper should exhibit a bluish-green centre with orange-yellow margins, no outer zone of red being visible. Grübler (Leipzig) sells the stain ready prepared ; dissolve 1 or 2 gms. in 100 c.c. of distilled water.

Israel mentions another of Heidenhain's solutions. Dissolve 12 gms. of Grübler's Biondi-Heidenhain staining powder in 100 c.c. of distilled water ; this forms a stock solution.

Staining mixture :—

| | | |
|---|---|---|
| Stock solution | . . | 1 c.c. |
| Water | . . . | 30 c.c. |
| Watery acid fuchsin, $\frac{1}{2}$ per cent | | 3 c.c. |
| Acetic acid, 2 per cent | . | 0·2-0·3 c.c. (5-6 drops). |

Proceed as follows:—

(1) Stain in the solution, 2-24 hours.

(2) Wash in 90 per cent alcohol.
(3) Dehydrate in absolute alcohol.
(4) Xylol ; balsam.

Resting nuclei, bluish ; mitoses and fragments of the nuclei of leucocytes, dark green ; red blood corpuscles, orange red ; protoplasm and connective tissue elements, fuchsin red.

The staining is somewhat capricious, and Heidenhain, in the *Festschrift for Kölliker*, p. 116, gives directions for the addition of acetic acid, etc., in order to make the method less variable.

The mixture is, in fact, a slight modification of Ehrlich's tri-acid stain, and it may be replaced by the latter with good effect.

3. **Fixation in mercuric chloride, staining with Heidenhain's iron hæmatoxylin.**—The process is conducted as follows, paraffin sections being most suitable :—

(1) Immerse the section in 1·5-4 per cent solution of iron-alum sulphate (violet-coloured salt) or iron-ammonium sulphate, $\frac{1}{2}$-3 hours.
(2) Wash in water.
(3) Stain in 0·5 per cent watery hæmatoxylin or hæmatein, 12-18 hours.
(4) Wash in water.
(5) Differentiate in the iron-alum or iron-ammonium solution until the section becomes deep blue (best controlled under low objective) and the nuclear structures stand out sharply.
(6) Wash in running water, 15 minutes.
(7) Dehydrate in absolute alcohol ; xylol ; balsam.

The hæmatoxylin solution may be used repeatedly ; the iron introduced with the section improves it. The time for staining may be shortened or lengthened ; in the former case the section is steel-grey or light blue ; in the latter, dark blue.

Instead of watery hæmatoxylin solution, Heidenhain has lately recommended the following :—Hæmatoxylin, 1 c.c. ; alcohol, 10 c.c. ; water, 90 c.c. The solution should be kept for four weeks before being used, and it stains better when it has been used for some time. Sections are left in it for 24-36 hours after being treated with 2·5 per cent iron-alum solution for 6-12 hours, differentiation being completed as in the method just described.

The centrosome is still more clearly defined, according to Heidenhain, if the section is stained for 24 hours in a weak solution of Bordeaux red before proceeding with the iron-alum and hæmatoxylin method. The chromatin and plasma are coloured by the Bordeaux red, but the centrosome is unaffected ; during the after-staining with

hæmatoxylin and differentiation, the chromatin and plasma give up the second stain more easily than the centrosome, and the latter thus stands out more prominently. (So-called *preoccupation* method.)

4. **Fixation and hardening in osmic acid, sublimate or other mixtures; staining by Benda's new iron-hæmatoxylin method.**

(1) Place the section in "liq. ferri sulfur. oxyd," Pharm. Germ. (diluted with double its volume of water) for 24 hours.

(2) Wash carefully in distilled water.

(3) Wash in ordinary tap water.

(4) Stain in 1 per cent watery hæmatoxylin until the section is black.

(5) Wash in water.

(6) Differentiate in 10-30 per cent acid or in "liq. ferri sulfur. oxyd." diluted with distilled water (1-20).

Lee considers Benda's method to be quite as good as Heidenhain's process and more stable. Mayer recommends a 10 per cent solution of ferric sulphate instead of Benda's persulphate of iron.

5. **Baumgarten's method for the simultaneous staining of mitotic figures and tubercle bacilli.**

(1) Fix and harden for several weeks in 0·2 per cent chromic acid solution; wash; after-harden in alcohol; dehydrate; imbed.

(2) Stain in aniline gentian violet (aniline water 100 c.c., saturated absolute alcoholic methyl violet 11 c.c.), 24 hours.

(3) Decolourise in acid alcohol until only the tubercle bacilli are still visible.

(4) Wash in 70 per cent alcohol.

(5) Stain in saturated alcoholic fuchsin 5-10 minutes, or in dilute fuchsin (8-10 drops of saturated alcoholic fuchsin, water 10 c.c.), 24 hours.

(6) Wash rapidly in absolute alcohol.

(7) Counterstain in saturated watery methylene blue, 4-5 minutes, or if stained with dilute fuchsin solution, 5-10 seconds.

(8) Dehydrate in absolute alcohol, 5-10 minutes.

(9) Xylol; balsam.

Bacilli, blue violet; mitoses, red.

Of these methods we have found the first—fixation in Flemming's fluid and staining with safranin—to yield the most uniform results. The cells of rapidly growing carcinoma form useful material for demonstration of the several methods and for the necessary practice therein.

## E. Nucleoli

The nucleolus, in contrast to the basic affinities of the nucleus, has affinities for acid dyes. It is thus stained otherwise than the nucleus when using Ehrlich's tri-acid or Romanowsky's stains; with the latter dye (or its several modifications) the nucleolus is red, the nucleus blue.

## F. Methods for the Demonstration of Special Protoplasmic Structures

1. **Cell granules—Altmann's method** :—

   (1) Fix very fresh, small, thin pieces of tissue in equal parts of 5 per cent potassium bichromate and 2 per cent perosmic acid (Altmann's mixture), 24 hours. Wash in running water for several hours. Harden in alcohol. Imbed in paraffin. Section; mount; remove paraffin with xylol; put first in absolute alcohol and then in dilute alcohol.

   (2) Stain, warming until the steam rises, in acid fuchsin 20 gms., aniline water 100 c.c.

   (3) When cool, remove the fuchsin with a mixture containing 1 part of saturated alcoholic picric acid and 2 parts of water.

   (4) Renew the picric acid solution and warm at 42° C. (best in an incubator or on the paraffin oven), 30-60 seconds.

   (5) Dehydrate in alcohol; xylol; balsam.

   Protoplasm, yellow; the so-called Altmann's granules, red; fat globules, black (from the osmic acid).

2. **Cell granules** may also be stained by the so-called "**vital**" and "**survival**" methods of staining which Arnold has especially used for this purpose. Such methods will be described later.

3. **Intra-cellular fibres and canals** (Holmgren).—Holmgren has employed a number of methods for the demonstration of these structures, which he has observed in glandular as well as in nerve cells. Formerly he used erythrosin extensively. We here give his later method :—

   (1) Fix in 2·5 per cent trichloracetic acid or in 5 per cent trichlorlactic acid, 8-24 hours.

   (2) Harden in 40, 50, 60, 70, 82, and 96 per cent alcohols, 24 hours each.

   (3) Dehydrate in absolute alcohol; imbed; make sections 2-5$\mu$ in thickness.

   (4) Stain in a **freshly** prepared dilute solution of Weigert's elastin stain (150-160 c.c. diluted to 200-210 c.c.) for 24 hours.

   (5) Differentiate in absolute alcohol; xylol; balsam.

4. **Cell inclusions** (after Russell) :—

   (1) Fix and harden in Müller's fluid; wash; alcohol; imbed in celloidin or, better, in paraffin; section. Remove paraffin; xylol; alcohol; water.

   (2) Stain in a saturated solution of fuchsin in 2 per cent carbolic acid, 10 minutes or longer.

   (3) Wash in water for some minutes.

(4) Wash in absolute alcohol, 30 seconds.

(5) Counterstain in iodine green (1 gm. dissolved in 100 c.c. of 2 per cent carbolic acid), 5 minutes. The section must be evenly spread out.

(6) Dehydrate rapidly in absolute alcohol.

(7) Xylol ; balsam.

Nuclei, green ; Russell's fuchsin bodies, light red ; Altmann's granules, light red.

5. A large number of hardening and staining methods have been proposed by **Pianese** for the demonstration of cell-contents of supposed parasitic origin. Some of the inclusions are hardened in special solutions, others in mercuric chloride or Zenker's solution. The method here given has been also used by Arnold for the staining of cell-granules.

(1) Harden small pieces in the following mixture for 6 hours :—

| | |
|---|---|
| 1 per cent watery solution of sodium-chloro-platinate | 15 c.c. |
| ¼ per cent watery solution of chromic acid | 5 c.c. |
| 2 per cent watery solution of osmic acid | 5 c.c. |
| Formic acid | 1 drop. |

Wash in running water, 12 hours ; harden in increasing strengths of alcohol ; imbed in paraffin ; section.

(2) Stain in the following mixture for 30 minutes :—

| | |
|---|---|
| Malachite green | 0·5 gm. |
| Acid fuchsin | 0·01 gm. |
| Martin's yellow | 0·1 gm. |
| Distilled water | 150 c.c. |
| Alcohol (96 per cent) | 50 c.c. |

(3) Dehydrate in absolute alcohol.

(4) Clear in xylol ; mount in balsam.

Nuclei, green ; protoplasm, reddish ; cell inclusions, generally light red.

## G. Staining of Cells characterised by Special Granules

Under this heading should be considered the different types of blood cells whose granules are distinguished by their staining affinities (Ehrlich), but for convenience they are deferred to Chapter XII., and the staining of mast and plasma cells is now alone more thoroughly described.

The eosinophile (acidophile) granules are deeply tinged with eosin when a preparation is stained with hæmatoxylin and eosin. In animals the granules of the polymorpho-nuclear leucocytes are also acidophile (instead of neutrophile, as in man), but the granules are much finer than those of the eosinophilous cells.

For simultaneous staining of the nucleus, protoplasm,

and granules, **v. Willebrand's** methylene blue eosin mixture is very useful. (See Chapter XII.)

1. **Mast cells** occasionally occur in normal blood, and are generally present in connective tissues during inflammatory processes. Their granules stain intensely with basic aniline dyes.

Ehrlich recommends the following procedure :—

(1) Harden in absolute alcohol ; imbed ; cut.
(2) Stain with saturated watery solution of dahlia.
(3) Wash in water.
(4) Dehydrate in absolute alcohol.
(5) Clear in xylol ; mount in balsam.

The mast cells may also be stained metachromatically by Unna's method, the plasma cells also being coloured.

2. **Plasma cells.**—Their origin and relation to blood or connective tissue elements is far from clear : Waldeyer introduced the term "plasma" cell for certain normal cells, such as the intermediate cells of the testis, while Unna has applied the same name to a type of cell which appears during the inflammatory reaction of the tissues, and the latter definition is now generally accepted.

**Unna's method for mast and plasma cells** :—

(1) Harden in absolute alcohol ; imbed ; cut.
(2) Stain in Unna's polychrome methylene blue, $\frac{1}{4}$-12 hours.
(3) Wash in water.
(4) Differentiate in Unna's glycerin-ether mixture (Grübler) 15 seconds to several minutes.
(5) Wash carefully in water.
(6) Dehydrate in absolute alcohol.
(7) Clear in xylol ; mount in balsam.

Mast cell granules, metachromatic red ; plasma cell granules, blue.

Polychrome methylene blue is a solution of methylene blue which also contains methylene violet and the red methylene azure. The mixture may be prepared in various ways. Unna's polychrome methylene blue may be obtained from Grübler.

Michaelis advises the following method of preparation :— Boil 200 c.c. of 10 per cent watery methylene blue solution with 10 c.c. of decinormal caustic soda for 15 minutes, and

when cool, neutralise exactly with 10 c.c. of decinormal sulphuric acid. (This may be obtained from Grübler as "azur-blau." Refer to chapter on "Blood.")

Although other methods exist, we can here only mention

**M. B. Schmidt's method for demonstrating the granules of some of the cells of the hypophysis cerebri.**—This method of staining granules is not effective for cells other than those of the hypophysis.

Harden in Orth's fixing fluid; transfer directly to alcohol without washing in water; imbed in paraffin; cut; or, fix and harden in formalin; freeze; cut. Stain in alum carmine, and then treat by Weigert's fibrin method. (Chapter XI. v.)

Nuclei, red; granules, blue.

Other cell contents, such as glycogen and pigment, are considered in the following chapter.

# CHAPTER XI

## SPECIAL CELL AND TISSUE STRUCTURES (PRESENTING NORMAL AND PATHOLOGICAL CHARACTERS)

It is impossible to separate these two conditions, since the excess of normal substances or the appearance of normal elements in abnormal positions both constitute pathological conditions. Fat may be cited as an example of such a combination.

### I. Connective Tissue Fibrils

Their identification—often necessary or interesting—is of special importance in connection with the absence or presence of fibrils between tumour cells, as, for instance, in carcinoma or sarcoma.

#### A. van Gieson's Stain

This is certainly the best stain for dense masses of connective tissue, but it does not sufficiently differentiate the finest fibrils.

#### B. Ribbert's Modification of Mallory's Axis Cylinder Staining Method

(1) Harden in alcohol; imbed; cut.
(2) Transfer the sections with glass needles to 10 per cent phosphomolybdic acid, 5-30 seconds.
(3) Wash rapidly in water.
(4) Stain in the following mixture, 1-5 minutes:—

| | |
|---|---|
| Phosphomolybdic acid, 10 per cent | 10 c.c. |
| Hæmatoxylin | 1·75 gm. |
| Water | 200 c.c. |
| Carbolic acid (cryst.) | 5 gms. |

(5) Wash in water.
(6) Dehydrate in absolute alcohol ; xylol ; balsam.
Connective tissue fibrils, blue ; other constituents, grey-green.

## C. Mallory's Aniline Blue Method

This method stains the connective tissue fibrils, according to our experiences, exceedingly well. Though differential, it may be used for general staining (*vide* Chapter XI.). A disadvantage is the necessity for fixation in sublimate solutions. The nuclei are not stained easily.

(1) Harden in sublimate solution or Zenker's fluid ; after-harden in alcohol ; imbed ; cut.
(2) Stain in $\frac{1}{10}$ per cent aqueous acid fuchsin, 5-10 minutes.
(3) Transfer directly to the following solution and stain for 20 minutes or longer :—

| | |
|---|---|
| Aniline blue, soluble in water (Grübler) | 0·5 gm. |
| Orange G (Grübler) . . . | 2·0 gms. |
| Aqueous solution of phosphomolybdic acid (1 per cent) . . . | 100 c.c. |

(4) Wash and dehydrate in several changes of 95 per cent alcohol ; dry with filter-paper.
(5) Clear in xylol or oleum origani.
(6) Mount in balsam.

**Blue.** Fibrils and reticulum of connective tissue. Amyloid and certain other hyaline substances.

**Red.** Nuclei. Protoplasm. Axis cylinders. Neuroglia fibres. Fibrin.

**Yellow.** Red blood corpuscles. Myelin sheaths.

**Pink or Yellow.** Elastic fibres.

The connective tissue stains more sharply if the staining with acid fuchsin is omitted, since the red tints are then not present. Both the nuclei and protoplasm stain yellow, and the blue fibrils and reticulum stand out more prominently.

As a rule, by this method the elastic fibres are only slightly stained, but the connective tissue fibres and fibrin stand out glaringly.

## D. Mall's Method for Reticulum

(1) Digest frozen sections of fresh tissue for 24 hours in the following solution :—

| | |
|---|---|
| Parke, Davis, and Co.'s pancreatin . | 5 gms. |
| Bicarbonate of soda . . | 10 gms. |
| Water . . . . | 100 c.c. |

(2) Wash carefully in water.

(3) Place sections in a test-tube half full of water and shake thoroughly to remove all the cellular débris.

(4) Spread out on a slide and allow to dry.

(5) Allow a few drops of the following solution to dry on the surface of the section :—Picric acid, 10 gms. ; absolute alcohol, 33 c.c. ; water, 300 c.c.

(6) Stain for 30 minutes in the following mixture :—Acid fuchsin, 10 gms. ; absolute alcohol, 33 c.c. ; water, 66 c.c.

(7) Wash in the picric solution for a second.

(8) Dehydrate in absolute alcohol ; xylol ; balsam.

### E. Mallory's Method for Fibroglia Fibrils

These fibrils bear the same relation to connective tissue cells that neuroglia fibrils bear to neuroglia cells. Fibrils staining by these methods are present also in the basement membranes of sweat glands, of mammary gland acini and tumours derived from them, of the tubules of the kidney, and beneath the endothelium of arteries and veins.

(1) Fix thin, small, fresh pieces of tissue in Zenker's fluid. Harden in alcohol ; imbed ; section.

(2) Stain celloidin or paraffin sections in 1 per cent aqueous acid fuchsin, for 12 hours in the cold or for 20-30 minutes at 50-56° C.

(3) Wash in water (5 seconds).

(4) Differentiate in 0·25 per cent aqueous potassium permanganate solution (10-20 seconds).

(5) Wash in water (5 seconds).

(6) Dehydrate in alcohol.

(7) Clear in origanum oil or xylol ; mount in balsam.

Fibroglia fibrils and cell nuclei, intense red ; the contractile elements of striated muscle cells, smooth muscle fibres, neuroglia fibres, cuticular surfaces of epithelial cells and fibrin are also red ; ordinary connective tissue fibrils, brownish yellow or colourless ; elastic fibres, unless degenerated, bright yellow.

## II. Collagenous Connective Tissue (Unna's methods)

### A. With Acid Fuchsin-Picric Acid (Unna)

1. Harden in absolute alcohol ; imbed ; cut.
2. Stain in 2 per cent watery acid fuchsin solution, 5-10 minutes.
3. Wash in water.
4. Stain in saturated watery picric acid solution, 1-2 minutes.
5. Dehydrate in a saturated alcoholic solution of picric acid.
6. Absolute alcohol ; clear in xylol ; mount in balsam.

Protoplasm, yellow ; nuclei, red ; collagenous connective tissue, red.

### B. With Polychrome-Methylene-Blue-Orcein (Unna)

1. Harden in absolute alcohol; imbed; cut.
2. Stain in polychrome methylene blue, 5-15 minutes.
3. Wash in water.
4. Differentiate in 1 per cent neutral orcein in absolute alcohol, 15 minutes.
5. Dehydrate in absolute alcohol.
6. Clear in xylol; mount in balsam.

Nuclei, dark blue; protoplasm, light blue; collagen, dark red; plasma cell granules, greenish blue; mast cell granules, red.

### C. With Water-Blue-Safranin (Unna)

1. Harden in alcohol (or in other media); imbed; cut.
2. Stain in 1 per cent watery solution of water blue, 20 seconds.
3. Wash in water.
4. Counterstain in 1 per cent watery solution of safranin, 5 minutes.
5. Wash in water.
6. Dehydrate and differentiate in absolute alcohol until the section is of a blue colour.
7. Clear in xylol; mount in balsam.

Nuclei, red; protoplasm, violet; collagenous connective tissue, sky-blue.

## III. Elastic Fibres

Elastic fibres are not clearly differentiated by ordinary nuclear or plasma stains. Even van Gieson's method does not yield sharp pictures. Carbol fuchsin colours them a diffuse violet. With Mallory's general stain the elastic fibres are said to assume a deep-red colour, but the appearances are far from distinctive.

Special methods have been therefore devised for the staining of elastic fibres. These fall naturally into two groups—(1) Those which are too complicated, inconstant, and not sufficiently elective, and thus are only of historical interest; and (2) those of modern origin which make the elastic fibres stand out prominently.

Amongst the first group may be mentioned v. Ebener's aniline-red method, Beneke's modification of Weigert's fibrin method, in which the elastic fibres are stained red;

Karl Herxheimer's methods with hæmatoxylin, and with aniline gentian violet and after-differentiation in menthol-vasogen; Martinotti's silver method and safranin method; Ferria's modification of Martinotti's method; Mibelli's method with safranin; Manchot's method with fuchsin and after-differentiation in a solution of sugar and sulphuric acid; Köppe's method; Lustgarten's method; an old one of Unna's with dahlia, and those of Bucci, Wolters, and many others.

A stain which marks the transition between the older and newer methods is the first one of Unna-Tänzer. It was composed of orcein dissolved in alcohol, water, and hydrochloric acid.

The second era commences with Unna's modification of the old Unna-Tänzer method.

### A. Unna's Modification of the Unna-Tänzer Method

1. Harden in absolute alcohol, formalin, sublimate, or Müller's fluid. Imbed; cut.
2. Stain in orcein, 1 gm.; hydrochloric acid, 1 c.c.; water, 100 c.c.; 10-15 minutes. The section should be just covered by the staining fluid and kept at about 30° C., viz. on the incubator. The liquid evaporates and a thick mass results.
3. Wash in 70 per cent alcohol.
4. Differentiate in acid alcohol (0·5-1 per cent HCl), for some seconds.
5. Wash in water.
6. Dehydrate in absolute alcohol; clear in xylol; mount in balsam. Elastic fibres, dark brown.

   The nuclei may be previously stained in lithium carmine, with subsequent differentiation in acid alcohol; the protoplasm may be stained during dehydration in absolute alcohol containing a few drops of alcoholic picric acid solution, the final dehydration being performed in absolute alcohol. The nuclei are then red, the protoplasm is yellow, and the elastic fibres are brown-black.

### B. Pranter's Modification of Unna's Stain

(1) Harden; imbed; cut.

(2) Stain in

| | |
|---|---|
| Orcein D (Grübler) | 0·1 gm. |
| Nitric acid | 2 c.c. |
| Alcohol, 70 per cent | 100 c.c. |

8-24 hours.

(3) Wash in water.

(4) Dehydrate in absolute alcohol.

(5) Clear in xylol; mount in balsam.

Or instead of (2), stain in Orcein D . . 1 gm.
Nitric acid . 5 c.c.
Alcohol, 70 per cent, 100 c.c., for 15-60 minutes.

Pranter prefers the first or slow method.

## C. Weigert's Method for Staining Elastic Fibres

**Preparation of the stain.**—In a porcelain basin place :—

| | |
|---|---|
| Resorcin . . . | 4 gms. |
| Fuchsin (Grübler) . . | 2 gms. |
| Water . . . . | 200 c.c. |

Boil; after the mixture has boiled for a few seconds, add 25 c.c. of "liquor. ferri sesquichlor." Pharm. Germ. III. sp. gr. 1·1; stir well and allow to boil for 5 minutes. When cool, filter. Carefully loosen the filter from the funnel, transfer it to the same porcelain basin (which still contains a small amount of sediment), and add 200 c.c. of 94 per cent alcohol. Boil and stir carefully. Remove the filter-paper when all the sediment is dissolved. Cool; filter; make up the filtrate with 94 per cent alcohol to 200 c.c., and to these 200 c.c. add 8 c.c. of hydrochloric acid. (The powder and solution may now be obtained from Grübler, but do not yield such good staining results as the freshly prepared solution.)

**Staining of elastic fibres.**

(1) Harden in any ordinary solution; imbed; cut.
(2) Stain with lithium carmine, about 1-5 minutes.
(3) Differentiate in acid alcohol, 1 to several hours.
(4) Wash in water.
(5) Stain in the resorcin-fuchsin mixture, 20-60 minutes.
(6) Wash rapidly in acid alcohol.
(7) Dehydrate and differentiate in absolute alcohol until the section appears red.
(8) Clear in xylol; mount in balsam.

Nuclei, red; elastic fibres, blue-black.

With celloidin sections, phenol xylol need not in this instance be used. The acid alcohol (No. 6) is not really necessary. The staining solution is very stable, although this has been denied; our experience has been that it will keep for years, but that as it becomes older it stains diffusely; this fault may, however, be remedied by differentiating with hydrochloric acid for a longer time. Weigert's instructions must, of course, be followed minutely.

To give the elastic fibres very sharp outlines, B. Fischer advises long differentiation in absolute alcohol.

If desired, the elastic fibres alone may be stained by omitting stages 2, 3, and 4, but nuclear staining is useful for diagnosis, and the best results are obtained by staining the nuclei before the resorcin fuchsin rather than afterwards. Stage 3 must be adjusted to the intensity of the carmine stain; as a rule it may be shortened, since the resorcin fuchsin contains hydrochloric acid. It may be omitted altogether, but its use sharpens the outlines.

**Weigert's** method for staining elastic fibres gives most durable preparations, is very practical, and is by far the best method we possess at present. It is peculiarly elective for even the finest elastic fibrils, does not stain the nuclei, and is perhaps the best example of elective staining; consequently it has been of late much used in the examination of elastic tissues, and has been subjected to numerous modifications.

**Minervini,** after staining according to Weigert's directions, places the sections in 0·5 per cent chromic acid solution for one hour, afterwards washing in water, dehydrating in alcohol, clearing in xylol, and mounting in balsam. The elastic fibres then stand out more prominently upon the unstained background.

**Pranter** has modified Weigert's method in almost the same way as he altered Unna's method. He prepares resorcin fuchsin, which may be obtained from Grübler. One of two solutions is used :—

| | | | | | |
|---|---|---|---|---|---|
| (1) | Resorcin fuchsin . . | 0·2 gm. | (2) | Resorcin fuchsin . . | 0·2 gm. |
| | Nitric acid . . . | 1·0 c.c. | | Nitric acid . . . | 3 c.c. |
| | Alcohol (70 per cent) . | 100 c.c. | | Alcohol (70 per cent) . | 100 c.c. |
| | Stain for 8-24 hours. | | | Stain for 15-60 minutes. | |

Pranter prefers the first solution. In both cases, the sections are, without previous differentiation, washed in water, dehydrated, cleared, and mounted in balsam. The staining results are not, however, improved by this modification. Weigert's original solution is not difficult to prepare, and has good keeping properties.

**Mayer** advises the after-addition of a small quantity of ferric chloride to Weigert's solution, in order to obtain more precise results and to avoid simultaneous staining of cartilage and mucin.

**Gustav Mann** adds sufficient of a saturated ferric chloride solution to precipitate the whole of the dye and to obtain a distinct yellow coloration of the supernatant solution, and, after filtration and the addition of alcohol, adds up to 10 c.c. (instead of 4 c.c.) of hydrochloric acid.

**Michaelis,** and more recently **Fischer**, have investigated the components of the staining solution from the theoretical standpoint. According to Michaelis the ferric chloride seems to act as an oxidising medium; the resorcin may be replaced by other phenols and the fuchsin by other basic dyes. **Fischer** finds that **vesuvin** is the best substitute

for fuchsin.[1] His careful researches show also that the ferric chloride and resorcin together form a mordant which promotes the combination between the fuchsin and elastin radicals, and that the staining results not from the fuchsin alone, but from a new fuchsin compound, formed during the preparation of the stain, and possessing specific affinities for elastic tissues.

The staining of elastic fibres may be combined with other specific methods, *e.g.* those for tubercle bacilli and fat.

### D. Staining of Elastic Fibres and Tubercle Bacilli
(Wechsberg)

(1) Harden ; imbed ; cut.
(2) Stain in carbol fuchsin for 1 hour at 37° C.
(3) Differentiate in acid alcohol.
(4) Wash in 70 per cent alcohol.
(5) Stain in Weigert's resorcin-fuchsin solution, 20-60 minutes.
(6) Wash in 96 per cent alcohol.
(7) Transfer to water.
(8) Stain in lithium carmine.
(9) Differentiate in acid alcohol.
(10) Wash in water.
(11) Dehydrate in absolute alcohol ; clear in xylol ; mount in balsam.

The red bacilli are not always easily differentiated from the red-tinged tissues, but the red of the bacilli is a somewhat different tint from that of the tissues.

### E. Staining of Elastic Fibres and Tubercle Bacilli
(G. Herxheimer)

(1) Harden ; imbed ; cut.
(2) Stain in lithium carmine, 1 to several minutes.
(3) Differentiate in acid alcohol, 1 to several hours.
(4) Stain in Weigert's resorcin-fuchsin solution, 1 hour.
(5) Wash rapidly in acid alcohol.
(6) Differentiate rapidly in 96 per cent alcohol.
(7) Stain in aniline gentian violet (*vide* Gram's or Gram-Weigert's stains) for some hours in the cold.
(8) Differentiate in acid alcohol.

[1] Fischer calls this colour vesuvelin, the syllable "el" indicating that Weigert's directions for preparing the solution are followed, except that vesuvin is used instead of fuchsin.

(9) Continue the differentiation in 96 per cent alcohol.
(10) Dehydrate in absolute alcohol ; xylol ; balsam.

Stage 3 is somewhat unnecessary, since the subsequent stain contains hydrochloric acid. If the celloidin is stained violet, it may be removed by treating the preparation with ether alcohol after absolute alcohol.

Nuclei, red ; elastic fibres, blue-black ; bacilli, light blue.

## F. Staining of Elastic Fibres and Tubercle Bacilli

(B. Fischer)

(1) Harden ; imbed ; cut.
(2) Overstain in alum hæmatoxylin.
(3) Wash in water, 30 minutes.
(4) Stain in carbol fuchsin, 24 hours.
(5) Wash in 70 per cent alcohol.
(6) Stain in vesuvelin (or Weigert's solution with the fuchsin replaced by vesuvin), 60 minutes.
(7) Wash in water.
(8) Transfer to dilute lithium carbonate solution, 5 minutes (until the hæmatoxylin becomes blue again).
(9) Wash in running water, 5 minutes.
(10) Dehydrate and differentiate in absolute alcohol, 1 hour.
(11) Clear in xylol : mount in balsam.

Nuclei, blue ; elastic fibres, brown ; bacilli, red.

## G. Staining of Elastic Fibres and Fat (Fischer)

The staining fluid is prepared as follows :—

| | |
|---|---|
| Weigert's resorcin-fuchsin solution | 74 c.c. |
| Water . . . . | 26 c.c. |

Saturate the mixture with Fett ponceau (or Sudan III.). Stain sections (cut by freezing—*vide* "Fat") in this solution for one hour. Differentiate in hot saturated solution of Fett ponceau (Scharlach R) in 70 per cent alcohol, in order to retain the staining of the fat. Mount in glycerin gelatin.

Fat, red ; elastic fibres, blue-black.

Weigert's resorcin-fuchsin stain may be also combined with van Gieson's solution, but the colour tones are less pure.

The staining for elastic fibres may be followed by Weigert's fibrin method ; then the elastic fibres are blue-black, the fibrin and bacteria light blue.

Fischer recommends safranelin (or Weigert's resorcin-fuchsin solution, in which the fuchsin is replaced by safranin) when a combined staining of elastic fibres and fibrin is desired. The elastic fibres are stained first with this method, and then the fibrin by Weigert's fibrin method.

## H. Staining of Elastic Fibres with Kreso-fuchsin

(Röthig)

This solution does not present any advantages over Weigert's staining mixture; in the hands of many workers it gave far less constant results.

**Unna** uses **special staining methods for elacins** (the changed elastin, which then reacts basophile).

I. (1) Stain sections with polychrome methylene blue, 10 minutes.
(2) Wash in water.
(3) Saturated watery tannin solution (about 33 per cent), 15-20 minutes.
(4) Wash thoroughly in water.
(5) Dehydrate in absolute alcohol.
(6) Clear in xylol; mount in balsam.

II. (1) Stain in 1 per cent watery solution of water blue, 2-5 minutes.
(2) Wash in water.
(3) Counterstain in 1 per cent solution of safranin in aniline water, 3 minutes.
(4) Wash in 70 per cent alcohol, 5 seconds; add to the vessel containing alcohol 1 drop of HCl alcohol.
(5) Dehydrate in absolute alcohol.
(6) Clear in xylol; mount in balsam.

To stain the **elacins** and **elastic fibres** at the same time, Unna employs the following methods:—

I. (1) Stain the elastic fibres by the Unna-Tänzer method.
(2) Wash in 70 per cent alcohol.
(3) Wash in water.
(4) Stain in polychrome methylene blue, 5-15 minutes.
(5) Wash in water.
(6) Concentrated watery tannin solution (about 33 per cent), 10 minutes.
(7) Wash thoroughly in water.
(8) Dehydrate in absolute alcohol; xylol; balsam.

II. (1) Stain with Unna-Tänzer's orcein.
(2) Wash in water.
(3) Stain with 1 per cent watery solution of safranin, 5 minutes.
(4) Wash in water.
(5) Dehydrate in absolute alcohol; xylol; balsam.

## IV. Fat

In a fresh preparation, when in large drops or in excess, fat is easily recognised by using a narrow diaphragm and by the addition of fluids, such as acetic acid or dilute caustic soda solution, which make it more distinct (see fresh methods).

Tissues hardened and stained by methods in which strong alcohol has been used, lose their fatty contents, and its previous presence is only suggested by the vacuoles left by the larger globules, as, for instance, in a van Gieson preparation of fatty liver. Fat cannot be therefore stained after ordinary hardening methods, and until recently osmic acid was the only reliable fixing and staining reagent.

The introduction of formalin has, however, furnished a hardening medium which preserves fat and at the same time permits the preparation of frozen sections stainable by dyes possessing special affinities for fat, such as Sudan III. or Fett ponceau. With these stains the tissues may be counterstained by nuclear and other dyes.

Alcannin (Dippel), and cyanin (Certes), and vesuvin (Brandt) have been also used for staining fat, but are not now of practical importance.

### A. The Staining of Fat with Osmic Acid

The usual strengths of osmic acid solutions are all suitable for the staining of fat. Very small pieces may be hardened in Flemming's or Marchi's fluids, washed thoroughly in water, and then hardened in increasing strengths of alcohol and imbedded in celloidin (or in paraffin, if the xylol is replaced by chloroform). Sections may then be made and stained in safranin, this being the most suitable dye for use after osmic acid fixation. The fat is stained black and the nuclei are red. This is the same method as given already for mitoses, except that the differentiation in acid alcohol is omitted, only absolute

alcohol being used; in this case it is the nuclei that are to be stained, not the mitoses. The method is not, however, very reliable, since some of the fat is removed by the alcohol ether or xylol used during the imbedding (Flemming, Altmann, Schmaus).

The osmic acid method should be combined with the freezing microtome method. Osmic acid only stains olein and oleic acid; it does not blacken palmitin and stearin. Starke thought that the latter could be also blackened by treating sections first with $OsO_4$ and subsequently with alcohol. The first blackening is due to the reduction of $OsO_4$ by the olein to $OsO_2$; the second darkening observed by Starke was confirmed by Schmorl, but was shown by Handwerck to be due to a hydration of $OsO_4$ into Os $(OH_4)$ by means of the alcohol, while the purer the stearic and palmitic material became, the less did it reduce osmic acid; oleic acid, if frozen, did not blacken osmium. With osmic acid, absolute alcohol should be avoided, since the fat may loosen and contribute to the formation of ring-shaped granules instead of real granules (Schmorl, Starke) and other false arrangements of fat, even if it be not entirely dissolved.

After the osmic acid has acted upon the tissues, the latter should be washed thoroughly in running water, or the alcohol acts upon and blackens any remaining osmium, so that black granular sediments appear in the preparation (Heidenhain).

The sections are to be mounted in glycerin gelatin. If desired, they may be mounted in warm melted Canada balsam, not in xylol balsam.

When these precautions are observed, good preparations of fatty tissues may be obtained by the following method:—

(1) Harden in formol, 24 hours.
(2) Wash. Freeze and cut on "freezing" microtome.
(3) Place the section in Flemming's or Marchi's fluids or in a 1 per cent watery solution of osmic acid—24 hours, or in Marchi's fluid for 2-3 hours at 30° C.
(4) Wash in running or frequently changed water, 4-6 hours.
(5) Transfer to 80 per cent alcohol for several hours.
(6) Wash in water.

(7) Place the section flat on a slide; dry it with filter-paper; add a drop of warmed, and therefore fluid, glycerin gelatin (Grübler); cover. The medium solidifies rapidly. "Ringing" is unnecessary.

The section may also be mounted in balsam:—

After (5),

[6] Dehydrate rapidly in absolute alcohol.

[7] Clear in pure benzene (not xylol).

[8] Mount in pure melted Canada balsam (not xylol balsam).

Some workers recommend the staining of fat by Heller's method for myelinated fibres (*vide* Chapter XII. VI.). The results are good.

Another method consists in both hardening and staining the sections in osmic acid solutions. The sections should be cut by the "freezing microtome," and then again treated with osmic acid according to the following

**Method of Sata**:—

(1) Harden and stain small pieces of tissue in Flemming's mixture, 2-3 days (in the dark).

(2) Wash in running water, 24 hours.

(3) Cut on freezing microtome; transfer the sections to 1 per cent watery osmic acid solution for 24 hours (or 4 hours at 37° C.) in the dark.

(4) Wash thoroughly for several hours, with frequent changes of water.

[(5) If necessary, stain the nuclei in aniline safranin and differentiate in acid alcohol.]

(6) Place in 80 per cent alcohol for several hours.

(7) Mount in glycerin gelatin or in pure balsam.

By this method the nuclei are stained red.

The osmic acid reaction for fat is not altogether reliable, since other substances, such as tannic acid, become black after contact with osmium. Similarly, some workers (*e.g.* Weidenreich, etc.) doubt if eleidin and para-eleidin are really fatty substances, although they yield the customary reaction for fat by Unna's method,[1] and it would seem that substances stained by Unna's method are not always to be regarded as fatty in nature.

[1] This method consists of a double treatment with osmic acid, in a manner similar to that of Sata.

## B. Staining of Fat with Sudan III. and Fett Ponceau (Scharlach R)

This method is more easy, more reliable, and expresses the total distribution of fat more strikingly than any other method.

Sudan III. was first used for commercial purposes by **R. Nietzki** in 1880, and introduced into histological work in 1896 by Daddi. Michaelis later investigated the action of Sudan III. and similar bodies, and found in Fett ponceau or Scharlach R (Kalle, Biebrich a Rh., and sold by Grübler) an even better stain than Sudan III.

Sudan III. is azo-benzene-azo-$\beta$-naphthol; Fett ponceau is azo-orthotoluol-azo-$\beta$-naphthol, and thus contains two methyl groups. Fett ponceau gives more precise results, but Sudan III. may be always substituted, the chief practical difference being the lighter yellow colour of the latter.

The pigments are dissolved in 70-80 per cent alcohol, since 96 per cent alcohol, as at first used, dissolves the fats. But the stains dissolve very slowly and slightly in the 70-80 per cent alcohol, and the solutions can only be used 24 hours later, while sections require at least 10-20 minutes for staining.

In order to obviate these drawbacks, the following methods of preparation have been introduced :—

1. **Fischer.**—Dissolve the stain in 70-80 per cent boiling alcohol, allow it to stand at 37° C. overnight, and use the solution warm. Stain for 20-30 minutes.

2. **G. Herxheimer** adds the pigment to the following solution :—

| | |
|---|---|
| Absolute alcohol . . . | 70 c.c. |
| Water . . . . | 100 c.c. |
| Caustic soda, 10 per cent solution | 20 c.c. |

A saturated solution of **Fett ponceau** in this solution is best prepared by slightly heating. This alkaline alcoholic solution is advantageous, because the alkali salt of the pigment is much more soluble in the alcohol than the simple pigment, and the solution itself is considerably darker than one made with the pigment alone. Stain for 2-3 minutes.

3. **G. Herxheimer.**

| | |
|---|---|
| Alcohol, 70 per cent . . | 50 c.c. |
| Pure acetone . . . | 50 c.c. |

This mixture is saturated with "Fett ponceau." Acetone dissolves very large quantities of the pigment. The addition of alcohol is necessary, otherwise the acetone affects the fat. Stain for 2-3 minutes.

All these solutions must be carefully filtered or decanted before use, and the staining vessel should be well covered, so as to avoid evaporation and subsequent formation of sediment.

Sections should be transferred from 70 per cent alcohol to the staining mixture. After staining they should not be transferred to water, but rapidly rinsed in alcohol, and then placed in water. The two latter solutions require somewhat longer differentiation in alcohol.

The nuclei of the tissue may be stained with hæmatoxylin. This is best performed after the fat is stained, but with the simple solutions it may be done previously. With the alkaline alcoholic solution, nuclear staining must follow that of the fat, for the alkali would tend to remove the hæmatoxylin. Rapid differentiation of the hæmatoxylin stain in acid alcohol is permissible with the two stronger solutions. If the sections are exposed to ammonia vapour or dipped in weak ammonia or lithium-carbonate solutions for a few minutes, the contrast between the blue nucleus and the red fat is accentuated, and time is saved.

After the fat is stained, and prior to nuclear staining, examine the preparation under the low power, since a small quantity of fat sometimes stands out more clearly when the subsequent nuclear staining is omitted.

These preparations of fat are mounted in glycerin or fluid glycerin gelatin; they cannot be mounted in balsam because of the solvent action of the alcohol and the xylol.

Of the three solutions the acetone mixture seems to give the best results. As the procedures may appear somewhat complicated, the following summary may be found useful:—

(1) Harden in formalin, 24 hours.
(2) Prepare sections by freezing microtome.
(3) Transfer the sections to 70 per cent alcohol.
(4) Stain in the acetone or alkaline-alcoholic solutions 2-3 minutes (in simple solution or Fischer's solution, 20-30 minutes).
(5) Wash in 50-70 per cent alcohol until the ground colour of the section is again more or less white. (With the simple and Fischer's solution, wash very rapidly or not at all.)
(6) Transfer to water.

(7) Dry with filter-paper and mount in glycerin gelatin;

or

(7) Stain in Ehrlich's or Delafield's or alum hæmatoxylin for a short time only, and differentiate rapidly in acid alcohol (if necessary).
(8) Wash in water.
(9) Place in weak ammonia or saturated lithium-carbonate solution.
(10) Wash in water.
(11) Arrange section on slide; dry with filter-paper.
(12) Mount in glycerin gelatin.

Fat, red, even the smallest intranuclear particles being visible; nuclei, light blue.

The nuclei may be also stained in lithium carmine, and the fat subsequently dyed blue in a saturated solution of *indophenol* in 70 per cent alcohol for 20 minutes (Herxheimer).

For the staining of fat in secretions and excretions, Rieder adds equal parts of saturated Sudan III. or Fett ponceau in 70 per cent alcohol to the material under investigation, centrifugalises or sediments, and examines the deposit.

**Levinson**, with the same object in view, adds Sudan III. in 70 per cent alcohol, 2 parts, and 10 per cent formol, 1 part.

**Michaelis** finds that when the alkaline alcoholic stains are used for cover-glass preparations, the film sometimes loosens, perhaps from the swelling of the tissues.

**Myelin** is considered in connection with fat, but the term has not yet been precisely defined. On the one hand it is regarded as chemically different from fat; on the other it is considered to arise from a physical alteration of the fat particles. Under the term "myelin" **Kaiserling** and **Orgler** include a group of fat-like substances, which stain with less intensity in solutions of fat dyes, and which, unlike fat, are doubly refractive.

**Fatty acids and soap.**—The staining of these substances is described in the section on fat necrosis (*vide* Chapter XIII.).

The use of fat stains in injections, in connection with the process for staining tubercle bacilli, and for the staining of amyloid or lardaceous material, is stated in the sections devoted to these subjects.

## V. Fibrin

Fibrin is stained by the acid aniline dyes (eosin, picric acid, etc.); after hardening in Flemming's fluid and staining in safranin, it is bright red; in van Gieson's mixture it becomes yellow; and in Mallory's stain, red. Fibrin-like threads which are stained yellow by van Gieson's stain suggest fibrin, but since other substances are

similarly stained, it is best to confirm by one of the following specific methods.

### A. Weigert's Fibrin Method

By this method, in addition to the fibrin, cocci and certain bacteria are stained a deep blue, as with Gram's method. The sections are stained in aniline gentian violet, then treated with iodine and dehydrated and differentiated in a mixture of aniline and xylol, the latter being the characteristic feature of the method. Absolute or strong alcohol is avoided, as it rapidly decolourises the fibrin.

The aniline gentian violet was formerly prepared as follows:—

Shake 10 c.c. of aniline oil with 100 c.c. of water; pass through a moist filter. To 90 c.c. of the filtrate add 11 c.c. of a saturated alcoholic solution of methyl violet. Filter. (Zenker adds the solid violet to the aniline water.) The solution keeps for 2-3 weeks only.

To obviate the difficulties of preparation, Weigert later introduced the following mixtures, which keep for years and only require to be mixed just before using:—

| **Stock Solution I.** | | **Stock Solution II.** |
|---|---|---|
| Absolute alcohol . . | 33 c.c. | Saturated watery solution of methyl violet. |
| Aniline oil . . . | 9 c.c. | |
| Methyl violet in excess. | | |

**For Staining Purposes:—**

Take of stock solution I. . 3 c.c.
Take of stock solution II. . 27 c.c. } Keeps for 14 days only.

It will be observed that methyl violet is used, not gentian violet, which is really an impure methyl violet.

The iodine solution used at first by Weigert was Lugol's solution (iodine, 1; potassium iodide, 2; water, 300), but later he employed a solution of 5 per cent watery potassium iodide saturated with iodine.

The aniline xylol was at one time composed of aniline oil, 2 parts; xylol, 1 part; recently, equal parts of aniline oil and xylol are advised. **Beneke** used 3 parts xylol and 2 parts aniline oil, in order to demonstrate elastic fibres, epithelium, etc., at the same time.

The section must be firmly fixed to a slide before staining, or it will shrink during the subsequent manipulations. Paraffin sections should be affixed in the usual manner. Celloidin sections are transferred from water to a slide previously cleaned and rubbed over with alcohol, arranged in order, dried thoroughly between folds of filter-paper, and pressed evenly. As a rule the section will then adhere to the slide. If not, the procedure may be modified by covering the section with a few drops of absolute alcohol and then with a **very dilute** celloidin solution, which, on drying, forms a fine film.

Nuclear structures may be stained in lithium carmine. When the tissues are hardened in chromic mixtures, and sometimes even after formalin, fibrin and bacteria do not stain well. Such tissues, however, when oxidised in potassium permanganate and then reduced in oxalic acid, permit successful staining (Weigert). The method is thus of very general application.

Proceed as follows :—

(1) Harden in absolute alcohol, formol, Müller's fluid, or mercuric chloride. Wash ; harden in alcohol ; imbed ; cut. (Good results may be also obtained with "frozen" sections, *q.v.*)

For special cases only (see above).

(2) Transfer to a 1 per cent solution of potassium permanganate to which 2 volumes of water have been added, 10 minutes (oxidation).

(3) Wash thoroughly in water.

(4) Immerse in 5 per cent watery oxalic acid solution, several hours (reduction).

(5) Wash in water.

(6) Stain in lithium carmine, 1 minute.

(7) Differentiate in acid alcohol, several hours.

(8) Wash in water. Dry thoroughly. (Unmounted sections to be fixed on the slide : see above.)

(9) Stain on the slide in aniline methyl violet, 20-30 seconds.

(10) Remove stain ; dry section with filter-paper.

(11) Iodine solution ; strong solution requires 20-30 seconds only.

(12) Remove iodine ; dry section thoroughly with filter-paper.

(13) Differentiate in aniline xylol until the dye commences to leave the nuclei and the ground colour is red. (Control, if necessary, under low-power objective.)

(14) Pour on a few drops of xylol ; dry with filter-paper. Repeat the process three times, or until the section is quite transparent when viewed on a dark background, viz. until

the water is completely removed. The aniline oil must also be entirely removed with xylol or the colour fades after a time.

(15) Mount in balsam.

Nuclei, red; fibrin, glistening blue; bacteria, a somewhat darker blue; mucin, keratin, clumped Altmann's granules (Lubarsch), also blue.

The times given for staining differ from **Weigert's** original directions, but are in accord with his later practice.

If the differentiation is carried too far, then only the fibrin and the bacteria will retain the stain. In such case, however, the finer fibrin threads will be decolourised. It is well to control the differentiation under the low power of the microscope and to stop the action of the aniline xylol when all the fibrin remains coloured. In such case the connective tissue, nuclear network, etc., are still stained, but they are so morphologically different that little difficulty will be experienced in differentiating them from the fibrin.

## B. Kockel's Fibrin Method

This is essentially a modification of Weigert's "myelin" method.

(1) Harden in any of the usual media; imbed; cut. Thin paraffin sections are best. Remove the paraffin; xylol; alcohol.
(2) Immerse sections in 1-5 per cent watery chromic acid solution, 5-10 minutes.
(3) Wash in water, until the section becomes pale but still distinctly yellow, 5-20 seconds.
(4) Stain in Weigert's hæmatoxylin solution for myelin (old recipe), 15-20 minutes.

| | | |
|---|---|---|
| Hæmatoxylin | 1 gm. | When solution is complete, add 1 c.c. of saturated lithium-carbonate solution. |
| Absolute alcohol | 10 c.c. | |
| Water | 90 c.c. | |

(5) Wash in water.
(6) Place in 10 per cent watery alum solution until the section becomes deep blue.
(7) Wash in water.
(8) Differentiate in Weigert's borax ferricyanide solution (myelin method), for 3-6 minutes, until the fibrin is black and the ground tone is yellowish brown (best controlled under the microscope).

| | | |
|---|---|---|
| Borax | 2 gms. | Dilute this solution with 3 volumes of water. |
| Ferricyanide of potassium | 2·5 gms. | |
| Water | 100 c.c. | |

(9) Wash in water, 3-5 minutes.
(10) Transfer the section to saturated watery solution of alum, 15-60 minutes.
(11) Wash in water.
[(12) If desired, stain the nuclei in borax carmine or dilute safranin solution.]
(13) Dehydrate in absolute alcohol; xylol; balsam.

