Pathological technique : a practical manual for workers in pathological histology including directions for the performance of autopsies and for microphotography / by Frank Burr Mallory.

PATHOLOGICAL TECHNIQUE

A Practical Manual for Workers in Pathological Histology

including

Directions for the Performance of Autopsies and for Microphotography

BY

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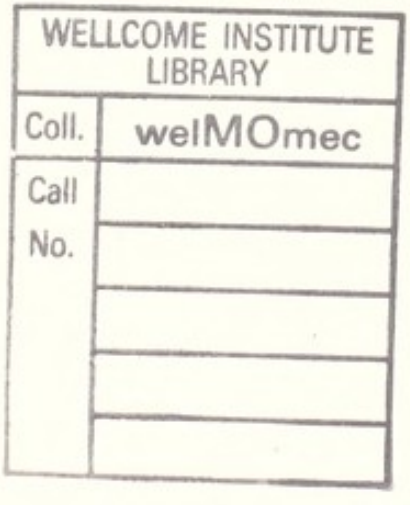

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To the Memory of
JAMES HOMER WRIGHT, A.M., M.D., S.D.

PREFACE

I HAVE undertaken the writing of this book as a result of many requests for a modern presentation of accepted *pathological techniques*. Technical methods are constantly changing in pathology as in other branches of science. The wisest plan in issuing a book like this seems to be to retain the best of the past and to add only the most promising methods of the present.

This book is not an encyclopedia of methods presented historically. Instead, it is a selection of those formulas that practical experience has shown to be of value. It is intended for pathologists in hospital laboratories and medical schools, for students and practitioners interested in pathology, and for technicians trained in that line of work.

The ideal function of the technique of pathological histology is so to fix tissues for microscopic examination that every tissue element and pathological product is perfectly preserved with all its morphological and chemical properties intact, and so to stain tissues that the various structures present can be readily differentiated from each other. In certain respects only has this ideal been reached, but the number of differential stains is increasing yearly.

A pathologist's work is of two types—the practical and the ideal—and the former is much the more abundant and time-consuming. He has to take tissues as they come and do the best he can with them. And always there is a rush to get them done. In spite of these drawbacks he should always have in mind his ideal—absolutely fresh tissue perfectly fixed in various reagents and stained by the best methods. Then his work becomes a joy and he can devote himself with pleasure to the study and interpretation of the lesions present because he has given himself every opportunity to understand them.

I am greatly indebted to a number of people for assistance and advice in the preparation of this book, small as it is, and desire to express here my obligations, especially to Dr. Frederic Parker, Jr., Dr. G. Kenneth Mallory, Mrs. Carol F. Mallory and Miss Lillian

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Conn, H. J.: Biological Stains, W. F. Humphrey Press, Inc., Geneva, N. Y., 1936, Ed. 3.

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Romeis, B.: Taschenbuch der Mikroskopischen Technik, R. Oldenbourg, München and Berlin, 1932, Ed. 13—an invaluable storehouse of histological methods.

FRANK BURR MALLORY.

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June, 1938.

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Pathological Technique

PART I. GENERAL MATERIALS AND HISTOLOGICAL METHODS

CHAPTER I

LABORATORY EQUIPMENT

1. GENERAL EQUIPMENT

THE modern pathological laboratory, especially if connected with a hospital, requires in its outfit a considerable number of instruments and utensils for the various kinds of work that must be performed. It is not the function of this book to appraise the relative merits of the different microscopes, microtomes or other instruments. The only suggestion that can be given is to obtain the best disinterested advice available before buying.

A few hints in regard to quarters and furnishings may be found useful in equipping a laboratory. Rooms intended for microscopic work should, if possible, face the north so as to obtain the best light. The windows should be wide and should extend to within 6 inches of the top of the desk. The panes of glass should be large, 2½ to 3 feet wide, so as to offer no obstruction to the light. Window desks should run the length of the room and be 2 feet wide and 30 inches above the floor. Shallow drawers, 3 inches deep, are convenient and useful at the center desks, and deeper drawers and cupboards can be placed at convenient points. The desks should be set 1 foot away from the wall