Fibrin, dark brown to dark blue ; ground colour, light grey to light brown or even colourless ; nuclei, after carmine and safranin staining, red.

Sometimes (especially after hardening in chromic acid), red blood corpuscles, muscle fibres, horn, hyaline masses, and sometimes elastic fibres and bacteria, are also stained brown.

The differentiation by this method is difficult. Thick sections should be allowed to remain a longer time in the potassium ferricyanide solution.

In using Kockel's method, we find, as a rule, that thick sections give better results, and that the fibrin stands out more clearly when the after-staining with carmine or safranin is omitted.

To avoid the effect of the hardening fluids (formol, sublimate), as manifested in the staining of the red blood corpuscles, etc., in this method, Kockel places the section in 5 per cent watery acetic acid for 5 minutes before staining. We have not been able by this means to entirely prevent the corpuscles from staining, without prolonging the acid bath and thus altering the staining affinities of the fibrin.

On the whole, although the preparations are very permanent, the method is less to be commended than that of Weigert. It is complicated, rather difficult successfully to complete, and does not yield the clear staining results which are obtained by following Weigert's directions.

The chief advantage of Kockel's method is said to lie in its application to chromic acid hardened tissues ; but this is not an advantage over Weigert's method, since the latter may be also similarly used after treating the sections with potassium permanganate and oxalic acid.

Kockel, however, rightly recommends this method as useful when it is necessary to confirm Weigert's method for the presence of fibrin.

C. Fibrin may also be stained by **Mallory's chloride of iron** hæmatoxylin method :—

1. Fix as preferred (formalin does not permit very good staining results).
2. Imbed in celloidin or paraffin ; cut.
3. Cover sections on the slide with 10 per cent aqueous ferric chloride solution, 3-5 minutes.
4. Drain and blot the section. Cover with 1 per cent freshly prepared aqueous solution of hæmatoxylin. If all the hæmatoxylin is precipitated by the excess of ferric chloride, pour off the solution and add a fresh supply. In 3-5 minutes the sections are coloured a dark blue-black.
5. Wash in water.
6. Decolourise and differentiate in ½ per cent aqueous solution of ferric chloride, keeping the sections constantly moving in the solution, a few seconds to 1 or more minutes.
7. Wash in water.
8. Dehydrate in alcohol ; origanum oil ; balsam.

Fibrin, greyish to dark blue ; nuclei, dark blue ; connective tissue, pale yellow.

When Zenker's fluid is used for fixation, the red blood corpuscles are greenish grey.

## VI. Mucin

The chemical characters of mucin are important, because they form the basis of its reactions in fresh preparations. Mucin is precipitated by alcohol and by acetic acid —pseudo-mucin is not affected by acetic acid—and the

acid precipitate is soluble in alkalies and in neutral salt solutions.

The fresh material is examined on a slide in water—not in neutral salt solution—and a drop of acetic acid is drawn underneath the cover-glass by means of blotting-paper placed at the opposite side.

Mucin often reacts in different ways, and the term represents a group of similar substances, rather than a single substance possessing specific characters. The various substances are generally acid in nature, and hence may be stained with basic aniline dyes, such as methylene blue, Bismarck brown, dahlia, methyl violet, thionin, toluidin blue, safranin, etc. With van Gieson's method mucin is, as a rule, coloured blue (hæmatoxylin); a similar appearance follows the use of Weigert's fibrin stain. After hardening in mercuric chloride, mucin is coloured light green by Ehrlich-Biondi's tri-acid stain.

There are several "elective" stains for mucin. They may be classified in two groups; the first depends upon meta-chromatic staining.

Mucin is stained by hæmatoxylin, but, according to Mayer, only when a small amount of alum is present, and when there is a complete absence of any free acid. These conditions are difficult to attain, on account of the acid properties of alum.

### A. Hoyer's Thionin Stain for Mucin

(1) Harden. Watery sublimate solution (2-8 hours) is better than alcohol. Wash rapidly in water; harden in 90-96 per cent alcohol, changing frequently (no iodine alcohol as in ordinary sublimate hardening); absolute alcohol; imbed in paraffin; cut; fix section on slide; remove the paraffin.

(2) Place the section in 5 per cent watery mercuric chloride solution, 3-5 minutes.

(3) Wash in alcohol (or water).

(4) Stain in dilute thionin solution (2 drops of hot saturated watery thionin solution to each 5 c.c. of water), 5-15 minutes.

(5) Wash in 96 per cent alcohol.

(6) Dehydrate rapidly in absolute alcohol.
(7) Clear in xylol or in a mixture of 4 parts of oil of thyme and 1 part of oil of cloves.
(8) Mount in balsam.

Nuclei, blue; mucin, mast cell granules, cartilage, and lardacein or amyloid, red.

The colours are most striking at (5), and the section may then be examined in water, instead of being mounted. Toluidin blue may be substituted for thionin; preparations stained with toluidin blue are more permanent.

## B. Unna's Polychrome Methylene Blue Stain for Mucin

(1) Harden in alcohol; imbed; cut.
(2) Stain in polychrome methylene blue, 10 minutes.
(3) Wash rapidly in slightly acidified water.
(4) Fix the section in 10 per cent potassium-bichromate solution, 30 seconds.
(5) Wash in water. Dry on slide with filter-paper.
(6) Differentiate in aniline oil containing 1 per cent hydrochloric acid, 10-30 seconds.
(7) Dehydrate in absolute alcohol; xylol; balsam.

Nuclei, blue; mucin, cartilage, amyloid or lardacein, red.

## C. Mayer's Muc-hæmatein Stain for Mucin

**Staining solution :—**

| | |
|---|---|
| Hæmatein | 0·2 gm. |
| | (mixed with a few drops of glycerin). |
| Aluminium chloride | 0·1 gm. |
| Glycerin | 40 c.c. |
| Water | 60 c.c. |
| or | |
| Hæmatein | 0·2 gm. |
| Aluminium chloride | 0·1 gm. |
| Alcohol, 70 per cent | 100 c.c. |
| Nitric acid | 1-2 drops. |

Proceed as follows :—

(1) Harden in absolute alcohol; imbed; cut.
(2) Stain in either of the above solutions, 5-10 minutes. The first is the most rapid and the best for ordinary use; the alcoholic solution is preferable if the mucin swells rapidly.
(3) Wash in water.
(4) Dehydrate in absolute alcohol; xylol; balsam.

Mucin, blue; no other tissues are stained. The nuclei may be counterstained with carmine.

### D. Mayer's Muci-carmin Stain for Mucin

**Staining solution :—**

| | | |
|---|---|---|
| Carmine . . | 1 gm. | Heat over a flame for 2 minutes and continue the staining until the mixture darkens. |
| Aluminium chloride . | 0·5 gm. | |
| Water . . | 2 c.c. | |
| Add alcohol, 50 per cent | 100 c.c. | |

Filter after 24 hours.

The stock solution may be diluted with water (1-10). If the mixture does not stain well, add 0·5-1 gm. of aluminium chloride. Only mucin is stained a red colour. The nuclei may be counterstained with hæmatoxylin or hæmatein if desired.

Method A is the simplest and the most extensively used; C and D give the most delicate reactions.

## VII. Colloid and Hyaline Substances

Colloid material is normally present in the acini of the thyroid gland, and occurs pathologically as a degeneration. Hyaline is a substance which, according to von Recklinghausen, resembles amyloid in its physical characters, but does not give the chemical reactions of the latter. "Hyaline" is also applied to structureless masses associated with several forms of degeneration. The two substances cannot be sharply differentiated, and the terms probably include substances of varying chemical composition.

There are no specific stains for these two substances.

Colloid stains with eosin, picric acid, and other acid dyes; it also stains slightly with basic aniline dyes. With van Gieson's stain, colloid takes the mixed colour orange, although it may at times stain a pure yellow or pure red.

Hyaline is readily recognised in unstained preparations by its clear, transparent appearance. With eosin or van Gieson's stains it is coloured a brilliant red; with Weigert's fibrin stain it assumes the same blue colour as does "colloid." van Gieson's stain gives the best results, and may be specially recommended for the staining of hyaline and colloid, as well as for the colloid of the thyroid gland. **Ernst** supposed that with this method pure hyaline, which is said to originate from connective tissues, stains only

with the red fuchsin, while colloid, for instance, derived from the glandular epithelium, absorbs both protoplasmic colours, and is thus stained orange; but these differences, according to **von Kahlden** and **Lubarsch**, shed no certain light on the exact origin of the substances. **Pianese**, by means of his five methods (one of which is stated in Chapter X. II. F), has stained the "colloid" of the thyroid gland quite a different colour from the "hyaline" of hyaline glomeruli. But whether such differences can be demonstrated in all such similar substances is questionable. **Unna** has also given some methods for these substances.

With **Russell's** method (Chapter X. II.) hyaline is stained red.

### VIII. Amyloid or Lardacein

Amyloid or lardacein is only met with under pathological conditions, and in composition is somewhat akin to the hyaline substances. **Lubarsch** has demonstrated the transition stages between amyloid and hyaline, as well as the staining affinities between amyloid and mucin. Amyloid has the same glassy, refractive appearance as hyaline, and with van Gieson's stain assumes a yellow colour; it may, however, be sharply separated from hyaline and allied substances by characteristic and specific staining reactions.[1] If, therefore, when using general stains, *e.g.* van Gieson's stain, the presence of yellow masses suggests amyloid, resort must be had to the "special" stains in order to exclude other bodies which are stained "yellow" by van Gieson's method. However, it must be pointed out that the amyloid material sometimes stains capriciously, and does not always give all the usual reactions; this is especially the case with the so-called localised amyloid tumours, which may give one or another reaction, but not a third one.

Fairly fresh material permits the best staining results—tissues preserved in alcohol for a long period stain poorly,

[1] Hyaline and amyloid are both stained by methyl violet: on differentiation in acetic acid, the stain is removed from the hyaline material but not from the amyloid, which retains its red tint.

and sometimes not at all. Formol, acting for a long time, seems to diminish the staining properties still more markedly.

Lubarsch states (*Encyclopädie*) that imbedded tissues containing amyloid also stain less electively than when they are hardened in formalin for 24 hours and then cut on the freezing microtome. We have observed the same effects.

The chief reactions may now be given: first must be described the classical method to which amyloid owes its name:—

## A. Reaction of Amyloid with Iodine

(1) Stain in Lugol's solution (diluted with three volumes of water, and, if desired, containing an added 25 per cent glycerin), 5-10 minutes.
(2) Wash thoroughly in water.
(3) Examine in water or glycerin. Permanent preparations may be obtained by mounting in glycerin gelatin. (Potassium iodide lowers the "setting" point of gelatin.)

Amyloid, mahogany brown; all else, yellow.
The staining is very unstable.

According to Lubarsch, the tissue may be previously stained in Mayer's alcoholic carmine solution.

If the sections, after being placed in iodine, are transferred to a 1 per cent solution of sulphuric acid for 3-5 minutes, then the amyloid assumes a bluish tone; instead of the blue, other similar shades may appear, the reaction being then irregular and not distinct. This further treatment may be interposed in the above described method between stages 2 and 3. Cholesterin (*q.v.*) also gives these iodine reactions; starch granules are stained blue by iodine alone; cellulose and the corpora amylacea of the central nervous system and the prostate are similarly affected.

Langhans' iodine method for glycogen (*q.v.*) is the same in principle, but yields more permanent results:—

(1) Stain in Lugol's solution, 5-10 minutes.
(2) Dehydrate in absolute alcohol, 4 parts; official tincture of iodine, 1 part.
(3) Clear and mount in origanum oil.

Ring the preparation with paraffin, gold size, or shellac.

This iodine reaction will always be used for the macroscopic identification of amyloid in post-mortem work, but

whether it is more reliable for microscopic purposes than the methods of staining now to be described is a moot point, and one, in fact, which has been, as well as now is, the subject of much discussion. We consider the following methods, based on the fact first observed by **Jürgens**, that certain aniline dyes stain amyloid a different colour from that which they stain other substances, viz. metachromatically, to be the more useful and important. Other substances, however, give similar reactions, and somewhat detract from the diagnostic value of these stains.

The most widely known is :—

## B. The Staining of Amyloid with Methyl Violet

(1) Stain in dilute methyl-violet solution (best 1 per cent diluted with equal parts of water), $\frac{1}{2}$ to more minutes.

(2) Wash in water.

(3) Differentiate in 2 per cent acetic acid solution, 1 to more minutes.

(4) Wash thoroughly in water.

(5) Mount in glycerin or glycerin gelatin.

Amyloid, red ; tissues, blue violet.

Dilute hydrochloric or nitric acid may be substituted for the acetic acid.

The colour is more permanent when the preparation is mounted in lævulose (Weigert).

The nuclei may be stained, in addition to the amyloid, by Birch-Hirschfeld's or Green's methods.

**Birch-Hirschfeld's method** :—

(1) Stain the section with Bismarck brown (2 per cent alcoholic solution), 5 minutes.

(2) Rinse in absolute alcohol.

(3) Wash in water.

(4) Stain in 2 per cent watery methyl-violet solution, 5 minutes.

(5) Differentiate in 1 per cent acetic acid until the section is brown again.

(6) Wash thoroughly in water.

(7) Mount in glycerin, in glycerin gelatin, or in lævulose.

Nuclei, brown ; amyloid, red.

**Green's method : Staining solution :—**

| | | |
|---|---|---|
| Hæmatein | 1 gm. | Dissolve by placing in a warm incubator; when dissolved, add 1000 c.c. of distilled water. |
| Alcohol . | 50 c.c. | |
| Alum . | 60 c.c. | |

To a few c.c. of this solution in a watch-glass add a saturated solution of methyl violet, drop by drop, until the mixture shows a faint purple-red tinge at the edge of the glass.

(1) Stain sections in the solution, 15-30 minutes.
(2) Differentiate in acid alcohol ($\frac{1}{100}$) until the purple begins to fade.
(3) Wash for 2 minutes in several changes of water.
(4) Mount in Farrant's solution or in glycerin ; or
  (4*a*) Remove excess of water with filter-paper.
  (5*a*) Dehydrate in pure liquid paraffin (best lamp oil).
  (6*a*) Remove the paraffin with xylol.
  (7*a*) Dry with blotting-paper to remove the xylol.
  (8*a*) Mount in pure white vaseline.
    (Iodine preparations may also be mounted in this way.)

Nuclei, blue ; amyloid, red.

Amyloid also stains metachromatically with other blue and also green aniline dyes, assuming, as a rule, a red or red-violet colour. The following methods exemplify this :—

## C. Staining of Amyloid with Methyl Green

The procedure is the same as in method B, the methyl green being substituted for the methyl violet.

Amyloid, red violet ; nuclei, green.

## D. Staining of Amyloid with Iodine Green (Stilling)

(1) Stain in $\frac{1}{3}$ per cent watery solution of iodine green, 24 hours.
(2) Wash in water.
(3) Mount in glycerin, glycerin gelatin, etc.

Amyloid, red violet ; nuclei and tissues, green.

We find it more useful to stain in a 1 per cent, or even stronger iodine-green solution, and to differentiate in acetic, hydrochloric, or nitric acids.

## E. Staining of Amyloid with Thionin (Kantorowioz)

(1) Harden in alcohol or mercuric chloride.
(2) Stain in saturated watery solution of thionin, 3-5 minutes.
(3) Wash in water.
(4) Spread section on slide ; dry with filter-paper.
(5) Clear in phenol xylol (or aniline xylol 2 : 1).
(6) Cover with xylol a few times and dry.
(7) Mount in dammar.

Amyloid, light blue ; nuclei, violet ; mucin, red.

In our experience the phenol xylol in number (5) causes the colour almost to disappear.

## F. Staining of Amyloid with Polychrome Methylene Blue

Formol hardening is the best, but other media may be used.

(1) Stain in polychrome methylene blue, 10-15 minutes.
(2) Wash in water.
(3) Treat the section with ½ per cent acetic acid, 10-20 seconds.
(4) Place in saturated watery alum solution 1 part, water 1 part, 2-5 minutes.
(5) Dehydrate rapidly in absolute alcohol, 30 seconds.
(6) Clear in xylol ; mount in balsam.

Amyloid, red ; nuclei, etc., blue. The colours are relatively permanent.

The following method does not depend upon metachromatic staining :—

## G. Staining of Amyloid with Fett Ponceau

(G. Herxheimer)

This method is based on the fact that the affinities between amyloid and Fett ponceau are stronger than those of the normal tissues for the pigment ; the amyloid is overstained, and the colour then removed from the tissues by differentiation. The amyloid is stained bluish red, the fat yellow-red, and these tints are quite distinctive. The method does not always give successful results, but when it does, the nuclear staining with hæmatoxylin is very sharp, and the fat, so often present in amyloid kidneys, etc., is also stained, the three substances standing out quite clearly.

Harden the tissue in formol, cut on the freezing microtome :—

(1) Stain in a hot, saturated solution of Fett ponceau in

| | |
|---|---|
| Absolute alcohol . . . | 70 c.c. |
| Caustic soda (10 per cent solution) . | 20 c.c. |
| Water . . . . | 10 c.c. |

(½ to more hours)

(2) Differentiate in 70 per cent alcohol until the non-amyloid portions of the sections are light in colour.
(3) Stain in hæmatoxylin solution.
(4) Wash in water.
(5) Place the section in weak ammonia water or saturated lithium carbonate until blue.
(6) Wash in water ; dry.
(7) Mount in glycerin gelatin.

Amyloid, red ; nuclei, blue ; fat, red.

## IX. Glycogen

Although some of the reactions of glycogen are similar to those of amyloid, the two substances can be sharply differentiated. Glycogen, in marked contrast to amyloid, except in certain special structures, such as cartilage cells, is readily soluble in water, easily digested by saliva, and does not give the iodine-sulphuric acid reaction. It is normally present in almost all the organs of the body, especially in the liver, muscles, kidneys, and cartilage. Under pathological conditions it is found in diabetic kidneys (Ehrlich), in leucocytes (Ehrlich), and in some tumours. Rarely occurring in the interstitial tissues, it is met with, as a rule, in the form of diffuse masses or small or large globules lying in the cellular protoplasm, and probably attached to, or combined with, the granules of the cell (Lubarsch, *Encyclopädie*, ii. p. 440).

For a study of the reactions of glycogen a diabetic kidney should be obtained, or the liver of a freshly killed animal may be substituted. Fixation and hardening in almost all the methods must be performed in absolute alcohol since water dissolves the glycogen, and the organs must be placed in the fixing fluid as *rapidly* as possible after death, since glycogen is broken up and disappears from the cells very quickly.

The following methods are based upon the iodine reactions of glycogen :—

### A. Langhans' Method

(1) Harden in absolute alcohol ; imbed (best in paraffin) ; cut.

(2) Stain in Lugol's solution, 5-10 minutes.

(3) Dehydrate in 1 part of tincture of iodine and 3 or 4 parts of absolute alcohol.

(4) Clear and mount in origanum oil. Ring the preparation with paraffin or gold size and shellac.

Glycogen, dark brown.

## B. Barfurth's Method

1. Harden as in A.
2. Spread and fix section on slide.
3. Stain and mount in a mixture of

| | |
|---|---|
| Glycerin . . . . | 1 part. |
| Potassium iodide, 3; iodine, 1; water, 500 | 1 part. |

Stronger solutions also may be used.

## C. Ehrlich's Method

This method avoids the contact of the glycogen with water almost entirely, and is therefore the most reliable of these methods :—

(1) Harden as in A.
(2) Arrange and fix section on slide.
(3) Stain and mount in gum arabic, 100 parts, with the addition of Lugol's solution, 1 part (or gum arabic may be added to Lugol's solution until a thick syrupy fluid results).

Cover-glass preparations may be placed in stoppered vessels containing a few crystals of iodine. The glycogen is then also stained (Ehrlich).

## D. Delépine's Method

1. Harden in absolute alcohol; imbed in paraffin or celloidin; cut.
2. Stain in

| | |
|---|---|
| Iodine . . . . | 1 |
| Absolute alcohol . . . | 4 |

3. Clear in oil of cloves.
4. Replace the oil of cloves by chloroform containing iodine.
5. Mount in chloroform balsam, in which iodine is dissolved.

The success of the method depends upon the rapid fixation of the cover-glass. If the procedure is slowly performed the iodine crystallises out.

**Driessen** (*Centrlb. f. Allge. Path.* 1905, s. 131) stains sections from absolute-alcohol-hardened tissues in saturated alcoholic cochineal solution or in Mayer's carmine solution, decolourises in 96 per cent alcohol, transfers to absolute alcohol for 3 minutes, and then places the section in phenol xylol which has been saturated with Lugol's solution and the water removed. He differentiates, if necessary, in phenol xylol, and mounts in Canada balsam.

## E. Lubarsch's Method

(1) Harden in absolute alcohol; imbed in paraffin; cut.
(2) Stain sections for 5 minutes in

| | |
|---|---|
| Delafield's hæmatoxylin | 10 c.c. |
| Gram's iodine | 10 c.c. |
| Water | 5 c.c. |

Filter. Keep in cupboard away from sunlight.

(3) Dehydrate in absolute alcohol.
(4) Clear in xylol; mount in balsam.

Glycogen, yellow brown to mahogany brown; nuclei, blue.

Lubarsch has also proposed another similar staining solution.

## F. Best's Iodine Method

(1) Harden in absolute alcohol; imbed in paraffin; cut.
(2) Stain somewhat deeply with hæmatoxylin.
(3) Wash in water.
(4) Stain in iodine, 1; potassium iodide, 2; water, 100.
(5) Dehydrate in iodine, 2; absolute alcohol, 100.
(6) Differentiate in origanum oil, 1-2 hours.
(7) Wash thoroughly with xylol.
(8) Arrange and fix on slide; allow to dry in the air.
(9) Mount in melted pure Canada balsam (not xylol balsam).

Glycogen, brown; nuclei, blue.

This method gives somewhat permanent results.

Glycogen may be also stained by fibrin stains, and the next method is a

## G. Modification of Weigert's Fibrin Stain by Lubarsch

(1) Harden in absolute alcohol; imbed in paraffin; cut.
[2] Stain in Mayer's alcoholic carmine.
[3] Differentiate in acid (HCl) alcohol.
[4] Wash in absolute alcohol.
(5) Stain with an old and strong aniline-methyl violet solution, slightly warming, 2 minutes.
(6) Wash rapidly in water.

(7) Dip into Gram's iodine, repeating the procedure several times, 5-10 seconds in all.
(8) Dry with filter-paper (see Fibrin Stain).
(9) Differentiate in aniline oil, 2 parts; xylol, 1 part.
(10) Add xylol several times, drying between each addition.
(11) Mount in balsam.

Glycogen, blue to violet; nuclei, red.

The preparations, according to Lubarsch, are more permanent if, after 1-2 days' exposure to daylight, they are kept in the dark. The method is not very reliable.

**Best** has recently investigated the conditions under which glycogen may be stained and demonstrated by the use of watery dyes. He has evolved two methods, both of which yield sharp, well-stained preparations. A slight drawback is the number of solutions, etc., required, but the results are well worth the trouble. The second method is a modification of the first; the solutions used are more reliable, and constitute an improvement upon the earlier process. For such reason the second method is here alone described.

## H. Best's Carmine Method for Staining Glycogen

Glycogen is stained by carmine dissolved in lithium-carbonate solution when the mixture has reached a certain maturity; the latter process may be hastened and made constant by the addition of chlorate of ammonium and boiling. Alcohol, added in quantity just sufficient to bring the solution within the border-line of precipitation, also assists the staining. Sections treated with such a mixture are differentiated in alcohol, not in water, which would wash out the stain.

**Staining mixture :—**

| | | |
|---|---|---|
| Carmine . . . | 1 gm. | Bring to boiling point, and when cool, add 20 c.c. of strong liquid ammonia. |
| Chlorate of ammonium | 2 gms. | |
| Lithium carbonate . | 0·5 gm. | |
| Water . . . | 50 c.c. | |

The solution should be kept in the dark. It may be used for staining after standing for 2 or 3 days, and gives good results up to 8-14 days.

The tissues should be imbedded in celloidin, which hinders the diffusion of glycogen in watery fluids (Best).

The details of the method are as follows :—

(1) Harden in absolute alcohol (formol, sublimate, etc., may also be used). Imbed in celloidin ; cut.

(2) Stain in hæmatoxylin ; differentiate in acid (HCl) alcohol (this previous staining is absolutely necessary).

(3) Wash in water.

(4) Stain in—

| | |
|---|---|
| Filtered carmine mixture . | 2 parts. |
| Strong liquid ammonia . . | 3 parts. |
| Methyl alcohol . . . | 6 parts. |

This mixture should be freshly prepared each time it is required, and not filtered ; only a few sections should be stained at once. A carmine precipitate soon falls. Stain $\frac{3}{4}$-1 hour.

(5) Differentiate for a few minutes in several changes of—

| | |
|---|---|
| Methyl alcohol . . . | 2 parts. |
| Absolute alcohol . . | 4 parts. |
| Water . . . . | 5 parts. |

(6) Wash in 80 per cent alcohol.

(7) Dehydrate in absolute alcohol.

(8) Clear in xylol or oil ; mount in Canada balsam.

Glycogen, red ; nuclei, blue ; dense connective tissue, sometimes mast-cell granules and secretions and protoplasm of gastric glands, red.

The latter substances cannot be confused with glycogen morphologically, and they give distinctive chemical reactions, not yielding the iodine reaction of glycogen and not being digested by saliva, whereas glycogen, after being treated with saliva, is not stained by carmine.

This method gives pretty as well as reliable pictures, the relations and positions of the glycogen being accurately delineated. Lubarsch also considers this to be the best method for the staining of glycogen, and we have always obtained good results with it.

## X. Cholesterin

Cholesterin is soluble in absolute alcohol, xylol, ether, and glacial acetic acid. With iodine or Lugol's solution it assumes a brown colour, which turns blue-violet after the addition of strong sulphuric acid, and changes therefrom to blue, and sometimes to green, and finally to red.

Concentrated or unmixed strong solutions of sulphuric acid, when poured upon sections or masses containing cholesterin, cause the crystals to become yellow and then rose pink in colour. This reaction is intensified by slightly heating the preparation.

Cholesterin crystallises in characteristic small and large rhombic plates. The crystals often lie in small masses, and a square notch is sometimes present at one of the corners.

The contact of the iodine or the sulphuric acid with the section should be effected by drawing these reagents under the cover-glass. The entire reaction can then be observed through the low or high objective.

## XI. Horn, Keratohyalin, Eleidin

**Horn** may be stained by the plasma stains, *e.g.* by acid dyes, such as picric acid, eosin, etc. The normal cuticle, cutaneous horns, and the "cell nests" of epithelioma all stain well with picric acid, and therefore van Gieson's method is useful for this purpose. The Ehrlich-Biondi-Heidenhain's method stains horn red, and Gram's method yields a blue colour. Ernst first called attention to this combination, by means of which the horny substance is stained thoroughly and resists the after-differentiation with acid alcohol. This latter method is thus of great value for the demonstration of horny structures.

Horn may be also stained in safranin or gentian violet solutions, after preliminary hardening in Flemming's solution, in accordance with the method for mitoses previously described (Reinke).

**Keratohyalin** occurs as fine granules in cells of the stratum granulosum. In weak alkalies these granules swell up; in strong mineral acids they are dissolved. They may be stained in hæmatoxylin, carmine, by Gram's process, and by Weigert's fibrin method.

## Special Staining Methods for Keratohyalin

### A. K. Herxheimer's Method

(1) Harden ; imbed ; cut.
(2) Place the section in absolute alcohol, 24 hours.
(3) Stain in aniline methyl violet, 30 minutes.
(4) Differentiate with 2 per cent menthol vasogen (from Pierson, Hamburg), 15-30 minutes.
(5) Clear in xylol ; mount in balsam.

Keratohyalin granules, blue-violet.

### B. Unna's Method

(1) Harden ; imbed ; cut.
(2) Overstain in hæmatoxylin.
(3) Place the section in $\frac{1}{2}$ per 1000 potassium-permanganate solution, about 10 seconds.
(4) Differentiate and dehydrate in absolute alcohol.
(5) Clear in xylol ; mount in balsam.

Keratohyalin granules, dark blue.

Instead of the potassium permanganate, 33 per cent iron sulphate solution may be used (10 minutes). The nuclei may later be counterstained with safranin.

### C. Fick's (K. Herxheimer) Method

(1) Harden in alcohol ; imbed ; cut.
(2) Stain in saturated watery solution of Cresylecht violet (Leonhardt and Co., Mülheim a/M.), 4 minutes.
(3) Wash thoroughly in water.
(4) Differentiate in 95 per cent alcohol until the interstitial tissues are colourless and the protoplasm of the epithelium is light violet.
(5) Dehydrate in absolute alcohol.
(6) Clear in xylol ; mount in balsam.

Keratohyalin, red ; nuclei, blue-violet ; plasma, light blue-violet ; keratin, dark violet.

**Eleidin,** which occurs in the basal layers of the cuticle (stratum lucidum), differs from keratohyalin both morphologically and chemically, but according to some authors it may be derived from keratohyalin.

Eleidin, like keratohyalin, stains with carmine, but, in contrast to keratohyalin, does not stain with hæmatoxylin. It is also coloured in the same manner as fat, but its fatty nature is disputed (Weidenreich).

## Special Staining Methods for Eleidin (or Eleiden and Kerato-hyalin)

### A. Buzzi's Method with Congo Red

(1) Harden; imbed; cut.
(2) Stain in Congo red (2-3 drops of a 1 per cent watery solution of Congo red, added to a small basinful of water, 2-3 minutes.
(3) Wash in water.
(4) Dehydrate in absolute alcohol.
(5) Clear in xylol; mount in balsam.

Eleidin, deep red; tissues, rose-coloured.

If the section is stained in hæmatoxylin between 3 and 4, the eleidin will be red, the keratohyalin blue.

When the Congo red stained section is treated with dilute acids, the red of the eleidin is changed to blue. Buzzi has described several other methods with nigrosin, etc.

### B. Dreysel and Oppler's Method with Carmine

(1) Harden in absolute alcohol 2-3 days; imbed in celloidin; cut in a dry condition.
(2) Stain in—

| | | |
|---|---|---|
| Carmine | 1 gm. | Stand the vessel containing the mixture on a water-bath, or expose to the air for several days so that some of the ammonia may evaporate. Then filter. |
| Strong liquid ammonia | 1 c.c. | |
| Saturated watery picric acid solution | 1 c.c. | |
| Water | 200 c.c. | |

Stain about 2-6 minutes for eleidin, 5-6 minutes for keratohyalin.

(3) Spread the section on a slide if not already there; remove the stain with filter-paper, examine in glycerin; or

[3] Transfer the section to $\frac{1}{2}$ per cent alcoholic solution of picric acid, about 1 minute.
[4] Dehydrate in absolute alcohol.
[5] Clear in xylol; mount in balsam.

The section may be stained in hæmatoxylin if washed in water after 2; the eleidin will then be red, the keratohyalin blue.

## XII. Calcareous Infiltration

The granules of chalk which occur as pathological deposits in necrosed and hyaline tissues are usually recognised during the examination of fresh tissues by their gritty, refractive appearance. (By reflected light they are light, by transmitted light, dark.)

An important diagnostic point is their solubility in acids. If the passage of dilute hydrochloric acid underneath the cover-glass is accompanied by the formation of gas bubbles ($CO_2$), the granules probably contain calcium carbonate; if concentrated sulphuric acid is added, fine slender needles of calcium sulphate appear.

With alum hæmatoxylin the calcareous deposits stain a deep blue or light red. On this is based the following

### A. Method of Leutert with Hæmatein

(1) Harden; imbed in celloidin; cut.
(2) Stain in saturated alcoholic hæmatein, 15 minutes.
(3) Wash in running water, 15 minutes.
(4) Stain in 1 per cent watery solution of safranin, 5-8 seconds.
(5) Wash in water.
(6) Differentiate in 96 per cent alcohol until the nuclei are defined.
(7) Dehydrate in absolute alcohol.
(8) Clear in xylol; mount in balsam.

Calcareous deposit, dark blue; nuclei, red.

For the finest granular deposits the following method may be employed:—

### B. Kossa's Silver Method for Calcium Phosphate

(1) Harden in alcohol or formol, etc.; imbed; cut.
(2) Place section in 1-5 per cent solution of silver nitrate; expose the section to daylight for 5 minutes to 1 hour.
(3) Wash in distilled water.
(4) Transfer the section to a 5 per cent solution of sodium hyposulphite (to remove the superfluous silver nitrate).

(5) Wash thoroughly in water.
(6) Dehydrate in absolute alcohol.
(7) Clear in xylol; mount in balsam.

Calcareous deposit, black (phosphate of silver being formed and the silver reduced by the action of light). The nuclei may be dyed red by staining in alum carmine between 1 and 2, or with safranin between 5 and 6. Tissues which contain much calcium must naturally be decalcified before being imbedded or sectioned.

## XIII. Pigments

Under the term "pigments" are included a number of substances, differing in their origin, form, and colour. In unstained sections the pigments stand out by virtue of their own natural colours. To render these pigments more prominent in sections of tissues, it is well to stain only with simple nuclear dyes, omitting those generally used for the protoplasm.

The yellow and brown pigments (the majority exhibit these colours) contrast better with carmine than hæmatoxylin; lithium carmine is therefore the best mixture for this purpose.

A special group is formed by the so-called **lipochromes.** They are fat-containing pigments, which are not well described by the term "lipochrome," since they do not give the necessary reactions with concentrated sulphuric acid and potassium iodide, which such named botanical substances should yield (Mühlmann, Lubarsch). These so-called lipochromes give the usual fat reactions with osmic acid, Sudan III., and Fett ponceau, but they do not stain so intensely as pure fat; unlike fat, they appear as pigmented granules in unstained preparations and in those in which the nuclei only are stained, as well as in those which have been treated with alcohol and ether, so that they are fatty pigments.

The pigments which contain **iron** are important from the standpoint of their micro-chemical reactions, viz. whether the pigment does or does not contain iron. Those containing iron and derived from hæmoglobin are included under the term "hæmosiderin." The tests for iron are not

always easy to obtain; in some cases the iron appears to be too closely combined with the cell albumins to give a positive result, and the age of the pigment somewhat determines the intensity of the reaction.

Glass needles should be employed for the following methods, since iron or steel instruments may introduce iron into the section and so give false results. Similarly all bottles, slides, and other glass utensils must have every trace of iron removed with hydrochloric acid, distilled water, and alcohol.

Several different groups of methods are employed for the demonstration of iron: (*a*) with ferrocyanide of potassium; (*b*) with sulphide of ammonium.

In both the selection of the hardening fluid is of importance. Falckenberg considers that alcohol is the best, formol, Orth's fluid, and Zenker's fluid not being so useful. It is advisable to harden in alcohol and then to imbed; or to harden in alcohol, then transfer to formol (which does not then interfere with the method), and to cut on the freezing microtome.

## A. Potassium Ferrocyanide Methods for Iron

The sections used for staining may be cut from fresh tissue or after hardening in formol or in alcohol, and imbedding in paraffin (*vide* Falckenberg's results).

(1) Place the section in 2 per cent watery solution of potassium ferrocyanide for some minutes.
(2) Transfer the section to $\frac{1}{2}$-1 per cent hydrochloric acid, or to acid alcohol.
(3) Examine in water or in glycerin.
Or [2] Examine the section in glycerin to which $\frac{1}{2}$ per cent hydrochloric acid is added.
[3] Dehydrate in absolute alcohol.
[4] Clear in xylol; mount in balsam.

Iron pigment, blue. The nuclei may be stained with carmine.

Wicklein recommends to put the sections in 25 c.c. of 1 per cent HCl, and then to add with a pipette 3 drops

of a cold saturated watery (fresh) solution of potassium ferrocyanide. The sections stay therein 5 minutes, and are then thoroughly washed.

Falckenberg uses the following solutions :—25 c.c. of 1 per cent HCl are mixed with 8-10 drops of a 2 per cent solution of potassium ferrocyanide. He leaves the sections in this mixture for $\frac{1}{2}$-1 hour, then washes them thoroughly in water, and counterstains in alum carmine, etc.

A procedure similar to Wicklein's is more commendable than that of Falckenberg.

**Stieda's method for iron.**—A good and reliable method :—

(1) Stain sections in lithium carmine for several hours.
(2) Wash in acid alcohol and then rapidly in water.
(3) Place in 2 per cent aqueous solution of potassium ferrocyanide, 2-3 hours.
(4) Transfer to acid alcohol, 6-12 hours.
(5) Wash rapidly in water.
(6) Dehydrate in absolute alcohol.
(7) Clear in xylol ; mount in balsam.

Iron pigment, blue ; nuclei, red. Alum carmine may be used instead of lithium carmine ; in such a case stain after stage 5.

## B. Quincke's Ammonium Sulphide Method for Iron

(1) Harden in alcohol ; imbed ; cut.
(2) Stain in a fairly old but yet yellow solution of ammonium sulphide, 5-60 minutes.
(3) Wash very rapidly in water.
(4) Examine in glycerin containing $\frac{1}{2}$ per cent hydrochloric acid. The details appear in 30-60 minutes.

Or [4] Dehydrate in alcohol.
[5] Clear in xylol ; mount in balsam.

Iron, grey-black to black. The nuclei may be stained with carmine.

Silver, lead, and mercury give similar reactions.

## C. Combined Method of Hall

In this method, during the stage of hardening, the iron is changed to the insoluble $Fe(OH)_2$, so that iron is not removed by the alcohol, as in the other methods. For "traces" of iron it is therefore the most reliable method.

(1) Harden the piece of tissue in

| | |
|---|---|
| Ammonium sulphide . . | 30 c.c. |
| Absolute alcohol . . . | 70 c.c. |

For liver, spleen, and marrow.

| | |
|---|---|
| Ammonium sulphide . . | 5 c.c. |
| Absolute alcohol . . . | 70 c.c. |
| Water . . . . | 25 c.c. |

For intestine.

After-harden in increasing strengths of alcohol; imbed in paraffin; cut.

(2) Place the section in the ammonium sulphide solution, or for 20 minutes in

| | |
|---|---|
| Potassium ferrocyanide . | 1·5 gm. |
| Hydrochloric acid . . | 0·5 c.c. |
| Water . . . | 100 c.c. |

(3) Wash in water.
(4) Dehydrate in absolute alcohol.
(5) Clear in xylol; mount in balsam.

Iron, blue, or black after further treatment with ammonium sulphide. Nuclei may be stained in lithium carmine before 2.

## D. Methods for Combined or "Masked" Iron

The above methods answer very well with free iron, but do not react with "masked" iron, or iron so combined that it is unaffected by ordinary staining processes.

Delépine finds that by leaving the sections in a mixture of 2 parts of proof spirit with 1 part of glycerin for 12-24 hours, tissues which previously did not yield the reactions for iron, or only slightly reacted, then give very distinct colour changes. MacCallum found that ammonium hydrogen sulphide ($NH_4HS$) is able to unmask organic iron, but that by its prolonged action the tissues suffer considerably. He therefore uses acid alcohols for the same purpose:—

| | | | | | |
|---|---|---|---|---|---|
| Sulphuric acid . | 4 | or | Nitric acid, sp. gr. 1·4 | 3 |
| Alcohol (95 per cent) | 100 | | Alcohol (95 per cent) | 100 |

The tissues are first subjected to the action of Bunge's fluid (95 per cent alcohol, 95; 25 per cent hydrochloric acid, 10) for 1-2 hours at 50-60° C., or 8-10 hours at 35° C. This removes the inorganic iron.

The organic iron is next unmasked in the following manner :—

(1) Place the tissues in the acid alcohol.
(2) Wash the sections in pure alcohol.
(3) Wash the sections in distilled water.
(4) Transfer them to ammonium sulphide for 5-60 minutes ;
or [4] Place them in a freshly prepared mixture of 1·5 per cent potassium ferrocyanide (not older than a week), 1 part ; 0·5 per cent hydrochloric acid, 1 part, for 5 minutes.
[5] Wash in water.
[6] Stain in 1 per cent eosin in 30 per cent alcohol, 3 minutes ; or in 1 per cent safranin in 30 per cent alcohol, 30 minutes.
[7] Dehydrate in absolute alcohol.
[8] Clear in cedar-wood oil ; mount in benzene balsam.
Preserve the preparations in the dark.

Or (4) Place the tissues in $\frac{1}{2}$ per cent hæmatoxylin in pure distilled water ; the iron then combines with the dye to form a deeply coloured, insoluble oxide. The remainder of the section is yellow-brown. The uncombined hæmatoxylin may be removed by steeping the preparation in a mixture of equal parts of absolute alcohol and ether for 30-60 minutes ; only the blue-black iron compound then remains. Clear in oil of cloves ; mount in balsam.

**Bile pigments** are readily recognised by their yellow colour. Formol and sublimate (Ziegler) change them to a green shade. After hardening tissues in formol, brown-black deposits frequently occur in the neighbourhood of hæmoglobin or its derivatives (Heile's pseudo-ochronosis, Browicz).

When masses of pigment obscure the finer histological details, they may be removed by carefully transferring the sections to fluids containing chlorides for 1-2 days, or by oxidisation and subsequent reduction (permanganate of potassium and oxalic acid ; see Fibrin Method). Mineral acids and alkalies may be also used to dissolve out the pigments (see MacCallum's Methods).

## XIV. Various Chemical Constituents

**Glycogen** and **iron** may be mentioned here, the means for their demonstration being already stated.

**Silver** (argyria) and **lead** yield black sulphides with ammonium sulphide (see Iron, Quincke's Method, etc.).

**Copper**, treated with potassium ferrocyanide and hydrochloric acid, gives a dark yellow-brown coloration (details of the reaction are stated under "Iron"). With hæmatoxylin copper assumes a dark-blue coloration.

**Phosphorus.—MacCallum** fixes fresh tissues in alcohol, treats sections with freshly-prepared nitric molybdate for 10 minutes to 48 hours at 35° C., washes them for 1-2 minutes in dilute nitric acid or distilled water, and then transfers them to watery solutions of 1-4 per cent phenyl-hydrazin hydrochloride; any phospho-molybdate is reduced to the dark-green oxide of molybdenum in 2-10 minutes. The section is then dehydrated, cleared in cedar-wood oil, and mounted in balsam. To distinguish between inorganic and organic phosphorus, reference should be made to MacCallum's papers.

**Iodine.—Justus** has recently described a special method for the demonstration of iodine, which substance he considers as present in all cell nuclei. The following are the details given :—

(1) Harden in absolute alcohol; imbed in celloidin; cut.

(2) Remove all alcohol from the section by placing it in a basin of water.

(3) Transfer the section to a wide-mouthed, stoppered vessel in which distilled water is present (about two fingers high).

(4) Remove the water; replace with freshly prepared green-coloured chlorine water; stopper well; in 1-2 minutes the section is decolourised.

(5) Transfer the section with a glass needle to a vessel containing 500 c.c. of water and 1 c.c. of a 1 per cent solution of silver nitrate, 2-3 hours. The section is coloured yellow-green, and a precipitate of silver chloride appears.

(6) Place the section in a saturated warm solution of sodium chloride. The section assumes a light colour.

(7) Wash in distilled water.

(8) Transfer to a concentrated (4-5 per cent) solution of mercuric chloride.

(9) Examine in pure glycerin.

The iodine is stained red.

The chemistry of the method is as follows :—The chlorine frees the iodine from its combination with albumin; the iodine forms silver iodide when placed in the silver nitrate solution; silver chloride also arises, the amount being lessened by using very weak silver nitrate, and is removed during the next bath of silver chloride. During stage 8 the silver iodide is changed to the red mercuric iodide, in order to make the compound more visible.

We have not been able to obtain satisfactory results by this method, the failure possibly arising from the lack of favourable material for the conditions of the procedure, a reason which Justus in a private communication also suggests.

**Uric acid and purin bases.**—Saint Hilaire has described a method based upon the affinity of cuprous oxide for purin bodies, but as the reaction itself is now not considered as purely elective, other substances being precipitated at the same time, the process is not here included.

The precipitation of uric acid by silver in the presence of magnesium and ammonia is a better method, and has been used for some time by one of the writers in micro-chemical work. Of late it has been described as a special method. It is only applicable to free purins; if it is desired to demonstrate the purins "bound" to albumins, the latter must first be split off.

**Courmont and André's method** :—

1. Fix tissues in absolute alcohol; imbed; cut.
2. Place sections in $\frac{1}{100}$ ammonia solution, or in very weak sodium hyposulphite solution.
3. Transfer to $\frac{1}{100}$ nitrate of silver solution.

4. Wash in water.
5. Develop with a photographic "developer."
6. Wash in water.
7. Stain with hæmatein and eosin ; dehydrate ; xylol ; balsam.

The uric acid and xanthin or purin bases stand out as black granules.

**Potassium.**—When a solution of potassium nitrite is added to a solution of cobalt, an orange-coloured double salt is precipitated (Fischer). This reaction forms the basis for the following method.

**MacCallum's micro-chemical test for potassium** (*Journal of Physiology*, 1905, p. 95) :—

1. Place teased preparations of fresh tissues or sections, made from fresh tissues by means of the freezing microtome, in the following reagent for 20 minutes.

Dissolve 20 grams of cobalt nitrite and 35 grams of sodium nitrate in 75 c.c. of dilute acetic acid (10 c.c. glacial acetic acid, 65 c.c. of water). Filter after some hours, and dilute to 100 c.c.

2. Wash in ice-cold water till the washings are uncoloured.
3. Mount in equal parts of 50 per cent glycerin and saturated ammonium-sulphide solution.

The preparations will keep for two months.

## APPENDIX TO CHAPTER XI

### Staining Tissues in Bulk

So far we have only treated of the staining of sections. As earlier stated, pieces of tissue may be stained prior to imbedding, and then only require to be sectioned and mounted. This method is more frequently used in normal than in pathological histology, since in the examination of morbid tissues it is difficult to forecast before the exact lesions are known whether one or more stains are necessary. The method thus calls for but a brief allusion.

Harden *small* pieces of tissue in formol or other fixing medium. When this stage is completed transfer the tissue to the selected staining solution, and leave it for several days ; wash well ; harden in increasing strengths of alcohol ; imbed ; cut ; mount the sections without further staining.

For thorough penetration of the stain into "bulk" tissue, alcoholic solutions are preferable. Friedländer's or Heidenhain's hæmatoxylin, hæmatein, hæmalum, Mayer's alcoholic carmine, Beale's carmine or alcoholic solutions of aniline dyes, such as Bismarck brown, will all give good results.

**Serial sections from celloidin-imbedded tissues stained in bulk.**—**Langhans** recommends the following procedure :—

Moisten the knife with origanum oil instead of alcohol. Place the sections in order on a slide previously covered with a thin film of the same oil. The mixture of the oil with the 70 per cent alcohol contained in the celloidin results in a milky cloudiness, but this soon disappears. The surplus oil is then removed, and the sections are mounted in balsam. **Schmorl** avoids the appearance of the cloudiness by substituting a mixture of 3 parts of oil and 1 part of absolute alcohol for the pure oil.

### Metallic Impregnations

The use of such metals as gold or silver for the demonstration of tissue structures is not widely practised amongst pathologists. Moreover, it is not, strictly speaking, a staining process, and we accordingly devote but little space to its consideration.

Fresh tissues are placed in metallic solutions, the latter being reduced by certain tissues, and especially by light, metallic deposits thus being formed ;

or, by the aid of reducing solutions the metals are precipitated and deposited in the tissues.

In "negative" impregnations the cells are unaffected, the deposit taking place in the intercellular substance; in "positive" impregnations the reverse order obtains. Gold and silver are chiefly used, rarely iron; osmic acid is, however, very extensively employed, and its action, etc., have been already fully described.

For this purpose it is best to take membranes or fresh sections which have been cut on the freezing microtome.

Gold salts—first used by **Cohnheim**—are chiefly applied to nerve tissues and tactile corpuscles. The preparations, before being placed in solutions of choride of gold or of gold potassium chloride, are treated with organic acids. As an example, **Loewit's method** may be given :—

(1) Small pieces of membrane or fresh sections are placed in formic acid, sp. gr. 1·12, until they are transparent, 3-5 minutes.
(2) Transfer to 1-1·5 per cent watery chloride of gold solution, 5-8 minutes.
(3) Wash in water.
(4) Reduce in formic acid, 1 part; water, 3 parts, 24 hours.
(5) Further reduce in formic acid, 1 part; water, 1 part, 24 hours.
(6) Wash thoroughly in water.
(7) Mount in glycerin gelatin.
All the stages except No. 1 should be conducted in the dark.

The **silver** salts (after **von Recklinghausen**) are chiefly used for the outlines of endothelial cells, and thus find a place in pathological technics for the demonstration of false or doubtful membranes.

For this purpose preparations are gently laved in distilled water or isotonic potassium nitrate solution (1·3 in 100), and then placed in watery silver nitrate solution (1 : 300) until they are light grey in colour. They are then washed with distilled water and transferred to a 2 per cent solution of sodium hyposulphite for 5-10 seconds (to avoid after-darkening), again washed in distilled water, and mounted in glycerin gelatin. The endothelial borders (intercellular ground substance) are stained brownish black. The sections may be also placed in 50-70 per cent alcohol (avoiding stronger alcohol, which causes too much shrinkage), then passed through aniline oil, xylol, and mounted in balsam.

The majority of authors recommend that these metallic solutions should be kept in brown bottles and away from the light, but Lee (in Lee-Mayer, p. 228) states that light appears to improve the particular impregnating qualities of these solutions, so that the older advices as to light, etc., may be neglected.

To demonstrate the endothelium of blood-vessels, it is best to inject a 1 : 200 watery silver nitrate solution, to every 100 c.c. of which one drop of nitric acid is added, and allow it to act for 2-3 minutes. Then the vessels are washed out with 5 per cent warm gelatin. The tissues are fixed in 95 per cent alcohol for 24 hours, then imbedded in gum, sections cut on the freezing microtome and exposed to sunshine until the outlines of the cells are visible (**Gustav Mann**).

The metallic impregnations are, as a rule, somewhat capricious, and much care and experience is necessary in order to avoid illusory appearances. The same is the case with Golgi's silver methods, which will be described in connection with the nervous system.

### Vital and Survival Staining

In these methods, with which **Ehrlich** has so richly endowed our technique, only those pigments may be used which, while staining the tissue constituents, do not in any way disturb them. Chief among these, according to **Fischer**, are certain basic dyes, such as Bismarck brown, methylene blue,

neutral violet, and especially neutral red. Methylene blue and neutral red are chiefly employed.

These methods have yielded brilliant results in investigations upon the structures of the nervous system and upon cell granules. By injections of the mentioned stains into living tissues, or by placing the surviving tissues in the stains, **Arnold** has been able to demonstrate the presence and the position of Altmann's granules in their unchanged or living condition. **Ehrlich** first employed the method for the nervous system, and it has since been successfully modified by Bethe (see Nervous System, Chapter XII. VI.) Herter and Richards have recently shown that chloroform and ether should not be used during the process of injection, since they seriously interfere with the reducing powers of the tissues, and a large quantity of the stain remains in the liver, stomach, and nervous system in an uncombined form.

For details of "survival" staining, see "Blood."

## Injections

In normal histology injections are often used for the demonstration of blood-vessels, lymphatics, glandular ducts and passages, but it is only under exceptional conditions that they are used by pathologists.

Both cold and warm staining solutions may be employed, one example of each type being now described.

### Cold Injection Masses

**Beale's glycerin carmine.**—Dissolve 0·3 gm. of carmine in a small quantity of water containing 5 drops of ammonia; add 15 c.c. glycerin and shake; add drop by drop a further 15 c.c. of glycerin containing 8-10 drops of glacial acetic acid.

Then still add:—

| | |
|---|---|
| Glycerin . . . . | 15 c.c. |
| Alcohol . . . . | 8 c.c. |
| Water . . . . | 24 c.c. |

### Warm Injection Masses

**Thiersch's Berlin-blue gelatin.**—Solutions:—

(*a*) Dissolve 1 part of gelatin in 2 parts of water by allowing it to swell for 24 hours, and then warming. Filter through flannel.
(*b*) Saturated watery solution of ferrous sulphate.
(*c*) Saturated watery solution of red ferricyanide of potassium.
(*d*) Saturated watery solution of oxalic acid.

Take of

| | | | |
|---|---|---|---|
| (1) | *a* . . | 30 c.c. | |
| | *b* . . | 12 c.c. | |
| (2) | *a* . . | 60 c.c. | |
| | *c* . . | 24 c.c. | |

Mix each separately at 30° C.

At 30° C. add to mixture 2, first, 24 c.c. of *d*, and then mixture 1, constantly stirring, so that the Berlin blue is precipitated. Heat on a water-bath to 90° C.; filter through flannel.

The warm mixtures are more complicated in their preparation and actual use, but are preferable to the cold ones.

For the injections the organs must be fresh—we treat here only of organs, not of animals, which are best bled during narcosis—and the vessels must be washed out with normal saline, or with a fibrin-dissolving fluid, such as a freshly filtered 8 per cent solution of sodium sulphate or nitrate, followed by normal saline. A cannula is introduced and secured in a free vessel and then

connected with a syringe, and the staining fluid is then injected under a low pressure. Instead of a syringe, special forms of apparatus to provide a constant stream, and with mercurial manometers to regulate the pressure, such as Toldt's, may be used.

The injection may be commenced when the injection mass is free from air-bubbles, and should be ended when the organ appears diffusely stained and the stain emerges from the venules.

For injections into **lymphatic vessels** (which are much more difficult), but very slight pressure must be employed, and the cannula should be introduced at the periphery of a blood-vessel, where the larger lymphatics are to be found.

When the cold injections are completed, harden the tissues in alcohol for a few hours, then cut into small pieces and imbed, etc. After the warm injection is made, immerse the organ in icy-cold 10 per cent formol or water, in order to hasten the solidification of the gelatin. Then transfer to 80 per cent alcohol and imbed after hardening.

For staining, those nuclear stains should be selected which contrast with the colour of the injection mass.

Considerable practice is necessary in order to regulate the pressure; if the latter is too high, extravasations may easily occur.

Recently, an **injection method** has been formulated by **Fischer**, quite different in type from those already in use, which is said to yield very good results. The procedure is as follows :—

Wash out the vessels with 8 per cent sodium nitrate or sodium sulphate, then inject them with milk. (The injection of milk is a relatively easy process.) When the injection is finished, harden the tissue for 24 hours or more in

| | |
|---|---|
| Water . . . | 1000 c.c. |
| Formalin . . . | 75 c.c. |
| (40 per cent formaldehyde). | |
| Pure glacial acetic acid . | 15 c.c. |

(The acetic acid is added in order to coagulate the milk.)

Freeze, cut, and stain with Sudan III. or Fett ponceau; the vessels are shown by the position of the fat globules.

Vascular tissues may be demonstrated without injecting the vessels, by subjecting them to the action of certain fixing and hardening reagents. Formol and chromic acid fluids are amongst the best of these. Eosin stains the blood very well. Blood corpuscles are sometimes very deeply dyed by the myelin and fibrin methods, and with these the capillaries often appear as if injected.

A type of self-injection in animals was used by **Silbermann** to determine the existence of thrombi in the smallest blood-vessels. He first employed indigo carmine and later eosin and phlosin-red. The pigment circulated in the normal blood-vessels, but the thrombotic areas were, of course, free from pigment. Kaufmann used this method for the demonstration of capillary thrombi in sublimate poisoning.

# CHAPTER XII

## SPECIAL TISSUES AND ORGANS

### I. Blood and Blood-forming Tissues

The microscopical examination of the blood consists in

1. Enumeration of the red and white corpuscles.
2. Preparation and examination of blood films for { I. Unstained films. II. Stained films.

*a.* Changes in the red corpuscles.
*b.* Changes in the white corpuscles and differential counts.
*c.* Presence of parasites, etc.
*d.* Medico-legal investigations.

Detailed instructions for the enumeration of the red and white corpuscles are supplied by the makers of the Thoma-Zeiss', Gower's, Oliver's, and Reichert's hæmocytometers. The differential count of the white corpuscles will be described later.

### Collection of the Blood

The blood may be obtained from the pulp of a finger or from the posterior aspect of the lobule of the ear.

The skin should be washed with soap and water, then cleansed with $\frac{1}{1000}$ mercuric chloride solution, and finally swabbed, first with alcohol, and then with ether.

A puncture is made with a triangular needle, or with a piece of broken glass tubing, or with a pen nib from which one prong has been removed, each being previously sterilised by heat.

When the pulp of the finger is selected and the puncture has been made, press gently with the ball of the thumb over the

furrow just above the terminal phalanx. A sufficient quantity of blood will then be easily obtained. If a second portion is required, remove the remains of the first drop with a piece of blotting-paper, and clean the edges of the wound.

In some instances it is permissible to place a drop of fixing fluid or of stain upon the skin and puncture through it, thus ensuring perfect fixation or "vital staining" (*q.v.*).

When a large quantity of blood is required, it is best to collect it in a narrow S-shaped tube with capillary ends, and to seal up the ends immediately after collection. The shape of the tube then allows immediate or later centrifugalisation.[1]

If it is desired to prevent coagulation or to retain the shape of the formed elements, the blood should be run into a tube containing about one-third of its capacity of any of the following solutions :—

(1) 0·2 per cent ammonium oxalate solution; (2) 2 per cent sodium citrate solution; (3) Hayem's fluid :—

| | |
|---|---|
| Mercury perchloride . . | 0·25 gm. |
| Sodium chloride . . . | 0·5 gm. |
| Sodium sulphate . . . | 5·0 gms. |
| Distilled water . . . | 100 c.c. |

(4) Isotonic saline solution (0·8-0·9 per cent solution of sodium chloride or distilled water); (5) Pacini's fluid :—

| | |
|---|---|
| Sublimate . . . | 1 gm. |
| Sodium chloride . . . | 2 gm. |
| Water . . . . | 200 c.c. |

(6) A very weak solution of iodine in potassium iodide solution; (7) serum obtained from the blood of the same individual or animal.

The blood may be imbedded *en masse* in celloidin, but the method offers no advantages. Rindfleisch's suggestion to spread already fixed blood upon a cover-glass by the aid of glycerin and Müller's plan of coating a cover-glass with thin celloidin solution do not give any better results than the ordinary film preparations, and they alone, therefore, will be described.

## Film Preparations

It is essential that the slides and cover-glasses be perfectly clean and their surface tension corrected. Any of the following methods may be used.

Directions for the cleaning of cover-glasses and slides are given in Chapter I.

[1] The papers of A. E. Wright, *Lancet*, 23rd Jan. 1904, etc., will well repay reference by those interested in the collection and manipulation of the blood for purposes of histological examination.

For the examination of blood and bacteria the slides, etc., must not only be clean, but must be freed from all greasy materials, so that the drops, etc., spread easily and evenly.

1. Boil for 10 minutes in a mixture of 20 per cent potassium bichromate and 20 per cent sulphuric acid. Wash well in running water, then in repeated changes of alcohol and absolute alcohol, and preserve in the latter fluid.
2. Place in concentrated sulphuric acid, or nitric acid, or hydrochloric acid, or glacial acetic acid, for one hour. Wash well in running tap water, then in distilled water, pass through alcohol and ether, and preserve in alcohol.
3. Boil in 10 per cent chromic acid solution for 30 minutes, wash well in tap water, then in three or four changes of spirit, then in absolute alcohol, and preserve in absolute alcohol.
4. Cover the slide with damp "whitening," and then remove it and dry and polish with a clean soft duster.

### Preparation of the Blood Film

(1) On cover-glasses. (2) On slides, making a film two-thirds of the length of the slide (this is the more satisfactory method).

Prick the skin, remove the first drop of blood, dry two cover-slips and place them in two pairs of Cornet's forceps; when the drop of blood is about the size of a pin's head touch it with one cover-glass and immediately place the two covers in apposition. The blood spreads out into a thin film; slide the glasses apart, do not press or squeeze; dry in the air. A piece of cigarette-paper cut at right angles to its length and, after smearing the ungummed end with blood, drawn steadily along the cover-glass also makes a good film. The same methods may be used for the preparation of films upon slides. Do not touch the films with the hands, and so avoid a current of warm, moist air, which alters the relations and arrangements of the corpuscles. Dry in the air. The blood should dry as quickly as it is spread. Ordinary films will be too thick if the blood stands in slow-drying pools.

**Marking the film.**—The film should be marked as soon as it is prepared. Labels may be used, but they are sometimes not at hand when wanted. It will be found sufficient to mark the number and date of the film with ink. The device of Powell of Bombay is useful: with a pin or needle he scratches the number and date on the film itself. This does not harm the film to any extent, and the markings are, of course, permanent.

For *preserving the freshness of the blood* or for the *despatch of films by post* the method of Braddon is useful:—Two cover-slips, or a slide and a cover-slip, are accurately superimposed. White cement or vaseline is painted around the edges, leaving a small area on one side and the entire edge of another side quite open. On touching the drop of blood with the free

edge, the blood enters and forms a thin uniform film, having suffered the minimum of exposure to air and of handling and of pressure. The free edges are closed by applying cement. The blood retains its freshness for some days.

**Removal of hæmoglobin from the corpuscles.**—It is sometimes useful to remove the hæmoglobin from the corpuscles before examination. The following methods are available :—

(1) Wash the film in distilled water.
(2) Place the film in 2 per cent formol solution, and then wash it in distilled water.
(3) Place the film first in 2 per cent formol solution and then in 2 per cent acetic acid.

## I. Examination of Unstained Film

Useful for
1. General appearances of corpuscles.
2. Amœboid movements of white corpuscles.
3. Platelets.
4. Vital staining of corpuscles.

1. **General appearances.**—The edge of the concave depression in a "hanging drop" or "moist chamber" slide is ringed with vaseline or cement. A drop of blood is placed on a cover-glass and the latter rapidly lowered over the depressed area.
2. The **amœboid movements** are best observed by collecting the blood on a cover-glass previously coated with a thin layer of agar-agar solution (boil 5 gms. agar-agar in 500 c.c. distilled water, filter hot, and to each 100 c.c. of the filtrate add 0·6 gram NaCl) and examined on a warm stage. At any moment the blood may be fixed by running in 1 per cent $OsO_4$ solution, and the preparation, after washing in water and 95 per cent alcohol, may be stained with hæmatoxylin and eosin (Deetjen).

Dekhuyzen collects the blood in isotonic salt solution. For mammals this is 0·9-0·95 per cent sodium-chloride solution. All glass-ware is cleaned in strong nitric acid, and after washing is heated until perfectly dry. The punctured finger is placed in a conical glass containing 50 c.c. of the isotonic solution. The corpuscles sink to the bottom. The sediment may be withdrawn in a pipette, a film prepared and examined in the fresh condition or fixed in osmic acid (2 per cent) 1 part, acetic acid (6 per cent) 1 part, washed in water, hardened in 96 per cent alcohol, and stained in methylene blue.

3. **Platelets.**—The following methods are useful :—

(*a*) *The cover-slip is coated with Deetjen's platelet fluid.*—To each 100 c.c. of the solution obtained in No. 2, 6-8 c.c. of 10 per cent solution of sodium metaphosphate and 5 c.c. of a 10 per cent solution of sodium diphosphate are added. The solution must not be further heated.

A drop of blood is examined on a warm stage and the platelets observed in the living condition. Or, the agar smear is allowed to harden and a thin strip about 2 mm. cut out; into this the blood is placed, and examined on a warm stage. If a permanent preparation is desired, the film may be fixed with $OsO_4$ (1 per cent), then washed and stained.

(*b*) *The blood is passed directly into a fixing fluid :—*

(i.) 2 per cent formalin containing 1 per cent sodium chloride.
(ii.) 1 per cent watery solution of osmic acid.
(iii.) 1 per cent watery solution of osmic acid (1 part).
·6 per cent of sodium chloride (2 parts).
(iv.) 14 per cent solution of magnesium sulphate.
(v.) A solution of equal parts of alcohol and ether and Romanowsky's stain. (The centre of the platelet is red-violet and the periphery blue.)
(vi.) Afanassiew's solution :—Peptone, 0·6 gm. ; methyl violet, 0·01 gram ; NaCl solution (0·6 per cent), 100 c.c. This must be sterilised before using.

(*c*) *The blood is passed directly into a fixing and staining fluid.*—A drop of the following mixture is placed upon the skin and the puncture made through it (Kemp and Calhoun). The platelets are blue.

| | |
|---|---|
| Formalin . . . . . | 5 c.c. |
| Sodium chloride . . . . . | 1 gm. |
| Methyl violet . . . . . | 0·1 gm. |
| Water . . . . . | 100 c.c. |

4. **Vital staining** (Wolff, Plato, etc.).—An ordinary fresh film is prepared. The stain is introduced and the degree and rapidity of the staining observed—

i. To an ordinary fresh film a few granules of neutral red or pure methylene blue are added.

ii. Place a drop of alcoholic solution of pure methylene blue, or pyronin, or methyl green, or eosin, or brilliant kresyl blue (Richberger) on a cover-glass. When dry, make a blood film on the same surface. Invert the preparation over the cavity of a "hanging drop slide" and examine on a warm or cold stage. While alive, the blood constituents show but little staining; the staining proceeds rapidly as the cells die. (Rosin, Bibergeil, Ito, etc.)

Fresh films may also be stained by placing a few drops of 0·1-0·5 per cent watery eosin at one border of the cover-glass and drawing it through by placing a fragment of filter-paper at the opposite side. The red corpuscles are yellow-red ; the eosinophilous granules of the leucocytes red. Dilute methylene blue stains the basophile granules, and a saline solution of neutral red stains the nuclei of the red blood corpuscles.

*For the preservation of the elements of the blood and of tissues generally Barjon and Regaud recommend :—*

1. Fixation of blood or tissue fluids in a few drops of 1 per cent $OsO_4$ or 10 per cent formalin contained in an ordinary centrifugalising tube. Fill up the tube with water.
2. Centrifugalise and remove the supernatant fluid.

3. Wash the sediment with distilled water and again centrifugalise.
4. Remove the distilled water; add equal parts of alcohol and ether; centrifugalise.
5. Remove alcohol and ether; add 10 per cent celloidin.
6. Imbed; cut; stain.

## Fixation of Films

**A. Without drying.**—The film is placed, immediately after making and while still wet, directly over a vapour or into a fixing fluid, and after fixation is always washed thoroughly.

1. **Formalin vapour** (40 per cent), 5 seconds, or $OsO_4$ vapour (2 per cent), 2 seconds. The film is dropped, while still wet, into absolute alcohol. (Is said by some to alter the staining properties of the cells.)
2. **Formol alcohol.**—Formalin (40 per cent), 1 part; absolute alcohol, 10 parts. Fix for 5-10 minutes.
3. Chromic acid (1 per cent), 15 parts; osmic acid (2 per cent), 4 parts; glacial acetic acid, 1 part (Flemming). Wash very thoroughly.
4. Corrosive sublimate (saturated in 0·6 per cent NaCl solution) (Muir). Wash very thoroughly.

**B. After the film is air-dried.**—Wait until the film is air-dried, and then treat it by the following methods:—

1. **Heat.**—110-115° C. Time varies with stain. With Ehrlich's tri-acid stain, 5-10 minutes; with methylene-blue-eosin stains, 2 hours. If a temperature of 120° C. be employed, 30-60 seconds are sufficient.

   **Pappenheim** recommends that heat-fixed films should be dipped for a moment into a saturated solution of corrosive sublimate, then well washed before staining.
2. **Methyl alcohol.**—Free from acetone, 1-2 minutes.
3. Absolute alcohol, or equal parts of absolute alcohol and ether, 30 minutes to 2 hours (Nikiforoff).
4. Absolute alcohol (25 c.c.), ether (25 c.c.), solution of corrosive sublimate in absolute alcohol (2 gms. in 10 c.c.), 5 drops (Gulland), 2-5 minutes.
5. Rectified spirit and saturated corrosive sublimate in 0·6 per cent NaCl (Durham).
6. Formol (25 c.c.), absolute alcohol (75 c.c.). Methyl alcohol may be substituted, and is really better.
7. Formalin vapour.

   The beginner is advised to commence with the method of formalin-vapour fixation. He may next use formol alcohol or methyl alcohol.

   Fixation by heat gives good results as regards the granules,

but is not good for the spongioplasm. It is the rapid removal of the water that is essential, therefore the film should be at once brought into a temperature of 110-120° C. The slower method of gradually raising the temperature from 80° C. to 120° C. permits some coagulative changes to occur, and the after-staining is not so definite.

Formalin gives useful results as a rule, but for use with Ehrlich's tri-acid stain, formol alcohol is preferable (Muir).

Sublimate fixation brings out the leucocytic structures very well, but for the red blood corpuscles and the granules is less useful than the other fixatives.

Thorough washing is always necessary.

#### Classification of White Blood Corpuscles

Ehrlich divides the white blood corpuscles into six varieties :—

1. **Small lymphocytes.**—Small round cells, with a large, round, deeply staining nucleus surrounded by a narrow zone of cytoplasm.

2. **Large lymphocytes.**—Large cells, with much protoplasm, and with a round nucleus which does not stain so deeply as that of the small lymphocyte ; the cytoplasm is larger in extent than No. 1.

3. **Large mononuclear cells.**—Large round or oval cells, with a weakly staining, round or oval nucleus, and with much non-granular protoplasm.

4. **Transitional forms.**—Large round or oval cells, with slightly granular cytoplasm and a more or less indented nucleus.

5. **Polymorphonuclear cells.**—Large cells, lobed nuclei. The cytoplasm is crowded with neutrophile granules.

6. **Eosinophile cells.**—Lobed nuclei, staining less deeply than the polymorphonuclears. Granules stain red with eosin.

Ehrlich classifies the leucocytic granules as follows :—

**Eosinophile** or **acidophile** ($\alpha$) granules, which from a mixture of acid, neutral, and basic dyes select the acid stain, and are therefore called **acidophile** and **eosinophile**, because the acid eosin dye is the one principally used.

**Amphophile** ($\beta$) granules, which stain with both acid and basic dyes.

**Basophile** ($\gamma$) granules (mast-cell granules) stain with basic dyes only.

**Basophile** ($\delta$) granules occur in mononuclear cells, are small, and stain with basic dyes only.

**Neutrophile** ($\epsilon$) granules occur chiefly in polymorphonuclear cells ; stain with neutral dyes only. These correspond to the pseudo-eosinophile or acidophile granules of animals ; they may be easily differentiated from the true eosinophile granules, because the latter are much larger.

## II. Preparation and Examination of Stained Films

The student will find a bewildering array of stains for his selection. Each has its devotees. Each group of stains represents some stage in the progress of research, and more combinations may be expected as our knowledge of the

micro-chemical reactions of tissues increases. It is thus advisable to select at first those staining methods which are easy to manipulate, and, with this purpose in view, we may recommend formalin fixation, followed by eosin and methylene blue and Jenner's or Leishman's stains. Later, any one of the other procedures may be followed, according to the structures it is desired to stain.

The stains available for staining blood films may be tentatively divided into five groups :—

(1) Hæmatoxylin and eosin.
(2) Eosin and methylene blue.
(3) Mixtures of eosin and methylene blue, containing new neutral compounds arising from the mixture, and dissolved in excess of the base or special solvents ; or the compounds themselves are separated and dissolved in suitable media.
(4) Neutral dyes, formed by the combination of equimolecular proportions of the basic and acid dyes, methylene blue and eosin, the exact constitution of the resultant dyes being determined, and certain of them being removed or isolated.
(5) Special elective stains for certain structures and substances.

## Stains for General Use [1]

1. Ehrlich's acid-hæmatoxylin-eosin :—

| | |
|---|---|
| Crystallised eosin | 0·5 gm. |
| Hæmatoxylin | 2·0 gms. |
| Absolute alcohol | 100 c.c. |
| Glycerin | 100 c.c. |
| Glacial acetic acid | 10 c.c. |
| Distilled water | 100 c.c. |

Saturate the mixture with alum, and prepare at least a week before using. The solution keeps well in a closed flask.

Fix film in absolute alcohol or heat. Stain 2-24 hours. Wash thoroughly in tap water, dry, and mount in xylol balsam.
Red blood corpuscles, eosin red.
Nuclei, deep blue.
Protoplasm, light blue.
Protoplasm of neutrophilous cells, rose.
Eosinophilous granules, red.

2. Formalin fixation, followed by eosin and methylene blue :—
   1. Fix the dried film over 40 per cent formalin for 1 minute.
   2. Place in absolute alcohol for 1 minute.
   3. Stain for 5 minutes in 1 per cent watery eosin.
   4. Without removing previous stain, place in watery methylene-blue solution for 2 minutes.
   5. Dry in air ; mount in balsam.

[1] The majority of the stains used in blood examinations are prepared and sold ready for use by Grübler and Co., Leipzig.

If the preparation is too blue, place it in a watery solution of methylene blue to which a trace of 1 per cent acetic acid has been added.

Nuclei and platelets are blue; bacteria, basophilous and mast-cell granules are dark blue; eosinophilous granules and red blood corpuscles are red; the neutrophilous granules are violet-red.

3. Of the numerous modifications of a mixture of eosin and methylene blue to form compounds soluble in certain solvents, etc., only a few can be mentioned. As a rule, the staining mixtures keep for a few days only, and the results are variable.

(*a*) **Chenzinski's solution** :—

| | |
|---|---|
| Saturated watery solution of methylene blue | 40 c.c. |
| 0·5 per cent solution of eosin in 70 per cent alcohol | 20 c.c. |
| Glycerin | 40 c.c. |

Filter before use. Solution will only keep 4-6 days.

Fix by alcohol for 5 minutes, or by heat for 2-3 hours.
Stain for 24 hours.
Nuclei, blue.
Mast granules, dark blue.
Plasmodia, light blue.
Eosinophilous granules, red.
Red blood corpuscles, red.

(*b*) **v. Willebrand's solution.**—The addition of acetic acid yields another methylene-blue compound which is not so deeply blue.

| | |
|---|---|
| 0·5 per cent eosin in 70 per cent alcohol | 50 c.c. |
| Saturated watery methylene blue | 50 c.c. |

Add 1 per cent acetic acid (15-30 drops) until a control solution of eosin is quite yellow.

Fix by heat, alcohol, or formalin. Stain 10-15 minutes, and warm until steam just rises. Wash in water. Dry, and mount in balsam.

Nuclei, Mast granules } dark blue.
Neutrophilous granules, violet.
Eosinophilous granules, Red blood corpuscles } red.

(*c*) **Michaelis's solution** :—

| | |
|---|---|
| 1 per cent watery solution of methylene blue (Free from zinc chloride.) | 20 c.c. |
| Absolute alcohol | 20 c.c. |

Will keep for 3 weeks.

| | |
|---|---|
| 1 per cent watery solution of pure eosin | 12 c.c. |
| Acetone (56-58° C. boiling point) | 28 c.c. |

Both solutions must be kept in well-stoppered bottles sealed with paraffin.

Stain in equal parts of the two solutions for 1-10 minutes. During the staining the colour changes from blue to red; when the blue tint disappears entirely the staining is completed. Dry without heat; mount in xylol balsam.

(*d*) Jenner collects the precipitate formed by the addition of eosin to methylene blue, and then dissolves it in pure methyl alcohol. The solution then fixes as well as stains the film.

**Jenner's eosin-methylene-blue stain** (May and Grünwald have proposed a similar stain) :—

| | | | |
|---|---|---|---|
| Aqueous eosin | 1·25 gm. | Methylene blue (medicinal) | 1 gm. |
| Distilled water | 100 c.c. | Distilled water | 100 c.c. |

Mix equal parts in an open basin and stir with a glass rod. After standing for 24 hours, filter and dry the residue at 50° C. Wash the residue well with distilled water and again dry it thoroughly.

Of the dried powder take 0·5 gm., and methyl alcohol pure (Merck), 100 c.c. Filter. The solution keeps well.

The powder may be obtained from Kanthack, 18 Berners Street, or from Baker or Frazer, Edinburgh. The stain is very sensitive, and the covers or slides must be entirely free from acids or alkalies and perfectly clean.

1. Prepare blood film. Dry in air. Do not fix.
2. Cover film with stain for 2 minutes, and place it under a watch-glass to prevent evaporation. This is necessary to avoid the appearance of granules in the film.
3. Wash in distilled water until the film has a pink colour.
4. Dry in the air. Mount in xylol balsam.

| | |
|---|---|
| **Red.** | Neutrophilous granules. |
| **Rose red.** | Eosinophilous granules. |
| **Terra-cotta.** | Red blood corpuscles.<br>Central portion of platelets. |
| **Violet.** | Basophilous granules. |
| **Blue.** | Nuclei of leucocytes.<br>Degenerated granules in red blood corpuscles. |
| **Light blue.** | Outer portion of platelets.<br>Protoplasm of nuclei. |

4. The next group of stains has resulted from Romanowsky's idea of uniting equimolecular proportions of methylene blue and eosin, the dyes obtained being dissolved in some suitable solvent. The researches of Ziemann, Rosin, Laurent, Zettnow, Nocht, Gustav Mann, and others have shown that by the exact unison of eosin and methylene blue there are formed eosinate of methylene blue, methylene violet, methylene azure, and a black dye, insoluble in ether, while a tetrabromfluorescin derived from the eosin can also be extracted. We can give only a few of the numerous modifications of the original method of

Romanowsky, and we select, therefore, those which in our hands have yielded the more constant results.

For general work we recommend the student to use Leishman's modification, which fixes at the same time as it stains, reserving the others for special purposes.

### Romanowsky Modifications

(*a*) **Nocht's earlier modification** of Romanowsky's stain :—

A. Methylene blue (Höchst pure.) . 1 gm.
Sodium carbonate . 0·5 gm.
Distilled water . 100 c.c.

Keep at 50-60° C. for 3 days. Remove the red colour ("*red out of methylene blue*") by extraction with chloroform. Syphon off the watery stain.

B. 1 per cent eosin solution . . . 3 drops.
(Eosin B. A. or extra P. D. Höchst.)
Distilled water . 2 c.c.

Cool solution A, and add it drop by drop to solution B until the mixture becomes blue-red. Stop when the colour is so dark that the original eosin colour has entirely disappeared. A fresh staining solution should be made for each occasion. Stain for 5-10 minutes in a porcelain dish, with the film side of the cover-glass downwards: if any precipitate forms, it then falls upon the unsmeared side of the preparation, and may be easily removed. Wash in water. Dry. Mount in balsam.

**Red.**
- Red blood corpuscles.
- Central portions of blood-plates.
- Eosinophile granules.

**Violet to red.**
- Nuclei of leucocytes.
- Nuclei of plasmodia malariæ.
- Neutrophile granules.

**Blue.**
- Protoplasm of leucocytes.
- Platelets.
- Degenerated portions of red blood corpuscles.

**Hastings** proposes to dissolve the powder obtained by Nocht's method in pure methyl alcohol.

He adds 0·3 gm. of the powder to 100 c.c. of methyl alcohol. The stain retains its properties for two years.

(*b*) **Michaelis's modification** :—

Medicinal methylene blue 2 gms.
Distilled water . . 200 c.c.
} Dissolve.

Add $\frac{1}{10}$ normal caustic soda solution, 10 c.c. Boil for 15 minutes. Cool. Add $\frac{1}{10}$ normal sulphuric acid, 10 c.c. Filter.

To the solution obtained add 5 parts $\frac{1}{1000}$ watery eosin solution; shake well; stain for 15 minutes. Wash in water. Dry. Mount in neutral balsam.

This preparation may be obtained from Grübler, Leipzig, as **azur-blau**.

(*c*) **Reuter's modification** :—

| | |
|---|---|
| Methylene-blue solution, 1 per cent | 100 c.c |
| Sodium bicarbonate . . | 0·5 gm. |

Keep the mixture at 40-60° C. for 3 days; shake out the red colour with $CHCl_3$; cool and filter; add saturated watery eosin in excess; filter; wash precipitate with distilled water; dry; dissolve 0·2 gm. of the dried precipitate in 100 c.c. absolute alcohol; add 2 c.c. aniline oil. Of this stock solution add 30 drops to 20 c.c. of distilled water. Stain in an open basin.

(*d*) **Giemsa** in his modification of Romanowsky's stain combines azur-blau with eosin :—

| | | |
|---|---|---|
| Watery azur-blau solution | 1 per cent. | For staining, take 1 c.c. eosin solution, add water, 10 c.c., and then 1 c.c. of the azur-blau solution. |
| Watery eosin solution . | 1 per cent. | |

Stain 10 minutes to 1 hour.

(*e*) **Laveran** modified Romanowsky's method as follows :—

A crystal of $AgNO_3$ is placed in 50-60 c.c. of distilled water, and when dissolved, 100 c.c. of caustic soda solution are added. The resultant silver oxide, after washing with distilled water to remove the excess of soda and sodium nitrate, is placed in a saturated watery solution of medicinal methylene blue (Höchst), prepared 14 days previously. This blue is now called "**Bleu Borrel**" and may be obtained ready prepared from Grübler, Leipzig.

For staining, take :—

| | |
|---|---|
| $\frac{1}{1000}$ watery eosin solution . | 4 c.c. |
| Distilled water . . . | 6 c.c. |
| Bleu Borrel . . . | 1 c.c. |

Stain preparation for 20-30 minutes, wash in distilled water, then treat it for 10-15 minutes with 5 per cent solution of tannic acid and again wash. Dry. Pass through xylol, and mount in balsam.

Nocht now uses a method similar to that of Laveran.

(*f*) **Leishman's modification** of Romanowsky's method.

Leishman's method gives good results for general blood work, fixing at the same time as it stains. It has also the advantage that it stains the red blood corpuscles infected by the malarial parasite in a special manner.

Solution A.—1 per cent medicinal methylene blue (Grübler) in distilled water; add 0·5 per cent $Na_2CO_3$ until alkaline.

Heat to 65° C. in paraffin oven for 12 hours; allow to stand at room temperature 10 days before use.

Solution B.—Eosin (extra B. A. Grübler), 1 gm.; distilled water, 1000 c.c.

Mix equal volumes of A and B in a large open vessel, allow to stand for 6 to 12 hours, stirring occasionally. Collect the precipitate on a filter, wash with distilled water until the washings become almost colourless, dry and powder the residue. (Grübler now makes this dye, and it may be also obtained in "soloid" form from Burroughs, Welcome, & Co.)

**To prepare the stain.**—Dried precipitate (green, metallic lustre), 0·3 gm.; pure methyl alcohol (Merck "for analysis"), 200 c.c.

The solution is of a clear dark-blue colour, shows a greenish iridescence by reflected light, and when kept in stoppered glass bottles does not deteriorate.

**Staining**:—

1. Prepare a thin film. Dry in the air.
2. Stain with 4 drops of the dye for 30 seconds.
3. Add to the alcoholic stain 6-8 drops of distilled water, and allow it to mix with the dye (by rotating the forceps).
4. Allow the film to stain for 5 minutes (if the film is very thick, 10 minutes).
5. Wash the stain away with distilled water. Allow a few drops of water to rest upon the film for 1 minute.
6. Dry in air. Mount in xylol balsam.

| | |
|---|---|
| **Red.** | Neutrophile, or fine eosinophile granules. |
| **Ruby red.** | Nuclei of polymorphonuclear and mononuclear leucocytes. |
| **Pink.** | Red blood corpuscles. Eosinophile granules. |
| **Violet to Purple.** | Basophile granules. |
| **Pale blue.** | Extra nuclear protoplasm of leucocytes and lymphocytes. |
| **Blue.** | Plasmodium malariæ. Bacteria. |

If the red blood corpuscles appear bluish instead of pink, the pink colour may be restored by washing the film in $\frac{1}{1500}$ acetic acid solution. Heat may not be used to dry the film, as it breaks up the stain and decolourises the chromatin.

If a granular deposit is deposited on the films, remove it by washing quickly in absolute alcohol, the film after a few seconds being plunged into distilled water to stop the decolourising effect of the alcohol.

For Schüffner's and Maurer's "dots," stain with the mixture of stain and water for 1 hour, placing the preparation under a watch-glass or the lid of a Petri dish to check evaporation.

(*g*) **Korck** adds Michaelis's acetone to the Romanowsky methylene blue eosin, and during the preparation substitutes for the alkali the same amount of collargol.

(*h*) **Berestneff** has further modified Romanowsky's stain.

1 per cent aqueous methylene blue containing 0·3 per cent $Na_2CO_3$ is heated for 3 hours on a water bath and then filtered. 1 c.c. of the filtered solution is added to 1·5 c.c. of a 1 per cent aqueous solution of methylene blue, and to this mixture are added 5 c.c. of a 1 per cent aqueous solution of eosin (extra B. A. Höchst). Fresh preparations fixed in absolute alcohol are stained 15-20 minutes and old preparations 15-20 hours, both at laboratory temperature, and then further stained for 15-20 minutes with gentle heat. The red corpuscles are decolourised in a solution of 10 c.c. methylene blue, 1 per cent; 200 c.c. distilled water; 0·2 c.c. acetic acid. They are then dried and mounted in neutral balsam.

**Wet films.—Scott's method.**—The wet film method is not now extensively used, but a modification by Scott has been much employed and yields good results. In order to prevent the changes of shape in the corpuscles and of the solution of portions of the film in the fixing or dehydrating agent, Scott first coagulates the protoplasm by a vapour fixative.

1. Hold film over 40 per cent formalin for 5 seconds.
2. Drop, while still wet, film downwards into absolute alcohol; leave in the alcohol for 1 hour to 2 days.
3. Blot off excess of alcohol.
4. Stain with eosin methylene blue (Jenner's) for 2 minutes.
5. Wash in distilled water until the film is a red-violet colour.
6. Blot off excess of water.
7. Dehydrate rapidly in absolute alcohol; xylol; balsam.

## 5. Special elective stains for certain structures.

### Staining of Ehrlich's Granules

1. **Eosinophilous or acidophilous granules** (*a* granules) :—

*a*. Ehrlich's hæmatoxylin-eosin stain (p. 136).

*b*. 1 per cent watery eosin, or 0·5 per cent alcoholic eosin, 5-10 minutes.

*c*. Eosin, 2 gms.; indulin, 2 gms.; orange, 2 gms.; glycerin, 30 c.c., 12-24 hours. Wash in water; dry; mount in balsam.

*d*. Saturated orange G in glycerin, 1 part; glycerin, 2 parts; add eosin and nigrosin to saturation by long shaking, stain 5-12 hours. Wash in water; dry; mount in balsam. Granules, red; nuclei, black.

Eosinophilous granules stain with glycerin solutions of eosin, indulin, and orange G; the pseudo-eosinophilous granules stain only with the first named.

2. **Basophile granules** ($\gamma$ and $\delta$ granules).—**The $\gamma$ granules are large and occur** chiefly in mast cells; they are met with in leuchæmic blood, and stain with the ordinary basic dyes.

**Ehrlich** recommends:—

| | |
|---|---|
| Saturated watery solution of dahlia | 10 c.c. |
| Absolute alcohol . . . | 50 c.c. |
| Acetic acid, glacial . . | 12·5 c.c. |
| Distilled water . . . | 100 c.c. |

Stain 4-8 hours, differentiate in alcohol. Granules are dark violet.

To obtain distinct nuclear staining **Westphal's** method may be used:—

| | |
|---|---|
| Saturated alcoholic solution of dahlia . . . | 100 c.c. |
| Grenacher's alum-carmine solution | 100 c.c. |
| Glycerin . . . . | 100 c.c. |
| Glacial acetic acid . . | 20 c.c. |

Stain 4 hours. Wash in water; dry; mount in balsam. The nuclei are blue to violet; the granules, red-violet.

The granules may be also well demonstrated by staining in a saturated solution of 50 per cent thionin for 10 minutes and differentiating in 50 per cent alcohol. Then dry and pass through xylol, and mount in balsam.

Loeffler's methylene blue, or methylene blue in 70 per cent alcohol, or Ehrlich's tri-acid mixture, or Heidenhain-Biondi mixture, with differentiation in weak acetic acid solution, or a 1 per cent solution of polychrome methylene blue (1-12 hours) with differentiation in equal parts of glycerin and ether, also give good results. (For the details of staining mast cells elsewhere consult Unna, *Monat. prakt. Derm.* Bd. 13, 19, and 33.) The mast cells are best stained in tissue sections (see Chapter II.).

**The fine basophile ($\delta$) granules** occur chiefly in mononuclear transitional leucocytes, and may be stained with a saturated watery solution of methylene blue for 10-20 minutes, then washed in water, dried, and mounted in balsam.

3. **Neutrophile ($\epsilon$) granules—Ehrlich's tri-acid solution**:—

| | |
|---|---|
| Saturated watery solution of orange G . | 120 c.c. |
| ,, ,, acid fuchsin | 80 c.c. |
| ,, ,, methyl green | 100 c.c. |
| Glycerin . . . . | 50 c.c. |
| Distilled water . . . | 300 c.c. |
| Absolute alcohol . . . | 180 c.c. |

The solution should not be used until it is 6 months old; it should be neither shaken nor filtered, the required stain being removed by a pipette. Stain for 5-10 minutes.

**Aronson and Philipp's modified tri-acid solution :—**

| | |
|---|---|
| Saturated watery solution of orange G . | 55 c.c. |
| ,, ,, rubin S . | 50 c.c. |
| Distilled water . . . | 100 c.c. |
| Absolute alcohol . . . | 50 c.c. |

Add to the above solution :—

| | |
|---|---|
| Saturated watery solution of methyl green | 65 c.c. |
| Distilled water . . . | 50 c.c. |
| Absolute alcohol . . . | 12 c.c. |

After standing for 2 weeks the stain is ready for use. Add 1 drop of the concentrated solution to 25 c.c. of distilled water, and stain for 24 hours.

The preparations may be fixed at 120° C. on a copper plate, but fixation in 10 per cent formol alcohol (Muir) is much more convenient and reliable. Equal parts of alcohol and ether for 30 minutes may also be employed.

Nutting states that pure methyl alcohol, acting for 5 minutes, is followed by good staining reactions.

The results are somewhat variable.

The nuclei are greenish blue; the neutrophilous granules, violet; the eosinophilous granules, fuchsin red.

**Lefas** fixes the film in absolute alcohol; after washing, he stains with hæmatoxylin, and again washes in distilled water. The preparation is then further stained in vesuvin S (Marque B. Anilin Soda Fabrik de Stuttgart), (vesuvin, 3 gms.; water, 100 gms.; filter). The film is again washed, then dried and mounted in balsam.

Neutrophilous granules, sharply defined, maroon.

Eosinophilous granules, dark maroon (or red with prolonged staining).

### The Staining of Lymphocytes

Any one of the preceding stains may be used. A saturated watery solution of methyl green, or an alcoholic solution of fuchsin, gives sharp contrast staining.

**Pappenheim** recommends a mixture of methyl green or polychrome methylene blue, 3-4 parts; pyronin, 1-2 parts. The nuclei are blue-green, the protoplasm is bright red. The film should be fixed in absolute alcohol; with formalin, the colours mingle, and are not sharply defined.

**Beattie recommends the following modification of Benda's stain :—**

1. Stain films in the saturated aniline-safranin solution, 2-3 hours.
2. *Gradually* decolourise with $\frac{1}{4}$ per cent solution of light green.
3. Wash; dry; mount in balsam.

Nucleus, nucleoli, and network are well shown.

## Enumeration of Blood Corpuscles

**Differential count of leucocytes.**—If possible, a stand with a moving stage should be used. A film is prepared and stained on a square cover-glass; it is much better, however, to make a film which covers the entire length of a slide.

Commencing at the left-hand upper corner of the film, count the leucocytes, and enumerate them under their respective headings, *e.g.* lymphocytes (small and large), neutrophiles, eosinophiles, mast cells, etc. The field is then moved to the left, and respective areas counted until the right edge of the cover-glass or slide is reached, when a return journey is made at a lower level. The counting is continued until 250 or 500 leucocytes have been examined, and then their percentage quantities are estimated.

### Enumeration of blood corpuscles in stained preparations

**Einhorn and Laporte** examine blood films stained by Jenner's stain with a Zeiss D.D. objective and ocular 2, and employing a specially devised diaphragm (*vide* original paper), count the red and white corpuscles in the field visible, and multiply the result by special factors.

Photography may be used for this purpose. For enumeration of the erythrocytes the negative plate answers equally well with the positive picture.

### The staining of blood corpuscles in sections of organs and tissues

The tissues should be fixed in concentrated sublimate solution and well washed out afterwards. Formol may also be used, but the granules of the leucocytes do not stain so sharply. Imbed in paraffin or celloidin, or freeze and cut.

### The staining of red corpuscles in diabetic blood (Bremer's reaction)

This reaction, which results in a greenish-blue coloration of the erythrocytes, is a constant phenomenon of diabetic blood, but has been also observed in some cases of leuchæmia, Hodgkin's disease, exophthalmic goitre, and multiple neuritis.

Take a clean cover-glass, smear one half with normal blood, and the other half with the supposed diabetic blood. Fix for 2 hours at 120° C., or in equal parts of absolute alcohol and ether at 60° C. (place the bottle in hot water at 60° C.) for 4 minutes. Stain in 10 per cent watery methylene blue for 2 minutes, wash off the stain in water, and stain for 10 seconds in $\frac{1}{8}$ per cent watery eosin. Wash, dry, and mount in Canada balsam.

Or, dissolve 0·025 gm. eosin blau (Grübler) in 10 c.c. of 30 per cent alcohol, and stain for 3-5 minutes.

In diabetic blood the red blood corpuscles are green, in normal blood they are red.

Congo red (1 per cent) and methylene blue (1 per cent) stain diabetic blood elements slightly, normal blood elements intensely.

Biebrich scarlet (1 per cent) stains diabetic blood intensely, normal blood slightly.

Alkaline methylene blue (1 per cent), placed in the concavity of a hanging drop slide and mixed with diabetic blood, is rapidly decolourised (modification of Williamson's test-tube reaction).

## The Staining of Glycogen in Blood Corpuscles

The glycogen may be stained with watery solutions of iodine, with a solution of iodine in gum (Ehrlich), or a granule of iodine may be placed in the concavity of a hanging drop slide, and after the cover-glass has been inverted, the slide is heated and the glycogen granules stained by the iodine vapour. Best's method (Chapter XI. 9) is not suitable for blood films.

**Lovell Gulland's directions :—**

| | | |
|---|---|---|
| Iodine . . | 1 gm. | Add gum acacia or gum arabic until the mixture is of syrupy consistence. Keep the bottle tightly corked to prevent loss of staining power. |
| Potassium iodide . | 3 gm. | |
| Distilled water . | 100 c.c. | |

1. Place a large drop of the solution upon an air-dried film.
2. After one minute remove the surplus fluid with blotting-paper, or remove the film to the other end of the slide.
3. Examine with $\frac{1}{12}$ oil-immersion lens.

   Red corpuscles, yellow ; white corpuscles, lemon yellow ; lymphocytes, almost invisible ; eosinophiles show large refractive granules ; neutrophiles, rather dark in colour because of their closely set granules. The reaction when "positive" occurs in the polymorphs as a brown or reddish-brown colour, (1) as a diffuse coloration ; (2) as fine granules scattered throughout the whole or part of the cell body ; (3) as coarser granules occurring at the periphery. The substance is not pure glycogen, but a combination of glycogen with some form of proteid.

   The reaction occurs in anæmia, in toxæmias of metabolic origin, in suppuration, in some bacterial infections, and respiratory disturbances.

## The Staining of Fat in Blood

(1) The blood film is placed while wet in a 1 per cent solution of osmic acid for 24 hours ; it is then washed and stained with 1 per cent watery eosin. As other substances than fat reduce osmium tetraoxide, it is necessary to make a control film, fixing it in equal parts of ether and alcohol for 24 hours and then placing it in 1 per cent osmic acid for a similar period, and, after washing, counterstaining it with 1 per cent watery eosin. The two films are then compared.

(2) The film may be placed in solutions of Scharlach R or Sudan III. in 70 per cent alcohol. The fat globules are stained red or scarlet.

## The Staining of Parasites occurring in the Blood

The majority of the parasites which occur in the blood-stream may be stained by the general blood stains. The following additional directions should, however, be carefully observed :—

### Malarial Parasites

**Preparation of films.**—The blood films are preferably made on a large cover-glass, and should not be too thin. Fix in equal parts of alcohol and ether or in absolute alcohol for ½-1 hour. Ruge uses a mixture of ½ per cent acetic acid and 2 per cent formalin for this purpose.

**Staining of films.**—The film may be stained by the "vital" or "survival" method, or by single or double staining.

1. **Vital or survival staining.**

A clean cover-glass is coated with a 1 per cent alcoholic solution of methylene blue, and, when dry, a drop of freshly drawn blood is placed on the stain, and the preparation then inverted over the cavity of a hanging drop slide and the edges closed by a ring of vaseline.

2. **Single staining.**

(1) *Manson's Borax-Methylene Blue Solution.*

| | | |
|---|---|---|
| Methylene blue (Höchst or Merck) | 0.5 gm. | Will keep for 6 weeks. |
| Borax . . . . | 5 gms. | |
| Water . . . . | 100 c.c. | |

Stain the air-dried, fixed film for ½-1 minute; wash in water; dry; mount in Canada balsam.

The plasmodium malariæ, blue; nuclei, dark blue; red blood corpuscles, green.

(2) *Carbol Thionin.*

| | | |
|---|---|---|
| Saturated solution of thionin in 60 per cent alcohol . | 20 parts. | Allow to stand 4 days before using. |
| Watery 2 per cent carbolic acid solution . . . | 80 parts. | |

Fix a fresh air-dried film in equal parts of absolute alcohol and ether; stain 5 - 10 minutes, differentiate in alcohol, dry, and mount in balsam.

The parasites are red-violet, the nuclei of white and red corpuscles, deep violet.

3. **Double staining.**—Either successive staining with hæmatoxylin and eosin, or eosin and methylene blue, or

Ehrlich's "tri-acid," or the eosin-methylene-blue compounds may be used. The latter possess many advantages, but under ordinary conditions can only be employed for film preparations. Romanowsky's method (see p. 139) is the most convenient for general results, and it is valuable in differentiating the protozoic parasites from the metazoic cells.

Specimens of blood obtained within 3-4 weeks require 7-10 minutes for staining; older specimens should be left in the dye for 12-24 hours.

Details as to the preparation and use of the Romanowsky stain and its modifications are given under "Blood" stains, since these dyes are so commonly employed for blood films. All the modifications are, in fact, in daily use for the demonstration of the malarial parasites. Perhaps in England the Leishman-Romanowsky stain is more generally used. It fixes at the same time as it stains, and that is a great convenience. It may be also obtained in "soloid" form, and the preparation of small quantities is thus greatly facilitated.

Lightly stained films will be found the best for subsequent examinations. When deeply stained the particles of pigment, etc., are often obscured.

In order to rapidly find the parasites

**Ross** advises the use of a **thick film**, and takes about 20 cm. of blood for each preparation :—

1. Make a thick film; dry at low temperature so as not to fix the hæmoglobin.
2. Spread Romanowsky's aqueous eosin solution over the film with a glass rod; allow it to stain for 15 minutes (this removes the hæmoglobin as well as stains the other elements).
3. Wash with a very gentle stream of water.
4. Stain with Romanowsky's methylene blue solution for 2-3 seconds.
5. Wash with water. Examine in water or, when dry, mount in balsam.

**Ruge** points out that with Ross's method much of the film is lost. To avoid this he first fixes the film for a few minutes in a 2 per cent solution of formalin containing ½ to 1 per cent acetic acid. The hæmoglobin is thus easily removed, and the other elements are fixed. The film may then be stained with Manson's, Romanowsky-Leishman's, or Ross's staining mixtures.

**Laporte** has modified Jenner's stain (*q.v.*) so that it can be used for the malarial parasite. Since the neutral eosin methylene blue does not well stain the chromatin of the parasites, he adds polychrome methylene blue and acetic acid.

The film is stained by Jenner's solution for 2 minutes, and then, without removing the stain, 10 drops of polychrome-methylene-blue solution (2 drops of saturated solution to 15 c.c. of water) are added. The staining is continued for 5 minutes, the preparation washed in distilled water, placed in dilute acetic acid (1 drop of acetic acid to 300 c.c. water) until it becomes red or rose-red; it is then washed in water, dried, and mounted in balsam.

The corpuscles are stained as with Jenner's solution; the body of the plasmodium is blue, and its chromatin is just a shade lighter than that of the leucocytes.

To facilitate the search for the **crescentic** bodies, a thick film may be fixed in alcohol, then immersed in 0·1 per cent acetic acid to remove the hæmoglobin, and stained in methylene blue. The crescents may be recognised with a comparatively low objective.

To stain the **flagellated malarial body**, strips of thick blotting-paper $3 \times 1\frac{1}{2}$ inches are cut with an oblong hole $1 \times \frac{3}{4}$ inch in the centre. The strips are slightly moistened with water and laid on a flat glass sheet. A thick film is prepared and placed film downward over the blotting-paper cell and pressed down. After 30-60 minutes the slides are removed and dried over a spirit-lamp, fixed in absolute alcohol for 5 minutes, placed in 10 per cent acetic acid until all the hæmoglobin is dissolved out, then washed, dried, and stained with weak carbol fuchsin for 6-8 hours. They are then well washed, dried, and mounted in balsam.

To overcome the difficulties of **staining after** the administration of **quinine** when there are few parasites in the blood, **Dantec** mixes 1 c.c. of blood with 20 c.c. of distilled water in a centrifuge tube and centrifugalises. The sediment contains the blood cells and plasmodia.

**Staining of plasmodium malariæ in imbedded tissues.**—The tissue should be fixed in formalin or mercuric chloride.

**Bignami** recommends

| | | |
|---|---|---|
| Mercuric chloride | 1 gm. | Fix for 2 hours. |
| Sodium chloride | 0·75 gm. | |
| Acetic acid | 0·75 gm. | |
| Water | 200 c.c. | |

Harden in alcohol and iodine alcohol, changing the alcohol each day during 7 days. Then place in absolute alcohol and imbed in celloidin or paraffin. Stain in a saturated watery solution of magenta or in a mixture of equal parts of saturated alcoholic mixtures of magenta and orange G.

Saturated watery solution of methylene blue or Loeffler's methylene-blue solution, for 5-10 minutes, give, however, less variable results. If double staining is required, 1 per cent watery eosin may be used; the section is stained for 1-2 minutes, washed in water, passed through alcohol and xylol, and mounted in balsam.

### Leishman's Modification of Christopher's Method for Chromatin Staining in Tissues

1. The tissues are imbedded in paraffin in the usual manner, and sections prepared, of an average thickness of 5$\mu$.
2. Remove the paraffin very thoroughly with alternate baths of xylol and absolute alcohol 3 or 4 times.
3. Remove the alcohol with distilled water and the water by blotting with tissue-paper.
4. While still moist, place a drop or two of fresh blood serum on the section and allow it to remain for 5 minutes.
5. Remove the excess of serum.
6. Stain for 1-1½ hours with Leishman's stain 2 parts, distilled water 3 parts, avoiding evaporation.
7. Wash with distilled water. The cell nuclei should be almost black.
8. Decolourise alternately with $\frac{1}{1500}$ acetic acid and $\frac{1}{7000}$ caustic soda, freshly prepared with distilled water. The alkaline solution removes the eosin; the acid solution removes the blue and brightens the red of the chromatin.
9. When the cell nuclei are deep red in colour and the protoplasm a pale pink or light blue, wash the section in water and remove all surplus water with blotting-paper.
10. Dehydrate very rapidly with absolute alcohol.
11. Clear in xylol; mount in balsam.

The **Trypanosoma** [*T. Brucei*, *T. equiperdum* (tsetse disease in horses and cattle), *T. Lewisii* (in rats); *T. Evanii* (Surra disease)], the **Sporidia** and the **Piroplasma bigeminum** ("dum-dum" parasites), and the so-called Leishman-Donovan bodies (Trypanosomata?) may all be stained by the Romanowsky method or by any of its modifications. Leishman's stain gives very satisfactory results.

For **trypanosomes** stain as for malaria parasites for 10-15 minutes, and allow the film to soak in water a little longer than usual. The macro-nucleus is red; the micro-nucleus, black; the flagellum, red; the basophile granules, black; the protoplasm, blue.

Elmassion and Migone recommend the following process for **trypanosomes**:—

1. Fix blood film in absolute alcohol for 12 hours.
2. Place film in 5 per cent potassium bichromate for 1-3 hours.
3. Wash thoroughly in water.

4. Stain for 15 minutes in the following staining mixture :—

| | | | |
|---|---|---|---|
| (1) Hæmatin . | 0·5 gm. | (2) Magenta red . | 1 gm. |
| Ammonia alum . | 5·0 gms. | Absolute alcohol . | 10 c.c. |
| Distilled water . | 100 c.c. | Distilled water . | 100 c.c. |
| Glycerin . | 20 c.c. | | |

Mix one drop of solution 2 with 5 c.c. of solution 1.

Protoplasm, purple-red ; flagellum and membrane, bright red ; nucleus, violet ; centrosome, rose or light brown.

Levaditi recommends Bismarck brown and polychrome methylene blue for the staining of **trypanosomes** and of **spirilla.**

1. Fix film in absolute alcohol (10 minutes) without warming, place on the film a few drops of ether, and allow it to evaporate.
2. Stain the fixed preparation in a saturated solution of Bismarck brown (vesuvin) for 2 minutes.
3. Remove the surplus stain with blotting-paper, and stain in Unna's polychrome-methylene-blue solution (half strength).
4. Wash ; dry over flame and mount in Canada balsam.

Spirilla, blue-brown ; nuclei, dark brown ; protoplasm, dark yellow.

**Filaria sanguinis hominis.** — On examination of the freshly drawn blood the presence of the filaria is often shown by the movements of the red corpuscles in its vicinity.

Leishman's modification of the Romanowsky method shows considerable differentiation of structure. No separate process of fixing the films is necessary, as the methyl alcohol does this. The best results are got by staining for 15 minutes, and subsequently washing rapidly in alcohol.

**To make a permanent preparation,** prepare a film which is very thin at one end and moderately thick at the other. Fix in equal parts of absolute alcohol and ether, and stain with hæmatoxylin and eosin. The sheath and internal structure of the parasite are well shown, and at the thin part of the film the leucocytes can be examined.

**Bacteria in the blood-stream.**—The following micro-organisms may occur in the blood-stream :—The pyogenic cocci, pneumo-cocci, bacillus pneumoniæ, Fraenkel's gas bacillus and other anærobes (in puerperal fever), micro-coccus tetragenus, bacillus proteus, bacillus diphtheriæ, meningo-coccus intracellularis of Weichselbaum, bacillus lepræ, bacillus anthracis, bacillus typhosus and paratyphosus, bacillus coli communis, bacillus tuberculosus, spirillum of relapsing fever.

**Spirillum Obermeieri.**—Carbol fuchsin stains this organism satisfactorily, but better results will be obtained by using Leishman's stain for 15-20 minutes, and proceeding as in the method for staining malarial parasites.

As a rule, however, the student should not attempt to stain the blood for these organisms. Cultures should be almost invariably made.

**Bacillus typhosus.**—For Gruber-Widal's and Ficker's reactions, see Bacteriology, Chapter XIV.

**Estimation of the phagocytic power of leucocytes.**—Leishman's method consists of the enumeration of the micro-organisms engulfed within a definite time by the polymorpho-nuclear leucocytes of the blood of the individual under examination. The result is then compared with a similar count made with the observer's own blood.

1. Prepare an emulsion of a fresh agar culture of the germ in a few drops of normal saline in a watch-glass. Stir with a platinum loop until turbid.
2. Take 5 cm. of freshly-drawn blood and 5 cm. of the emulsion in a capillary tube; expel the blood and emulsion on to a clean slide, and mix well. Take up 5 cm. of the mixture with a capillary pipette; expel it on to a clean slide, and at once cover with a cover-glass.
3. Make a similar preparation with the blood of the observer.
4. Place both preparations in a moist chamber, and incubate at 37° C. for 30 minutes.
5. Loosen cover-glasses from slides by applying a little normal saline solution around the edges; gently slide off the cover glass from the slide, as in making ordinary film preparations.
6. Dry film in incubator for 1 minute, and then fix and stain by the Leishman-Romanowsky method.
7. Count the micro-organisms in 20 cells as they come into view, and compare the average number of germs with those contained in the cells of the observer's blood.

## Medico-legal Examination of Blood

This has for its object the identification of the corpuscular elements or the blood pigment, generally in scrapings or stained fabrics. For proof of the corpuscles a small portion of the material is moistened with normal saline solution or glycerin and water (1:10), the fluid then expressed and examined. If the stained fabric is old, the colouring matter will have been changed into hæmatin, and will be much less soluble. If distilled water or normal saline solution do not dissolve out the pigment, the material may be treated with a cold saturated solution of borax; if still insoluble, a few drops of ammonia may be added, in which case a solution of hæmatin will be obtained. Some writers recommend special macerating fluids, in which a few strands of the suspected material are placed for some minutes, and then examined under the microscope:—

| | |
|---|---|
| **Virchow.** | 32 per cent caustic potash. |
| **Puppe.** | 32 per cent caustic potash and 40 per cent formalin. |
| **Hoffman-Pacini** | Mercury perchloride, 1 part; sodium chloride, 2 parts; glycerin, 100 parts; water, 300 parts. |
| **Raissini.** | Glycerin, 3 parts; concentrated sulphuric acid, 1 part. |
| **Dragendorf.** | Sodium chloride, 1 gm.; sodium sulphate, 5 gms.; water, 94 c.c. |
| **Moser.** | Alcohol and ether (equal parts). |
| **Lesser.** | 15 per cent tartaric acid. Pepsin glycerin (does not give constant results). |

**Schmorl** recommends the following procedure :—

Moisten a small piece of the fabric in water and stain it with hæmatoxylin. Differentiate in HCl alcohol, wash thoroughly in water, stain with $\frac{1}{1000}$ watery eosin solution, wash in water (3-6 hours), place in alcohol, and then again in water. Gently tease into fine threads on a slide, and examine the isolated fibres in a drop of glycerin. The red blood corpuscles are then easily found, and the nuclei of the leucocytes or of the red corpuscles of birds and amphibia stand out clearly. A permanent preparation may be obtained by passing the specimen through alcohol and xylol, and mounting in balsam.

In some cases a portion of the stained fabric may be imbedded in celloidin, and the sections therefrom stained with hæmatoxylin and eosin.

The **red blood corpuscles** may be measured by the ocular micrometer. The red corpuscles of camel's blood are oval, and those of the sheep are smaller than those of man (5-7·7$\mu$), but the differences in the size of the red corpuscles are so slight as to render this method of diagnosis useless for medico-legal purposes.

If red blood corpuscles cannot be found, the stains must be tested for the presence of **blood pigment**. It is well also to confirm the previous examination. The pigment may be demonstrated by obtaining hæmin crystals (Teichmann's crystals), proceeding as follows :—

Place a small piece of the fabric or a scraping upon a clean slide, and saturate it with glacial acetic acid ; express the fluid ; add to the fluid a small crystal of sodium chloride or sodium iodide. Cover. Heat gradually until active ebullition occurs ; cool slowly. Examine with $\frac{1}{6}$-$\frac{1}{8}$ inch objective. The hæmin crystals are brown or claret-coloured, usually take the shape of superimposed rhombic plates, and are insoluble in water, alcohol, and ether.

Use a minute crystal of sodium chloride (the size of a pin's head). Employ a large quantity of glacial acetic acid. Boil slowly to avoid evaporation of the fluid around the cover and the subsequent projection of the cover-glass to some distance during ebullition. To avoid overheating Wachholz recommends a mixture of alcohol (90-95 per cent) and concentrated sulphuric acid ($\frac{1}{1000}$) instead of the acetic acid, as such a mixture has a lower boiling-point.

If the fabric shows any signs of contamination with fat or grease, treat it first with ether, since the presence of fat interferes with the production of the crystals.

Very old stains should be allowed to steep in a small quantity of acetic acid for 12-24 hours before attempting to obtain the hæmin crystals.

When the suspected stain occurs on iron or steel, sand, coal or clay, the crystals cannot, as a rule, be obtained, and a solution of the pigment in a saturated solution of borax should be made and submitted to spectroscopic examination.

The spectroscopic appearances of blood pigments will be found in physiological and medico-legal text-books, and the proposed biological method of distinguishing human from animal blood by the action of precipitins upon albumins will be found in the papers by **Uhlenhuth**, **Wassermann**, and **Nuttall** (*British Med. Journal*, 1903, and *Blood Immunity and Blood Relationship*, Cambridge University Press, 1904).

## Bone Marrow

**Bone marrow** may be examined in film preparations or in sections. The location of the cells and their quantitative relations can only, of course, be well examined in sections : for thorough investigation of the specific granules film prepara-

tions are preferable. The usual nuclear and plasma stains may be employed, but the methods given under "Blood" give the best results as regards the granules of the cells, etc.

Marrow may be obtained from living animals by perforation of a bone and, after removal, filling the hole with paraffin, further quantities being removed at pleasure by simply removing and returning the paraffin (Wolff).

The fixation of the film is best obtained by plunging it into a mixture of equal parts of alcohol and ether (Nikiforoff). The ether dissolves out the fat which otherwise hinders perfect drying. Other fixing fluids are 1 per cent osmic acid or a saturated sodium-chloride solution with mercuric chloride (Muir).

Muir and Lovell Gulland both recommend the "wet" method of fixation for marrow films, and employ formol alcohol for such purpose, preferably the formol methyl alcohol (p. 134).

For staining sections, any of the nuclear and protoplasmic stains may be used. It is well to employ several stains, in order to bring out the several features of the different elements.

To examine isolated cells, stain a thin celloidin section; place a drop of Canada balsam on the section; invert it over the concavity of a hanging drop slide; press slightly upon the cover-glass, and the cells will be easily dislocated.

**Price Jones** (*British Med. Journal*, 1905, p. 409) contends that dissociation of the cells is necessary for the identification and enumeration of the non-granular and granular groups of cells. He proceeds as follows:—

(1) Squeeze the marrow from ribs or vertebræ as soon as possible after death.

(2) Place a platinum loopful of the marrow in 10 per cent neutral glycerin solution in a watch-glass.

(Dilute the glycerin with ammonia-free distilled water to form a 10 per cent solution, titrating against $\frac{n}{10}$ caustic soda solution, using phenol-phthalein as an indicator.)

(3) Place a loopful of 10 per cent glycerin on a clean cover-slip. Add a loopful of the emulsion in the watch-glass. Make a film, and allow it to dry in the air, without heating (5 minutes).

(4) Fix and stain in Jenner's (or Leishman's) stain, $2\frac{1}{2}$ minutes.

(5) Wash in ammonia-free distilled water; dry thoroughly; xylol; balsam.

### Spleen and Lymphatic Structures

These tissues may be suitably examined in a fresh condition. Plunge a trocar into the spleen or lymph gland, place a portion of the material upon a cover-glass and examine it in 0·6 per cent sodium-chloride solution.

Films prepared by the methods given under "Blood" yield good results, and may be fixed and stained by the same reagents and mixtures used for bone marrow.

The **reticulum** should be examined after imbedding and stained by Mallory's method (Chapter XI. I.).

To stain **bacteria in situ** the usual aniline dyes may be used. For tubercle bacilli, aniline water should be added to the carbol-fuchsin mixture (1-4) and the stain fixed by treatment with the tannin-orange or tannin-blue solutions of Unna-Delbanco.

Typhoid bacilli are usually demonstrated by staining sections of the spleen in Loeffler's methylene blue.

## II. Liver

For general staining purposes van Gieson's method may be used; for fat, the methods described in Chapter XI. IV. may be applied.

Special methods have been devised for the bile capillaries, reticulum, and Kupffer's stellate cells.

### A. Bile Capillaries

All the methods depend much upon the freshness of the tissue. Bile capillaries are often well stained by Heidenhain's iron-alum-hæmatoxylin method and by Biondi-Heidenhain's stain (red). The following are special methods:—

(*a*) **Schmorl** uses a modification of Weigert's fibrin method (somewhat similar to Beneke's modification):—

| | |
|---|---|
| Crystal violet . . . | 1 gm. |
| Alum (10 per cent) solution . | 100 c.c. |

Sections are stained in this solution and differentiated with aniline xylol (1:4), following the general procedures of Weigert's fibrin method.

(*b*) **Boehm's method** (after Golgi's nerve methods) :—

(1) Harden pieces of tissue, about 1 cm. in size, for 12 hours in

| | |
|---|---|
| Potassium bichromate, 3 per cent | 4 parts. |
| Osmic acid, 1 per cent . . | 1 part. |

(2) Transfer to 0·75 per cent silver nitrate solution, 24-48 hours.
(3) Wash in water.
(4) After-harden in alcohol ; imbed ; cut ; stain with a nuclear stain.

Bile capillaries, black on a yellow ground (precipitates of chromate of silver). The method is not very reliable.

(*c*) **Browicz's method.**—Browicz considers that for the demonstration of bile capillaries the tissues should be hardened in formalin. Sections should then be cut on the freezing microtome and stained according to van Gieson's method. The walls of the capillaries are visible as fuchsin-coloured streaks.

(*d*) **Weigert's neuroglia method** (*q.v.*).—This and the following method give the best results. According to v. Jagić the neuroglia method may also be used with sections cut on the freezing microtome after being hardened in formol. In such a case the sections are placed in a $\frac{1}{2}$ per cent solution of chromic acid for 1 hour, transferred to the neuroglia mordant for 5-6 hours, then washed well with water, and treated exactly as in the neuroglia method.

In connection with this part of the subject, it may be pointed out that Weigert's neuroglia stain is also exceedingly useful for the excretory and secretory capillaries of other organs.

(*e*) **Eppinger's (Jun.) method.**—This is a combination of Weigert's myelin and neuroglia methods :—

(1) Harden in formol (5 days).
(2) Place in Weigert's neuroglia mordant (*q.v.*), 10 days, or 5 days in a warm oven.

Stages 1 and 2 may be combined by placing the sections in

| | |
|---|---|
| Formalin (commercial, 40 per cent) . | 1 part |
| Neuroglia mordant . . . | 10 parts |

for 10 days, or 5 days in a warm oven.

(3) After-harden in alcohol ; imbed ; cut.

(4) Stain in 1 per cent watery hæmatoxylin solution, ½ to more hours; in freshly prepared solutions, 12-24 hours.
(5) Transfer directly to a saturated watery solution of copper acetate for exactly 5 minutes.
(6) Wash in distilled water for several hours.
(7) Differentiate in

| | |
|---|---|
| Potassium ferricyanide . . | 2·5 gms. |
| Borax . . . . | 2 gms. |
| Water . . . . | 100 c.c. |

until the section is brown (about 10 minutes).

(8) Wash in water.
(9) Place in saturated lithium-carbonate solution, 3-5 minutes.
(10) Dehydrate in absolute alcohol.
(11) Clear in xylol; mount in balsam.

Bile capillaries and nuclei, brown.

The bile capillaries may also be demonstrated by means of injections, especially intra-vital. They then appear like the "naturally injected" bile capillaries in icterus.

## B. Methods for Demonstrating the Reticular Structures

(*a*) **Boehm's method** (modification of Golgi's method for nerve tissues, *q.v.*):—

(1) Harden pieces about 1 cm. square in ½ per cent chromic acid solution, 48 hours.
(2) Transfer to 0·75 per cent silver nitrate solution, 72 hours.
(3) Wash in distilled water for several hours.
(4) After-harden in alcohol; imbed very rapidly in celloidin or paraffin; cut.
(5) Wash; dehydrate; clear in xylol; balsam.

Reticular fibres, black.

(*b*) **Oppel's method** (also a modification of Golgi's method for the central nervous system):—

(1) Harden in alcohol.
(2) Transfer the piece of tissue to 10 per cent solution of potassium bichromate, 24 hours.
(3) Wash in very dilute silver nitrate solution (a few drops of 0·75 per cent solution to 30 c.c. of water), using a large quantity of fluid.
(4) Transfer to 0·75 per cent silver nitrate solution, changing the fluid several times, 24 hours.
(5) Wash in distilled water.
(6) Dehydrate in absolute alcohol; imbed; cut.

**C. Methods for demonstrating Kupffer's stellate cells** in their relation to the intra-lobular capillaries of the portal vein.

(*a*) **Kupffer's gold method :—**

(1) Cut fresh sections on the freezing microtome.
(2) Wash in 0·05 per cent chromic acid solution.
(3) Transfer to

| | |
|---|---|
| Gold chloride . . . | 1 part |
| Hydrochloric acid . . | 1 part |
| or formol (0·4 per cent) | |
| Water . . . | 1000 parts, |

and allow the sections to remain until they are red or violet, 36-48 hours.

(4) Reduce in 0·1-0·2 per cent formic acid.
(5) Dehydrate in absolute alcohol.
(6) Clear in xylol ; mount in balsam.

Stellate cells, black ; liver cells, red-violet. The method is not very satisfactory.

**Rothe** impregnates frozen sections in gold chloride 1 part, hydrochloric acid 1 part, distilled water 10,000 parts, for 48 hours, and reduces them with 0·1-0·2 per cent formic acid solution.

(*b*) **Method of injection.**—The stellate cells may be isolated and impregnated by injections of cinnabar or carmine (Asch) or Chinese ink (v. Kupffer). Cohn obtains better results by injecting the vein of a rabbit's ear with "argentum colloidale" (Crede) 1 gm., water 5 c.c. The livers of animals killed three minutes after the operation already exhibit the black-coloured stellate cells.

## III. Skin

Pieces of skin must not remain too long in alcohol, or they become very brittle and difficult to cut. For the same reason it is better to imbed them in celloidin than in paraffin.

Keratohyalin and eleidin, which occur in epidermal structures, are considered in the previous chapter ; similarly, mast and plasma cells (for instance in lupus), and pigment, all of which here play important parts, are included in earlier sections.

Amongst the special constituents of the skin for which distinctive methods have been devised, the epithelial fibrillæ deserve the first consideration.

(*a*) **Kromayer's method for the epithelial fibrillæ.**—This is a modification of Weigert's fibrin method, and akin to the already mentioned Beneke's method :—

(1) Harden in alcohol; imbed in paraffin; prepare very fine sections (this is important, according to Kromayer); remove the paraffin with xylol; transfer to absolute alcohol; add water gradually until the alcohol is diluted; arrange the section on the slide; dry well with filter-paper.
(2) Stain in equal parts of aniline water and saturated watery methyl violet 6 B (the mixture should be always freshly prepared), 5 minutes.
(3) Wash thoroughly.
(4) Place in weak Lugol's solution—1 part Lugol, 2 parts water—for about 30 seconds.
(5) Wash in water; dry the section thoroughly with filter-paper.
(6) Differentiate in aniline xylol (1 : 2), then in xylol until the colour ceases to come away in clouds, controlling the further differentiation under the microscope until the fibrillæ are prominent.
(7) Clear thoroughly in xylol; mount in balsam.

Epithelial fibrillæ, blue. The nuclei may be previously stained in lithium- or alum-carmine.

In a similar manner K. Herxheimer stained the fibrillæ by Gram and Gram-Weigert's methods prior to Kromayer.

(*b*) **Unna's method for the epithelial fibrillæ.**—Unna has devised a number of methods for this purpose, employing water-blue and orcein, hæmatoxylin and picric acid, hæmatoxylin and orcein, gentian violet and iodine, the most recent procedure being a modification of Weigert's fibrin method.

He stains celloidin sections in

| | | |
|---|---|---|
| Gentian violet | . . | 1·5 gm. |
| Alum | . . . | 10 gms. |
| Water | . . . | 100 c.c. |

transfers them to an iodine solution and differentiates first in aniline xylol (1 : 4), then in equal parts of aniline and xylol for 30 minutes, clears in xylol and mounts in balsam.

Here may be also recorded

(*c*) **Unna's water-blue orcein method :—**

(1) Harden; imbed; cut.
(2) Stain in 1 per cent watery solution of water-blue, 10 minutes.
(3) Wash in water.
(4) Transfer to a neutral 1 per cent alcoholic orcein solution (Grübler), 5-10 minutes.
(5) Dehydrate in absolute alcohol.
(6) Clear in xylol; mount in balsam.

(*d*) **K. Herxheimer's method for epithelial fibrillæ :—**

(1) Harden ; imbed ; cut.
(2) Stain in a saturated watery solution of cresylecht violet.
(3) Dehydrate in absolute alcohol.
(4) Clear in clove oil.
(5) Mount in balsam.

**Kolossow** has recommended several methods for the demonstration of **intercellular bridges;** of these we quote the following :—

Inject 0·6 per cent normal saline solution into the vessels of a recently killed animal, and then continue the injection for several minutes with the following solution :—

| | |
|---|---|
| Osmic acid, ½ per cent watery solution | 100 c.c. |
| Nitric acid (30 per cent) . . | ½-1 c.c. |
| Glacial acetic acid . . . | 1 c.c. |
| Potassium nitrate . . | 10-12 gms. |

Place small pieces of the injected tissue in ½ per cent osmic acid, 24 hours ; transfer to 10 per cent tannin solution, 24 hours (changing several times).

Wash in water and in 70 per cent alcohol ; harden in alcohol ; imbed in paraffin ; prepare sections ; mount without staining.

The metallic impregnations described in the last chapter are useful for certain skin structures. Silver nitrate is often selected to demonstrate the superficial layers of the epidermis, while for nerves and tactile corpuscles the gold chloride methods give good results (Ranvier), as also for the "prickle" cells and the intercellular bridges.

The intercellular bridges may also be demonstrated by ordinary staining methods, such as van Gieson's, with which they remain unstained ; in this condition careful illumination is required for their recognition, a remark that also applies to their recognition in unstained sections and in macerated tissues.

## IV. Muscle

For the demonstration of muscle fibres van Gieson's stain is perhaps the best we possess (not excepting even eosin), since the fibres take up the yellow and are thus strongly contrasted with the deep red of the connective

tissues. It may be remarked, however, that very old, dense, connective tissue usually stains yellow, but the shade can be easily distinguished from that of the muscles.

Striated muscle fibres are very evident, when some of the light is cut off by using a narrow diaphragm, in unstained as well as in stained preparations.

Smooth muscle fibres are very well defined by van Gieson's stain, with which they assume a bright yellow.

For the definite differentiation of single or isolated smooth muscle fibres from connective tissue fibres no method at present exists.

Next to van Gieson's method for muscle fibres, Mallory's aniline-blue stain (*q.v.*) is perhaps the best; by deeply staining with the acid fuchsin the connective tissues are a brilliant blue and the muscle fibres are light red.

The **myoglia** fibrils stain well with Mallory's stain for fibroglia fibrils (*q.v.*).

## V. Bone

As a rule, bony tissues are decalcified before making sections, but in those pathological conditions in which the calcium salts are entirely or almost entirely removed from the bone, decalcification is unnecessary.

The decalcifying fluids are given in Chapter V. Here again the sulphurous or trichloracetic acids may be advised, and the necessity for thorough washing be emphasised.

Celloidin imbedding is best; the heat necessary for paraffin imbedding frequently damages the osseous tissues.

The stains must act for a longer period than usual, because of the possible "traces" of acid in the tissues, and it is well always to place the sections in alkaline fluids before staining, so that any remaining acid may be neutralised; this is especially necessary for nuclear stains.

To obtain **preparations of bone without decalcification**, pieces of bones are first fixed, hardened, and stained in bulk if desired. They are then sawn in the dried condition,

being afterwards polished with a hone, pumice stone, or fine emery powder; or sections may be cut with a double saw, used like a double knife (*Präcisionssäge* of Arndt). These methods are but rarely employed in pathological histology, and we only just mention them. Details of procedure are given in Boehm und Davidoff and in other histological text-books.

As a **general stain for bony tissues** van Gieson's fluid is very useful, the bony areas becoming yellow to red and the cartilage blue.

A number of **special methods** have been devised to demonstrate the various constituents of fully formed bone, to differentiate the calcified and non-calcified areas, and to display the exact relations of the various structures of growing bone. These may be conveniently considered in three separate sections.

(The reader is referred to Schaffer's two papers in the *Zeitschrift f. Wiss. Mikr.* Bd. 5 and 10, for more exhaustive details.)

## I. Individual Constituents of Bone

### A. Sharpey's Fibres

(*a*) **Ebner's method.**—Sections of decalcified bone are treated with a saturated solution of sodium chloride or strong hydrochloric acid. Sharpey's fibres appear dark when the iris diaphragm is almost closed, and the illumination is thus lessened.

(*b*) **Kölliker's method**:—

(1) Harden; decalcify; after-harden; imbed; cut.
(2) Place the section in strong acetic acid until it is transparent.
(3) Stain in a saturated watery solution of indigo carmine, 15-16 seconds.
(4) Wash in water.
(5) Mount in glycerin.

Fibres, red; ground substance, blue.

(*c*) **Beneke's modification of Weigert's fibrin method.**—*Vide* Elastic Fibres (Chapter XI. 3). Sharpey's fibres are stained blue.

### B. Lacunæ and Canaliculi

A large number of methods are available for this purpose, and we select the following for general use :—

(*a*) **Recklinghausen's method.**—Place sections or shavings in a strong solution of alum for 10 minutes. The acid portion of the alum liberates $CO_2$ and penetrates to the canaliculi and lacunæ. The process should be controlled under the low power, using a small diaphragm, and stopped as soon as the acid reaches the lacunæ. The preparation may then be examined in glycerin. If alum-carmine solution containing additional alum is used, the tissues are stained at the same time.

(*b*) **Zimmermann's method.**—Heat shavings of non-decalcified bone in xylol for two minutes (to remove the fat); dry thoroughly, heat for a further two minutes in an alcoholic solution of fuchsin. Place the section, which should be covered with the staining solution on all sides, on the prong of a pair of forceps, and arrange it so that the dye may dry on the preparation. Two or three days later, carefully scrape away the dried stain, heat once more in xylol, and mount in balsam.

The canals are stained red.

(*c*) **Schmorl's method with thionin and picric acid.**—This, as well as the following method, yields almost perfect demonstrations of the lacunæ. Being applicable to sections of decalcified bone, it has superseded the older methods (which were performed with bone shavings), and has caused their relegation to the shelf of history. The method is as follows :—

(1) Harden in formol, Orth's mixture, or less preferably in other media (except sublimate).
(2) Decalcify. Schmorl advises Müller's nitric acid, formol nitric acid, or Ebner's alcoholic hydrochloric acid solution.
(3) Wash thoroughly in water. After-harden in increasing strengths of alcohol; imbed in celloidin (not paraffin); cut.
(4) Transfer the section to water for 10 minutes at least.
(5) Stain the section (well spread out) for 5-10 minutes or more in

| | |
|---|---|
| Saturated solution of thionin in 50 per cent alcohol . . . | 2 c.c. |
| Water . . . . | 16 c.c. |

(The results are better if 1 or 2 drops of liq. ammoniæ are added.)

(6) Wash in water.
(7) Stain in hot saturated and cold filtered watery solution of picric acid, 30-60 seconds.
(8) Wash in water.
(9) Differentiate in 70 per cent alcohol until the colour ceases to come away in blue-green clouds, 5-10 minutes or more.
(10) Dehydrate in 96 per cent alcohol (celloidin preparations).
(11) Clear in phenol xylol; xylol; balsam.

Lacunæ and canaliculi, dark brown to black; bone cells, red; ground substance, yellow (brownish yellow).

This method, which yields such sharply defined pictures, consists in an impregnation with fine sediment rather than a distinct staining. If the deposits are too numerous in the other portions of the section, Schmorl removes them by washing the section in water for 30-60 minutes between stages 9 and 10. Then the ground substance is not yellow, but colourless or blue according to the methods of hardening and decalcification. Previous staining (overstaining in hæmatein because of the decolourising action of the picric acid) in this case renders the cells more prominent, but in such a case better results may be obtained by the following method:—

(*d*) **Schmorl's thionin and phosphotungstic or phosphomolybdic acid method**:—

(1) Fix small fresh pieces of bone in formol.
(2) Harden in Müller's fluid, 6-8 weeks, or 3-4 weeks in the incubator.

1 and 2 are best conducted at 37° C.

(3) Transfer to Ebner's alcoholic hydrochloric acid solution; when entirely decalcified, wash thoroughly. After-harden in alcohol. Imbed in celloidin or paraffin. Make very thin sections.
(4) Place in water, 10 minutes at least.
(5) Stain in the alkaline-thionin solution, previous method, 3 minutes.
(6) Wash in water.
(7) With glass needles transfer the sections to a saturated watery solution of phosphotungstic or phosphomolybdic acid for a few seconds (the tissues are not harmed by slightly longer immersion).
(8) Wash—5-10 minutes—until the section is sky-blue in colour.

(9) Fix the stain for 3-5 minutes in

| | |
|---|---|
| Liq. ammoniæ . . . | 1 part. |
| Water . . . . | 10 parts. |

(10) Transfer directly to 90 per cent alcohol; change twice.

(11) Dehydrate in 96 per cent alcohol (celloidin); absolute alcohol (paraffin).

(12) Clear in phenol xylol (celloidin); xylol (paraffin).

(13) Mount in balsam.

If the ground substance is of too dark a colour, place the section in acid alcohol ($\frac{1}{100}$ HCl alcohol) for 5 minutes between stages 10 and 11, then wash in water before dehydration.

The outlines of the lacunæ and the canaliculi are dark blue, the ground substance is stained a light or greenish blue, the cellular elements a diffuse blue.

**Schmorl** considers the previous method the more reliable, and advises this one for growing bones only. He states that in rickets the well-ossified areas alone stain well. **Donati** has shown that Schmorl's methods yield good results with bones that have lain macerating for years.

### C. Osteoblasts and Osteoclasts

For the demonstration of their basophile characters, **Askanazy** advises the following method:—

(1) Harden; decalcify; after-harden; imbed; cut.

(2) Stain sections in Loeffler's methylene blue, 5-10 minutes.

(3) Wash in distilled water.

(4) Counterstain in 95 per cent alcohol containing 2 drops of a saturated solution of alcoholic eosin or 5 drops of a saturated watery solution of orange, 2-5 minutes.

(5) Dehydrate in absolute alcohol, 30-60 seconds.

(6) Clear in xylol; mount in balsam.

Nuclei, light blue; protoplasm of osteoblasts and osteoclasts, dark blue; ground substance, yellow to red.

This method should be generally useful for growing cells, since the granules of young **granulation tissue** cells as well as those of endothelial cells are stained by basic dyes.

## II. Methods for the Differentiation of Calcified and Non-calcified Areas (Rickets, Osteomalacia, etc.)

Since this distinction is not with certainty recognised in completely decalcified bones, methods have been devised which enable sections to be made, although decalcification is incomplete.

**Pommer** places small pieces of bone in Müller's fluid and allows them to remain there until they can be easily cut with a razor (this stage generally requires a few weeks to a few months). They are then washed, hardened in alcohol, imbedded in celloidin and cut. In unstained sections the areas containing lime salts appear refractive and homogeneous, while those without lime salts present a fibrillar structure.

When sections are placed in dilute ammonia carmine (6 parts of ammonia carmine to 100 parts of water) for 6-12 hours and then examined in glycerin, the areas that do not contain lime are stained more deeply.

If sections are treated with 2-5 per cent silver nitrate solution for 15-30 minutes and exposed to sunlight, washed in water, then transferred to 10 per cent sodium sulphate solution (to remove the excess of silver) and subsequently stained in hæmatoxylin and eosin, the calcified areas are black, osteoid tissue red, and the nuclei blue.

As a rule, the areas which contained lime salts prior to decalcification may also be demonstrated in entirely decalcified tissues by the following method of Pommer :—

(1) Harden in Müller's fluid.
(2) Decalcify in Ebner's watery fluid ; wash ; after-harden ; make sections without imbedding.
(3) Transfer the sections from water to the following solution and leave them to stain for 12-18 hours :—

Methyl violet, 0·002 per cent watery solution,

or Dahlia . . 0·004 per cent watery solution,

or Safranin . 0·01-0·016 per cent watery solution.

(4) Wash in water.
(5) Mount in glycerin containing the stain.

The areas which contained lime before decalcification are stained, the others are unstained.

With Schmorl's first thionin method the calcified parts are stained a darker yellow than the non-calcified areas, the lacunæ being more distinct in the former ; and thus the

calcified and non-calcified areas are also by Schmorl's method easily recognised.

The above-described method of Recklinghausen for the demonstration of Sharpey's perforating fibres also makes the reticular fibres more visible, the latter occurring, according to Recklinghausen, in non-calcified areas (Halisteresis).

## III. Methods for the Demonstration of Normal and Pathological Formation of Bone

For this purpose double staining in hæmatoxylin and neutral carmine or eosin yields useful results. After decalcification, celloidin imbedding and cutting, sections are washed well for 12-24 hours, stained in hæmatoxylin, again washed for 12-24 hours, and finally stained in dilute neutral carmine solution (6-12 hours). When counter-staining with eosin, allow the sections to lie in water for some time after staining, and then transfer them directly to alcohol, completing the preparations in the usual manner.

The decalcified areas and osteoid tissue are stained red; cartilage varies in colour; as a rule the ordinary cartilage is red, the growing zones and the osteoblasts and bone cells dark blue.

**Klaatch** stains sections in a saturated watery solution of picric acid for 1-2 minutes, transfers them to glacial acetic acid for 30 seconds, washes in water, dehydrates, clears and mounts.

The cartilage is bluish; newly formed bone, intense yellow.

In good van Gieson preparations the red-stained osteoid tissue is somewhat sharply differentiated from the yellow-coloured bony (calcified) areas.

For the study of developmental changes in bone, Schaffer's modification of Bouma's method is exceedingly useful:—

(1) Fix; decalcify in $\frac{1}{2}$ per cent chromic acid (after fixation in picric acid sublimate solution); after-harden; imbed; cut.
(2) Stain in watery solution of safranin, 24 hours (1 drop of 1 per cent safranin solution to 20 c.c. of water).
(3) Wash in water.
(4) Fix the section to the slide and dry it with blotting-paper.
(5) Clear with benzene.
(6) Mount in balsam.

The slightest trace of lime in the cartilaginous matrix is stained yellow and so is readily recognised.

**Cartilage** is stained deeply by hæmatoxylin, and growing areas absorb the colour even more intensely.

With Weigert's fibrin and elastin stains cartilage maintains, as a rule, its original blue colour; with Hoyer's stain for mucin and with similar dyes the cartilage stains metachromatically and becomes red. According to Roethig it is also stained red by kreso-fuchsin (see Elastic Fibres).

Cartilage and bone, both from their normal structure and from their respective staining affinities, are easily differentiated.

**Bone Marrow,** see p. 153.

**Teeth.**—The methods which have been cited for the investigation of bony structures are also applicable to the study of dental tissues.

## VI. Nervous System

**Fixation and hardening.**—Until recently, Müller's fluid was universally employed for the preservation of tissues taken from the central nervous system and eye, and even now it may be used with advantage for these two organs. After fixation, the tissues are washed in water for a very short time and hardened in increasing strengths of alcohol in the dark. The process requires much time: for spinal cord, 3-4 months, for brain, 6-12 months; both these periods may be shortened by keeping the tissues at 30-37° C. The fluid must be changed frequently during the first few days.

The time may be considerably shortened by employing Orth's fluid (Müller's fluid and formol). Hardening in formol alone is even better practice. Afterwards any of the other hardening methods may be used, or the tissues may be mordanted with chromic acid solutions, which absorbs less time than when fixing in Müller's fluid from the first.

With nervous tissues, in order to obtain good staining results small pieces of tissue should be placed in the hardening fluid as early as possible after death, and laid upon cotton-wool or tow. If large pieces are necessary, they must be cut in several places to permit the penetration of the fixing medium.

**Onuf** injects 12 per cent formalin by lumbar puncture and through the foramen magnum immediately after death, in order to fix the nervous structures at the earliest hour possible.

When removing the spinal cord, the tissues must be handled

with exceeding care, pulling or tearing should be avoided, and the cord must be transferred to the fixing fluid in a very gentle manner. If these precautions are not observed, numerous artefacts may result. A very readable account of these is given by van Gieson in his well-known papers.

**Imbedding.**—For the imbedding of nervous tissues celloidin is better than paraffin, but paraffin may be used for general purposes.

**Staining.**—As with other organs, nuclear and plasma stains are used for general impressions. For such purpose van Gieson's method is particularly appropriate, because, in addition to the nuclei and protoplasm, it stains certain other nervous structures, of which we shall presently treat.

The following modification of the Ehrlich-Heidenhain-Biondi tri-acid staining method by **Rosin** is also useful :—

(1) Stain celloidin sections for 1 minute in

| | |
|---|---|
| Ehrlich's tri-acid solution . . | 0·4 c.c. |
| Acid fuchsin solution, $\frac{1}{2}$ per cent . | 32 c.c. |
| Water . . . . | 100 c.c. |

(2) Wash in water for about 1-2 minutes (until no more dense clouds of the dye are given off).

(3) Transfer to acetic acid solution $\frac{1}{100}$, 5-10 seconds (at most).

(4) Wash in water.

(5) Rapid differentiation and dehydration in absolute alcohol, 1-2 minutes.

(6) Clear in xylol ; mount in balsam.

Nuclei of connective tissue, vessels and glia, blue ; connective tissue and vessel walls, deep red ; protoplasm of all cells, red-violet ; red blood corpuscles, orange ; neuroglia tissue, axis cylinders, violet ; myelin (after formol and chrome hardening), yellow-orange ; nerve cells (after formol and alcohol hardening), rose, with blue Nissl's granules ; chromatin of their nuclei, red-violet ; nucleolus, violet.

After chrome hardening the chromatin of the nucleus and the nucleolus of the nerve cells are stained red.

A large number of processes have been devised for the specific staining of the more important nervous structures, and some of them constitute the most important procedures of pathological histology. In some instances these elective stains are in themselves quite sufficient for an examination of diseased tissues ; in others, several must be used, as for instance when a determination of the age and extent of degeneration of nerve fibres is necessary. As a rule, it is

not possible to definitely select methods until certain stains have been tried.

The most important special methods may be included under the following heads:—

| | | | | |
|---|---|---|---|---|
| I. | Methods | for | the staining of | ganglion cells. |
| II. | „ | „ | „ | myelin sheaths. |
| III. | „ | „ | „ | axis cylinders. |
| IV. | „ | „ | „ | neuro-fibrillar structures. |
| V. | „ | „ | „ | several nerve structures at the same time (Golgi : intra - vital methylene blue). |
| VI. | „ | „ | „ | neuroglia. |
| VII. | „ | „ | „ | degenerated nerve fibres. |
| VIII. | „ | „ | „ | the peripheral nervous system. |

## I. Methods for Staining Ganglion Cells

In addition to the general staining methods, that of **Nissl** has been largely used for the demonstration of the so-called Nissl's or tigroid bodies:—

Very small pieces of fresh tissue are hardened in 96 per cent alcohol for 5 days ; 2-3 days are sufficient for hardening purposes, but it is better to extend the time and to change the alcohol frequently, in order to remove the cleavage products of the myelin (see below).

After thus hardening the tissue, Nissl imbeds the tissue neither in celloidin nor in paraffin. The glossy under-surface of the object is rapidly dried with filter-paper, and lightly pressed on to a block of wood previously covered with a thin layer of gum arabic, and the block and tissue then placed in 96 per cent alcohol. The gum soon hardens, turns white, and sections may be cut, the knife being well sprinkled with 96 per cent alcohol during the process. The sections are placed in the same alcohol, and should spread themselves out quite evenly. The block may be kept, but fresh sections must be cut whenever it is necessary to stain them.

The sections are overstained by heating, differentiated with aniline alcohol, and then treated with oil of cajuput. The

whole process must be performed as rapidly as possible. The following is perhaps the best staining mixture :—

| | |
|---|---|
| Methylene blue B . . . | 3·75 gms. |
| Venetian soap shavings . . | 1·75 gm. |
| Water . . . . | 1000 c.c. |

Shake well.

The solution should be kept for 3 months before using, and stains better when even still older. Before using, it should be always well shaken and then filtered.

The watch-glass or basin containing the sections should be heated quickly over the flame until bubbles appear. The sections should swim freely in the solution. They are then differentiated in the following mixture :—

| | |
|---|---|
| Aniline oil . . . . | 10 parts. |
| Alcohol, 96 per cent . . . | 90 parts. |

The aniline oil should be almost as light and as clear as water ; it is best to obtain it from the "Höchster Farbwerken," and to protect it from the daylight.

The section may not remain long in the aniline alcohol, but should be rapidly spread upon a slide, dried with blotting-paper, and covered with cajuput oil. Up to this stage the manipulations should not require more than 5 - 20 seconds. The colours will not now change further. The oil of cajuput is to be removed, and the section dried thoroughly. Benzene is then poured over the section in excess (to remove the oil), the superfluous benzene drained off, and the wet section covered with a few drops of xylol colophonium.[1] If a milky cloudiness occurs, it is harmless, and will disappear on slightly warming the preparation.

It is best to place a large drop of the colophonium on the section (see above), to warm slightly (when the medium is thinned), and to pour off the superfluous colophonium and cover. The preparation should be again warmed slightly ; press gently upon the cover-glass ; if colophonium still emerges, remove it and repeat the proceeding. During these procedures the section is not allowed to become dry, and all the details are to be performed rapidly. For the beginner Nissl advises a very thin solution of colophonium. If the preparations are protected from the sunlight, they keep well.

In exceptional cases celloidin imbedding may be practised, but then the pieces must not remain long in the celloidin, and

[1] The xylol colophonium is prepared by half-filling a glass with powdered colophonium, then filling it nearly to the brim with xylol, and allowing it to stand exposed under a bell-jar. The upper layer of clear, thin fluid is decanted and used. Benzene may be substituted for the xylol.

the block should be rapidly transferred to 80 per cent alcohol; the staining mixture in this case should not be so strongly heated, and differentiation must be performed in 96 per cent alcohol.

Toluidin blue, thionin, dahlia, Bismarck brown, and neutral red may be used instead of the methylene blue, and they sometimes yield better results.

Nissl describes certain artefacts which may appear; swellings, ball forms, and distended cells, due to the cleavage of myelin by the alcohol. Fortunately these again disappear during the stages of aniline alcohol and cajuput oil.

(*a*) **Nissl's method** may be shortly summarised as follows:—

(1) Harden in 96 per cent alcohol for about 2-5 days; preparation of the block with gum arabic; cut; place the sections in 96 per cent alcohol.
(2) Stain in the methylene-blue soap mixture until bubbles arise.
(3) Differentiate very rapidly in aniline alcohol.
(4) Arrange section on a slide; dry; clear in cajuput oil.
(5) Dry; add benzene.
(6) Remove benzene; mount the wet section in xylol colophonium, slightly warming.
(7) Cover; press gently; remove excess of colophonium.

The structure of the ganglion cells is well shown; granules, dark blue; nuclei, light blue.

Other methods, which are more or less modifications of Nissl's method, are as follows:—

(*b*) **van Gehuchten's method** (described by Lee).—Imbed in paraffin; prepare sections; stain in Nissl's methylene-blue mixture for 5-6 hours at 35-40° C. Differentiate as in Nissl's method and mount in xylol dammar.

(*c*) **Gothard, Luithlen and Sorgo's method.**—Harden as usual; imbed in celloidin; stain in Unna's polychrome methylene blue (24 hours); wash in water for 24 hours; differentiate in—

| | |
|---|---|
| Creosote . . . . | 5 c.c. |
| Xylol . . . . | 5 c.c. |
| Oil of cajuput . . . | 4 c.c. |
| Absolute alcohol . . . | 16 c.c. (Gothard), |

or in Unna's glycerin-ether mixture (Luithlen and Sorgo), in both cases until the grey and white substances are plainly distinguished with the naked eye. Then wash well with absolute alcohol, remove the alcohol thoroughly in several changes of origanum oil, and mount in balsam.

(*d*) **Held's method** :—

(1) Harden in 96 per cent alcohol or in picric-sulphuric acid (200 c.c. saturated watery picric acid, 4 c.c. sulphuric acid ; filter ; add 100 c.c. water). After-harden in rising strengths of alcohol ; imbed in paraffin ; cut.

(2) Stain for 1-2 minutes, slightly warming, in

| | |
|---|---|
| Erythrosin . . . | 1 gm. |
| Water . . . . | 150 c.c. |
| Glacial acetic acid . . | 2 drops. |

(3) Wash in water.

(4) Stain in equal parts of Nissl's methylene-blue soap solution and 5 per cent watery solution of acetone. Warm gently until no odour of acetone is given off.

(5) When the slide is cool, differentiate in $\frac{1}{1000}$ alum solution until the section is again red, a few seconds to a few minutes.

(6) Wash in water.

(7) Dehydrate rapidly in absolute alcohol.

(8) Clear in xylol.

(9) Mount in benzene colophonium.

Nissl's granules, deep blue (with light violet shade) ; nucleolus, blue ; protoplasm and nuclei, light red.

(*e*) **Rosin's method** :—

(1) Fix in formol ; imbed in celloidin ; cut.

(2) Stain in saturated watery solution of neutral red, some hours.

(3) Wash in water.

(4) Dehydrate in absolute alcohol.

(5) Clear in xylol ; mount in balsam.

Nissl's granules and nucleolus (basophile), red ; protoplasm of cells and nuclei (acidophile), yellowish.

(*f*) **Lenhossék's method.**—This method is the best for general work and particularly for the beginner :—

(1) Harden in equal parts of saturated watery picric acid and mercuric chloride (Rabl's mixture) ; after-harden in absolute alcohol ; imbed in paraffin ; cut.

(2) Stain in saturated watery solution of toluidin blue overnight.

(3) Wash rapidly in water.

(4) Differentiate carefully in absolute alcohol.

(5) Transfer to phenol xylol.

(6) Clear in xylol (very rapidly).

(7) Mount in balsam.

Nissl's granules, blue.

50 per cent formalin may be used for hardening, 2 days. A saturated solution of thionin blue may be substituted for toluidin blue; stain for 5 minutes.

Aniline alcohol (1-10) may also be used for differentiation; it is a trifle slower in action.

Sections may be cut on the freezing microtome without imbedding, and good results will be obtained

(*g*) **Bielschowsky-Plien's method**:—

(1) Harden in formol or alcohol; imbed; cut.
(2) Stain in 6-10 drops of saturated watery cresyl-violet R. R. solution added to 50 c.c. of water, 24 hours.
(3) Wash rapidly in water.
(4) Differentiate and dehydrate in increasing strengths of alcohol.
(5) Clear in xylol.
(6) Mount in balsam

The stain appears to be more permanent than the others which are used for this purpose.

(*h*) **Cox's method** (specially for spinal ganglion cells):—

(1) Fix in saturated mercuric chloride solution . 30 parts.
or in " " " " . 15 parts.
mixed with Platinic chloride solution . 15 parts.
Osmic acid, 1 per cent . . 10 parts.
Glacial acetic acid . . 5 parts.
for 2-3 days.

After-harden in alcohol; imbed in paraffin; cut; fix section on slide; remove paraffin.

(2) Place in 20-25 per cent tannin solution, 8 hours.
(3) Wash in water.
(4) Mordant in iron-ammonium-sulphate solution (2·5 per cent) for 5-10 minutes.
(5) Wash in water, 10 minutes.
(6) Stain in

| | |
|---|---|
| Methylene blue . . . | 1 gm. |
| Potassium carbonate . . | 1 gm. |
| Water . . . . | 100 c.c. |

Mix shortly before using. Take of this solution 2 parts, and 2 per cent solution of carbolic acid, 5 parts. Stain for 5 minutes on a water bath, with gentle heat.

(7) Remove excess of stain by drying the section on the slide; dehydrate in 90 per cent alcohol, 90 parts, mixed with xylol, 60 parts.
(8) Clear in xylol; mount in balsam.

(*i*) **Kodis' method** :—

(1) Fix thin pieces in saturated watery solution of cyanide of mercury, 1-2 days.

(2) Harden (without washing) in 10 per cent formol, 1-3 days. Cut on the freezing microtome.

(3) Stain for 1-2 minutes in molybdic acid hæmatoxylin :—

| | |
|---|---|
| Hæmatoxylin (crystals) . | 1 gm. |
| Pure molybdic acid (Merck) . | 1·5 gm. |
| Water . . . | 100 c.c. |
| Hydrogen peroxide . . | 0·5 c.c. |

Allow the solution to stand for one day after being prepared. For staining, take of the mixture 1 part, water 4 parts.

(4) Wash in water, 1-2 minutes.

(5) Counterstain in alcoholic solution of light green (if desired).

(6) Dehydrate in absolute alcohol.

(7) Clear in xylol ; mount in balsam.

Ganglion cells and processes, violet ; neuroglia fibres, reddish ; neuroglia nuclei, red.

The tissue may be also imbedded.

Kodis states that with the same hardening and subsequent staining in Heidenhain's iron hæmatoxylin, only the myelin is coloured blue-black.

(*j*) **Robertson's and Orr's method** with methyl violet for cortical nerve cells and neuroglia.—The essentials for success in this method are **freshness** of tissue and preparation of sections immediately the fixation and hardening are completed, viz. on the fourth or fifth day. When the tissues have been 4 or 5 weeks in alcohol the staining results are unsatisfactory.

(1) Fix small pieces of tissue in a saturated solution of mercuric chloride in 0·5 per cent sodium-chloride solution, 24 hours. Wash in water, a few minutes. Place in 80 per cent alcohol containing alcoholic solution of iodine, 12 hours. Transfer to 95 per cent alcohol, also containing iodine. Place in 95 per cent alcohol ; water ; dextrine solution (1 in 2) or "gum and sugar."

(2) Cut on freezing microtome. Place sections first in water, then in alcohol.

(3) Transfer sections directly from alcohol to 1 per cent methyl violet 6 B in water, 5-10 minutes.

(4) Wash in water, 10-15 seconds.

(5) Place in saturated solution of iodine in 1 per cent potassium iodide, 10 minutes.

(6) Wash sections in water ; up to 1 hour.

(7) When the iodine ceases to come out, place section on slide ;

blot; cover immediately with a few drops of a mixture of equal parts of turpentine and benzole. Renew the turpentine benzole after a few seconds.

(8) Place the slide at 60° C. until completely dehydrated (the black and opaque tissue becomes dark blue and faintly translucent).

(9) Allow slide to cool; remove the turpentine benzole.

(10) Decolourise in perfectly anhydrous aniline benzole, until the colour ceases to come away.

(11) Wash in several changes of pure benzole.

(12) Mount in balsam in benzole.

(*k*) **Low's rapid method :—**

(1) Cut sections on the freezing microtome without previous fixation.

(2) Transfer sections to water; then place them on slides.

(3) Cover section for 5-15 seconds with the following solution :—

| | |
|---|---|
| Saturated watery picric acid solution | 50 c.c. |
| Formol, 6 per cent . . | 50 c.c. |

(4) Return sections to water.

(5) Place section on slide; cover with 5 per cent methylene-blue solution; warm until bubbles appear; allow to cool.

(6) Wash in water.

(7) Differentiate in

| | |
|---|---|
| Aniline oil . . . | 10 c.c. |
| Absolute alcohol . . . | 100 c.c. |

until the colour ceases to come away.

(8) Dry with filter-paper.

(9) Clear in origanum oil; remove the oil with benzene.

(10) Mount in colophonium.

(Melt colophonium in the smallest quantity of benzene; with a glass tube place a drop of the mass on the section; cover and warm the preparation until the cover-glass is evenly affixed.)

For Azoulay's, Roncoroni's and Rehm's methods the worker is referred to their original papers (see Bibliography).

For demonstration of the **lipochromes**, *e.g.* the fatty pigments of ganglion cells (Rosin), any or all of the stains for fat may be employed.

**The pigment may be also stained by v. Lenhossék's method :—**

(1) Harden; imbed in paraffin; cut; affix sections on slide.
(2) Stain in a saturated alcoholic solution of aniline blue, 12 hours or more.
(3) Wash in water.
(4) Differentiate in absolute alcohol.
(5) If desired, counterstain in eosin.

Pigment, bluish black.

**For mitoses in the central nervous system**

**(*l*) Weigert's method may be used.**

(1) Harden in 96 per cent alcohol ; imbed ; make thin sections.
(2) Place in tinct. ferri Rademacheri, ½ hour.
(3) Wash quickly in water.
(4) Stain in Hæmatoxylin . . . . 1 gm.
Absolute alcohol . . . 10 c.c.
Water . . . . . 100 c.c.
(5) Wash in water.
(6) Differentiate in 1 per cent acid alcohol.
(7) Wash 10 minutes in water.
(8) Dehydrate ; clear ; mount.

## II. Methods for Staining Myelinated Structures

By far the best of these methods, and one which yields almost mathematically precise results, is Weigert's method, or rather methods, since he devised several processes as well as frequently modified that one to which his name is more generally attached. All his processes are based, however, on the same principle, so that our restriction to the newer and best modification, as used and recommended by Weigert in his last days, will obviate the risk of confusion with any of the others. This principle may be shortly stated :—Pieces of tissue are mordanted, first with potassium bichromate, then with copper acetate ; sections are overstained with a myelin-staining hæmatoxylin solution, and differentiated in a solution of ferricyanide of potassium made alkaline with borax.

The primary mordanting is best performed by placing formol-hardened tissue (Müller's fluid was originally used, but its action is very slow) in a mixture of potassium bichromate and fluorchrom (replacing the earlier used chrome alum) for 4-5 days. Weigert preferred to mordant the pieces of tissue, not the sections.

The best secondary mordant is that employed in Weigert's neuroglia method (*q.v.*), a combination of copper, fluorchrom, and acetic acid.

Weigert at first stained in hæmatoxylin, 1 gm. ; alcohol, 10 c.c. ; water, 90 c.c. ; cold saturated solution of lithium carbonate, 1 c.c. (the latter being used to rapidly oxidise the hæmatoxylin to hæmatein). With this simple solution the alum lake can be avoided, since in the mordanted tissues there is already a metal in combination.

In his last years Weigert again made use of an hæmatoxylin lake; this time it was not an alum lake, but a mixture of iron and hæmatoxylin in which both the constituents were in contact with the tissues at the same time. By these means he obtained exceptionally brilliant and well-defined staining. Thus Weigert was led to formulate his **iron hæmatoxylin** (*q.v.*), which is now one of the most useful general nuclear stains we possess. When employed for the present purpose it is not *necessary* to add the hydrochloric acid, which is a constant constituent of the stain when used for nuclear staining.

The necessary solutions may be tabulated as follows:—

**Primary mordant**:—

| | |
|---|---|
| Potassium bichromate | 5 gms. |
| Fluorchrom | 2·5 gms. |
| Water | 100 c.c. |

Boil; filter.

**Secondary mordant**:—

| | |
|---|---|
| Neutral copper acetate | 5 gms. |
| Fluorchrom | 2·5 gms. |
| Water | 100 c.c. |

Boil; then add

| | |
|---|---|
| Acetic acid (about 36 per cent solution) | 5 c.c. |

**Staining fluid**:—

| | | |
|---|---|---|
| (1) | Hæmatoxylin | 1 gm. |
| | Alcohol, 96 per cent | 100 c.c. |
| (2) | Liq. ferri sesquichlorati (Pharm. Germ.) | 4 c.c. |
| | Water | 100 c.c. |

(Hydrochloric acid, 1 c.c., may be used if desired.)

Mix equal parts of 1 and 2 just before staining.

**Differentiating fluid**:—

| | |
|---|---|
| Potassium ferricyanide | 2·5 gms. |
| Borax | 2 gms. |
| Water | 100 c.c. |

All these solutions keep for long periods; the staining fluid is best preserved in separate stock solutions.

(*a*) **Weigert's method** may be shortly summarised as follows :—

(1) Harden in formol, 2-3 days.

(2) Primary mordant (potassium chromate, fluorchrom mixture), 4-6 days.

(3) Without washing, after-harden in increasing strengths of alcohol (in the dark).

(4) Imbed in celloidin.

(5) Secondary mordant (copper acetate mixture), 1 day at 37° C.

(6) Transfer to 70-80 per cent alcohol.

(7) Cut.

(8) Stain in iron-hæmatoxylin mixture, 24 hours.

(9) Wash in water, 30 minutes or longer.

(10) Differentiate in the borax-potassium ferricyanide mixture until the grey substance appears yellow to white. (Control under microscope.)

(11) Wash thoroughly in water.

(12) Dehydrate in absolute alcohol.

(13) Clear in xylol.

(14) Mount in balsam.

Myelinated fibres, blue-black. The fibres are sharply defined by this method, and stand out prominently upon a colourless or light yellow background. Red blood corpuscles are sometimes also stained blue-black.

Of the many modifications of Weigert's method by other workers we select the following :—

(*b*) **Pal's modification of Weigert's method.**—The method of differentiation in this method is based upon the decolorisation with permanganate of potassium and sodium sulphite, a procedure originally introduced into histology by Lustgarten.

(1) Harden, preferably in Müller's fluid or potassium bichromate ; after-harden ; imbed in celloidin ; cut.

(2) Stain according to Weigert (the copper mordant is not used) ; or, after Pal, in $\frac{3}{4}$ per cent watery hæmatoxylin, prepared with heat, and after cooling, mixed with "some alcohol," to which, just before staining, 2 c.c. of a saturated watery solution of lithium carbonate are added for each 100 c.c. (Pal's latest formula).

(3) Wash in tap water (containing, if desired, 1-2 per cent lithium carbonate), until the section is of a dark blue colour.

(4) Transfer to $\frac{1}{4}$ per cent potassium permanganate solution, 20-30 seconds.

(5) Wash in water.

(6) Place in

| | | |
|---|---|---|
| Oxalic acid . . | 0·5 gm. | for some seconds, until the "grey" substance becomes colourless (the "white" substance remains blue). |
| Potassium sulphite | 0·5 gm. | |
| Water . . . | 100 c.c. | |

(7) Wash thoroughly in water. Counterstain, if wished, in lithium carmine or eosin, etc.
(8) Dehydrate in absolute alcohol.
(9) Clear in phenol xylol (celloidin sections).
(10) Mount in balsam.

The appearances are the same as in Weigert's method.

The differentiation is more rapid than in Weigert's method, and therefore more difficult to carry out successfully. The results are not constant. It is claimed for this modification that the ground colour is white and that the fibres thus appear more prominent, but with the new iron-hæmatoxylin stain the contrasts by Weigert's process are at least quite as pronounced. These reasons will account for the present short description of the method.

### (*c*) Fraenkel's modification of Weigert's method :—

(1) Harden in Weigert's potassium bichromate, fluorchrom formol mixture, or in Müller's fluid ; after-harden ; imbed in celloidin or paraffin ; cut.
(2) Stain in Unna's polychrome methylene blue, 24 hours.
(3) Wash in distilled water.
(4) Place in a saturated watery solution of tannic acid until the grey and white substances are well differentiated (to the naked eye).
(5) Wash in distilled water ; then repeat 2, 3, 4, and 5, using in 2 excess of stain and few sections.
(6) Dehydrate in 96 per cent alcohol or in absolute alcohol (paraffin).
(7) Clear in phenol xylol or xylol ; mount in balsam.

Myelinated nerves, blue-black.

### (*d*) Kulschitsky's modification of Weigert's method :—

(1) Harden in Erlitzky's medium (Chapter IV.), 1-2 months. Wash in water, 1-2 days ; harden in alcohol ; imbed in celloidin ; cut.
(2) Stain for several hours in

| | |
|---|---|
| Hæmatoxylin . . . | 1 gm. dissolved in a little absolute alcohol. |
| Acetic acid, 2 per cent . . | 100 c.c. |

or in

| | |
|---|---|
| Carmine . . . . | 2 gms. |
| Acetic acid, 10 per cent . . | 100 c.c. |

Boil for several hours ; when cold, filter.

(3) Decolourise for several hours in

| | |
|---|---|
| Saturated watery solution of lithium carbonate | 100 c.c. |
| Potassium ferricyanide, 1 per cent . . | 10 c.c. |

(4) Wash thoroughly in water.
(5) Dehydrate in absolute alcohol.
(6) Clear in xylol ; mount in balsam.

Myelinated nerve fibres, blue.

(*e*) **Wolters' modification of Weigert's method** :—

(1) Harden in Müller's fluid, some months ; after-harden ; imbed in celloidin ; cut.
(2) Stain in Kulschitsky's hæmatoxylin (see above), 24 hours at 37° C.
(3) Place in Müller's fluid.
(4) Further treatment as in Pal's method (*b* 4-10).

Myelinated nerve fibres, blue-black ; ganglion cells, yellow to brown.

(*f*) **Schrötter's stain for myelinated fibres** :—

(1) Harden in Müller's fluid ; imbed ; cut.
(2) Stain the section in a cooled, saturated solution of gallein in spring water (the solution is not permanent), 15-20 minutes.
(3) Differentiate in 5 per cent sodium-carbonate solution, or better, in very dilute caustic soda solution.
(4) Immerse in a light violet solution of potassium permanganate for a few seconds.
(5) Wash in water.
(6) Dehydrate in absolute alcohol.
(7) Clear in xylol.
(8) Mount in balsam.

Myelinated nerve fibres, violet.

In a similar manner Aronson used gallein prior to Schrötter.

(*g*) **Mosse's method for myelinated fibres** :—

(1) Harden in Müller's fluid ; transfer directly to alcohol (without washing in water) ; imbed in celloidin ; cut.
(2) Place the section in Müller's fluid, 24 hours.
(3) Transfer to 2 per cent argentamin (silver preparation) solution, 10 minutes.
(4) Wash in water.
(5) Reduce in 10 per cent solution of pyrogallic acid, until the section is entirely black, 1-2 minutes.
(6) Wash in water ; differentiate by Pal's method (*b* 4-10).

Myelin sheaths, brown-black ; nerve cells, lemon-yellow.

In a similar manner Mosse stains the Nissl bodies of nerve cells with argentamin black.

For other modifications of Weigert's method the reader is referred to the papers of Vassale, Flechsig, Heilmeyer, Rossi, Haug, Berkley, Kaiser, van Walsem, Marcus, Karusin and Tschernyscheff, Hill, Schafer, Gudden, Bolton, Boehm and Oppel, Allerhand, Adamkiewicz, and Nikiforoff.

After all, Weigert's method, especially when used in accord-

ance with the directions just given, yields most reliable results, and the other methods do not afford any additional information.

**For the rapid staining of medullary sheaths** Benda recommends hardening in formol, cutting sections on the freezing microtome, then staining in Böhmer's hæmatoxylin for 24 hours, differentiating for one hour or more in Weigert's borax-ferricyanide of potassium mixture, dehydrating, clearing in xylol, and mounting in balsam.

## Osmic Acid Methods for Staining Medullated and Peripheral Nerve Fibres

The following methods depend upon the principle that when the lecithin of the myelin substance is liberated it is able to reduce osmic acid. The tissues are first placed in solutions of chrome salts to form an insoluble lecithin compound. The lecithin is now liberated and reduced by pyrogallic acid or sodium sulphite, etc.

These methods are much prized by some workers, and after some practice and experience reliable and constant results may be expected.

### (*h*) Exner's method for myelinated nerve fibres :—

(1) Harden small pieces in 1 per cent osmic acid solution (about 10 times the volume of the tissue), 5-10 days, renewing the fluid on the second day ; after-harden in alcohol ; imbed ; cut ; wash in water.
(2) Place in glycerin for a few minutes.
(3) Mount the section in glycerin containing a drop of strong ammonia, and cover when a few minutes have elapsed.
Myelinated nerve fibres, black.
The preparations are not permanent.

### (*i*) Heller's method for myelinated fibres and peripheral nerves :—

(1) Harden in formol or in Müller's fluid ; cut on freezing microtome. The sections should not be too thin.
(2) Wash the sections in water.
(3) Place in 1 per cent osmic acid at 37° C., 24-48 hours.
(4) Reduce in the following solution for 15-30 minutes in the incubator :—

| | |
|---|---|
| Sodium sulphite . . . . | 25 gms. |
| Sodium carbonate . . . | 1·5 gm. |
| Distilled water . . . . | 100 c.c. |
| Pyrogallic acid . . . . | 3 gms. |

The sections become black.
(5) Place in a light violet coloured solution of permanganate of potassium.
The sections turn brown. Re-oxidisation of the osmium which is not combined with medullated nerves or with fat.
(6) Transfer to 1-2 per cent oxalic acid solution.
The sections become yellowish green, the brown colour of the reduced permanganate being removed by the oxalic acid.
(7) Dehydrate in 30, 60, 95 per cent alcohol ; clear ; mount.
Nerves and fat, black ; tissue, yellowish green.

Shorter and better is

### (*j*) Robertson's modification of Heller's method :—

(1) Fix and harden in Weigert's mordant for neuroglia fibrils, containing 10 per cent formalin, 8-10 days.
(2) Wash in water. Dehydrate ; imbed in celloidin ; cut.
(3) Stain sections in 1 per cent osmic acid solution, half an hour in the dark.
(4) Transfer to a 5 per cent watery solution of pyrogallic acid, about 30 minutes.
(5) Differentiate in $\frac{1}{4}$ per cent aqueous solution of potassium permanganate, about 30 minutes.
(6) Remove the brown colour in 1 per cent watery solution of oxalic acid, 3-5 minutes.
(7) Dehydrate in 96 per cent alcohol ; phenol xylol ; balsam.

The sections must be washed very thoroughly between each stage. The method, after some practice, gives very good results, but the beginner is recommended to first master the details of Weigert's method.

Consult also the papers of Azoulay.

### (*k*) Orr's method for staining medullated fibres "en bloc" :—

(1) Place *fresh* tissue from cerebral cortex or cord, not exceeding $\frac{1}{8}$ inch in thickness, in

| | |
|---|---|
| Osmic acid, 2 per cent . . . | 8 c.c. |
| Acetic acid, 1 per cent . . . | 2 c.c. |

for 48 hours. If the mixture is darkened at the end of 24 hours it should be renewed.
(2) Transfer to 10 per cent formol, 3 days, in order to complete reduction and hardening.
(3) Imbed in celloidin or paraffin ; cut sections.
(4) Remove paraffin ; alcohol ; water ; differentiate in $\frac{1}{8}$-$\frac{1}{12}$ per cent potassium permanganate solution.
(5) Transfer to 1 per cent oxalic acid, as in Heller's method (*q.v.*).
(6) Wash ; dehydrate ; xylol ; balsam.

The acetic acid enables the osmium thoroughly to penetrate the tissues, and the results obtained are reliable and satisfactory.

## III. Methods for the Staining of Axis Cylinders

This section forms a contrast to the previous one in that, although a large number of methods have been proposed, the problem of staining has not been solved, since we do not possess a dye which is "elective" for the axons.

van Gieson's stain is here very useful, the axis cylinders being stained brown, while the glia and sclerotic tissue are dyed a different tint.

Mallory's method with phosphomolybdic acid, etc., and the modification of Ribbert stain the axis cylinders, glial fibres, and ganglion cells, blue.

The best known methods for axis cylinder staining are the following :—

**Carmine methods.**—These are the oldest, and slow staining is preferable. Dilute solutions of ammonia or lithium carmine may be used.

Axis cylinders, interstitial tissue and cells, red.

Among others, the following may be specially cited :—

### (*a*) Schmaus's uran-carmine stain for axis cylinders :—

(1) Harden in Müller's fluid ; after-harden in alcohol (without washing in water) in the dark ; imbed ; cut.
(2) Stain for 5-60 minutes in

| | |
|---|---|
| Sodium carminate . . | 1 gm. |
| Uranium nitrate . . . | 0·5 gm. |
| Water . . . . | 100 c.c. |

Divide the powder finely ; boil for 30 minutes ; cool ; filter. Just before using, add 2 drops of 1 per cent acid alcohol (HCl) to each c.c. of the stain (Chilesotti).

(3) If overstained, differentiate in acid alcohol, a few seconds.
(4) Wash in water.
(5) Dehydrate in absolute alcohol.
(6) Clear in xylol ; mount in balsam.
Axis cylinders, interstitial tissue and cells, red.

### (*b*) Sahli's methylene-blue stain for axis cylinders :—

(1) Stain decelloidinised sections (prepared from tissues which have been hardened in 3-4 per cent potassium bichromate solution, and then after-hardened and imbedded) in a saturated watery solution of methylene blue, for some hours.
(2) Wash rapidly in water.
(3) Stain in a saturated watery solution of acid fuchsin, 5 minutes.
(4) Rapidly rinse in water.
(5) Differentiate in weak alkaline alcohol, a few seconds.
(6) Wash thoroughly in water.
(7) Dehydrate in alcohol.
(8) Clear in xylol ; mount in balsam.
Axis cylinders, red ; myelin fragments, blue and red.

### (*c*) Nigrosin stain for axis cylinders :—

Stain in 0·2 per cent watery solution of nigrosin, 5-10 minutes. Further treatment as usual : alcohol ; xylol ; balsam.

### (*d*) Wolters' method for axis cylinders :—

(1) Harden in the dark for 24 hours in a saturated solution of copper sulphate and potassium bichromate in 50 per cent alcohol, to each 100 c.c. of which 5-6 drops of glacial acetic acid have been added. After-harden ; imbed in celloidin.
(2) Place the sections for 24 hours in a mixture of 10 per cent vanadium chlorate, 1 part ; 8 per cent aluminium acetate, 4 parts.
(3) Wash in water, 10 minutes.
(4) Stain for 24 hours at 37° C. in

| | |
|---|---|
| Hæmatoxylin . . . | 2 gms. |
| Absolute alcohol . . . | sufficient to dissolve the hæmatoxylin. |
| Acetic acid, 2 per cent . . | 100 c.c. |

(5) Differentiate in weak acid alcohol until the sections are light blue to red.
(6) Transfer to dilute alcohol.
(7) Dehydrate in absolute alcohol ; xylol ; balsam.
Axis cylinders, also ganglion cells and neuroglia, blue.

(*e*) **Stroebe's method for axis cylinders** :—

(1) Harden in Müller's fluid ; without washing in water, after-harden in alcohol ; imbed in celloidin ; cut.
(2) Stain in a freshly saturated watery aniline-blue solution, 30-60 minutes.
(3) Rinse in water.
(4) Place in a small vessel containing alcohol, to which 20-30 drops of caustic potash, 1 gm. ; 70 per cent alcohol, 100 c.c., are added. Stain 1 or more minutes until the section becomes a transparent light brownish red.
(5) Wash in distilled water for about 5 minutes, until the sections are light blue.
(6) Counterstain in a half-saturated watery solution of safranin, 15-30 minutes.
(7) Differentiate and dehydrate in absolute alcohol.
(8) Clear in xylol ; mount in balsam.

Axis cylinders, blue ; myelin, mostly yellow-red ; nuclei, generally dark red.

These older methods are but rarely used, and are chiefly of historical interest ; the important modern methods now follow :—

(*f*) **Chilesotti's new method for axis cylinders** :—

(1) Harden in Müller's fluid for several months, or in Orth's mixture, 4 days.
(2) Place the piece of tissue in Weigert's primary myelin mordant, 5 days.
(3) After-harden in the dark in alcohol ; imbed in celloidin ; cut ; transfer sections to water.
(4) Stain in the following solution for 20 hours or more :—

Boil 1 gm. of finely powdered carmine nacarat (Merck) in 250 c.c. of tap water for 30 minutes ; allow to stand for 24 hours ; decant. To each 100 c.c. add 3 c.c. of 1 per cent hydrochloric acid in 70 per cent alcohol ; shake thoroughly ; allow to stand for 24 hours ; decant.

(5) Wash in water.
(6) Place in $\frac{1}{2500}$ potassium-permanganate solution (*i.e.* 1 part of Pal's solution with 5 parts of water), 30 seconds.
(7) Transfer to a saturated watery solution of sulphurous acid (about 5 per cent), 10-60 seconds.
(8) Wash well in water. Repeat 6, 7, and 8 until the sections are of a rose colour (5-10 times), gradually shortening the stay of the section in 8. The latter is the crucial point of the whole proceeding.
(9) Dehydrate in 96 per cent alcohol ; clear in phenol xylol ; balsam.

Axis cylinders and ganglion cells, red ; red blood corpuscles, nuclei, and connective tissue, partly dyed red ; myelin and neuroglia, unstained.

(*g*) **Fayersztajn's method for axis cylinders** :—

(1) Harden in 5-10 per cent formol, 1 day to several weeks ; cut on freezing microtome.
(2) Mordant in 0·25-0·5 per cent chromic acid, 5-24 hours.
(3) Wash in (warm) water several times, 10 minutes.

(4) Stain in Weigert's or Kulschitsky's hæmatoxylin solution, 2-6 hours.

(5) Differentiate and treat further according to Pal's modification of Weigert's method for medullated fibres.

Axis cylinders, dark blue.

The method is based upon the fact that by avoiding the use of alcohol and ether in Weigert's method (*i.e.* during the imbedding), the dye remains in the axis cylinders and not in the myelin.

Fayersztajn has also worked out another silver impregnation method for axis cylinders. It belongs, however, more to normal than to morbid histology.

(*h*) **Strähuber's method for axis cylinders** :—

(1) Fix in formol or other fixing media (except alcohol).
(2) Mordant in Weigert's solution, 5 days :—

| | |
|---|---|
| Potassium bichromate . . | 5 parts. |
| Fluorchrom . . . | 2 parts. |
| Water . . . . | 100 parts. |

(3) After-harden in alcohol ; imbed in celloidin ; cut.
(4) Stain sections in saturated watery aniline-blue solution, about 12 hours.
(5) Differentiate by Pal's method or in water containing a few drops of sodium hypochlorite.
(6) Rinse in water.
(7) Dehydrate in 96 per cent alcohol.
(8) Clear in xylol ; mount in balsam.

Axis cylinders, blue.

Counterstaining in alcoholic eosin (for 24 hours) may be interpolated between 3 and 4.

(*i*) **Kaplan's method** :—

(1) Harden in Müller's fluid, 3 months or more ; after-harden in alcohol ; imbed ; cut. (If in celloidin, imbed as rapidly as possible.)
(2) Stain in a 10 per cent freshly prepared watery solution of "Anthracene eisen gallus-tinte" (Grübler) for 3 days, if possible at 35° C.
(3) Rinse rapidly in water.
(4) Differentiate in ¼-1 per cent potassium-permanganate solution ; bleach in sulphurous acid after Pal's method. (Repeat the two stages several times.)
(5) Wash in water.
(6) Counterstain in 0·1 per cent fuchsin solution or carmine, etc.
(7) Dehydrate in alcohol, 80 per cent, 95 per cent, absolute alcohol.
(8) Phenol xylol ; xylol ; xylol colophonium.

Axis cylinders, dark steel-blue on a colourless background.

(*j*) **Bielschowsky's methods.**—Bielschowsky has formulated several modifications of his original method, and we select the ones containing the latest and most obvious improvements.

The principle of the method is as follows :—Sections are impregnated with silver nitrate, and silver diammonium nitrate

is then formed by transferring the tissues to a solution of ammonia. Formerly Bielschowsky directly impregnated the tissues with silver-ammonia compounds. The silver diammonium nitrate is next reduced by aldehyde, the latter being assisted in its action by formol. The silver salt being soluble in alcohol, xylol, etc., the section is treated with gold (which also hastens the differentiating processes), then fixed and passed through the usual stages of mounting. Glass needles must, of course, be employed, metal instruments being entirely avoided.

The stages of the process are as follows :—

(1) Fix the tissues (best within 24 hours after death) in 12 per cent formol. Cut on the freezing microtome (sections should not be thicker than 20$\mu$).

(2) Place the sections in 2 per cent watery solution of silver nitrate, 12-24 hours.

(3) Transfer to 3 per cent ammonia solution (*i.e.* ammonium chloride, 1 part; water, 10 parts), 10-20 seconds. The section turns yellow.

(4) Place the section in 20 per cent formol solution (spring water if possible), containing a few drops of saturated lithium-carbonate solution, 10 minutes.

(5) Pass the section through the 3 per cent ammonia solution.

(6) Immerse the section in 0·5 per cent silver nitrate solution and allow it to remain until it becomes brownish, about 30 seconds. A metallic precipitate arises from the silver di-ammonium nitrate, and this must be removed by filtration. Renew the fluid for every two or three sections.

(7) Reduce in 20 per cent formol until the section is a dark brown (white clouds appear in the fluid).

(8) Pass through 3 per cent ammonia solution ; the section becomes brownish black.

(9) Transfer to 20 per cent formol, a few minutes.

(10) Wash in distilled water.

(11) Allow the section to lie in the following solution until it becomes grey.

To 10 c.c. of water add a few drops of a 1 per cent solution of gold chloride, and to the total quantity taken add 2-3 drops of glacial acetic acid.

(12) Transfer to the following solution for a few seconds :—To 10 c.c. of a 5 per cent solution of sodium thiosulphate (fixing soda) add 1 drop of a saturated solution of sodium sulphite.

(13) Wash rapidly in distilled water.

(14) Dehydrate in absolute alcohol.

(15) Clear in xylol or phenol xylol ; mount in balsam.

Quite recently **Bielschowsky and Pollack** have modified the method so that it may be also used for imbedded tissues.

(*k*) (1) Harden in 12 per cent formol.
(2) Transfer to 2 per cent watery silver nitrate solution, 24-48 hours.
(3) Rinse in distilled water.
(4) Place in the following solution for 30-60 minutes :—

Silver nitrate solution (2 per cent) 20 c.c.
Sodium hydrate, 40 per cent . 2-3 drops.

Add, while stirring with a glass rod, liquid ammonia until the precipitate of $Ag_2O$ is dissolved. The ammonium silver nitrate salts are formed. The solution should be freshly prepared.

The tissue becomes brownish black.

(5) Rinse rapidly in distilled water.
(6) Transfer to 20 per cent formol, 12-24 hours.
(7) Rapidly dehydrate in increasing strengths of alcohol ; imbed in paraffin.
(8) Cut ; affix section ; remove paraffin, etc.

Proceed as in 11, 12, 13, 14, 15 of previous method.

According to Bielschowsky, the intracellular fibrils and Golgi's network are stained, as well as the axis cylinders. The connective tissue and some glia fibrils are also often stained. Bielschowsky also recommends a slight modification of this method, viz. a combination of pure and ammoniacal silver solutions.

By this method the axis cylinders appear somewhat broader than by other processes ; its value lies in the demonstration of non-medullated fibres.

(*l*) **Williamson's modification of Bielschowsky's method.**—This is a short, practical procedure which can be well recommended for rapid and ordinary work. The results obtained are reliable and satisfactory.

1. Harden in Müller's fluid ; imbed ; cut.
2. Place sections in 10 c.c. of tap water containing a few drops of formalin (40 per cent formaldehyde), 5 minutes.
3. Wash in water.
4. Place in the following silver bath for 5-10 minutes :—

3 drops of liq. ammoniæ B. P. are dropped into a test tube. Add 10 per cent nitrate of silver solution, drop by drop, until a brownish precipitate is obtained. Dissolve the latter by adding ammonia, drop by drop, until the fluid is quite clear. Add tap water up to 10 c.c.

5. Wash thoroughly in water.
6. Transfer to the dilute formalin solution (stage 2) and allow them to remain until they become greyish black (about 1-3 minutes).
7. Place in the following solution for a few minutes :—

   To 10 c.c. of water add two drops of 1 per cent watery solution of chloride of gold, a few drops of saturated borax solution, and a few drops of a 10 per cent solution of carbonate of potash.
8. Transfer to a 10 per cent watery solution of sodium hyposulphite for a few minutes.
9. Wash in water ; dehydrate in alcohol ; clear in oil of cajuput ; xylol ; Canada balsam.

(*m*) **Methods of Ramon y Cajal** :—

(1) Harden for 4 days at 36° C. or 10 days or longer at room temperature in

| | |
|---|---|
| Formol . . . . | 30 c.c. |
| Water . . . . | 70 c.c. |
| Pyrogallol . . . | 3 gms. |

(2) After-harden in alcohol ; imbed in celloidin ; cut.
(3) Pass the sections through several changes of water.
(4) Transfer to 3 per cent solution of pyrogallol, 1-2 hours.
(5) Wash in 3-4 changes of distilled water.
(6) Place in water to which a few drops of ammonia have been added, or better, in a saturated filtered solution of lithium carbonate, 30 seconds.
(7) Wash in 3-4 changes of distilled water.
(8) Transfer to chloride of gold solution ($\frac{1}{1000}$-$\frac{1}{3000}$), 2-12 hours (best in the dark).
(9) Differentiate in Lugol's solution for 5-60 minutes, until the grey substance commences to be transparent.
(10) Place in 20 per cent sodium hyposulphite solution.
(11) Wash thoroughly in water.
(12) Dehydrate in alcohol ; clear in xylol ; mount in balsam.

Axis cylinders, violet ; cells, dark violet ; myelin, unstained. In other similar methods tannin and chloride of gold are used. For gold chloride Ramon y Cajal has also substituted silver nitrate, previously treating with tannin or hydroquinone.

Lately Ramon y Cajal has published three new methods, the first for myelinated, the second for myelin-free fibres, and the third for the end structures of nerve fibres. With these methods the neuro-fibrils are stained, but the results are not so satisfactory as may be obtained by Cajal's special methods (see next section).

The **first method** (for myelinated fibres) is as follows :—

(1) Harden pieces of tissue not thicker than 5 mm. in 96 per cent alcohol, 24 hours.
(2) Wash in distilled water.
(3) Place in 1-1·5 per cent silver nitrate solution and keep at 30-35° C. for 3-5 days.
(4) Wash in distilled water.
(5) Reduce in the following solution :—

| | |
|---|---|
| Hydroquinone or pyrogallol | 1-2 gms. |
| Formol, 40 per cent | 5 c.c. |
| Sodium sulphite | 0·25-0·5 gm. |
| Distilled water | 100 c.c |

(6) Wash in distilled water, several seconds.
(7) Dehydrate ; imbed ; cut, etc.
(8) Dehydrate sections in alcohol ; phenol xylol ; balsam.
Axis cylinders, red-brown.

**Second method** (for myelin-free nerves and neuro-fibrils) :—

(1) Place small pieces 3·5 mm. thick, 24 hours to 3 days, in

| | |
|---|---|
| Alcohol (96 per cent) | 100 c.c. |
| Ammonia | $\frac{1}{2}$-1 c.c. |

(2) Wash thoroughly in distilled water.
(3) Place in 1-1·5 per cent silver nitrate solution, and keep at 30-35° C. for 3-5 days.
(4) Reduce in

| | |
|---|---|
| Formol | 5 c.c. |
| Pyrogallol | 2 gms. |
| Distilled water | 100 c.c. for 24 hours. |

Further treatment as in 6, 7, and 8 of the first method.
Myelin-free and the finest medullated fibres, red.

In both methods a gold bath may be added if desired ; the axis cylinders are then brownish black.

**Third method** (for end structures of nerve fibres) :—

(1) Place small pieces for 24-48 hours in

| | |
|---|---|
| Formol | 25 c.c. |
| Ammonia | 100 c.c. |
| Distilled water | 0·25-1 c.c. |

(2) Wash several hours in running water.

(3) Place in 1-3 per cent silver nitrate solution, and keep at 30-35° C. for 3 days.

(4) Wash for some seconds in distilled water.

Reduce as in the last method, etc. The pericellular plexus and terminating bulbs or clubs of fibrillæ, grey or black; nerve cells, yellow.

## APPENDIX

The modifications of **Cohnheim's gold method** for the demonstration of axis cylinders are very numerous. As, however, they are but rarely used in pathological work, it is here not necessary to do more than to refer the reader to p. 126, and to the works of Gerlach, Flechsig, Freund, and Upson.

**Rubaschkin's method** may be included here, although it is used only for the central nervous system of small mammalia. The vessels of the brain are first injected with the following mixture :—

| | |
|---|---|
| Bichromate of potash solution (2½ per cent) . | 100 c.c. |
| Neutral acetate of copper . . . | 0·5-1 gm. |
| Glacial acetic acid . . . . | 2·5-3 gms. |
| Formalin . . . . . | 10 c.c. |

Pieces of central nervous tissue are also fixed in the same fluid for 6-7 days at 35-40° C. Dehydrate rapidly; imbed in paraffin.

(1) Stain sections on a slide (not attached to it) in a saturated watery solution of methyl violet, 6-12 hours.
(2) Wash thoroughly in water.
(3) Differentiate in Lugol's solution, ½-1 minute.
(4) Place in 95 per cent alcohol, ½ minute.
(5) Differentiate in oil of cloves; xylol; balsam.

Neuroglia fibres, dark violet; nuclei and Nissl's bodies of nerve cells, light violet.

The method does not seem to be quite reliable.

## IV. Methods for the Demonstration of Neuro-fibrillar Structures

These methods, which interest the normal rather than the morbid histologist, are here only included for the sake of completeness.

These fibrils are stained by Heidenhain's iron-hæmatoxylin method, by acid fuchsin after osmic acid hardening in vertebrates, and by many other dyes, such as toluidin blue, after mordanting in molybdic acid (Mönckeberg and Bethe).

With Bielschowsky's method (see above) the fibrils also may be demonstrated.

Of special methods space only permits a description of Apathy's gold method, the molybdic method of Bethe, the last published one of Ramon y Cajal, and the silver method of Robertson. S. Meyer's process of impregnation with Berlin blue can only be alluded to.

### (*a*) Apathy's gold method :—

(1) *a.* Fix in Saturated solution of mercuric chloride,
or in Sublimate alcohol,
or in Osmic acid, 10 per cent
Saturated mercuric chloride solution } equal parts.

(Fixation in osmic acid and sublimate should be performed in the dark.)

*b.* Wash in water.

*c.* Remove the sublimate by iodine (1 per cent potassium iodide, ½ per cent iodine), 12 hours.
*d.* Place directly into 95 per cent alcohol, 8-12 hours.
*e.* Transfer to alcohol containing 1 per cent potassium iodide, and ½ per cent iodine, 8-12 hours.
*f.* Dehydrate in absolute alcohol.
*g.* Imbed in paraffin (through chloroform) or in celloidin.
*h.* Cut ; if paraffin sections, remove the paraffin with chloroform ; absolute alcohol.
*i.* Distilled water for some hours.

(2) Place the sections in a tube containing 1 per cent solution of chlorate of gold (Merck), 12-24 hours.
(3) Wash in water.
(4) Place the slide, with the section on its under surface, in a glass vessel containing 1 per cent formic acid solution, 6-8 hours. The section should be well exposed to the light on all sides, but the temperature should not exceed 21° C.
(5) Remove the gold precipitate from the upper surface of the slide ; wash in water.
(6) Dehydrate in absolute alcohol.
(7) Clear in chloroform ; mount in balsam.

The fibrils are dark blue to black on a light background, and stand out prominently.

This method, as well as the following one, does not always yield successful results. It is better for non-vertebrate than for vertebrate animals. For the latter the following method is more useful :—

### (*b*) Bethe's method :—

In this process the neuro-fibrils combine with molybdic acid and then with basic dyes to form an insoluble compound. The simultaneous staining of other structures is avoided by differentiating first in alkalies and then in hydrochloric acid.

(1) *a.* Harden the piece of tissue in 3·5-5 per cent solution of concentrated nitric acid, 24 hours.
*b.* Transfer directly to 95 per cent alcohol, 12-24 hours.
*c.* Place the tissue in ammonia (sp. gr. 0·95), 1 part ; water, 3 parts ; 96 per cent alcohol, 8 parts, 24 hours.
*d.* 96 per cent alcohol, 6-12 hours.
*e.* Hydrochloric acid, 1 part
Water, 3 parts
Alcohol (96 per cent), 10 parts
} 12-24 hours.
*f.* Alcohol, 10-24 hours.
*g.* Water, 2-6 hours.
*h.* 4 per cent solution of white ammonium molybdate, 24 hours.
*i.* Wash rapidly in water.
*j.* 96 per cent alcohol ; dehydrate rapidly in absolute alcohol ; imbed in paraffin ; cut ; affix section to slide with egg-albumin mixture ; remove paraffin ; alcohol ; distilled water (rapidly).

(2) Dry the slide on its under surface ; cover the section with water, and place it in an oven at 60° C. for 2-10 minutes.
(3) Remove the water ; wash rapidly in water.
(4) Cover the slide and section with a watery toluidin-blue solution ($\frac{1}{300}$), and stain at 60° C. for 10 minutes.
(5) Wash in water.
(6) Differentiate in 96 per cent alcohol until clouds cease to be given off from the dye, ¾-2 hours.
(7) Dehydrate in absolute alcohol.
(8) Clear in xylol ; mount in balsam.

Only the fibrils and nuclei are stained.

(*c*) **Ramon y Cajal's method** :—

(1) Place thin pieces of tissue in 1·5-6 per cent solution of silver nitrate for 4 days or more at 30-40° C.
(2) Wash in water, 1-2 minutes.
(3) Place in

| | | |
|---|---|---|
| Pyrogallol . . . | 1 gm. | for 24 hours. |
| Distilled water . . . | 100 c.c. | |
| Formalin (40 per cent) . . | 5-10 c.c. | |

(4) Wash in distilled water, 1-2 minutes.
(5) Harden in 90 per cent alcohol and upwards ; imbed ; cut.
(6) Treat as usual, mounting in Canada balsam (use phenol xylol) or dammar.

Ramon y Cajal recommends fixation in 3 per cent nitrate of silver, 4-6 days, for general work, and in 6 per cent solution, 2-3 days, for the spinal cord and medulla oblongata.

The neuro-fibrils are brown on a yellow ground.

v. Tellyesniczky has modified this method by transferring the sections to a solution containing 4 c.c. of a 1 per cent solution of chloride of gold, and 150 c.c. of distilled water, 5-30 minutes, until the preparation is steel-grey when viewed with the naked eye or under the microscope. The ground substance and the nuclei of nerve cells are unstained ; the neuro-fibrils are intensely black.

It has been already remarked that neuro-fibrils are also stained by Cajal's methods for axis cylinders.

**Ford Robertson's silver method ; after toning with gold or platinum.**—This is one of the best of the methods available for the demonstration of neuro-fibrils, etc., and its author advises its application to other tissues, since it minimises the harmful tissue effects which follow the ordinary silver impregnations.

1. Wash thin slices of formalin-hardened tissues (5 to 10 per cent solution of formalin in water ; change on second day) for about 24 hours in water. Change the water at least once.
2. Transfer to an ammonio-nitrate of silver solution, prepared by adding to a 1 per cent solution of silver nitrate in distilled water a 5 per cent solution of ammonia in distilled water, drop by drop, until the precipitate which forms is nearly but not entirely dissolved, and then filter. The fluid should measure at least 50 times the volume of the tissues. Cork the bottle or specimen tube and put it in the dark. The pieces are ready for examination in from 1 to 10 weeks.
3. Place a piece of the impregnated tissue in water (500 c.c.) to which about 3 c.c. of 5 per cent ammonia have been added. Renew the fluid after an hour, and leave the tissue in it for 3 or 4 hours longer.
4. Transfer to dextrine solution (dextrine, 5 ounces or 140 grammes, water, 10 ounces or 280 c.c. ; dissolve by boiling ; filter through cotton-wool while still hot ; after it has cooled add

1 per cent of carbolic acid) to which ammonia has been added in the proportion of 10 drops of a 5 per cent solution to 1 ounce, immediately before use. Allow the tissue to remain in this for from 12 to 24 hours.

5. Cut thin sections on the freezing microtome. Transfer them from the knife to a bowl of water to which about 10 drops of 5 per cent ammonia have been added. After about 5 minutes transfer the sections to another bowl of ammonia and water, and after a similar period give them a third wash.

6. Transfer the sections to a bowl of water to which there have been added from 5 to 10 drops of a saturated solution of citric acid in water, and allow them to remain in this for 4 or 5 minutes.

7. Place the sections in a bowl of water, and after a few minutes transfer them to a second bowl of water. They are now ready for toning.

8. To 10 c.c. of a $\frac{1}{4}$ per cent solution of gold chloride in distilled water add a single drop of a 1 per cent solution of citric acid in water, and filter the fluid, preferably into a flat-bottomed white porcelain dish. Transfer the sections from the water to this toning bath by means of a glass rod or platinum needle. Allow them to remain spread out for about 1 hour. About a dozen sections of ordinary size may be toned in this amount of the gold solution.

9. Wash the sections for 10 minutes in a bowl of tap water, and then transfer to a bowl of water to which 10 drops of 5 per cent solution of ammonia have been added, where they should remain for 2 or 3 minutes.

10. Transfer to a bowl of tap water.

11. Dehydrate the sections with absolute alcohol, clear in equal parts of turpentine and benzole, remove turpentine with pure benzole, mount in benzole balsam.

Platinum toning of the silvered sections may be carried out as follows :— To 1 ounce of freshly prepared $\frac{1}{2}$ per cent solution of potassium chloroplatinite in water add 10 drops of a 5 per cent solution of phosphoric acid in water (citric acid may be used instead). Filter this solution and place the sections in it for about half an hour. Wash the sections shortly in water and then clear them in a watch-glassful of 1 per cent solution of platinum bichloride ($PtCl_4$) in water for about 10 minutes. Wash well in water, dehydrate, clear, and mount in balsam.

The acidophile tissue elements, such as the neuro-fibrils (intra-cellular and extra-cellular) are deeply impregnated.

## V. Methods for the Simultaneous Demonstration of several Nervous Structures

This section is chiefly concerned with Golgi's methods and their modifications, and Ehrlich's vital methylene-blue method. They are not extensively used in pathological work.

### A. Golgi's Method

The principle of the method is that when small fresh pieces of tissue are treated with a solution of potassium bichromate and then transferred to a bath containing silver or mercurial salts, a precipitate of chrome silver or chrome sublimate is formed and deposited in certain structures.

For cerebral tissues this method has been largely employed, and the deposits there occur in the ganglion cells and their processes, in the glia cells, nerve fibres (axis cylinders and myelin), and also in vessels, glia and connective tissue. In other organs nerve fibres and nerve endings, epithelial borders and secretory capillaries, as for instance, the bile capillaries (*q.v.*), may also be demonstrated.

Numerous deposits occur, and the results obtained need very careful interpretation, and are inconstant.

(*a*) **Golgi's slow silver method** :—

(1) Harden in Müller's fluid, in brown glass vessels, 3 months in cold or 1 month in warm weather. Every 8 days increase the bichromate (up to about 5 gms. to 100 c.c. water).

Or, harden in Erlitzky's fluid, or in a mixture consisting of Erlitzky's fluid, 20-50 per cent, and Müller's fluid, 10-50 per cent. Perform the following proceedings every third or fourth day, until a good result is obtained.

(2) Wash in distilled water (rapidly).

(3) Place the tissue in $\frac{1}{2}$-1 per cent silver nitrate solution, 24-48 hours, changing the solution when it becomes yellowish.

(4) Make sections with a sharp razor, or cut on the freezing microtome. Thick sections will show more than thin ones. If desired, the tissue may be imbedded in celloidin or paraffin by putting it in absolute alcohol for 1-2 hours, transferring it to thin celloidin for 1-2 hours, then imbedding in celloidin, or transferring it from the alcohol to xylol and then imbedding it in paraffin.

(5) Wash the sections well in alcohol, in order to remove the silver nitrate.

(6) Transfer to 96 per cent alcohol.

(7) Clear in creosote for a few minutes, and in oil of turpentine, 10 minutes.

(8) Mount in dammar (or balsam) and allow the medium to dry in the air, protected, of course, from dust. No cover-glass is required.

Appearances as described above.

(*b*) **Golgi's quick method.**—Harden in

| | |
|---|---|
| Potassium bichromate, 2 per cent . | 8 parts. |
| Osmic acid, 1 per cent . . . | 2 parts. |

or

| | |
|---|---|
| Potassium bichromate . . . | 2 parts. |
| Osmic acid, 1 per cent . . . | 1 part. |

on the second or third and daily up to the twelfth day try pieces of tissue by treating further as in Method *a* until the results are satisfactory.

(*c*) **Golgi's combined method.**—The tissue is hardened in Müller's fluid and then transferred to the osmium-bichromate mixture (*vide* Method *b*). After remaining in Müller's fluid for 3-4 days, the procedures are performed daily up to the 25th-30th day until successful. In the osmium-bichromate solution it is well to leave the objects for 2-3 days and then to try daily up to the 10th-12th day, treating them as in Method *a*. The time necessary for human tissues to remain in the second solution depends upon the elements to be impregnated ; neuroglia cells generally require 2-4 days, nerve cells, 3-5 days, and nerve fibres and collaterals, 6-9 days.

(*d*) **Golgi's sublimate method.**—This is the most convenient method, and larger pieces of tissue may be impregnated.

(1) Harden in Müller's fluid, 20-30 days.
(2) Transfer to ¼-1 per cent watery solution of mercuric chloride, 8 days to 1 or 2 months. Change the solution at first daily, and afterwards, whenever it turns yellow. Cut, etc. (as in Method *a*).
(3) Wash the sections in distilled water.
(4) Place the sections in the following mixture (well known in photographic work) for 1-2 minutes. They become black.

| | |
|---|---|
| *a*. Sodium hyposulphite | 175 gms. |
| Alum | 20 gms. |
| Ammonium sulphocyanide | 10 gms. |
| Sodium chloride | 40 gms. |
| Water | 1000 c.c. |

Allow to stand for 8 days, then filter.

| | |
|---|---|
| *b*. Gold chloride | 1 gm. |
| Water | 100 c.c. |

Mix 60 parts of *a* with 7 parts of *b*.

(5) Wash thoroughly in water.
(6) Counterstain with acid carmine.
(7) Wash in water.
(8) Dehydrate in alcohol ; clear in xylol ; mount in balsam.

Of the numerous modifications only the following can be included.
In place of osmic acid formol may be used. For the central nervous system it gives very good results, and the "deposits" are slight.

(*e*) **Kopsch's modification.**—The tissues are hardened in freshly prepared

| | |
|---|---|
| Potassium bichromate (3·5 per cent) | 40 c.c. |
| Formol (40 per cent) | 10 c.c. |

for 1 day and then transferred to potassium bichromate for 3-6 days.

(*f*) **Ramon y Cajal's modification.**—The impregnation is repeated 2 or 3 times, the preparations after the silver treatment (especially when good results are absent) being again placed in the osmium-bichromate solution (or in a mixture of 20 c.c. of the bichromate and 2-3 c.c. of 1 per cent osmic acid), and then returned to the silver solution.

(*g*) **Sehrwald's modification.**—**To avoid the peripheral deposits** the tissue is coated with thin gelatin before being placed in the silver nitrate solution, and prior to cutting sections the block is dipped in a warm saturated solution of silver chromate to remove the gelatin. Filter-paper and blood are by others recommended for the same purpose.

(*h*) **Cox's modification.**—Potassium bichromate and mercuric chloride are allowed to act at the same time; hence fixation is rapidly completed.

The solution used is as follows:—

| | | |
|---|---|---|
| Mercuric chloride (5 per cent) | 20 parts | Mix. |
| Potassium chromate (5 per cent) | 16 parts | |
| Water | 30-40 parts. | |
| Potassium bichromate (5 per cent) | 20 parts. | |

Add the potassium bichromate last.

(1) Harden small pieces, 1-6 months. Change the fluid after the first day. Wash in water.
(2) Cut on the freezing microtome or imbed rapidly.
(3) Place the sections in 5 per cent solution of sodium carbonate, 1-2 hours. Dilute ammonia solution or saturated lithium-carbonate solution may also be used.
The precipitate is black.
(4) Wash in water.
(5) Dehydrate rapidly in absolute alcohol.
(6) Clear in anise oil, removing it with filter-paper.
(7) Mount in balsam, or in Canada balsam dissolved in benzole. When the solvent of the balsam has evaporated and the balsam become hard, a drop of castor oil may be placed on the preparation and a cover-glass gently lowered on to it. The specimen, however, may be preserved without a cover-glass.

(*i*) **Shaw Bolton's method**:—

(1) Fix pieces of tissue about $\frac{1}{8}$ inch in thickness in 5 per cent formol—weeks to months.
(2) Transfer to 1 per cent ammonium-bichromate solution, 5 days.
(3) Wash in water.
(4) Place in 1 per cent silver nitrate solution, 16-24 hours.
(5) Harden in 60 per cent alcohol, a few hours.
(6) Dry with filter-paper.
(7) Imbed, without soaking, in melted paraffin. Cut, keeping the razor wetted with 60 per cent alcohol.
(8) Pass sections through methylated spirit, absolute alcohol, chloroform, and xylol.
(9) Mount in xylol balsam, without a cover-slip.
Or, after cutting, transfer the sections to water and develop and fix them by the method of Kallius, p. 198.

We may here mention still another process which yields results similar to those of Golgi's method and its modifications. It can be strongly recommended for the central nervous system.

(*j*) **Kronthal's method (impregnation with lead sulphide).**—The hardening fluid is prepared as follows:—Add pure formic acid, drop by drop, to a saturated solution of lead acetate, until the fine white crystals of lead formiate fill the vessel. Filter. Dissolve the crystals in water until a saturated solution is obtained. Mix equal parts of the lead-formiate solution with 10 per cent formalin.
(1) Harden small pieces in this fluid, 5 days.
(2) Transfer (without washing) to a mixture of equal parts of 10 per cent formol and sulphuretted hydrogen, 3-5 days. Pour off the first discoloured portion of the mixture, replacing it with new solution.
(3) Harden in increasing strengths of alcohol; imbed in celloidin; cut.
(4) Dehydrate sections in 96 per cent alcohol; clear in phenol xylol; mount in balsam.
Nerve structures and neuroglia, black.

**Corning** fixes tissues first in 10 per cent formalin and then proceeds according to Kronthal's method. He uses, however, lead formiate prepared by Merck.

As a rule, it will be found advantageous to employ the freshly prepared hardening solution.

Of the many proposals to render Golgi preparations more permanent and to permit counterstaining, we cite only that of Kallius, which we know to yield good results.

(*k*) **Kallius's modification.** — Dilute commercial hydrochinon developer with 12 times its volume of water, and allow the resultant solution to stand in the dark for several days (it keeps for several weeks).

Just before using, again dilute it with $\frac{1}{3}$-$\frac{1}{2}$ its volume of absolute alcohol.

After 5 (method *a*), place the sections in the diluted hydrochinon solution for some minutes; wash in 70 per cent alcohol, 10-15 minutes; transfer to sodium hyposulphite solution (10-50 water), 5 minutes. Wash frequently in distilled water (24 hours); dehydrate in absolute alcohol, clear in xylol, mount in balsam, and cover with a cover-glass.

**Ford Robertson,** after washing in water sections obtained from Golgi preparations, places them for 5 minutes in

| | |
|---|---|
| Chloride of gold . . . . | 1 grain. |
| Sulphocyanide of potassium . . | 12 grains. |
| Hyposulphite of soda . . | ½ grain. |
| Distilled water . . . . | 4 ounces. |

The toned specimens are then washed well in water, dehydrated, cleared, and mounted in balsam with a cover-glass. As a rule, the preparations are fairly permanent.

## B. Vital Methylene-Blue Method (Ehrlich, 1886)

When methylene blue is injected into the circulation of a living or dying animal, the ganglion cells, their branches and finest processes, and the nerve fibres and nerve endings are stained blue; the same result follows if a piece of fresh tissue is placed in a methylene-blue solution and kept at 37° C. for 1-3 hours. The injection fluid is generally a ¼-1 per cent normal saline solution of purified methylene blue. The methylene blue is rapidly (after the death of the animal) reduced to the colourless leuco-methylene by the tissues (Aronson), and this substance must be oxidised by exposure to the air; the original blue colour then returns.

In order to imbed and to prepare thin sections, a number of fixing fluids have been proposed.

**Dogiel** first used ammonium picrate for this purpose, but the solution, although fixing well, did not harden sufficiently.

A medium possessing both properties was devised by **Bethe,** who in 1895 introduced ammonium molybdate, which permits the formation of methylene blue molybdate.

To oxidise the leucobase, Bethe added hydrogen peroxide, and in order to produce an acid-ammonium molybdate, hydrochloric acid was

included. For hardening it was found possible to use either chrome solutions, which do not alter the salt, or osmic acid, which makes the methylene blue molybdate a darker blue and on the addition of alcohol forms an insoluble compound.

**Bethe** at first used the following fixing fluid for vertebrate animals :—

| | |
|---|---|
| Ammonium molybdate | 1 gm. |
| Water | 10 c.c. |
| Hydrogen peroxide | 1 c.c. |
| Official hydrochloric acid | 1 drop. |

The tissue was hardened at 0° C. for 2-5 hours, and then for some time at room temperature; it was then washed for ½-2 hours in distilled water, dehydrated in cold absolute alcohol (12 to 24 hours), cleared (when the piece was so small that it could be directly examined) in xylol, and mounted in balsam.

Better, however, and more convenient (since it may be entirely carried out at room temperature) is **Bethe's later method**, which is a combination of his earlier method with that of **Dogiel**.

The tissue is placed in a saturated watery solution of ammonium picrate, 10-15 minutes (the blue thus becoming more violet), and then is directly transferred to a solution of ammonium molybdate. The choice of six molybdate solutions is offered ; we select two (Bethe's **3** and **6**), which are the most useful when the tissue is to be imbedded afterwards :—

| | | |
|---|---|---|
| (3) | Ammonium molybdate | 1 gm. |
| | Osmic acid solution (½ per cent) | 10 c.c. |
| | Water | 10 c.c. |
| | Official hydrochloric acid | 1 drop. |
| (6) | Sodium phosphomolybdate | 1 gm. |
| | Osmic acid (½ per cent) solution | 10 c.c. |
| | Water | 10 c.c. |
| | Official hydrochloric acid | 1 drop. |

1 c.c. of hydrogen peroxide may be added to each solution.

In either of these solutions the tissue already fixed in ammonium picrate is allowed to remain for 4-12 hours. It is then thoroughly washed, dehydrated in cold absolute alcohol (not too long), and imbedded in paraffin or celloidin. The sections may be counterstained with alum carmine, etc., passed through absolute alcohol and xylol, and mounted in balsam.

**Dogiel** now fixes in 5-8 per cent watery solution of ammonium molybdate, ½-1 hour (according to the size of the piece of tissue), washes for 1 hour in cooled water, dehydrates in cooled absolute alcohol, and imbeds rapidly in celloidin.

**Ramon y Cajal** fixes according to **Bethe**, without the addition of hydrogen peroxide, washes in water, and then places the tissue in the following mixture :—

| | |
|---|---|
| Formol | 40 c.c. |
| Platinic chloride (1 per cent) | 5 c.c. |
| Water | 60 c.c. |

After washing, the piece of tissue is transferred to absolute alcohol containing ⅛ per cent platinum chloride, and finally imbedded in paraffin. The sections are later similarly treated with alcohol containing ⅛ per cent of platinic chloride.

Turner (*Brain*, 1901, vol. xxiv. p. 242) has devised a similar method.

## VI. Methods for the Demonstration of Neuroglia

No existent method stains every single neuroglial fibre under all conditions. The best process is that of Weigert, which is the most convenient for studying this structure, and which, in the hands of the inventor, led to the demonstration of the basal facts of our knowledge of the subject. Weigert later modified and improved his older method, obtaining beautiful and constant results, but the process was not entirely applicable to the neuroglial tissues of lower animals. Sadly enough, Weigert died before his modifications were published, so that we can quote only the old formulæ, etc.

(*a*) **Weigert's method of staining neuroglia** :—

(1) Fix small fresh pieces of tissue in formol (10 per cent), 1 day.

(2) Mordant in the following mixture, 8 days at room temperature, 4 days at 37° C.

| | |
|---|---|
| Copper acetate . . . | 5 gms. |
| Fluorchrom . . . | 2·5 gms. |
| Acetic acid . . . | 5 c.c. |
| Water . . . | 100 c.c. |

Or, harden and mordant at once in 9 parts of the copper mordant and 1 part of commercial formaldehyde for 8 days, changing on the second day and once again later.

(3) Wash in water ; after-harden in alcohol ; imbed in celloidin ; cut.

(4) Place sections in $\frac{1}{3}$ per cent watery solution of potassium permanganate (oxidation and a sort of mordanting process).

(5) Wash in two changes of water.

(6) Place in the following *reducing* mixture, 2-4 hours :—

| | | |
|---|---|---|
| Chromogen . . | 5 gms. | Filter. To 90 c.c. add 10 c.c. of 10 per cent sodium sulphite solution just before using. |
| Formic acid (sp. gr. 1·2) | 5 c.c. | |
| Water . . | 100 c.c. | |

(7) Wash twice in water.

(8) Place sections in 5 per cent carefully filtered aqueous chromogen solution, 10 - 12 hours. (The glia fibres become darker, and a yellowish contrast is obtained for the ganglion and ependymal cells and for the thicker axis cylinders. The connective tissue is stained red. This step is advisable but not necessary.)

(9) Wash in water.

(10) Place the section on a slide which has been freshly cleaned with alcohol; dry with filter-paper (see Fibrin Method); stain in the following mixture for about 30 seconds :—

| | |
|---|---|
| Saturated solution of methyl violet in 70-80 per cent alcohol | 100 c.c. |
| Oxalic acid (5 per cent solution) | 5 c.c. |

or in any other alcoholic methyl-violet solution. (The oxalic acid makes the preparations more permanent.)

(11) Remove the excess of stain; dry with filter-paper; cover the slide with iodine solution (saturated solution of iodine in 5 per cent potassium-iodide solution; see Fibrin Method), for about 30 seconds.

(12) Remove iodine solution; dry with filter-paper; differentiate in a mixture of equal parts of aniline oil and xylol until no more heavy clouds of stain are given off. (Best to control under the microscope.)

(13) Dry the section with filter-paper; add xylol; dry again; repeat the procedure three times.

(14) Mount in Canada balsam, or in turpentine colophonium, which preserves the colours better.

Neuroglia fibres and nuclei, blue; connective tissues, blue-violet; thicker myelin sheaths, ganglion and ependymal cells, yellowish.

If desired, sections may be preserved for future use by placing them after step 9 in the following mixture :—

| | |
|---|---|
| Alcohol (80 per cent) | 90 c.c. |
| Oxalic acid (5 per cent) | 10 c.c. |

They may then be stained when required, and the staining is then often better and more permanent.

Of all the many modifications of Weigert's method in relation to the several stages of hardening, mordanting, imbedding, oxidation, reduction, and staining, none has constituted any material advance upon this method. The reader is referred for the details of these modifications to the works of Benda, Krause, Storch, and Aguerre.

(*b*) **Benda's method of staining neuroglia** :—

(1) Fix fresh material in 90 per cent alcohol, 2 days or more.
(2) Place thin pieces in 10 per cent nitric acid, 24 hours.
(3) Transfer to 2 per cent aqueous solution of potassium bichromate, 24 hours.
(4) Place in 1 per cent aqueous solution of chromic acid, 2 days.
(5) Wash in running or frequently changed water, 24 hours.
(6) Harden in increasing strengths of alcohol; imbed in paraffin, avoiding long exposure to strong heat; cut; affix sections to slide.

(7) Stain the section in Heidenhain's iron hæmatoxylin,
or in van Gieson's staining mixture,
or according to Weigert's myelin-staining method,
or in Benda's stains.

The first is a modification of Weigert's method.

$(7)_1$ Place the sections (from water) in $\frac{1}{2}$ per cent aqueous potassium permanganate solution until they are dark brown.

$(8)_1$ Reduce by Pal's method (see Myelin) until white; stain, and further treat as in Weigert's method (go on to step 10).

Instead of Weigert's stain Benda advises

| | |
|---|---|
| Saturated solution of crystal violet in 70 per cent alcohol . . | 1 part. |
| Acid alcohol (HCl, 1; 70 per cent alcohol, 100) . . . | 1 part. |
| Aniline water . . . | 2 parts. |

Or $(7)_2$ Place the section in 4 per cent iron-alum solution, 24 hours (or in liq. ferri sulf., 1 : 2 parts water).

$(8)_2$ Wash well in running water.

$(9)_2$ Place the sections in an amber-coloured watery solution of sodium sulphalizarate (Kahlbaum), 24 hours.

(10) Dip in water; dry with filter-paper (or before 10 dip in potassium-bichromate solution).

(11) Stain in 1 per cent watery solution of toluidin blue for 1-24 hours in the cold, or warm until the steam rises and then allow to stand for 15 minutes.

(12) Dip in 1 per cent acetic acid or in very dilute solution of picric acid.

(13) Dry with filter-paper; dip in absolute alcohol.

(14) Differentiate in beech creosote, about 10 minutes, controlling progress under the microscope.

(15) Dry with filter-paper; clear in xylol; dry; again add xylol, etc.

(16) Mount in balsam.

Neuroglia, blue; axis cylinders, red; cell nuclei, blue.

With Benda's method centrosomes, striated muscle fibres, fibrin, and many secretory granules also may be stained.

(*c*) **Mallory's method of staining neuroglia** :—

(1) Harden small pieces in formol (10 per cent), at least 4 days.

(2) After-harden in saturated watery picric acid solution, 4-8 days; or 1 and 2 may be combined :—

| | |
|---|---|
| Saturated watery solution of picric acid | 90 c.c. |
| Formalin (commercial 40 per cent) . | 10 c.c. |

(3) Place in 5 per cent watery solution of ammonium bichromate, 4-7 days at 37° C., changing the solution on the second day, or 3-4 weeks at room temperature.
(4) Without washing, harden in alcohol; imbed in celloidin; cut.
(5) Place sections in ½ per cent aqueous solution of potassium permanganate, 15-30 minutes (oxidation).
(6) Wash in water.
(7) Immerse in 1 per cent watery solution of oxalic acid, 15-30 minutes (reduction).
(8) Wash in several changes of water.
(9) Stain in the following mixture, 24 hours to several days :—

| | |
|---|---|
| Hæmatoxylin | 0·1 gm. |
| Phosphotungstic acid, 10 per cent | 20 c.c. |
| Hydrogen peroxide | 0·2 c.c. |
| Water | 80 c.c. |

(10) Wash rapidly in water.
(11) Differentiate in freshly prepared 30 per cent alcoholic solution of ferric chloride, 5-20 minutes.
(12) Wash in water.
(13) Dehydrate in 95 per cent alcohol.
(14) Clear in origanum oil; mount in balsam.

Neuroglia, nuclei, and fibrin, dark blue; all else is pale yellow or grey.

Tissues hardened according to Mallory's method may be stained by Weigert's neuroglia or fibrin-staining mixtures.

If differentiation 11 is omitted, the axis cylinders and ganglion cells are rose-pink; the connective tissues, dark red-pink.

(*d*) **Yamagiva's method of staining neuroglia** :—

(1) Harden small pieces in Müller's fluid, 1 month, repeatedly changing. Without washing, harden in absolute alcohol, frequently renewed (some days); imbed in celloidin; cut.
(2) Stain sections in saturated alcoholic eosin solution, 12 hours.
(3) Counterstain in saturated watery aniline-blue solution, 4-6 hours.
(4) Differentiate in 70 per cent alcohol (to which 1 per cent potassium hydrate or carbonate has been added until it is slightly alkaline) till the blue-coloured section again appears reddish brown.
(5) Wash in distilled water until the section is again blue.
(6) Differentiate in 70 per cent alcohol until the red colour reappears.
(7) Dehydrate in absolute alcohol.
(8) Clear in origanum oil; the section again appears bluish.
(9) Mount in balsam.

Neuroglia (and red blood corpuscles), dark red; axis cylinders, deep blue; myelin, yellow-red; connective tissue, blue-green; glia cells, pale violet; ganglion cells, blue-grey; nuclear membranes, bluish; nucleoli, deep violet to deep blue.

(*e*) **Anglade's method** :—

(1) Fix pieces of tissue for 48 hours at 37° C. in

| | | |
|---|---|---|
| 1 per cent osmic acid | 2 vols. | 3 parts. |
| 1 per cent chromic acid | 25 vols. | |
| 2 per cent acetic acid | 5 vols. | |
| Water | 68 vols. | |
| 7 per cent mercuric chloride solution | | 1 part. |

(2) Wash in water; dehydrate in alcohol.
(3) Dehydrate in acetone, 24 hours.
(4) Transfer to paraffin, 3 hours; imbed.
(5) Stain thin sections in a warm saturated solution of Victoria blue (Grübler), until it steams.
(6) Cover the sections with Gram's iodine for a few seconds.
(7) Differentiate in aniline xylol (2 : 1).
(8) Wash in absolute alcohol containing erythrosin.
(9) Clear in xylol; mount in balsam.

Campbell considers that none of these processes alone stain neuroglia satisfactorily. He therefore combines Bevan Lewis's method with one of the other neuroglial methods.

(*f*) **Bevan Lewis's methods for fresh nervous tissues.**—For microchemical reactions, and where the microtome is not at hand or cannot be employed, this method is very valuable.

**Neuroglia** is well stained by this method.

**Cord** :—

(1) Make a fine section with a razor through the anterior cornua.
(2) Immerse it in Müller's fluid (on the slide) for a few minutes.
(3) Cover; compress with needle; examine and arrange cells as desired by further pressure.

**Brain** :—

(1) Cut sections with razor blade saturated with methylated spirit.
(2) Place on slide; immerse in Müller's fluid for a few seconds.
(3) Cover with cover-glass so as to cover the film by one-half its diameter; press, flattening the section to a transparent film.
(4) Remove Müller's fluid by rinsing in water. Place the slide in a flat dish containing methylated spirit, 30-40 seconds.

(5) Remove cover from slide by passing a needle under it and gradually elevating it. Wash films with stream of water from a large camel-hair brush.

(6) Stain in 1 per cent aniline blue-black until the cells are deeply stained ; wash in water.

(7) Allow preparations to dry under bell jar.

When dry add chloroform, then mount in balsam.

The films may also be treated with 0·25 per cent osmic acid solution for one second, washing immediately in water, or treated with gold, platinum, or silver salts or aniline dyes.

As an Appendix we add :—

(*g*) **Ford Robertson's platinum method for mesoglia, etc.**—The method is based upon the fact that when platinum bichloride is slowly reduced in the presence of a tissue, the platinum tends to be deposited first in certain tissue elements. The reducing agent employed is formalin. Many additional factors also come into play, and these are not yet entirely understood. The process gives histological pictures quite different from those of silver and sublimate depositions, and the mesoglia, nerve cells, and vessels are well brought out.

The following directions differ from those contained in the original paper, in that, through the kindness of Dr. Ford Robertson, we are here enabled to embody his as yet unpublished, newer modifications :—

(1) Harden pieces of tissue, $\frac{1}{16}$ of an inch thick, in 10 per cent formalin.

(2) Transfer to a large volume of the following mixture, and place the tightly corked bottles in the incubator at 37° C. :—

Formalin, 2 per cent . . } equal parts.
Platinum bichloride, 1 per cent }

(Prepare freshly ; filter before using.)

The deposition of platinum black begins in 3-4 days, and the tissues are ready for cutting in from 4-6 weeks.

If the yellow colour of the platinum bichloride disappears, remove half of the fluid and replace it by $\frac{1}{2}$ per cent solution of the salt.

(3) Wash rapidly in water.

(4) Place in watery dextrine solution (1 : 2) ; cut on freezing microtome.

(5) Dehydrate ; clear ; mount in balsam.

For mesoglia, 10-20 per cent formalin (previously neutralised by pure calcium carbonate) should be added to the 1 per cent platinum bichloride solution.

In conclusion, it may be observed that although van Gieson's stain was first devised for the staining of neuroglia, it is not specially appropriate for such purpose.

Kulschitsky has modified it by substituting rubin for the acid fuchsin.

## VII. Methods for Nerve Degeneration.

When considering these methods a sharp line must be drawn between those intended for recent degeneration, when the degenerated products are present, and those designed for older lesions, when the degenerated products have been removed and are replaced by sclerotic tissue. At the autopsy it is not always easy to distinguish the two conditions, and it is thus important, as well as customary, to preserve the tissue in such different media as will allow the respective methods to be completed. Formol, however, may be used in almost all cases, its action but very rarely interfering with subsequent treatment by other fluids.

For early degeneration (days to weeks or months) of nervous tissues Marchi's method is available.

(*a*) **The Marchi-Algeri method.** — With Weigert's myelin method the result attained is a negative one, since the degenerated nerve fibres have lost their capacity for stains, and the process is only useful for large areas.

By Marchi's method the broken-up constituents of the myelin are stained (viz. the fat itself); and in the examination of small necrosing foci this method is without a rival. It must be pointed out, however, that the presence of fat granules in small quantities does not constitute a pathological process, since, according to the investigations of V. Tiling, S. Mayer, and Loewit, such may occur physiologically in normal nervous tissues; they may also result from slight or very slight mechanical injuries.

The principle of Marchi's method is as follows:—Myelin and fat both reduce osmium tetraoxide to the black hydroxide, but when mordanted with chrome salts (Müller's fluid, etc.), the myelin loses its property of reducing osmic acid. Consequently when myelin and fat are placed in a mixture of osmic acid and potassium bichromate after primary mordanting in the latter fluid, the fat alone appears black, the myelin being combined in some way with the potassium salts and so losing its

affinity for the osmic oxide (Senger and Münzer, Redlich). Thus the use of Marchi's method permits the differentiation of normal and broken-up myelin sheaths.

Halliburton and Mott have shown that *peripheral* nerves may be placed directly into Marchi's fluid, the time for the previous mordanting thus being saved. The same procedure cannot be employed for the central nervous system. (The chemistry of this procedure is fully discussed by Mann, *Physiological Histology*, p. 315.)

The details of the method are as follows :—

(1) Harden small fresh pieces of tissue in Müller's fluid for at least 8 days. Take care to avoid mechanical injuries. Formol (10 per cent) may also be used, the tissue being later transferred to Müller's fluid.

(2) Place in freshly prepared Marchi's fluid (Müller's fluid, 2 parts ; 1 per cent watery osmic acid solution, 1 part) for about 8 days in the incubator at 37° C. The brain requires a longer time. When the mixture loses the osmic acid smell, renew it.

(3) Wash in running water, 24 hours.

(4) Harden in increasing strengths of alcohol.

(5) Imbed, best in celloidin ; cut.

Sections are then dehydrated, cleared, and mounted in balsam. Degenerated nervous tissues (fat) are black ; all else, brownish grey. By staining in lithium carmine, or by van Gieson's method, the nuclei or other structures may be also demonstrated.

Halliburton and Mott state that after division of nerve fibres in cats the reaction first appears about 92 hours after injury, etc., is most intense on the 10th-13th day, is plainly evident on the 27th day, but cannot, as a rule, be obtained after the 44th day.

### (*b*) Modifications

**Schaffer**, in order to demonstrate the earliest appearance of the products of degeneration, hardens the tissues for a very long period in Müller's fluid, transfers them to Marchi's fluid, and then washes for 8-14 days.

**Busch** adds sodium iodate to the osmic acid mixture, so as to increase the slight penetrating power of the osmium tetraoxide. After hardening in Müller's fluid he places the tissues for 6 or 7 days in a mixture containing osmic acid 1 gm., sodium iodate 3 gms., water 300 c.c.

**Campbell** uses this method after formol hardening and counterstains with rubin and picric acid.

**Teljatnik** places pieces, not thicker than 1½ cm., first in Müller's fluid and then in rising strengths of osmic acid. Sections are bleached after Pal's method for medullated fibres (*q.v.*). Blackened areas which are not degenerated are said to be removed by such procedure.

The addition of acetic acid ensures a better penetration of the osmic acid. This is the basis of

(*c*) **Orr's modification of Marchi's method** :—

1. Place nervous tissues in 2 per cent potassium bichromate, containing 5 per cent formol, for 24 hours.
2. Transfer to 2 per cent potassium-bichromate solution for 10 days.
3. Place pieces $\frac{1}{8}$ inch thick in

| | |
|---|---|
| Osmic acid, 2 per cent . . | 8 c.c. |
| Acetic acid, 1 per cent . . | 2 c.c. |

for 48 hours. Renew the mixture if it becomes darkened.

4. Without washing in water, pass into alcohol and imbed in paraffin or celloidin ; cut sections.

(*d*) **Langley and Anderson's method** :—

(1) Fix in 2 per cent potassium bichromate solution.
(2) Place in a syrupy, thick solution of gum in 2 per cent potassium-bichromate solution.
(3) Cut on freezing microtome.
(4) Place sections in 2 per cent potassium-bichromate solution, to remove the gum.
(5) Transfer to a mixture of 2 per cent potassium bichromate and 2 per cent osmic acid, 1-3 weeks.
(6) Dehydrate in 70 per cent alcohol ; stain in picro-carmine.
[7] If desired, treat according to Weigert-Pal's method.
[8] Dehydrate ; clear ; mount.

A combination of the methods of Marchi and Weigert is used in

(*e*) **Schafer's method** :—

1. Prepare sections by freezing or collodion methods.
2. Place in Marchi's solution, a few hours.
3. Wash in water.
4. Stain in acid hæmatoxylin (Kulschitsky), 12 hours. The sections become quite black.
5. Wash in water.
6. Transfer to 0·25 per cent permanganate of potash solution, 5 minutes.
7. Rinse in water and place in Pal's solution, a few minutes to half an hour. (If at the latter period the differentiation is not complete, repeat stages 6 and 7.)
8. Wash in water ; dehydrate ; oil of bergamot ; xylol balsam. Medullated fibres, black. Degenerated areas, unstained or Marchi-black, according to the age of the degeneration.[1]

[1] Starlinger has devised an apparatus which allows slices of uniform thickness being cut without danger of damaging the nervous tissues. (It is made by Reichert, Vienna, at a cost of £2.)

(*f*) **Benda** stains the fat set free during the breaking up of the myelin segments with Scharlach R. He hardens the tissue in formol, prepares sections by the freezing microtome, stains with Weigert's method for medullated fibres, and then in Scharlach R (see Fat). The fat appears red, the myelin is blue-black.

In order to demonstrate the degenerative changes in nerve fibres which occur from the second day onwards, viz. those changes which are not made prominent by Marchi's methods, a mordanting with potassium bichromate and tin or copper, after the procedures of Donaggio, may be employed. The results are excellent.

(*g*) **Donaggio's methods for early degenerative changes** :—

Method I.

(1) Fix in Müller's fluid or in 4 per cent potassium-bichromate solution. The tissue may remain in the fluid for any length of time, even years.
(2) Transfer directly (without washing) to alcohol. Dehydrate. Imbed in celloidin. Cut sections about 20-30$\mu$.
(3) Place sections in distilled water for a few seconds.
(4) Transfer to the following mixture for 10-20 minutes. To 20 per cent solution of ammoniated chloride of tin add an equal amount of 1 per cent aqueous hæmatoxylin. Allow to stand for five days. Keep in the dark and in a cool place.
(5) Wash rapidly in distilled water.
(6) Differentiate in Pal's solution until the normal fibres are entirely decolourised.
(7) Dehydrate ; xylol ; neutral balsam.

Degenerated fibres, blue ; normal fibres, decolourised.

Method II.

(1) Fix in Müller's fluid ; imbed as in Method I.
(2) Place sections in 0·5-1 per cent aqueous hæmatoxylin solution, 10-20 minutes.
(3) Transfer directly to a saturated aqueous solution of neutral acetate of copper, 30 minutes. Renew the copper solution once.
(4) Decolourise as in Method I.

(5) Wash rapidly in distilled water.
(6) Dehydrate in rising strengths of alcohol ; xylol ; balsam.

Degenerated fibres, black ; normal fibres unstained, except for a narrow circle at periphery.

If the differentiation is pushed, the degenerated fibres vary from grey to black according to the extent of degeneration.

Method III.

(1) Fix and imbed as in Method I.
(2) Stain in 0·5-1 per cent aqueous hæmatoxylin solution, 10-20 minutes.
(3) Transfer directly to 10-20 per cent solution of perchloride of iron. The section becomes black. After a few seconds they lose their colour. If washed in water they regain their colour.
(4) Without washing, differentiate in acid alcohol (0·75 c.c. HCl in 100 c.c. alcohol).
(5) Dehydrate in absolute alcohol ; xylol ; balsam.

Degenerated fibres appear as small black streaks or circular areas.

For the demonstration of the so-called "granule" cells which are filled with fatty particles, the tissues may be examined in the fresh condition, or stained by the ordinary fat stains, the material being placed in Marchi's or Flemming's mixtures, or in formol (10 per cent) with subsequent staining in Sudan III. or Fett ponceau.

In long-standing degeneration the fat is removed and Marchi's method is therefore useless. Weigert's myelin method (*q.v.*) demonstrates the absence of myelinated fibres, but the effect is only well-marked when large areas are involved. The seat of the degeneration is now, however, occupied by newly formed neuroglia or connective tissue, and these may be stained so as to demonstrate the position of the lesion.

For this purpose van Gieson's stain is useful, in that it dyes the degenerated areas a dark red, although it does not differentiate neuroglia (for which it was first devised) and connective tissue.

By combining van Gieson's stain with Weigert's myelin method very useful results may be obtained, the van Gieson being used after differentiation. One precaution is necessary. The myelinated fibres lose some colour in van Gieson's solution, thus it is well to differentiate in diluted borax-ferricyanide mixture (see Weigert's Myelin-sheath Method)

for a short time only. The myelinated fibres are blue-black, the degenerated areas, deep red.

In some cases it may be necessary to ascertain the relative parts played by the neuroglia and the connective tissues in the formation of the sclerotic patch.

To demonstrate the former, recourse may be had to Weigert's or other neuroglia methods.

The connective tissue can be stained as just stated. Weigert ha shown that by staining in his iron-hæmatoxylin solution and counterstaining in van Gieson's mixture the connective tissue is stained red, the neuroglia remaining unstained, whereas when using van Gieson with the ordinary hæmatoxylin solutions the neuroglia and connective tissue both stain red. This is a further and special advantage of van Gieson's staining mixture when used in combination with Weigert's iron-hæmatoxylin solution (*q.v.*).

**Paraffin sections for Weigert's method** (Laslett) :—

(1) Harden nervous tissues for 14 days in Müller's fluid ; cut into slices about 2 mm. thick and place in Marchi's fluid for a week. Wash ; imbed in paraffin ; cut ; fix sections on slide.
(2) Stain sections in acetic acid hæmatoxylin overnight in a warm oven.
(3) Wash in water.
(4) Transfer to a saturated solution of sodium or lithium carbonate. The colour changes to blue-black.
(5) Differentiate by Pal's method.

## VIII. Peripheral Nervous System

The methods already described for use in connection with the central nervous system, viz. for myelin sheaths, axis cylinders, ganglion cells, etc., are also applicable here.

For the demonstration of peripheral nerves and nerve-endings the "vital" methylene blue and "gold" methods are very useful. Of the large number of methods available, Kühne's modifications of Loewit's and Golgi's methods, and those of May and Drasch, yield perhaps the best results. Schmorl advises the latter processes. Ranvier's and Frey's methods are also in general use. **Loewit's method** has already been described under the heading of "Metallic Impregnations."

(*a*) **Kühne's** modification consists in reducing the gold by a mixture of equal parts of glycerin and water to which $\frac{1}{5}$-$\frac{1}{4}$ volume of formic acid is added.

(*b*) **May's method** is as follows :—

(1) Place the piece of tissue in 2 per cent acetic acid until it is thoroughly swollen (about 12 hours).

(2) Transfer to the following freshly prepared mixture for 2-3 hours :—

| | |
|---|---|
| Potassium gold chloride solution (1 per cent) . | 1 part. |
| Osmic acid solution (2 per cent) . . | 1 part. |
| Acetic acid (2 per cent) . . . | 20 parts. |

(3) Clear and examine (after several hours) in

| | |
|---|---|
| Hydrochloric acid (25 per cent) . . | 1 part. |
| Water . . . . . | 20 parts. |
| Glycerin . . . . . | 40 parts. |

(*c*) **Drasch's method** :—

(1) Place the material in a low temperature for 12-24 hours. This avoids the previous action of acids and the devices of other methods by permitting the post-mortem production of reducing substances to assist reduction.

(2) Put small pieces in 0·5 per cent watery solution of gold chloride, 15-60 minutes (best in the dark).

(3) Reduce in dilute formic acid (20 : 100 water), controlling under the microscope, until the nerves are dark blue or dark violet.

(4) Examine ; mount in glycerin, repeatedly changing the latter until the acid reaction disappears.

(*d*) **Ranvier's method** :—

(1) Tease fresh material into small pieces ; place them in the juice of a fresh lemon for 5-10 minutes, when they will become transparent.

(2) Wash in water.

(3) Place in 1 per cent watery solution of gold chloride, 10-60 minutes.

(4) Wash in water.

(5) Expose to daylight for 1-2 days in dilute acetic acid solution (2 drops of acetic acid to 50 c.c. of water).

(6) Harden in alcohol. Tease further or imbed and cut ; dehydrate ; clear ; mount.

(*e*) **v. Frey's method** :—

(1) Harden small pieces in 2 per cent watery solution of ammonium bichromate for about 2 weeks in the cold.

(2) Wash in running water, 10 minutes.

(3) Transfer to 1 per cent solution of chloride of gold containing 1 per cent hydrochloric acid, 1 hour.

(4) Wash rapidly in water.

(5) Place in 1 : 50 chromic acid solution for about 24 hours in the dark.

(6) Fix in sodium hyposulphite solution.

(7) Wash in water ; dehydrate ; imbed ; cut ; mount.

(*f*) **Mönckeberg and Bethe's method** for myelin sheaths and fibrils of peripheral nerves :—

(1) Fix in 0·25 per cent osmic acid (nerves stretched out), 24 hours.
(2) Wash in 90 per cent alcohol, 4-6 hours.
(3) Wash in water, 4 hours.
(4) Place in 2 per cent sodium-bisulphite solution, to each 10 c.c. of which 2-4 drops of concentrated HCl are added just before use, 6-12 hours.
(5) Wash in water, 1-2 hours.
(6) Dehydrate in alcohol ; imbed in paraffin ; cut thin sections and affix them to the slide with albumin glycerin.
(7) Stain in 0·1 per cent solution of toluidin blue in distilled water, 10 minutes at 50-60° C.
(8) Wash in water, 1-2 minutes.
(9) Place in 1 per cent ammonium - molybdate solution, a few seconds to a few minutes.
(10) Dehydrate in alcohol ; clear in xylol ; mount in neutral balsam (Grübler).

Or [7] Place in 1-4 per cent solution of ammonium molybdate, 5-10 minutes at 10-30° C.
[8] Wash in distilled water, immersing the slide 5-6 times ; dry the slide.
[9] Cover the preparation with 0·05-0·01 per cent solution of toluidin blue ; place in paraffin stove at 50-60° C. for 5 minutes.
[10] Wash in water ; dehydrate in alcohol ; xylol ; balsam.

For rapid demonstration of the axis cylinders of peripheral nerves and pathological processes connected with them the following procedure is useful :—

(*g*) **Platner's method** :—

(1) Harden in 25 per cent solution of liq. ferri sesquichlor., 1-5 days.
(2) Wash in water until the addition of KCNS to the water yields no reaction.
(3) Place in 75 per cent alcohol containing an excess of di-nitro-resorcin, 2-30 days, according to the size of the piece of tissue.
(4) Dehydrate in absolute alcohol.
(5) Imbed ; cut ; dehydrate ; clear ; mount.

Axis cylinders, emerald green.

## APPENDIX TO CHAPTER XII

It may be here mentioned that, in certain organs, cells are sometimes present which are stained yellow-brown in chrome solutions; because of these affinities they are termed **chromophilic** cells, masses formed of groups of these cells being known as chromophilic bodies or paraganglion (Kohn). They belong to the sympathetic system, and occur in the so-called medulla of the suprarenals and in the carotid and coccygeal glands. Otherwise they have been observed by Aschoff in the paroöphoron and paradidymis of the fœtus and of the newly born child.

Tumours may originate from these bodies (Manasse, Stangl), and thus these chromophilic structures possess a pathological importance.

These affinities are developed in all the chrome solutions, such as Orth's fluid (*q.v.*), etc., the chromophilic cells staining yellow to brown. When subsequently stained in polychrome methylene blue the cells assume a grass-green colour (Pecnik, Stangl).

# CHAPTER XIII

## SPECIAL PATHOLOGICAL PROCESSES

METHODS suitable for these purposes have already been described, chiefly in Chapter XI. It will be at once understood that tissues, the seat of fatty changes, and fat emboli may be stained by the methods for staining fat, and that patches of mucoid degeneration may be treated by the processes for demonstrating mucin; similarly amyloid, hyaline, and colloid changes may be recognised by the procedures stated under their respective headings.

The diagnosis of albuminous granules in cloudy swelling and their differentiation from fat are given in the chapters upon the examinations of fresh tissues; they should be always looked for in fresh untreated material.

In certain inflammations, *e.g.* of the lungs or of serous or mucous membranes, fibrin should be stained for by Weigert's method.

Suppurative foci require bacteriological examinations, and methylene blue, Gram's and other special methods for the pyogenic cocci, bacilli of diphtheria, typhoid, etc., may precede or accompany the use of culture media.

With caseous foci the usual stains for the tubercle bacillus may be usefully combined with the method for staining elastic fibres (*q.v.*). In gummatous formations which do not yield characteristic microscopic appearances, an examination for elastic fibres may be useful in determining the presence of endophlebitis (Rieder).

The staining of elastic fibres also will be found useful in the diagnosis of vascular lesions, and may frequently indicate other pathological processes.

For tumours, general staining methods should first be

used, and later those for mitotic figures. To assist in the differentiation of sarcomata and carcinomata, the use of the methods already described for the demonstration of connective tissue fibrils may be recommended.[1] The authors who support the parasitic theory of tumour formation have provided numerous methods for the demonstration of these so-called organisms. Feinberg uses methylene blue eosin (after the Romanowsky stain), Russell's method is described in Chapter II. F 4, and Plimmer's method is as follows :—

(1) Harden in Hermann's mixture, 12-24 hours :—

| | |
|---|---|
| Platinic chloride ($\frac{1}{2}$ per cent) . | 15 parts. |
| Osmic acid (2 per cent) . | 4 parts. |
| Glacial acetic acid . | 1 part. |

(2) Wash thoroughly in water, 12-24 hours. After-harden in increasing strengths of alcohol ; imbed in paraffin ; cut ; affix section to slide, etc.

(3) Transfer to hydrogen peroxide (15-30 minutes) until the section is not further blackened.

(4) Wash in water.

(5) Place in 4 per cent iron-alum solution, 2 hours.

(6) Wash in water.

(7) Stain in 0·5 per cent watery hæmatoxylin solution, 30 minutes.

(8) Differentiate in the iron-alum solution until the nuclei are dark and the protoplasm is colourless (best to control under the microscope).

(9) Wash in water, 3-6 hours.

(10) Counterstain in 1 per cent solution of Ehrlich's neutral red (it must be quite neutral) until the preparation is yellow-red.

The so-called parasites are yellow-red to copper-red ; nuclei, blue-black.

It is perhaps unnecessary to remark that these bodies are not generally recognised as parasites nor as a direct cause of carcinoma.

All the methods employed for general pathological processes may be used for the various organs of the body. The special methods for certain organs are detailed in Chapter XII. In order to avoid frequent repetition, the remaining organs have not been considered in detail. Their several structures and associated pathological processes can be demonstrated by the general and special methods already given.

[1] Sarcomata should be examined in the fresh condition (see p. 10) whenever possible. By such means the contents of the cells, such as glycogen, etc., are more easily demonstrated.

One point in regard to staining procedures deserves especial mention. Granted that the tissue is apparently in a good staining condition, and has not been too greatly altered by post-mortem changes, then an absence of nuclear staining in either the whole or separate parts of the section points to *necrotic* changes, as for instance tuberculous caseation, etc. With acid or plasma stains necrosed patches are stained diffusely. In early nuclear degenerative changes the nuclei often stain uniformly dark, or the chromatin is fragmented and the threads are disjointed and arranged irregularly. The tissue around the necrosed areas is frequently infiltrated with leucocytes, polymorphs, and mono-nuclears.

For one type of necrosis, viz. *fat necrosis*, a special staining method has been worked out by Benda.

**Benda's method** for the demonstration of the so-called fat necrosis of the pancreas and neighbouring adipose tissue :—

(1) Harden the tissue in 10 per cent formol.

(2) Transfer to Weigert's copper-fluorchrom mordant (Chapter XII. VI.), and place in the incubator, 2-4 days.

Or combine the formol and mordant (10 parts formalin, 40 per cent, 100 parts mordant).

The necrosed areas are stained green.

(3) Cut on the freezing microtome ; stain the sections in Sudan III. or Fett ponceau, and finally in hæmatoxylin.

Nuclei, blue ; normal fat, red ; necrosed fatty tissue, green.

According to Benda the reaction is due to the formation of a fatty acid copper salt.

**Fischler** has recently shown that the fatty acid copper salts (fatty acid potassium, sodium or calcium salts + copper) which are formed during Benda's method do not stain with watery solutions of hæmatoxylin, although in alcoholic hæmatoxylin solutions they stain black. The black compound is almost insoluble in Weigert's borax ferricyanide of potassium mixture.

Fischler stains sections in a 1 per cent solution of hæmatoxylin in 96 per cent alcohol for 24 hours, and then differentiates in Weigert's borax potassium ferricyanide solution. Only the areas containing fatty acids, viz. areas of fat necrosis are stained.

Soaps yield the same reaction when changed into insoluble salts. The latter may be obtained by hardening the tissue in a solution of formalin saturated with calcium salicylate. By the comparison of one portion of tissue hardened in this mixture with another portion hardened in formalin alone the soaps and fatty acid salts may be differentiated.

# CHAPTER XIV

## BACTERIA, MOULDS, AND OTHER FUNGI

BACTERIA may be examined in films or in sections, the former being, as a rule, the easier method. The general procedures to be followed in the making of film preparations have been already described in the section which deals with blood.

All instruments and apparatus must be absolutely sterile. It is best to use new cover-glasses and slides, to clean them thoroughly, and to remove all traces of fatty materials from their surfaces (see "Blood," Chapter XII. 1.).

### A. EXAMINATION OF BACTERIA IN FILM PREPARATIONS

The preparations obtained may be examined before or after staining.

#### 1. Unstained Preparations

The organisms may be examined in fresh films, in hanging-drop preparations, or in interlamellar films.

**Hanging-drop preparations.** — Clean slides and cover-glasses are sterilised by heat. On the under surface of the cover-glass place a very small drop of the suspected fluid or culture (with a small sterilised platinum loop or capillary tube); invert the cover-glass on the slide (the under dust- and bacteria-free surface) over a cavity formed by placing a sterilised rubber ring or a piece of moist blotting-paper in which a small circular aperture has been punched or cut. Special "hanging-drop" slides also may be used; they are thick glass slides in which a small concavity is present. The edges of the cavity are generally "ringed" with vaseline before the cover-glass is inverted, so that the resultant chamber is air-tight.

**Interlamellar films.**[1]—This method is specially useful for studying the life-history of moulds and protozoa. A glass slide is made hot; on its upper surface three small drops of sealing-wax are placed, so arranged that they form the apices of an equilateral triangle the side of which measures about 1 inch. On a sterilised cover-glass a droplet of a suitable nutrient medium is placed, and this is inoculated with the organism to be observed.

[1] Delépine.

The slide is also sterilised in the Bunsen flame, a droplet of the nutrient medium is placed in its centre between the points of sealing-wax, and the cover-glass picked up with sterilised forceps, inverted, and lowered on to the slide. The nutrient medium is thus contained between the slide and the cover-glass, and by using a hot wire and so softening the sealing-wax it can be spread out to form as thin a layer as desired. The preparation is kept in a moist chamber to prevent evaporation, and can be studied when required.

If necessary, a drop of acetic acid or one of a 20 per cent solution of caustic potash may be added to the unstained preparation. These reagents dissolve or clarify the albuminous granules or threads of fibrin and connective tissue structures. Droplets of oil may be removed by the addition of ether or chloroform.

A small diaphragm (as in all unstained preparations) and a high objective (as a rule, an oil-immersion $\frac{1}{12}$) are essential.

## II. Stained Preparations

Almost all basic aniline dyes stain nuclei; methyl green, however, dyes nuclear structures only. The reason for these staining affinities is not clear, but it has been suggested that the nuclear structures of the bacteria may be diffusely spread throughout the protoplasm (not circumscribed as in animal cells), so that the entire organism stains like a nucleus (Weigert).

Some of the aniline dyes stain bacteria better than others, and are therefore more usually employed. Amongst the former are methylene blue, fuchsin, methyl violet,[1] thionin, and Bismarck brown; saturated alcoholic solutions, or such solutions diluted with alcohol, are generally selected. Differentiating fluids are, as a rule, unnecessary, and the films dry rapidly. The methods are thus simple and easily applied.

The stages are as follows :—

(1) Stain in one of the solutions (methylene blue or fuchsin) for 2-10 minutes. Warming in an incubator or heating over the flame shortens the time and intensifies the staining.
(2) Wash in running water.
(3) Dry between thick folds of blotting- or filter-paper.
(4) Mount in Canada balsam.

Or [3] Dehydrate and decolourise in absolute alcohol.
[4] Clear in xylol or in ethereal oils.
[5] Mount in balsam.

[1] Gentian violet may be used instead of methyl violet. Weigert recommended gentian violet when he first described his method, but later advised methyl violet, which is a purified gentian violet. Whenever gentian violet is mentioned methyl violet or crystal violet may be substituted.

Aniline oil and chloroform remove the aniline dyes, and are therefore avoided.

Blotting-paper is used for drying the films when absolute alcohol cannot be used on account of its property of dissolving out the dyes from the stained structures. After drying with blotting-paper it is well to pass the preparation slowly through a Bunsen flame, in order to ensure thorough dehydration.

In some cases it is needful to employ differentiating fluids in order to remove the dye from tissue structures which are stained in the same manner as the bacteria. The following reagents are used for such purpose :—

**Differentiation fluids :—**

| | |
|---|---|
| 1. Alcohol, 70-90 per cent .<br>2. Aniline and aniline xylol .<br>3. Alcoholic solutions of acid aniline dyes — eosin, fluorescin, tropæolin .<br>4. Acetic acid ($\frac{1}{2}$-1 per cent) . | These act mildly, remove the dye from cell protoplasm, intercellular tissues, and in some cases from fibrinous and mucoid substances, leaving the nuclei and bacteria deeply stained. |
| 5. HCl-alcohol (1-25 per cent)<br>6. Weak solutions of picric acid<br>7. Iodine - potassium iodide solution (1-2 to 300 water) . . . .<br>8. Solutions of potassium permanganate, potassium carbonate, chloride of iron, etc. . . . . | These act rapidly and strongly, and are used only in specific cases. |

[redacted] and 5 are chiefly used for general purposes, and 2, 3, and 4 for special methods.

Whenever these solutions are used the acids or alkalies must be thoroughly removed by repeated washings in water or alcohol, and the film or section be cleared with ethereal oils, the oil completely removed by xylol, and the preparation mounted in xylol balsam.

The differentiation fluids should not be allowed to act for too long, or the bacilli may be also decolourised. Retention of the nuclear staining is useful in some cases, as for instance for intracellular organisms. The process should be carefully controlled by frequent microscopic examinations.

The staining effects of the aniline dyes already enumerated are frequently accentuated by the addition of alkalies or aniline or phenol. Here may be suitably stated the combinations which, primarily devised for special purposes, are now used for the general staining of bacteria :—

**Loeffler's methylene blue solution** :—

| | |
|---|---|
| Saturated alcoholic solution of methylene blue | 30 c.c. |
| Potassium hydrate solution, 1 in 10,000 | 100 c.c. |

Stain for 5-10 minutes. Keeps well.

**Carbol methylene blue solution** :—

| | |
|---|---|
| Methylene blue | 1·5 gm. |
| Absolute alcohol | 10 c.c. |
| Carbolic acid (cryst.) | 5 gms. |
| Water | 100 c.c. |

**Carbol-thionin solution** :—

| | |
|---|---|
| Saturated solution of thionin in 50 per cent alcohol | 10 c.c. |
| Carbolic acid (cryst.) | 1 gm. |
| Water | 100 c.c. |

**Carbol-fuchsin solution** :—

| | |
|---|---|
| Fuchsin (basic) | 1 gm. |
| Absolute alcohol | 10 c.c. |
| Carbolic acid (cryst.) | 5 gms. |
| Water | 100 c.c. |

**Aniline gentian-violet solution** :—

| | | |
|---|---|---|
| Aniline oil | 10 c.c. | Shake till milky in appearance. |
| Water | 100 c.c. | Filter. |

| | |
|---|---|
| Add Saturated solution of crystal violet, or gentian violet, or methyl violet | 11 c.c. |
| Absolute alcohol | 10 c.c. |

The mixture will keep for 8-12 days only.

Stain 1-5 minutes.

A modified and much better preparation is described in Chapter XI. section v.

For simple staining Loeffler's alkaline methylene-blue method is the best; it stains more sharply than fuchsin and gentian violet. Some workers prefer for routine work **Pappenheim's** pyronin-methyl-green solution :—

| | |
|---|---|
| Saturated aqueous solution of methyl green | 3-4 parts. |
| Saturated aqueous solution of pyronin | 1-1½ parts. |

Keeps for several weeks. Bacteria, red; nuclei, blue or purple.

The stains just given (with the exception of Pappenheim's mixture) colour bacteria and nuclei somewhat similarly. There exists, however, a method called, after the name of its inventor, Gram's method, which consists in the decolorisation by alcohol of tissues stained in aniline methyl violet, and subsequently treated with an iodine potassium-iodide solution. Some bacteria are, however, not decolourised, and it thus constitutes a means of differential diagnosis between certain groups of bacteria. Certain contrast nuclear stains, which materially assist in the recognition of some micro-organisms, may be used without materially altering the procedures.

| *Stained by Gram's method.* | *Not stained by Gram's method.* |
|---|---|
| Staphylococcus pyogenes aureus. | Gonococcus. |
| ,, ,, albus. | Bacillus typhosus. |
| ,, ,, citreus. | Bacterium coli communis. |
| Streptococcus pyogenes. | Bacillus dysenteriæ (Shiga). |
| Micrococcus tetragenus. | Spirillum cholera asiaticæ. |
| Diplococcus pneumoniæ. | Bacillus pyocyaneus. |
| Anthrax bacillus. | ,, mallei. |
| Tubercle ,, | ,, pneumoniæ (Friedländer). |
| Lepra ,, | ,, proteus. |
| Tetanus ,, | ,, of influenza. |
| Diphtheria ,, | ,, of bubonic plague. |
| Bacillus of rhinoscleroma. | ,, of fowl cholera. |
| ,, aerogenes capsulatus. | ,, of rabbit septicæmia. |
| ,, mouse septicæmia. | ,, of swine plague. |
| ,, swine erysipelas. | ,, of chancroid. |
| Oidium albicans. | |
| Mycelium of actinomyces. | |
| Some bacteria of putrefaction. | |

**Gram's method is as follows** :—

(1) Fix film by heat. Stain 3-5 minutes in aniline gentian violet (or aniline methyl or crystal violet). Remove surplus stain. Wash rapidly in water.

(2) Treat the stained film with iodine solution (iodine, 1 gm. ; potassium iodide, 2 gms. ; water, 300 c.c.), until the preparation is dark brown in colour.

(3) Without washing, decolourise with alcohol until the film appears almost colourless.

(4) Dry and mount in xylol balsam.

Bacteria, blue ; cellular tissues, yellow ; nuclear tissues, blue ; mast-cell granules, blue ; keratin, light blue.

Gram's method has been modified in several ways. Weigert's modification, known as the Gram-Weigert method, is perhaps the most extensively used, and is certainly the best. It has been already described in Chapter XI. p. 99 ; the differentiation is made with aniline xylol, a slower but safer procedure.

Of the other modifications, Guenther's is sometimes used, and Czaplewski's use of carbol instead of aniline gentian violet has at least the merit of possessing better keeping qualities, although Weigert's mode of preparing his solutions (*vide* Chapter XI. section v.) makes such a modification unnecessary.

It is sometimes useful to employ a **contrast stain in Gram's method.** For this purpose a watery solution of Bismarck brown is useful. Czaplewski advises a carbol-fuchsin solution, which is prepared by mixing 1 gm. of fuchsin with 5 c.c. of liquid carbolic acid until the crystals are dissolved. 50 c.c. of glycerin are then added, the resultant solution well mixed and made up to 155 c.c. with water. For staining, add 1 c.c. of the staining mixture to 9 c.c. of water, and stain for 1 minute at about 50° C. Wash in water; dry; mount in xylol balsam.

Lorrain Smith saturates the aniline xylol with eosin, and thus counterstains during differentiation.

### B. Examination of Bacteria in Sections of Tissues

The essentials for success in staining bacteria in sections are rapid fixation and hardening. Alcohol fixation is said to be followed by the best staining results so far as bacteria are concerned, but formalin fixation is by no means to be despised, and as it is a general fixing medium, it may be used for this purpose with the certainty of good after-staining of micro-organisms. Sections obtained by the freezing microtome are applicable, but it is best to imbed in paraffin or celloidin, as very thin sections are necessary for successful demonstration of the bacteria.

The hardening fluids and methods are those already described in Chapter IV. Müller's fluid is to be avoided, as it hardens too slowly, does not inhibit bacterial growth, and allows the formation of fungi. If the tissues are left in alcohol for a long time, their staining affinities are diminished.

Should formalin fixation not give satisfactory staining results with Gram-Weigert's method, oxidise and reduce according to Weigert's directions, Chapter XI. p. 99, the results will then be good.

The staining mixtures used for film preparations are generally applicable. Methylene blue in watery solution is a good general stain, and Gram-Weigert's method is employed as a matter of routine.

The time for staining should be at least three times as long as for film preparations.

Methods which may be relied upon to produce good preparations are as follows :—

**Loeffler's methylene blue** :—

(1) Stain in Loeffler's solution, 5 minutes to 3 hours.

(2) Wash in water.

(3) Differentiate in 1 per cent watery acetic acid solution, 10-30 seconds.

(4) Wash in 90 per cent alcohol, 2-5 minutes, then dehydrate thoroughly in absolute alcohol ; clear in xylol ; mount in balsam.

Sections stained with Loeffler's methylene blue or with carbol methylene blue and then washed in water give better results with typhoid bacilli, and the bacilli of fowl and swine cholera and pseudo-tuberculosis, if they are placed in a 10 per cent solution of tannic acid for $\frac{1}{2}$-1 minute, then dehydrated in absolute alcohol, cleared in xylol, and mounted in balsam (Nicolle).

Glanders and influenza bacilli are very sensitive to the differentiating reagents, and very weak acidified water should be substituted for the acetic acid solution.

**Gentian violet.**—This stain yields excellent results with the majority of micro-organisms, but should not be used for the bacillus of glanders. The bacteria stain sharply and are not shrunken. The depth of stain must be regulated by repeated examination under the low power of the microscope.

1. Stain sections in a 2 per cent watery solution of gentian violet for 5-20 minutes.
2. Wash in water.
3. Decolourise in 70 per cent alcohol until the stain ceases to come away.
4. Dehydrate in absolute alcohol; clear in xylol; mount in balsam.

**Romanowsky's methylene blue eosin.**—Sections stained by Leishman's modification (p. 141) yield good results.

The stains just cited, while yielding useful stain pictures, give, however, a light blue background to the dark blue bacteria, and the colours are not alcohol-fast. Very careful differentiation is necessary to obtain good results.

**Zieler** has devised the following method, which is particularly useful for organisms such as the *B. mallei*, *B. typhosus*, *gonococcus*, etc., which are always rather difficult to stain deeply.

**Zieler's method for staining bacteria in tissues :—**

(1) Fix in Orth's mixture or other fixing fluids, except those containing osmic acid ; harden ; imbed in paraffin or celloidin ; cut.

(2) Stain sections in Pranter's mixture (p. 86), 8-24 hours :—

| | |
|---|---|
| Orcein D (Grübler) . . | 0·1 gm. |
| Hydrochloric acid . . | 2·0 c.c. |
| Alcohol (70 per cent) . . | 100 c.c. |

(3) Wash rapidly in 70 per cent alcohol (to remove the surplus orcein).

(4) Wash in water.

(5) Stain in polychrome methylene blue, 10-120 minutes.

(6) Wash in distilled water.

(7) Differentiate in glycerin ether (Grübler), until no more clouds of colour come away and the section is light blue.

(8) Wash in distilled water.

(9) Place in 70 per cent alcohol ; absolute alcohol, 5-10 minutes ; xylol ; balsam.

Protoplasm, grey-brown ; bacteria, dark blue. Background, absolutely colourless.

## C. Staining Methods for Certain Special Bacterial Structures

The capsules, granules, spores, and flagella of bacteria require special methods for their clear demonstration ; they all are stained with difficulty, and the procedures devised are most successful in film preparations.

### I. Spores

Spores can only be efficiently stained in film preparations. Enclosed in a dense membrane, they are more resistant to chemical substances than the adult forms, and must be subjected to special methods. Once stained, however, they are not easily decolourised

**Neisser and Hueppe's method :—**

1. Stain the air-dried and heat-fixed film for 1-5 hours in saturated aniline-fuchsin solution at 40-80° C. Add more aniline water from time to time to compensate for evaporation.
2. Decolourise for 5 seconds in 25 per cent $H_2SO_4$.

3. Place the film in alcohol until no more dye can be removed.
4. Wash in distilled water.
5. Stain in watery methylene-blue solution for 3-5 minutes. Wash in water; dry; mount in balsam.

Spores, red; bacilli, blue.

**Möller's method** :—

1. Place the heat-fixed film in 5 per cent watery chromic acid solution for 2-5 minutes.
2. Wash in water.
3. Stain with carbol fuchsin or aniline fuchsin, 1 minute at 80-100° C.
4. Decolourise in 5 per cent $H_2SO_4$ (5 seconds). Wash in water.
5. Stain in watery methylene-blue solution, 3 minutes; wash in water; dry; mount in balsam.

Originally, the fixed film was covered with chloroform for 2 minutes. This, although advisable, is not necessary.

Spores, red; bacilli, blue.

**Klein's method** (specially useful for anthrax bacilli) :—

1. Heat 1-2 c.c. of filtered carbol fuchsin and 2 c.c. of an emulsion of bacteria in 0·6 per cent sodium-chloride solution.
2. Make smears on cover-glasses or slides.
3. Decolourise in 1 per cent sulphuric acid for 1-2 seconds.
4. Wash in water.
5. Stain in watery methylene-blue solution, 3-4 minutes.
6. Wash rapidly; dry; mount in balsam.

Spores, red; bacilli, blue.

## II. Granules

**Ficker** recommends a combination of methylene blue and lactic acid. Methylene blue (Höchst), 1 gm.; distilled water, 100 c.c.

For staining, take 1 c.c. of the methylene-blue solution, 2 c.c. of pure lactic acid, and 100 c.c. of distilled water.

Some granules are stained by the methods already given for spores and also by the method of staining the tubercle bacillus. Others are stained a reddish colour with polychrome methylene blue, as for instance the granules of some spirilla.

The staining of granules is most essential for the differential diagnosis of real and pseudo-diphtheria bacilli, and will be considered later (p. 238).

## III. Capsules of Bacteria

The capsules of bacteria are stained best when the organisms are obtained from animal tissues or secretions. During cultivation the staining affinities are diminished, if not entirely lost.

Johne's method is perhaps the best for the beginner. All the others give equally good results.

1. **Johne's method** :—

(1) Stain heat-fixed film with 2 per cent watery gentian violet solution for 2 minutes, slightly warming. Wash in water.

(2) Place the stained film in 2 per cent acetic acid, 10 seconds. Wash in water. Examine in water; the deep blue bacilli are surrounded by a light blue capsule.

2. **Ribbert's method** :—

| | |
|---|---|
| Saturated watery solution of dahlia | 100 c.c. |
| Glacial acetic acid . . | 12·5 c.c. |
| Alcohol . . . . | 50 c.c. |

Stain for 1-2 seconds; wash in water; dry; mount in balsam. Bacteria, dark blue; capsules, light blue.

3. **MacConky's method** :—

(1) Stain films in the following mixture, heating until the steam begins to rise :—

| | |
|---|---|
| Dahlia . . . . | 0·5 gm. |
| Methyl green OO crystal . | 1·5 gm. |
| Saturated alcoholic fuchsin solution | 10 c.c. |
| Distilled water . . . | 200 c.c. |

Rub up the dahlia and methyl green in a mortar with a part of the water until dissolved, then add the fuchsin and the remainder of the water.

(2) Place aside for 5 minutes.

(3) Wash in water; dry; mount in balsam.

4. Any of the modifications of the Romanowsky eosin methylene blue may be employed, Zettnow's and Leishman's giving perhaps the best results.

5. **Muir's method** for staining the capsule of the pneumococcus :—

(1) Place an air-dried film in

| | |
|---|---|
| Watery solution of tannic acid, 20 per cent . | 2 c.c. |
| Saturated watery solution of mercuric chloride | 2 c.c. |
| Saturated watery solution of potash alum . | 5 c.c. |

for 2 minutes.

(2) Wash preparation in water, then in alcohol and again in water.

(3) Stain in carbol fuchsin for 2 minutes, warming gently until the steam rises.

(4) Wash ; treat with the tannin-mercury solution for 2 minutes and again wash.

(5) Stain in a saturated watery solution of methylene blue for 2 minutes.

(6) Wash in water ; differentiate in alcohol ; dehydrate in absolute alcohol ; mount in balsam.

Capsules are blue ; cocci and bacteria, red.

6. **Welch's method** :—

(1) Cover the preparation with glacial acetic acid for 5 seconds.

(2) Remove the surplus acid and stain in aniline gentian violet, repeatedly adding the dye in order to entirely remove the acid.

(3) Wash in 2 per cent solution of sodium chloride ; mount and examine in the same medium.

(Acetic acid produces a precipitate of the mucin-like substance of the capsules, insoluble in sodium chloride.)

For demonstration of the capsules of bacteria in sections

7. **Friedländer's method** may be used :—

| | |
|---|---|
| Concentrated alcoholic gentian violet solution . . . | 50 c.c. |
| Glacial acetic acid . . . | 10 c.c. |
| Water . . . . | 100 c.c. |

(1) Stain film or section, 2-24 hours.

(2) Decolourise in 1 per cent acetic acid, 1-2 minutes.

(3) Dehydrate in alcohol ; xylol ; balsam.

The capsule is light blue ; the bacillus, dark blue. Careful decolorisation is necessary.

## IV. Flagella

To stain flagella it is absolutely necessary that the cover-glasses should be free from fat and all organic material other than bacteria. Place new cover-glasses in pure sulphuric acid for 1 hour and then transfer them to a mixture of equal parts of alcohol and ether. Dry on soft linen or silk, and heat in the flame for a few minutes before using.

Flagella can only be stained in film preparations, and the results are somewhat uncertain.

The material for examination should be obtained from agar cultures.

Since the flagella do not take up the colours equally as the bacterium itself, it is necessary to use a mordant.

Hill (*Journal of Medical Research*, 1904) attributes the difficulties of staining to the broth or other culture medium which adheres to the flagella. He advises the use of 24-hours-old broth cultures. These are centrifugalised for 15 minutes, the supernatant fluid decanted, the tube filled with water and again centrifugalised, the process being repeated 3 or 4 times. From the last dilution preparations are made. The manipulations do not shake loose the flagella, but by cleaning them render them easier to stain.

1. **Loeffler's method** (modified by Bunge) :—

| *Mordant.* | *Stain.* |
|---|---|
| Tannic acid, saturated watery solution . 30 c.c.<br>Liq. ferri perchloridi (1 in 20 water) . . 10 c.c.<br>Concentrated watery fuchsin solution . 4 c.c.<br>Allow to stand in the air for some days or add to each 5 c.c. of the filtered mordant 0·5 c.c. of 3 per cent fresh peroxide of hydrogen.<br>The mixture must be freshly prepared for each occasion and then filtered. | Carbol fuchsin or carbol gentian violet. |

(1) Place the air-dried, heat-fixed film in the filtered mordant solution. Warm for 1-5 minutes until the steam rises.
(2) Wash in water.
(3) Stain in carbol fuchsin or carbol gentian violet for 5 minutes, warming slightly.
(4) Wash in water ; dry ; mount in balsam.

2. **van Ermengen's method** :—

*Mordant.*

Osmic acid, 2 per cent . 10 c.c.
Tannic acid solution, 10-20 per cent . . 20 c.c.
Glacial acetic acid . 1 drop.

*Solutions.*

**A.**
Silver nitrate . 0·5 gm.
Water . . 100 c.c.

**B.**
Gallic acid . . 5 gms.
Tannic acid . . 3 gms.
Sodium acetate, fused 10 gms.
Distilled water . 350 c.c.

(1) To a large drop of water add a small quantity of a 12-18 hours agar culture of the organism ; mix ; take a small loopful of the diluted culture and make a smear on a specially cleaned cover-glass ; dry in the air ; fix by heat.
(2) Cover the film with the osmic tannin solution and place it in an incubator at 50-60° C. for 5 minutes, or allow it to remain at the room temperature for 30 minutes.
(3) Wash well with water and then with alcohol (70 per cent).
(4) Place film for a few seconds in the 0·5 per cent silver nitrate solution.
(5) Transfer it directly to the gallic tannin solution for 10 seconds.
(6) Return the film to the silver nitrate solution and leave it there until it becomes quite black.
(7) Wash rapidly in water and examine under a high objective. If the flagella are not visible, again repeat the gallic acid and silver immersions.
(8) Dry ; mount in balsam.

Bacteria, dark brown ; flagella, black.

**Stephens** has shortened and modified Ermengen's method as follows :— He mordants the film for 30-60 minutes in the osmium-tannin mixture, then places some 0·1 per cent silver nitrate solution on the film, and adds a few drops of a mixture of equal parts of 5 per cent tannic acid and 5 per cent ammonia solution. When the film appears a deep red-brown (about 1 minute), the silver-tannin-ammonia mixture is removed and the film washed in water. The procedure may be repeated several times, viz. until the film is dark brown or black. Wash. Dry. Mount in balsam.

3. **Pitfield's method** (modified by Hunt and Smith) :—

**Mordant.**—Saturate hot saturated mercuric chloride solution with ammonia alum. Shake thoroughly. Cool.

Take of this fluid . . 10 c.c.
10 per cent tannin solution (freshly prepared) . 10 c.c.
Carbol fuchsin solution . . 5 c.c.
} Mix ; filter.

Cover film with the solution. Warm until steam is given off, avoiding boiling. Wash in distilled water. **Stain** in

Saturated alcoholic solution of gentian violet . 1 c.c.
Saturated solution of ammonia alum . . 10 c.c.

Wash. Dry. Mount in balsam.

4. **De Rossi's method** :—

De Rossi uses a 4-days-old agar culture. A small particle of culture taken on a platinum needle is gently mixed with a few drops of distilled water in a watch-glass, and a loopful of this added to a second lot of distilled water ; a drop of the latter is placed—not spread—on a cover-glass and then rapidly dried in an exsiccator ; when dry the centre is transparent, and there is a faint white ring at the margin of each drop.

| *Mordant.* | | *Stain.* | |
|---|---|---|---|
| Tannic acid . . | 25 gms. | Carbolic acid (cryst.) . | 5 gms. |
| Potassium hydrate, 1 per cent aqueous solution . | 100 c.c. | Alcohol . . . | 10 c.c. |
| (Dissolve by heat.) | | Fuchsin . . . | 0·25 gm. |
| | | Distilled water . . | 100 c.c. |

Pour one drop of the mordant on to the cover-glass and also 4-5 drops of the stain—a precipitate is formed ; leave the mixture on the film for 15-20 minutes ; wash with distilled water ; dry between thick folds of blotting-paper ; mount in balsam.

5. **Zettnow's method**, which gives good results, is specially preferred by some workers. It is described in Zettnow's paper in the *Zeitschrift für Hygiene und Infectionskrankheiten*, Bd. 30.

## D. Staining Methods for Certain Bacteria

### I. Tubercle Bacillus

The tubercle bacillus combines very closely with aniline stains, but does not easily yield up the dye to decolourising reagents. It is generally classified as acid-fast and alcohol-fast. These properties may have some relation to the high percentage of fat present. Young forms exhibit these characters in a much less marked degree. Successful staining of the organism depends upon a thorough impregnation of the dye and the careful removal of the stain from all other bacteria and tissue elements.

#### *a.* Film Preparations

Film preparations made from sputa, liquids (urine, stomach contents, exudates, lumbar puncture fluids), fæces, lymph glands, or other tissues, are to be dried in the air and fixed by heat.

When sputum is examined for tubercle bacilli, the small caseous masses should be selected (*vide* "Sputum").

Fluids should be centrifugalised or allowed to stand for 24 hours, and the film then made from the sediment.

It is better to use slides than cover-glasses ; at least three films should be always prepared, and the material should be

spread over the greater part of the slide, since it is sometimes necessary to examine several preparations before finding the bacilli.

When only few bacilli are present, additional measures are necessary for their demonstration. Of the numerous methods available, the following present many advantages and are easily carried out :—

1.

(1) Heat the sputum or other material in a test-tube with boiling water or in a steriliser for 15 minutes. The bacilli with the coagulated albuminous constituents fall to the bottom.
(2) Remove the supernatant liquid and examine the coagulum.

2. **van Ketel's method** :—

(1) Place about 10 c.c. of sputum in a wide-mouthed jar which will contain 100 c.c. of fluid.
(2) Add 10 c.c. of water and 6 c.c. of liquefied carbolic acid crystals. Shake for 1 minute.
(3) Fill the bottle with water and shake again.
(4) Allow the contents to stand for 24 hours ; examine the sediment.
(5) Place the films in a mixture of equal parts of alcohol and ether.
(6) Stain with hot fuchsin solution and decolourise with dilute sulphuric acid (p. 233).

3. **Nebel** mixes the sputum with 8-10 volumes of clear lime-water and centrifugalises after the calcium has acted for 2 minutes.

4. The group of methods associated with the names of **Mulhäuser, Czaplewski, Jochmann, Spengler,** and **Stroschein** yield more consistent results than those previously mentioned.

**Beitzke** has recently compared these procedures, and has introduced modifications which are embodied in the following well-recommended method :—

(1) Shake the sputum with 4 times its volume of 0·2 per cent caustic soda for 1 minute in a stoppered glass measure.

If the mucinoid or grey masses are not dissolved, add more 0·2 per cent caustic soda, shake vigorously, and repeat the procedure until a thin mucinous mixture results. Should such not be obtained within 5 minutes, pour the contents of the measure into a porcelain basin and heat to boiling, stirring until the fluid is free from tissue particles but retains slightly its mucinoid characters.

(2) Add 1-2 drops of phenol-phthalein solution.
(3) Add 5 per cent acetic acid, drop by drop, constantly stirring, until the red colour disappears. Too much acid must not be added, or the mucin will be precipitated and the object of the method thus frustrated ; this may be avoided by thorough mixing after the addition of each drop.
(4) Place the fluid in a centrifugalisation tube and add double its volume of 96 per cent alcohol.
(5) Centrifugalise and prepare films from the sediment.

### *b.* Staining Methods for Film Preparations of Tubercle Bacilli

1. **Ziehl-Neelsen's method :—**

(1) Stain in carbol fuchsin, 3-8 minutes, warming the solution until the steam rises. Deep staining is necessary.
(2) Wash in water.
(3) Decolourise in 25 per cent $H_2SO_4$ (30 per cent $HNO_3$ may also be used), 20-25 seconds.
(4) Wash in 90 per cent alcohol.
(5) Stain in watery methylene blue, 2-3 minutes.
(6) Wash in water ; dry ; mount in balsam.

Tubercle bacilli, red ; other structures, blue.

2. **Koch-Ehrlich's method :—**

(1) Stain in freshly prepared aniline fuchsin or aniline methyl violet, heating very gradually until the steam rises. When using slides, hold the slides above the flame with a pair of Cornet's forceps until the steam rises, then allow the stain to act for another minute without further heating.
(2) Decolourise in 33 per cent $HNO_3$, $\frac{1}{4}$-2 minutes.
(3) Wash in 60 per cent alcohol until the red colour disappears and the preparation becomes a light pink.
(4) Stain with watery methylene blue (or with Bismarck brown after methyl violet).
(5) Wash in water ; dry ; mount in balsam.

Tubercle bacilli, red ; other structures, blue.

3. **Gabbet's method :—**

(1) Stain in Ziehl-Neelsen's carbol fuchsin, heating until the steam rises, 1-3 minutes.
(2) Wash in water.
(3) Decolourise and stain for contrast in methylene blue, 2 gms. ; sulphuric acid, 25 c.c. ; water, 75 c.c., 1 minute.
(4) Wash in water.
(5) Absolute alcohol ; xylol ; balsam.

4. **Fraenkel's method :—**

(1) Stain in aniline fuchsin or gentian violet, heating until the steam rises, 1-2 minutes.
(2) Decolourise in 50 c.c. alcohol, 30 c.c. water, 20 c.c. $HNO_3$, added to a saturated solution of methylene blue or Bismarck brown.
(3) Wash in water.
(4) Absolute alcohol ; dry ; mount in balsam.

5. **Czaplewski's method :—**

Fluorescin is substituted for the mineral acids.

(1) Stain in Ziehl-Neelsen's carbol-fuchsin solution, 3 minutes.
(2) Pass the film through a concentrated solution of yellow fluorescin, saturated with methylene blue, 6-10 times, allowing the stain to drain off the slide after each immersion.
(3) Stain in saturated alcoholic methylene-blue solution.
(4) Wash rapidly in water.
(5) Dry ; mount in balsam.

Methods 4 and 5, which decolourise and contrast stain at the same time, are shorter than methods 1 and 2, and have thus been adopted ; they do not, however, allow an accurate control of the decolorisation, and when few bacilli are present the latter may be almost invisible. Koch-Ehrlich's and Ziehl-Neelsen's methods are more satisfactory in this regard, but with the former the staining mixture must be always freshly prepared. Ziehl-Neelsen's carbol fuchsin keeps almost indefinitely, and hence this stain is more generally used. Acid alcohol may with advantage be substituted for the acids, since it allows better control during decolorisation.

### *c.* Staining of Tubercle Bacilli in Sections

The methods already described for films may also be used for sections ; but instead of being warmed over the flame the sections should be placed in an incubator, or allowed to stand for a long time at room temperature, and for decolorisation acid alcohols, not mineral acids, should be used. It is an advantage to stain the nuclei in hæmatoxylin solution. We recommend the following procedure :—

1.

(1) Stain in aniline or carbol-fuchsin solution for 2-3 hours in the incubator, or for 24 hours at room temperature.
(2) Wash in water.
(3) Decolourise in acid alcohol until the sections are light pink in colour.
(4) Wash in water.
(5) Stain in Ehrlich's or Delafield's hæmatoxylin ; wash in water ; place in lithium-carbonate (5 per cent) solution, 1 second ; wash in water.
(6) Dehydrate in absolute alcohol.
(7) Dry ; xylol (not phenol xylol, which removes the stain from the bacilli).
(8) Mount in balsam.

2. **Schmorl's method** :—

(1) Stain in hæmatoxylin solution, 20-30 minutes.
(2) Wash in water, $\frac{1}{2}$-1 hour.
(3) Stain in carbol fuchsin, $\frac{1}{2}$-1 hour, at 37° C.
(4) Decolourise in acid alcohol, 1 minute.
(5) Wash in 70 per cent alcohol, 2-3 minutes, and then in water.
(6) Place in weak solution of lithium carbonate (1 part of saturated solution to 10 parts of water) until the section appears blue.
(7) Wash in water, 5-10 minutes.
(8) Dehydrate in absolute alcohol ; xylol ; balsam.

3. **Delépine's modification of Kühne's and Borrel's method** :—

(1) Fix in perchloride of mercury ; harden in alcohol ; imbed in paraffin.
(2) Stain with a solution of hæmatein, 20-30 seconds. Wash thoroughly.
(3) Stain with Ziehl's carbol fuchsin at 47° C. for 20-30 minutes in a moist chamber to prevent evaporation.
(4) Remove the surplus stain and treat the section with 2 per cent watery solution of hydrochlorate of aniline for a few seconds.
(5) Decolourise in 75 per cent alcohol until the section is apparently free from stain (20-30 minutes).
(6) Counterstain with a solution of orange (saturated watery solution of orange, 1 part ; 50 per cent alcohol, 20-40 parts).
(7) Dehydrate in absolute alcohol ; xylol ; balsam.

4. **Mallory and Wright's method** :—

(1) Stain in alum-hæmatoxylin solution and wash in water.
(2) Dehydrate in 95 per cent alcohol.
(3) Stain in carbol fuchsin, 2-5 minutes in the incubator. Wash in water.
(4) Differentiate in acid alcohol.
(5) Wash thoroughly in water to remove the acid.
(6) Remove the fuchsin with 95 per cent alcohol.
(7) Differentiate with aniline.
(8) Clear in xylol ; mount in balsam.

Celloidin, colourless ; nuclei, blue ; protoplasm, colourless ; tubercle bacilli, red.

For the demonstration of **tubercle bacilli and elastic fibres in the same preparation** the methods of Wechsberg or Herxheimer may be employed (*vide* Chapter XI. section iii.).

### *d.* Methods for Staining the Branched or Streptothrix Forms of Tubercle Bacilli

The Weigert-Gram method and the Birch-Hirschfeld method of staining actinomyces give satisfactory results.

The following method is specially devised for this purpose :—

**Friedrich and Noeske's method** :—

*Solutions.*

*Victoria Blue.*

| | |
|---|---|
| 33 per cent alcohol | 80 c.c. |
| Aniline | 1 c.c. |
| Saturated alcoholic Victoria-blue solution | 10 c.c. |

*Acid Alcohol.*

| | |
|---|---|
| 50 per cent alcohol | 50 c.c. |
| Distilled water | 50 c.c. |
| Hydrochloric acid | 1 c.c. |

*Alkaline Methylene blue Solution.*

| | |
|---|---|
| Saturated aqueous lithium-carbonate solution | 5 c.c. |
| Distilled water | 80 c.c. |
| Rectified spirit | 10 c.c. |
| Saturated alcoholic solution of methylene blue | 2 c.c. |

*Acid Methylene blue Solution.*

| | |
|---|---|
| Saturated alcoholic methylene blue solution | 5 c.c. |
| Acetic acid | 10 drops |
| Distilled water | 200 c.c. |

*Eosin Solution.*

| | |
|---|---|
| Eosin (watery) | 2 gms. |
| Distilled water | 100 c.c. |

(1) Stain the film or section in the Victoria-blue solution, warming slightly.
(2) When cool treat with Lugol's solution (iodine, 1 ; potassium iodide, 2 ; water, 300) for 2 minutes.
(3) Wash in water, 30 seconds.
(4) Wash rapidly in 80 per cent alcohol.
(5) Decolourise with pure aniline until the colour has almost entirely disappeared.
(6) Wash in alcohol, then in water.
(7) Stain in the eosin solution for 2 minutes. Wash in water.
(8) Stain in the alkaline methylene blue until the preparation is blue-red ($\frac{1}{2}$-1 minute). Wash in alcohol.
(9) Stain in the acid methylene blue, 5-10 minutes.
(10) Dehydrate in alcohol. Clear in xylol ; mount in balsam.

### *e.* Methods for the Differential Diagnosis of Tubercle Bacilli from other Acid-fast Bacilli

The acid-fast characters of the tubercle bacillus are also presented by the bacillus of leprosy, the smegma bacillus, Lustgarten's bacillus of syphilis, and the pseudo-tubercle bacilli of Timothy, Rabinowitch, and Moeller.

Some of these can be differentiated only by cultivation and inoculation. The differential diagnosis of the smegma and tubercle bacilli is often of great practical importance. In such cases the following methods will be found useful, although it may be sometimes necessary to resort to experiments on animals.

1. **Pappenheim's method** :—

(1) Stain in steaming carbol-fuchsin solution, 2-5 minutes.
(2) Remove the excess carbol fuchsin, and then stain in the following solution :—

| | |
|---|---|
| Corallin . . . | 1 gm. |
| Absolute alcohol . . | 100 c.c. |

Saturate with methylene blue, and then add 20 c.c. of glycerin.
(3) Wash rapidly in water ; dry ; xylol ; balsam.
Tubercle bacilli, red ; other bacilli, blue.

2. **Bunge and Trautenroth's method** :—

(1) Fix film in absolute alcohol for 3 hours.
(2) Treat with 5 per cent chromic acid for 15 minutes.
(3) Wash thoroughly in water.
(4) Stain in carbol fuchsin for 2 minutes, steaming.
(5) Decolourise in 25 per cent $H_2SO_4$, 3 minutes.
(6) Remove the acid with alcohol.
(7) Counterstain in a saturated alcoholic solution of methylene blue, 5 minutes.
(8) Wash in water ; dry ; balsam.

Of these acid-fast bacteria only the tubercle bacilli retain the dye.

3. A **simpler method** is to stain in Ziehl-Neelsen's carbol fuchsin, and decolourise in acid alcohol (HCl, 1 c.c. ; alcohol, 70 per cent, 100 c.c.) or in 20 per cent acetone alcohol. The smegma bacillus is decolourised, the tubercle bacillus retains the stain if the differentiation is not too protracted.

## II. Bacillus of Diphtheria

For the purpose of obtaining diphtheria bacilli directly from the diseased tissues, a sterilised swab is first prepared. This consists of a piece of rather stiff wire, around one end of which a pledget of cotton-wool is securely fixed, and then enclosed in a stoppered tube and sterilised. Remove the swab from its tube ; gently rub it over the surface of the affected mucosa, and then replace it in the tube. The material so gained may be used for making smear preparations and for inoculating culture media.

The organism stains by Gram's method, but the decolorisation must be very carefully controlled under the microscope, the stain being but poorly retained. It stains readily in the

ordinary basic aniline dyes, yielding the best results with Loeffler's methylene blue or with fuchsin.

Since, however, it is of importance to differentiate the diphtheria bacillus of Loeffler from the pseudo-diphtheria bacilli, it is necessary to employ special methods for the staining of the granules of the former in order to distinguish it from the other bacilli which stain similarly with general dyes.

**Cultures** should be stained by Neisser's method, and are best examined when from 9 to 24 hours old.

1. **Neisser's method** :—

*Solutions.*

| | |
|---|---|
| Methylene blue . . | 1 gm. |
| Alcohol, 90 per cent . . | 20 c.c. |
| Distilled water . . . | 950 c.c. |
| Glacial acetic acid . . | 50 c.c. |

Filter.

| | |
|---|---|
| Bismarck brown . . | 2 gms. |
| Water (boiling) . . | 1000 c.c. |

Filter.

(1) Stain for 1-3 seconds in the solution.

(2) Wash in water.

(3) Stain for 3-5 seconds in Bismarck brown.

(4) Wash in water; dry (balsam).

Bacilli, brown rods, the diphtheria bacilli, containing bluish black granules.

**Peck** recommends fixation of the film by heat, staining with Loeffler's methylene blue for 3-4 seconds and counter-staining with 0·2 per cent watery solution of Bismarck brown for 30 seconds, the preparation being later washed, dried, and mounted.

2. **Hunt's method** :—

(1) Stain in saturated aqueous methylene-blue solution. Wash in water.

(2) Place in 10 per cent aqueous tannic acid solution for 10 seconds. Wash in water.

(3) Stain in freshly prepared saturated aqueous methyl-orange solution for 1 minute. Wash in water.

(4) Dry; mount in balsam.

Bacilli, light green; granules, dark blue.

3. **Pitfield's method for staining chromatin points** :—

*Solutions.*

| | | |
|---|---|---|
| (*a*) | Silver nitrate . . . | 5 gms. |
| | Saturated alcoholic fuchsin . | 3 c.c. |
| | Distilled water . . | 5 c.c. |
| (*b*) | Acid pyrogallic . . | 1 gm. |
| | Sodium-hydrate solution . | 5 c.c. |
| | Distilled water . . | 10 c.c. |
| (*c*) | Carbol-fuchsin solution . | 10 drops. |
| | Distilled water . . | 10 c.c. |

(1) Fix by heat.
(2) Treat film successively with solutions *a*, *b*, and *c*, allowing them to remain for 1 minute, and then washing the preparations in water.
(3) Dry ; mount in balsam.

Bacilli, pink ; chromatin points, brilliant shining black ; cell membrane, grey-brown.

4. **Ljubinsky's method** :—

(1) Fix film preparations for $\frac{1}{2}$-2 minutes in

| | |
|---|---|
| Pyoktanin "Merck" . . | 0·25 gm. |
| Acetic acid (5 per cent) . . | 100 c.c. |

(2) Wash in water.
(3) Stain in $\frac{1}{1000}$ vesuvin solution, $\frac{1}{2}$ minute.

Bacilli, dark violet : granules, blue black.

Blumenthal and Lipskerow recommend $\frac{1}{300}$ chrysoidin solution instead of the vesuvin.

This method gives most excellent results, and is worthy of adoption for routine examinations.

## III. Typhoid and Coli Groups of Bacteria

These bacilli cannot be differentiated by their staining reactions. They are not stained by Gram's method. They may, however, be stained in Loeffler's methylene blue and in other aniline dyes. In all cases staining should be prolonged and accelerated by warmth.

Sections may also be similarly stained, then decolourised for 5 seconds in $\frac{1}{2}$ per cent acetic acid solution, transferred to alcohol for 2-3 minutes, dried, and mounted in balsam (Schmorl).

Or they may be stained for 10 minutes in

| | |
|---|---|
| Methyl violet | 5 gms. |
| Alcohol | 1 c.c. |
| Aniline | 2 c.c. |
| Water | 88 c.c. |

decolourised in $\frac{1}{1000}$ watery acetic acid, 10-15 minutes, dehydrated in oil of cloves, cleared in xylol, and mounted in balsam.

The typhoid bacilli are generally grouped in characteristic colonies. For differential diagnosis the use of specific culture media is necessary (refer to handbooks of bacteriology).

The **agglutination of typhoid bacilli** by agglutinins present in the blood of patients suffering from typhoid and similar conditions is now made use of for diagnostic purposes, the reaction, based on Gruber's work, being first practised by Grünbaum, but being known as the Gruber-Widal reaction, since the latter first introduced it into general practice.

**Gruber-Widal's reaction** :—

The procedure yields both naked-eye and microscopical evidence, and may be completed rapidly or slowly.

A positive naked-eye reaction consists in the formation of flocculent masses which sink to the bottom of the vessel containing the blood serum of the patient.

A positive microscopic reaction is indicated by the decreasing motility of the organisms and their ultimate "clumping" or adherence to one another.

According to the dilutions employed, the reaction may be completed within 1 or 12-24 hours.

(1) Collect blood (see Chapter XI. 1.) in a glass tube or pipette. Allow serum to form—by sedimentation or centrifugalisation.

(2) Transfer the serum in a marked capillary pipette to a small sterile test-tube; dilute the serum by adding 4 times its quantity of sterile broth (1 in 5).

(3) Prepare a "suspension of typhoid bacilli." Place a little sterile broth in a test-tube; add several loops of bacilli obtained from a 24-hours-old agar growth, so as to form a turbid suspension; mix well. Examine under microscope to see if the bacilli are actively motile.

(4) With a capillary pipette place a drop of the diluted blood on a slide. Add from another new capillary pipette a drop of suspended bacilli; the blood is now diluted 1 in 10. Mix; cover. Examine under high power. The clumping should be complete within 1 hour. This dilution is not sufficient.

Or [4] Add 10 pipettes of sterile broth to the diluted blood serum; then on the addition of the drop of bacilli suspension to a drop of the diluted serum the dilution is 1 in 30.

If still another 10 pipettes of sterile broth are added, then on the addition of the drop of diluted serum to the drop of the fluid containing the suspended bacilli the dilution will be 1 in 50. Some workers advise a dilution of 1 in 100.

In the two latter instances the reaction requires from 3 to 24 hours for its completion.

Recently **Ficker** has shown that a suspension of dead bacilli answers equally well. This permits a wider application of the reaction. Merck (Darmstadt) and Parke Davis (London) now prepare the emulsion of dead organisms, and the latter supply the necessary sterilised glass-ware, etc., for its completion.

The procedures are exactly the same as those already given under Widal's reaction, the fluid containing the dead organisms being substituted for 3 in the preceding method. The factors of the emulsion of dead bacilli give any special directions for their particular fluid.

Numerous observers report results quite equal to those obtained by Widal's reaction. Many recommend naked-eye observation alone. It is much safer to examine microscopically also, using a low-power objective in order to observe the small flocculi.

## IV. Influenza Bacillus

This organism may be stained either in films or sections in a very dilute carbol-fuchsin solution (about 30 drops of a saturated alcoholic solution added to 100 c.c. of water).

Sections, after staining, should be differentiated in acetic acid (1 c.c. acetic acid, 1000 c.c. absolute alcohol), then cleared in xylol and mounted in balsam.

Films, after staining, are washed in water, dried, and mounted in balsam.

The bacilli stain very slowly.

## V. Smegma Bacillus

The differentiation of this bacillus from the tubercle bacillus is given under the latter heading; the smegma bacilli are stained by the methods for the tubercle bacillus, if little or no decolorisation is performed.

The so-called **syphilis bacillus** of Lustgarten probably belongs to the same group. The following special methods have been devised for their demonstration :—

1. **Lustgarten's method** :—

(1) Stain in aniline gentian violet, 24 hours, or for 2 hours at 37-40° C.
(2) Wash in absolute alcohol, 2-5 minutes.
(3) Place in 1½ per cent potassium-permanganate solution for 10-30 seconds.
(4) Transfer to freshly made, pure sulphurous acid solution, 2-5 seconds (or to Pal's oxalic acid potassium-sulphite solution for nerve fibres).
(5) Wash in water. Repeat 3 and 4 if necessary, and again wash.
(6) Dry ; xylol ; balsam.

2. **Giacomi's method** :—

(1) Stain **films**, 5-10 minutes, in hot aniline fuchsin ; **sections**, 24 hours in cold aniline-fuchsin solution.
(2) Wash in very dilute solution of ferric chloride (a few drops in a glass of water).
(3) Decolourise in saturated solution of ferric chloride, 2-3 minutes.
(4) Wash and dehydrate in absolute alcohol ; xylol ; balsam.

3. **Doutrelepont and Schültz's method** :—

Harden in absolute alcohol ; freeze ; cut ; place sections in ½ per cent sodium chloride, 3-5 minutes, and then in absolute alcohol.
(1) Stain in 1 per cent watery gentian violet, 24-48 hours.
(2) Decolourise in $\frac{1}{15}$ watery nitric acid.
(3) Differentiate in 60 per cent alcohol (5-10 minutes) until the section is blue-violet.
(4) Counterstain in weak watery safranin, 2-3 minutes.
(5) 60 per cent alcohol ; absolute alcohol ; xylol ; balsam.

## VI. Bacillus Mallei (Glanders)

1. **Film preparations.—Loeffler's method** :—

(1) Stain in Loeffler's methylene blue (or in aniline gentian violet or aniline fuchsin mixed with an equal quantity of $\frac{1}{10000}$ NaOH or ½ per cent $NH_4OH$), slightly warming ; 5 minutes.
(2) Wash in water.
(3) 1 per cent acetic acid containing watery tropæolin O to a light yellow colour, 1 second.
(4) Wash in water ; dehydrate in alcohol ; dry ; balsam.

2. **Sections.—Loeffler's method** :—

(1) Stain in Loeffler's methylene blue, 5-10 minutes.
(2) Wash in water.
(3) Decolourise for 5 seconds in

| | |
|---|---|
| Water | 1 c.c. |
| Saturated watery sulphurous acid solution | 20 drops. |
| Oxalic acid, 5 per cent solution | 10 drops. |

(4) Wash and dehydrate rapidly in absolute alcohol.
(5) Xylol ; balsam.

3. **Abbott's method** :—

Imbed in paraffin. Do not remove the paraffin.
(1) Stain in Loeffler's methylene blue, 15 minutes.
(2) Stain in dilute carbol fuchsin (1 : 10), 30 minutes.
(3) Wash in 0·3 per cent acetic acid, 10 seconds.
(4) Wash in distilled water.
(5) Blot; dry by very gently heating.
(6) Clear in xylol; mount in balsam.

4. **Noniewicz's method** :—

(1) Stain in Loeffler's methylene blue, 2-5 minutes.
(2) Wash in water.
(3) Place for about 5 seconds in

| | |
|---|---|
| Acetic acid ($\frac{1}{2}$ per cent) . . . | 75 c.c. |
| Tropæolin ($\frac{1}{2}$ per cent watery solution) . | 25 c.c. |

(4) Wash in water.
(5) Dry between folds of blotting-paper and by slightly warming.

Bacilli, deep blue; tissues, light blue.

## VII. Bacillus of Leprosy

Gram's method and the ordinary aniline dyes may be employed for these organisms; they are also stained by the methods used for the tubercle bacilli, but they are more easily decolourised.

Baumgarten has devised the following special method :—
(1) Stain in dilute fuchsin solution (5 drops of a saturated alcoholic solution to 5 c.c. of water) for 5-7 minutes.
(2) Decolourise in nitric acid alcohol (1 : 10), 20-30 seconds.
(3) Wash in water; dry; mount in balsam.

Sections are treated in the same manner, except that they are dehydrated in alcohol and cleared in xylol before being mounted in balsam.

Leprosy bacilli, red; the tubercle bacilli are not stained in this short time.

## VIII. Bacillus of Rhinoscleroma

This organism is stained by Gram's method, and is thus easily differentiated from Friedländer's bacillus. It is morphologically somewhat similar to the latter organism. Both possess capsules.

**Wolkowitsch's method for tissues hardened in alcohol** :—

(1) Stain in aniline gentian violet, 24-48 hours.
(2) Wash in water.
(3) Place in Lugol's iodine solution, 1-4 minutes.
(4) Decolourise in absolute alcohol.
(5) Remove more colour by oil of cloves.
(6) Xylol ; balsam.

The hyaline substances of the rhinoscleroma are stained by basic stains (especially methylene blue, violets, fuchsin) and by eosin.

Schmorl says that if the tissues are hardened in osmic acid and stained by hæmatoxylin, the bacilli appear dark blue, their capsules light blue.

## IX. Ducrez-Unna's Bacillus (the so-called Chancroid Bacillus)

This bacillus may be stained in alcoholic solutions of fuchsin and violet and with Loeffler's methylene blue. In the latter case the differentiation in alcohol or aniline alcohol should be very rapidly performed.

**Unna's method** :—

*Staining Solution.*

| | | | | | |
|---|---|---|---|---|---|
| No. 1. | Methylene blue | 1 gm. | No. 2. | Water | 100 c.c. |
| | Potassium carbonate | 1 gm. | | Borax | 1 gm. |
| | Alcohol | 20 c.c. | | Methylene blue | 1 gm. |
| | Water | 100 c.c. | | | |

Mix the solutions together.

Harden in alcohol.
(1) Stain in the solution, 5-10 minutes.
(2) Place sections on slide ; blot.
(3) Decolourise in Unna's glycerin-ether mixture (Grübler), some seconds.
(4) Dry ; dehydrate in alcohol.
(5) Xylol ; balsam.

## X. Bacillus Pestis (Bubonic Plague)

**Film preparations** :—

1. Loeffler's methylene blue.
2. Aniline gentian violet.
3. Any of the Romanowsky solutions (for polar staining).

**Sections** :—

Fix in alcohol or mercuric chloride; formalin does not permit good staining reactions.

1. Loeffler's methylene blue.
2. Carbol thionin.
3. Carbol fuchsin, diluted, 1-10.
4. Gaffky stains in weak methylene blue, 2-3 hours, dehydrates rapidly in alcohol, and mounts in balsam; or stains in saturated solution of fuchsin in glycerin for 24 hours, decolourises in dilute acetic acid; dehydrates, clears, and mounts in balsam.

## XI. Bacillus of Pneumonia (Friedländer)

This bacillus stains with all the general stains, but not by Gram's method. The capsules are stained as already described (p. 228).

## XII. Comma Bacillus (Spirillum of Cholera) (see Fæces)

The comma bacilli are not stained by Gram's method. Fuchsin and methylene blue are generally employed.

It is best to use a saturated watery solution of fuchsin or diluted carbol fuchsin for films, staining for 10 minutes.

For sections Loeffler's methylene blue (5-10 minutes) or strong fuchsin (24 hours) solutions are to be advised.

It is practically impossible to differentiate the cholera vibrio from the harmless vibrios of Metschnikoff or Knieler, the *Vibrio berolensis*, etc., by staining reactions. Recourse must be had to experimental evidence such as that afforded by **Pfeiffer's reaction or phenomenon**, viz. :—

When cholera vibrios are injected into the peritoneal cavity of a guinea-pig, the temperature rapidly falls and the animal dies in 12-24 hours. If cholera spirilla are placed in the peritoneal cavity of an immunised guinea-pig, they disintegrate and are destroyed. The latter is called "Pfeiffer's Phenomenon."

For **recurrens spirillum**, see Blood (Chapter XII. 1.).

## XIII. Cocci

The majority of the cocci are stained by Gram's method, and this is the best general process for films as well as for sections. By this means the **streptococci**, the **staphylococci**, the **diplococcus of pneumonia**, the so-called **micrococcus of acute rheumatism**, are all stained. The general aniline dyes also may be employed.

Weichselbaum's **diplococcus** (micrococcus) **cellularis meningitidis** is also stained by Gram's method, but very careful differentiation is necessary. Loeffler's methylene blue gives the best results.

Fraenkel stains the cocci blue and the cells red by the following solution :—

| | |
|---|---|
| Water . . . . | 20 c.c. |
| Saturated watery methylene blue solution . . . | 8 drops. |
| Carbol fuchsin . . . | 50 drops. |

Stain in the mixture for 5 minutes ; wash in water ; dry ; balsam.

## Gonococcus

The gonococcus is not stained by Gram's method. This is important for differential diagnosis, and indicates morphological differences from the other cocci. They are best stained in Loeffler's methylene blue.

For films Fraenkel's method is useful :—

(1) Stain in saturated alcoholic eosin solution (warm), 3-5 minutes.
(2) Dry.
(3) Counterstain in saturated alcoholic methylene blue solution, 20 seconds.
(4) Wash in water ; dry ; mount in balsam.

Gonococci and nuclei, blue ; protoplasm and eosinophilous granules, red.

For **diagnostic purposes** the following procedure is helpful :—

Make a smear of the suspected gonococcal material at one end of the cover-glass or slide and a smear of staphylococci at the other end. Stain by Gram's method ; the gonococci are not stained. Stain the film again with fuchsin or vesuvin ; the staphylococci remain blue, the gonococci are red or brown.

For **sections** Schmorl recommends Loeffler's methylene blue or dilute carbol fuchsin (6 : 20), 1-2 hours. Place for a moment in $\frac{1}{1000}$ acetic acid, transfer to alcohol for 2 minutes ; dry ; mount in balsam. These methods give the best results.

We have only mentioned in this section the more important bacteria and those for which special methods are in use. The remainder may be stained by the general stains for bacteria.

## Actinomyces

It is necessary to stain the hyaline swollen sheaths which surround the ends of the filaments as well as the filaments themselves and the other forms of the organisms.

The following methods are devised in order to stain the mycelium and the clubs at the same time. It will be found, however, that while Gram-Weigert's method is the best for demonstration of the mycelium, van Gieson's or rubin and orange stains must be added in order to bring out the "clubs."

**Film preparations** may be prepared from the minute white-yellow granules contained in the purulent fluid by adding acetic acid or potassium hydrate, and then gently crushing. The latter, however, damages the structure, and a diagnosis cannot always be safely made therefrom. After drying and fixing by heat, the film may be stained in eosin and methylene blue.

**Sections.**—Fix in formalin and harden in alcohol. Imbed in paraffin or celloidin.

Stain in

1. **Hæmatoxylin and eosin** or van Gieson's stain.

   The clubs are made very prominent by van Gieson's stain or by rubin and orange.

2. **Gram's method.**—(Stains the mycelium well.)

3. **Schmorl's modification of the Gram-Weigert method** :—

(1) Stain after Gram-Weigert.
(2) After the aniline place the section in alcohol, 2-3 minutes.
(3) Counterstain in acid fuchsin (3 drops of a saturated watery solution to 15 c.c. of water) for 3 minutes.
(4) Wash in water for 2 minutes.
(5) Dehydrate in alcohol; xylol; balsam.

Mycelium, dark blue; swollen sheaths (clubs), fuchsin red.

4. **Mallory's method** :—

(1) Stain in alum cochineal.
(2) Transfer to saturated watery eosin for 10 minutes.
(3) Wash in water.
(4) Stain in aniline gentian violet, 2-5 minutes.
(5) Wash in normal saline solution.

(6) Place the section in Lugol's solution, 1 minute
(7) Pass rapidly through water.
(8) Dry thoroughly between folds of filter-paper.
(9) Cover section with aniline oil until quite clear.
(10) Xylol ; balsam.

Swollen cell membrane or sheath (club), pink ; mycelium blue.

5. **Weigert's method** :—

(1) Stain for 1 hour in absolute alcohol, 20 c.c. ; acetic acid, 5 c.c. ; distilled water, 40 c.c. ; extract of orseille until the solution is deep red in colour.
(2) Wash in absolute alcohol.
(3) Stain in 2 per cent solution of gentian violet.
(4) Wash in alcohol ; xylol ; balsam.

6. **Birch-Hirschfeld's method** :—

(1) Stain in hæmatoxylin or lithium carmine.
(2) Stain in warm 2 per cent crystal violet solution, 5 minutes.
(3) Dip in $\frac{1}{2}$ per cent alcoholic picric acid solution, $\frac{1}{2}$-1 minute.
(4) Wash in absolute alcohol until the preparation is blue-green, 10-30 minutes.
(5) Differentiate in origanum oil ; xylol ; balsam.

Mycelium, blue ; clubs, outer parts, yellow.

By staining in carbol or aniline water fuchsin after (1) for 5 minutes the central parts of the clubs are red.

7. **Boström's method** :—

(1) Stain in aniline gentian violet, 10-15 minutes. Drain off the stain.
(2) Stain in Weigert's picro-carmine, 5-10 minutes.
(3) Wash thoroughly in water.
(4) Wash in alcohol until the preparation is red-yellow.
(5) Clear in origanum oil. Mount in balsam.

Mycelium, pale blue ; clubs, red ; nuclei, red-yellow.

8. **Miller** uses a modification of Mann's methyl blue eosin and obtains good contrast staining of both mycelium and clubs.
(1) Harden in formalin, alcohol, or sublimate ; imbed ; cut.
(2) Stain sections in the following mixture, 5-10 minutes :—

| | |
|---|---|
| 1 per cent methyl blue in distilled water . | 35 c.c. |
| 1 per cent eosin in distilled water . . | 45 c.c. |
| Distilled water . . . . . | 100 c.c. |

(3) Wash in distilled water.
(4) Place in 1 per cent watery KOH solution until the preparation is pink, few seconds.
(5) Transfer to 2 per cent acetic acid until it is blue again.
(6) Rinse in water ; dehydrate ; xylol ; balsam.

## Streptothrix and Leptothrix

Methylene blue (Loeffler) and carbol fuchsin give the best results, but any of the aniline dyes may be used.

## Yeasts, Moulds, and some Fungi

**Yeasts.**—Hanging-drop preparations are useful when the yeasts are examined in the fresh state.

**Film preparations.**—Dilute the culture or material in water in order to isolate the cells.

1. Loeffler's methylene blue or any other aniline dyes.

**Sections.**—Harden in alcohol ; imbed ; cut.

1. Loeffler's methylene blue.

2. **Busse's method :—**

(1) Stain in hæmatoxylin solution, 15 minutes.
(2) Wash in distilled water.
(3) Stain in weak carbol fuchsin (1 : 20) for ½-24 hours.
(4) Decolourise in 95 per cent alcohol, ½-1 minute.
(5) Absolute alcohol ; xylol ; balsam.

**Moulds and fungi** are perhaps best examined in an unstained condition. It is sometimes necessary to remove the fat from the fungi and from their tissues in which they lie. This may be done by placing the material in a mixture of equal parts of alcohol and ether for a few minutes or hours, according to the size of the tissue, and then transferring them to 3 per cent sodium or potassium hydrate solution. The cells and tissues are thus clarified, and the organisms and spores are distinctly visible. For general staining purposes Loeffler's methylene blue is the best.

Sections are stained for 1-2 hours, and counterstained with eosin.

*Tiner favosa*, *Trichophyton tonsurans*, *Microsporon furfur*, *Mucor rhizo-podiformis*, etc., stain well in these dyes.

**Aspergilli** are best coloured with hæmatoxylin, vesuvin, or fuchsin.

**Oidium albicans** is not decolourised by Gram's method, and in sections the Gram-Weigert modification yields instructive pictures.

## Fungi on Skin and Hairs

For the examination of hairs or scales of epidermis for fungi, special methods have been devised in order to remove the fat and to diminish the strong affinity of the corneous material for aniline dyes.

### 1. Malcolm Morris' method :—

1. Ether and alcohol, equal parts.
2. Stain in 5 per cent gentian violet in 70 per cent alcohol, 5-30 minutes.
3. Transfer to Lugol's iodine solution, 1 minute.
4. Dry thoroughly ; place in aniline containing 2-4 drops of nitric acid.
5. Aniline ; xylol ; balsam.

### 2. Unna's method :—

1. Add glacial acetic acid to the hair or epidermis ; make cover-glass preparations, drying by heat.
2. Ether and alcohol, equal parts.
3. Stain in borax, 1 gm. ; methylene blue, 1 gm. ; water, 100 c.c., ½-5 minutes.
4. Wash in water ; dry ; balsam.

If the horny elements are too deeply stained, decolourise in Unna's glycerin-ether mixture (Grübler), 2 minutes, or in 1 per cent acetic acid, 10 seconds, or in 1 per cent oxalic, citric, or arsenious acid, 1 minute.

3. **Boeck**, after staining in borax methylene blue, washes the preparations in water containing a few grains of resorcin, then decolourises in alcohol, and, if necessary, transfers them for additional removal of colour to a watery solution of hydrogen peroxide, finally dehydrating in absolute alcohol and mounting in balsam.

# CHAPTER XV

## ANIMAL PARASITES

**Amœba coli** (see Fæces).

Examine **fresh material** (obtained from fæces or cultures) in normal saline solution on a warm stage (37° C.). Later the preparation may be irrigated with methylene blue and carmine; the latter stains the nucleus. Permanent specimens may be obtained by removing the excess of stain and running in some 50 per cent glycerin.

**Unna's method** gives better results with the discharges, which should be fixed and hardened in alcohol.

1. Stain in Unna's polychrome methylene blue solution (Grübler), $\frac{1}{4}$-12 hours.
2. Differentiate in water containing a few drops of Grübler's glycerin-ether mixture.
3. Wash well in water.
4. Alcohol; oil of bergamot; balsam.

**Sections.—Mallory's method :—**

(1) Harden portions of liver, intestine, or dysenteric discharges in alcohol.
(2) Stain sections in saturated aqueous thionin solution, 3-5 minutes.
(3) Differentiate in 2 per cent aqueous solution of oxalic acid, $\frac{1}{2}$-1 minute.
(4) Wash in water.
(5) Dehydrate in absolute alcohol; xylol; balsam.

Nuclei of the amœbæ are stained brownish red; other nuclei, blue; granules of mast cells, brown.

**Herpomonas Lewisii and allied forms; Trichomonas vaginalis and intestinalis; Cercomonas coli; Megastoma entericum; Paramæcium or Balantidium coli; Pyrosoma bigeminum; Trypanosomas.**—These organisms are best examined in the fresh condition in normal saline solution and at 37° C. Stained permanent preparations may be obtained by irrigating the preparation with methylene blue or carmine. The addition of a drop of ¼ per cent osmic acid or of 10 per cent formalin is of value in preventing distortion. Any one of the modifications of Romanowsky's stain may be used with good results; the chromatin is coloured red-violet.

## Coccidia

Examine in fresh condition in normal saline solution. Stain **cover-slip preparations** in carbol fuchsin or in van Gieson's stain, after fixation by heat or formalin vapour. For **sections**, fix the tissues in sublimate alcohol, formalin, Zenker's fluid, or in Pianese's solution (aqueous solution of sodium and platinic chloride, 1 per cent, 15 c.c.; ¼ per cent aqueous chromic acid solution, 5 c.c.; 2 per cent osmic acid solution, 5 c.c.; formic acid, 1 drop). Imbed in paraffin or celloidin.

Stain by the Ziehl-Neelsen carbol-fuchsin method for tubercle bacilli, or in the Ehrlich-Biondi staining mixture.

Weigert's iron hæmatoxylin, followed by van Gieson's stain, gives very good results.

Delépine stained sections in rubin, decolourised with acetic acid, and counterstained in methylene blue.

**Plasmodium malariæ** (see under "Blood").

The numerous methods for the demonstration of **protozoa, yeasts, cell inclusions,** etc., used in connection with investigations upon the ætiology of tumours are, for many reasons, not here included. A summary of these papers by Walker Hall may be found in the *Medical Chronicle*, 1904.

**Cestodes, Nematodes,** and **Trematodes.**—The heads and proglottides and ova are best examined in the fresh condition in either water, normal saline solution, or glycerin. Acetic acid may be added to the uterus and other contents.

Sections through the wall of the echinococcus cyst made with a knife or a pair of scissors reveal the **laminated structure** of the wall, and the latter may be further demonstrated by staining in carbol fuchsin.

**Scolices of echinococcus** may be scraped off from the wall of the cyst and examined, and mounted in glycerin or Farrant's solution.

**Hooklets of echinococcus** may be identified without staining by gentle teasing; they may be isolated from tissues or from calcified cysts.

**Scolices of cysticercus** may be set free by tearing the cyst wall. The suckers and hooklets are more evident after slight pressure on the cover-glass.

**Ova** may be demonstrated by pressing a ripe proglottis of a tapeworm or a female nematode or trematode between a cover-glass and slide.

**Filaria sanguinis hominis** (see under "Blood").

**Filaria medinensis** (guinea-worm).—The milky discharge from the ulcer usually contains many embryos. Spread it on a slide; fix by heat; stain in carbol thionin; dry; mount in balsam.

**Trichinæ** may be obtained by teasing muscle fibres, those nearest the tendons being usually selected. Squeeze small pieces of the tissue between two slides and examine with a low power.

> **Barnes** digests the trichinous material with pepsin and hydrochloric acid for 3 hours at 37° C.; the trichinæ are thus set free, and may be examined on a hot stage.

Calcified trichinæ should be treated with weak hydrochloric acid before examination.

The finer details may be studied by imbedding the tissue and staining the sections with alcoholic carmine solutions.

The other **nematodes** are recognised, as a rule, without staining, but for demonstration purposes **Mayer** and **Lee** recommend fixation in mercuric chloride and staining in the following mixture:—4 gms. of carmine dissolved in 15 c.c. of water and 30 drops of HCl; boil; when cool, add 95 c.c. of 85 per cent alcohol; filter while hot; when cold, neutralise with ammonia and again filter.

> To obtain permanent preparations of the cestodes spread the segments upon a glass plate or cork and fix for 2-3 hours in 5 per cent mercuric chloride or 10 per cent formalin. Transfer to 70 per cent alcohol for 24 hours. Stain in a saturated solution of orange G, containing a few drops of glacial acetic acid, for 24 hours, or in borax carmine or alum hæmatoxylin. Wash in distilled water. Alcohol; xylol; Canada balsam.
>
> If sections are desired, imbed in paraffin or celloidin. To demonstrate the excretory and genital organs inject Berlin or methylene blue through the genital pore; or place the worm in a watery solution of methylene blue to which is added a solution of albumin in normal saline; intravital staining results.
>
> Golgi's method of staining (Chapter XII. VI.) also brings out clearly the nerves, muscles, and excretory organs.

To make **permanent preparations of nematodes** it is necessary to make slight slits into the hard cuticle, so that the fixing fluids may enter; this is not, of course, necessary for the smaller varieties. Saturated aqueous mercuric chloride is the best general fixing medium; in the case of the ascaris it is well to place the worm in glacial acetic acid for ½-1 minute, and afterwards transfer it to saturated aqueous mercuric chloride containing 20 per cent of glacial acetic acid for 24 hours. Then imbed very carefully in paraffin or celloidin, or preserve entire in alcohol.

Fix the **ova** in Carnoy's solution (absolute alcohol, 6 parts; chloroform, 3 parts; glacial acetic acid, 1 part).

**Small nematodes** may be fixed in 10 per cent formalin, hardened in 70 per cent alcohol for 24 hours, transferred to glycerin containing rubin and orange or fuchsin, and, when transparent, mounted in glycerin gelatin, preferably on a "hanging-drop" slide.

**Trematodes.**—Permanent preparations may be made by fixing in Flemming's solution, in 2 per cent osmic acid, or in a saturated aqueous solution of mercuric chloride. Stain in alum cochineal.

**Loos** recommends fixing **Bilharzia hæmatobia** in 1 per cent mercuric chloride in 70 per cent alcohol at 50-60° C. The **ova** may be similarly treated.

The fixation of **cercariæ** is best performed in saturated mercuric chloride solution at 35-40° C.

**Arthropoda.**—The **Acarus scabei** and **Acarus folliculorum** are best examined in normal saline solution, serum, or glycerin. To demonstrate the appearances of the "burrow," remove the portion of skin involved, fix it in saturated picric acid solution containing 1 c.c. of sulphuric acid. Examine under a low power, or imbed and cut.

# CHAPTER XVI

## CLINICAL MICROSCOPY

THIS chapter in no way pretends to cover the field of clinical pathology. During pathological investigations the student and pathologist must often combine an examination of secretions and excretions with that of morbid tissues. Hence a *resumé* of the methods generally employed may save the reader's time and facilitate reference.

### Cyto-diagnosis

In order to examine the elements contained in fluids obtained by puncture of cavities (pleural, peritoneal, pericardial, spinal, or cranial) or of cysts (ovarian, renal, biliary, parasitic, etc.), the fluid should be collected in sterilised flasks or tubes, allowed to clot, then centrifugalised for 5-10 minutes and the supernatant fluid removed.

For the detection of micro-organisms, and of the tubercle bacillus in particular, the method of Jousset may be followed.

In this the fluid is permitted to clot; the clot is taken from the receiving vessel and washed on a piece of sterile linen or fine gauze until all the serum is removed, and then placed in another flask and digested with a sufficiency of the following solution:—Pepsin, 2 gms.; glycerin, 10 c.c.; hydrochloric acid, 10 c.c.; sodium fluoride, 3 gms.; distilled water, 1000 c.c. (Some workers find that a freshly prepared pepsin in 0·2 per cent HCl answers equally well.) Incubate at 37° C. until the clot is digested (3-4 hours). Centrifugalise for 5-10 minutes. Pour or pipette off the supernatant fluid.

To remove any excess of albuminous material, which may interfere with the staining, it is well, after removal of the fluid, to fill the tube with normal saline solution, and again centrifugalise for 5-10 minutes, repeating the same if necessary.

With a small platinum loop stir the sediment thoroughly, place a drop on a cover-glass, and allow it to dry in the air—heat above 40° C. should not be applied,—or fix in formol-alcohol (10-90). Stain with hæmatoxylin and eosin, Leishman's, Jenner's, carbol fuchsin, or by Gram's method.

If the sediment cannot be examined at once, add a few drops of formalin to preserve it, otherwise the cells rapidly break up.

A wet preparation should always be examined as well as a film preparation.

In **transsudates** the leucocytes and the sheets of degenerated endothelium may be stained in Leishman's stain, in eosin and methylene blue (consecutively), or in hæmatoxylin and eosin.

In **exudates** the following may be demonstrated :—

| | |
|---|---|
| **Fibrin** | By Weigert's method. |
| **Red blood corpuscles**<br>**Leucocytes**<br>**Endothelium** | Leishman-Romanowsky stain, eosin-methylene blue, etc. |
| **Micro-organisms** | Methylene blue, carbol thionin, Gram's method. |
| **Cholesterin**<br>**Pigment granules** | No special staining is necessary. |
| **Glycogen** | Iodine vapour or solutions. |
| **Fat** | Osmic acid or Sudan III. or Scharlach R solution. |

**Differential counts** of the white blood corpuscles should be made (*vide* Chapter XI.).

Excess of polymorphs indicates acute processes.

Excess of mononuclears indicates chronic processes, such as tuberculosis, or possibly the terminal stages of an acute process.

The sediment from **lumbar puncture** fluids.—Film preparations may be stained with methylene blue or thionin for the pyogenic cocci and Weichselbaum's diplococcus and with the specific methods for the tubercle bacillus (*vide* Bacteriology). In the former it is important to enumerate the polynuclear leucocytes and the lymphocytes in order to recognise an acute or chronic affection. Any of the ordinary blood stains will serve this purpose, but for the lymphocytes it is best to use Pappenheim's pyronin-methyl green, and for the other cellular elements hæmatoxylin and van Gieson form perhaps the best guides.

The contents of **ovarian** cysts, etc., are best shown by staining with hæmatoxylin and van Gieson, as by this procedure the colloid concrements, the large vacuolated cells which previously contained fat, and Bizzozero's cylindrical ciliated and beaker

epithelial cells are well brought out. For the red and white blood corpuscles eosin and methylene blue is useful, and the pigment, fat, and cholesterin are treated as described in Chapter XI.

The renal epithelium and casts in fluids obtained from **hydronephrotic** kidneys may be stained in hæmatoxylin and van Gieson (*vide* Urine).

The sediment from **biliary cysts** and the **gall-bladder.**—**Mucin** may be demonstrated by thionin, and **micro-organisms,** such as the *B. coli* and micrococci, should be stained by Loeffler's methylene blue and by Gram's method for purposes of differentiation.

Fluids obtained from **parasitic cysts** rarely require the addition of dyes during microscopic examination, the hæmosiderin, cholesterin, fatty cells, scolices, and hooks all being more or less self-evident.

## Sputum

**Red blood corpuscles, white blood corpuscles.**—See Blood, Chapter XII. p. 129.

**Epithelium.**—The squamous cells from the oral cavity, the cylindrical and ciliated cells from the trachea and bronchi, may be seen in fresh specimens, and may be demonstrated by adding methylene blue or by making a film and staining with hæmatoxylin and eosin. The alveolar epithelium containing pigment or carbon requires no special stain.

**Elastic fibres.**—These are most abundant in the **caseous masses.** Wash the mouth and brush the teeth carefully before collecting the sputum, so as to avoid contamination from retained food particles.

Add a little 10 per cent solution of caustic potash or soda to the portion under examination, or boil equal parts of sputum and 10 per cent caustic soda; mix the solution with five times its volume of water and allow to stand or centrifugalise. Examine the sediment, and if a permanent preparation is required, prepare a film and stain with Weigert's resorcin-fuchsin mixture (or with acid orcein).

**Fibrin** in the sputum of bronchitic or pneumonic patients may be demonstrated by picro-carmine, or better, by Weigert's fibrin stain.

**Crystals—Charcot-Leyden crystals.**—Permanent preparations may be made by fixation of a film in 5 per cent mercuric chloride solution (5-10 minutes) or in absolute alcohol (30 minutes). Wash thoroughly and stain in dilute

alcoholic fuchsin. If the film is air-dried and then fixed in absolute alcohol, the eosin methylene blue stains give good results. After removal of the excess stain the preparations are dried, cleared in xylol, and mounted in balsam.

The other crystals, viz. cholesterin, phosphates, blood pigments, and fatty acids are visible without staining. The rarely occurring tyrosin and leucin are also self-evident.

**Parasites.—Gram's** method stains *oidium albicans*, *actinomyces*, *diplococcus of pneumonia*, *streptococci* and *staphylococci*, *anthrax bacillus*, *diphtheria bacillus* and the *tubercle bacillus*. For selection of stains and of suitable material, see section on Bacteriology.

The bacillus of glanders, bacillus of influenza, actinomyces, aspergillus, mucor, and leptothrix are stained by Loeffler's methylene blue and the differential stains stated in the chapter on Parasites, which also see in regard to *echinococci*, *distomum pulmonale*, *cercomonas*, ova of *schistosoma*, *hæmatobium*.

Particles of silica, of coal dust, of iron, or of calcium compounds occur in sputum obtained from coal miners, stonemasons, grinders, etc.—They can be usually indentified without micro-chemical reactions. For the latter, consult Chapter X.

## Vomit and Stomach Contents

After sedimentation or centrifugalisation prepare wet and dry film preparations. Stain with hæmatoxylin and eosin for the cellular elements and with vesuvin or methylene blue for parasites.

If shreds of tissue are present, they may be fixed, imbedded, and cut in formalin.

**Blood.**—Test for Teichmann's crystals (p. 153).

**Yeasts, sarcinæ, cocci, cholera bacilli** may be stained by Bismarck brown or methylene blue and by the differential methods given under Parasites.

**Larvæ, oxyuris vermicularis,** and **anchylostoma duodenale** are best examined in fresh preparations.

**Pus corpuscles** from the mouth, tonsil, lung, or gastric abscess may be stained by Gram's method or with methylene blue.

**Epithelial cells.**—Stain in hæmatoxylin and van Gieson.

**Mucin.**—Use thionin (see Chapter XI. p. 102).

## Fæces

It is well to remember that fæcal material may be very infective, and that careful antiseptic precautions should always be taken.

**Collection of the fæces.**—The fæces, when passed, should be at once covered to prevent contamination by flies, dust, etc. They should be kept warm, especially when the *Amœba coli* is suspected. They should be examined fresh, and may be carried or sent to the laboratory wrapped in filter-paper.

**Examination of fæces.**—(1) Fresh preparations.
(2) Films.

Undigested muscle fibres, fat, crystals, are all easily recognised in a preparation made by streaking a cover-glass with a small quantity of fæces.

**Blood.**—Hæmin crystals are sometimes present when the blood is derived from the stomach.

When the blood is present in other forms Teichmann's test is not available, because of the presence of fat in the fæces. **Strzyzowski** therefore presses the dark-coloured patches between pieces of paper, places a small portion of the darkest patch on a slide, adds a drop of a solution of sodium iodide ($\frac{1}{500}$), warms, covers with a cover-glass, allows a few drops of glacial acetic acid to flow underneath the cover-glass, and then heats for 3-6 seconds, until bubbles appear. Black crystals of the iodic acid ester of hæmatin appear when blood is present.

The presence of iron in the fæces may also interfere with Teichmann's test. **Rossel** in this case shakes out the fæces with strong acetic acid and water, and then with ether containing chloral hydrate, finally distilling off the ether, precipitating the hæmatin by neutralisation, and then examining for the hæmatin crystals.

**Joachim** simply shakes up the fæces with ether containing acetic acid and then examines the acetate of hæmatin.

**Mucus.**—This may be stained by thionin (see p. 102), which will also dye the attached cells and micro-organisms.

**Casts, epithelial cells,** or collections of leucocytes may be examined in the fresh condition, or small portions may be placed in 10 per cent formalin, hardened in alcohol, and stained after imbedding in celloidin or paraffin.

**Bacteria.**—**Strasburger** recommends the following method for the isolation of bacteria from fæces. Mix a portion of the stool with water and centrifugalise. The micro-organisms are suspended and the other constituents form the sediment. Remove the fluid portion, add a little water, and centri-

fugalise ; the bacteria will now appear in the sediment and may be removed and stained or dried.

To isolate the **cholera vibrio,** allow the fæces to stand for some time with an equal quantity of alkaline bouillon in an open wide glass. The cholera bacilli will grow on the surface (Schottelius). See p. 245.

When examining for the tubercle bacillus, employ the acid-alcohol method for differentiation from the smegma and other acid-fast bacilli.

The typhoid and coli group of organisms must be isolated from the fæces before stains can be satisfactorily applied. For the tedious processes connected therewith reference should be made to handbooks of bacteriology. When isolated, the usual specific stains may be employed.

**Parasites.**—For the identification of **Megastoma entericum** and **Trichomonas intestinalis** P. Cohnheim collects the fæces by passing well into the rectum a small vaselined glass tube with an opening in its rounded end. After withdrawal the contents may be examined upon a warm stage.

**Amœba coli** (see Animal Parasites).—The fæces must be kept warm and should be examined on a warm stage at about 37° C. The organism at this temperature is motile, and contains granules, bacteria, and vacuoles.

**Worms. Cestodes, nematodes, trematodes** (see Animal Parasites).—Segments of the tapeworms and the nematodes and trematodes are, as a rule, visible to the naked eye. Sometimes a handglass will be found very useful.

The **heads of tapeworms** and of the **smaller round worms** may be collected for microscopic examination in the following manner :—

Cover the mouth of a large vessel with gauze. Place the fæces on it. Allow a gentle stream of water to flow on to it, stirring meanwhile with a glass rod.

Or, add a quantity of water to the vessel containing the fæces ; stir gently with a glass rod, so that the worm falls to the bottom. Remove one half of the liquid ; replace it with clean water ; repeat the process several times. Transfer the worm to a clear glass dish, drawing the large segments over the edge of the first dish and allowing them to fall into a second dish containing water. As the segments become smaller use the handglass and search for the head—about the size of a pin head—and examine in water, normal saline, or glycerin.

**Ova.**—Dilute the fæces with water and allow the sediment to form ; or better, centrifugalise.

The ova are often adherent to masses of mucus.

Place the fæces which contain ova in the incubator at 37° C. for a few days. Young embryos will probably be obtained.

**Ankylostoma duodenale.**—Dilute the fæces with water. Centrifugalise. Examine sediment. Fix in 10 per cent formalin. Mount in glycerin jelly or in Canada balsam after hardening in alcohol.

## Genito-urinary Secretions, etc.

The urinary sediment yielded after standing or centrifugalisation may contain blood corpuscles, mucus, epithelium, casts, pus cells, pigment (blood and melanin), all of which, in addition to the usual crystalline elements, are readily recognised without the use of stains. The addition of a drop of methylene blue or eosin solution is, however, a distinct advantage.

**Fragments of new growth** may be hardened, imbedded, and cut, but a diagnosis of their malignancy is rarely possible until portions of tissue are removed directly from the tumour.

**Uterine scrapings** may be fixed in formol, hardened in alcohol, imbedded (in paraffin or celloidin), and stained after preparing sections.

To determine the presence of **decidual cells** or **chorionic villi** in cases of abortion, in addition to imbedding, it is useful to tease fragments of tissue in normal saline solution, and to stain the capillary network or decidual cells in picro-carmine, or to make a film preparation and employ Leishman's stain.

**Spermatozoa.**—In **spermatorrhœa**, the long, stringy threads frequently exhibit Florence's sperm reaction.

A drop of an iodine solution (1·6 iodine, 2·5 potassium, 30 water) is mixed with a portion of the material on a slide. After covering, examination with a low power shows the presence of rhombic brown or violet crystals at the periphery of the fluid. They are probably due to some lecithin compound.

The reaction is also applicable to particles of supposed spermatozoal origin. It yields, however, negative rather than positive indications; other organic substances yielding similar crystals. When no crystals appear the absence of spermatozoa is most probable. Should, however, there be concomitant evidence of putrefactive changes, the reaction may give negative results, since spermatozoa are easily broken up by the organisms of putrefaction.

**Seminal stains.**—For the removal of the spermatozoa from any suspected fabric water should not be used, as it causes swelling and separation of the head from the filaments.

Dixon Mann recommends Ungar's method of placing the lower end of a strip of the fabric, carefully flattened out, in a watch-glass containing dilute HCl (1-40 distilled water) for 10 minutes to 5 hours, according to the age of the stain. The fabric is then removed with a pair of "dressing" forceps and gently dabbed on a slide, the smear being covered with a thin glass cover and examined with a $\frac{1}{6}$-$\frac{1}{12}$ inch objective.

Another method consists in moistening the fabric with glycerin, or with a mixture of equal parts of glycerin and normal saline solution; when the fabric is well moistened it is dabbed on a clean dry slide, and any entire spermatozoa readily adhere to the glass.

Permanent preparations may be made by drying the film in the air, fixing by heat, and staining in hæmatoxylin and eosin. The back part of the head is stained dark blue, the front part with the middle piece and filament, an intense red.

A simpler and more practicable procedure is to combine the softening and staining solutions in one. The strip of fabric with the suspected seminal stain is allowed to remain in the following mixture:—

| | |
|---|---|
| Methyl green . . . . | 1 gm. |
| Hydrochloric acid . . . | 15 drops. |
| Distilled water . . . . | 350 c.c. |

for 1-6 hours; it is then removed with the forceps and dabbed on a slide, and examined in the moist state or dried, fixed, and a permanent film prepared. The head is stained an intense green.

**Parasites.**—For the detection of bacteria the urine should be centrifugalised; in some cases, however, in which the organisms are present in large numbers, sedimentation is sufficient. Rieder recommends the addition of Sehlen's reagent (borax 4, boric acid 4, water 100) before sedimentation. The sarcinæ, yeasts, leptothrix, etc., are readily recognised without staining.

Gonococci, staphylococci, and streptococci may be stained according to the methods given in the chapter on bacteria.

Film preparations should be fixed by heat, or in equal parts of absolute alcohol and ether, then washed in hot water to remove the soluble salts before staining.

**Tubercle bacillus.**—Some workers prefer to collect the urine by catheter to avoid contamination of organisms which infest the outer urinary passages. If this is impossible, the urine should be centrifugalised. The ordinary carbol-fuchsin stain may be used, differentiating with acid alcohol to eliminate the smegma bacillus. Bunge and Trautenroth's method is also useful (p. 237). Several observers have recommended that the films should be placed in a warm solution containing 10 per cent of soda and 5 per cent of alcohol before staining.

Delépine recommends that the sediment, after being spread and fixed on a cover-glass, be washed, first in hot water, to remove the soluble salts, and then placed in a mixture (equal parts of alcohol and ether for 2 hours), which, after about 2 hours, is heated to the boiling-point of ether; the films are then washed rapidly with absolute alcohol and are ready for staining. Confirmation by culture and inoculation is always advisable.

**Typhoid bacillus: Bacillus coli communis.**—Both of these organisms stain with ordinary aniline dyes, but microscopical examination affords no positive evidence. Cultivation is necessary.

**Higher animal parasites.**—Hooklets and cysts of echinococcus, filaria sanguinis, eggs and embryos of distoma hæmatobium and infusoria are all recognised without the use of stains. Permanent preparations may be made according to the methods given in the chapter upon Parasites.

## Preparation of permanent specimens of urinary sediments:—

**Unorganised sediments.**—The following are abstracted from Delépine's methods, given in the *Atlas of Urinary Sediments*:—

**Dry method.**—Wash the deposit rapidly with distilled water (3 or 4 times) and dry at a temperature below 40° C. Keep the dust-like material in air-tight tubes. Examine by mixing a small portion with a

drop of water on a cover-glass. When dry, press gently upon thick Canada balsam.

"**Saturated solutions**" **method.**—A large excess of sediment is placed in water or glycerin fluid. Further details are given in the original.

**Organised sediments**:—

1. Remove the urine from the sediment and substitute Müller's solution. After 3 weeks, pour off the Müller's fluid and wash thoroughly with 50 per cent alcohol. Replace the alcohol by glycerin. Keep in stoppered tubes. To make preparations, add a small quantity to a drop of Farrant's medium or glycerin fluid and mix gently.
2. After washing the sediment with water, add a small quantity of 1 per cent osmic acid ; again wash and keep in glycerin fluid. The casts, etc., are olive brown in colour.
3. Orth's fluid may be used in place of Müller's solution, the sediment being treated in exactly the same manner.

The sediments may be stained with hæmatoxylin, carmine, or any of the aniline dyes, by using dilute solutions in the same way as the hardening solutions, the excess of stain being removed also by the washing method.

## Examination for Bacteria in Milk

(Revis and Moore, *Journal of Pathology*, 1900, p. 291.)

1. Place 50 c.c. of milk in a glass cylinder or rotation tube capable of holding 150 c.c.
2. Add 5 c.c. of methylated ether and stir well.
3. Add 20 c.c. of the following mixture and stir well.

| | | |
|---|---|---|
| Bleaching powder | 10 gms. | Triturate. |
| Water | 100 c.c. | |
| (Dry) Potassium carbonate | 20 gms. | Dissolve. |
| Water | 100 c.c. | |

Mix the two solutions. Allow to stand for 1 hour. The liquid becomes greenish yellow, and there is a heavy deposit of $CaCO_3$. Filter under pressure.

4. Place cylinder with contents in water at 100° F. for 15 - 20 minutes.
5. Cool, and add 30 c.c. of a mixture—

| | |
|---|---|
| Alcohol, rectified spirit | 33 c.c. |
| Methylated ether | 67 c.c. |

6. Mix well, and pour the semi-clear brown liquid into rotation tubes.
7. Rotate for 3 minutes at about 2000 revolutions per minute.
8. Remove supernatant liquid and ether.
9. Add to deposit 6 drops of 10 per cent solution of sodium carbonate, and fill up tube with distilled water and 15 c.c. of ether.
10. Rotate and examine deposit

    1. By Ziehl-Neelsen method.
    2. By any aniline dyes.

# CHAPTER XVII

## REPRODUCTION OF MICROSCOPICAL PREPARATIONS

In some instances it may be desired to obtain the microscopic appearances of a section in a form suitable for reproduction, so that the memory may be assisted or the appearances may be easily communicated to others. For such purpose we may apply photographic processes, or prepare drawings.

Micro-photography has the advantage of being absolutely objective; but in spite of the advances made in colour photography, it does not, as a rule, fully represent all the differential details of the preparation, or, at all events, it is at present very difficult to obtain such appearances. With film preparations of bacteria or of blood it gives, however, exceedingly useful results, providing the films are very thin. Its most useful field lies in the representation of low magnifications, and it thus plays a considerable part in investigations upon the central nervous system.

The best forms of micro-photographic apparatus are supplied by Zeiss or Winkel. Concerning the details of the method, the reader is referred to the works of Kaiserling, Neuhauss, Bagshaw, Cresbie, Mathet, and to the chapter on "Micro-photography" in Aschoff-Gaylord's *Atlas of Histology*. The latter book contains the best collection of micro-photographs of pathological preparations hitherto published.

In regard to drawings, these may be made in black or white, or in the original colours of the specimen, the

latter being the more instructive and even easier for the novice, since shading is unnecessary. They are naturally more expensive to reproduce. It is convenient and useful if the histologist makes his own drawings; much is learned by the necessarily close inspection during reproduction; there is, of course, the possibility of subjective renderings, but these may after all be totally avoided by paying special attention to this point. On the other hand, if the drawings are made by some one talented in the art of the brush or pencil but ignorant of the principles of histology, numerous explanations are necessary and much time may be wasted. If the artist and the histologist be combined, so much the better, but any one may learn in a short time to draw histological figures, even if he understands but little concerning perspective drawing.

As coloured figures of microscopical preparations present the fewest difficulties, the beginner will be well advised to commence with colour work. The first trials may be made upon a nucleus or an entire cell with a pencil and without shading.

If the paper and pencil are not discarded after the first attempt, a technique sufficient for all average demands will soon be attained. Thick paper or thin cardboard with a smooth surface should be selected. It is well to at first describe a circle the size of the figure intended; a watch-glass serves the purpose quite well. In regard to choice and production of colours, each individual must work out his own plans; it is scarcely possible to give general rules. The outlines are to be first marked out with a lead pencil, and Edinger's or similar drawing apparatus (with projection) will be found very useful for the enlargement and relative positions of the various structures; with a little practice, rapid and accurate representations will result.

Select a ground-colour and cover the whole area of the circle with it, or only those portions it is desired to colour; then commence to paint in the cells or nuclei. It is well to have a quantity of filter-paper at hand, and to dry each part just after painting it.

A number of fine, medium, and thick brushes are necessary, also drawing-pins.

For painting, it is best to use the same colours as were used for staining the sections, but only water-colours are permissible. Alcoholic solutions cannot be used.

If it is desired to mark a certain part of the preparation for future drawings, etc., this may be done by marking the cover-glass with ink, while examining the specimen under the low power. A little tubular instrument has been constructed by Zeiss for the same purpose; in place of an objective it contains a small diamond, by means of which a small circle can be made around the particular area.

# APPENDIX

## Solubilities of Stains

| | | |
|---|---|---|
| Saturated watery methylene blue ; 100 c.c. contains | 6·68 | gms. of the dye. |
| Saturated alcoholic methylene blue ; 100 c.c. contains | 0·66 | ,, ,, |
| Saturated watery gentian violet ; 100 c.c. contains | 1·75 | ,, ,, |
| Saturated alcoholic gentian violet ; 100 c.c. contains | 4·42 | ,, ,, |
| Saturated watery basic fuchsin ; 100 c.c. contains | 0·66 | ,, ,, |
| Saturated alcoholic basic fuchsin ; 100 c.c. contains | 2·92 | ,, ,, |

## Temperature

Conversion from one temperature scale to another—

$$\frac{C}{5}=\frac{R}{4}=\frac{F-32}{9}.$$

Thus to convert 100° F. to centigrade—

$$\frac{100-32}{9}=\frac{C}{5}. \quad \therefore C=37{\cdot}7°.$$

## Weights and Measures

| Metric. | English. |
|---|---|
| 1 gm. = 1 c.c. water at 4° C. = 15·4323 grains. | 1 grain = 0·648 gms. |
| 1 kilogm. = 2 lbs. 3 oz. 119 grains. | 1 oz. (avoirdupois) = 28·3595 gms. |
| | 1 minim = 0·06 c.c. |
| 1 c.c. = 16·63 minims. | 1 drachm (fluid) = 3·54 c.c. |
| 1 litre = 35·21 fluid ounces. | 1 oz. (fluid) = 28·39 c.c. |
| | 1 pint = 567·93 c.c. |

## Aqueous Alcohols

Specific gravity and percentage by weight at 15·5° C. (Fownes)—

| Per cent. | Specific gravity. |
|---|---|
| 50 | 0·9184 |
| 60 | 0·8956 |
| 70 | 0·8721 |
| 80 | 0·8483 |
| 90 | 0·8228 |
| 96 | 0·8061 |

# INDEX OF AUTHORS

*The numbers at the end of each reference are those of the pages upon which the Authors' names occur.*

Amongst others, the following abbreviations are employed :—

| | |
|---|---|
| B. M. J. . . . | British Medical Journal. |
| J. of Physiol. . . | Journal of Physiology. |
| Z. f. wiss. Mikr. . . | Zeitschrift für wissenschaftliche Mikroskopie und für mikroskopischen Technik. |
| V. A. . . . . | Virchow's Archiv. |
| Centr. f. allg. Path. . | Centralblatt für allgemeine Pathologie und pathologische Anatomie. |
| Centrlb. f. Bakt. . . | Centralblatt für Bakteriologie und Parasitenkunde und Infektionskrankheiten. |
| Ziegler's Beitr. . . | Ziegler's Beiträge zur pathologischen Anatomie und zur allgemeinen Pathologie. |
| Encycl. . . . | Encyclopädie der mikroskopischen Technik, 1903. |
| Neurol. Centralb. . . | Centralblatt für Neurologie. |

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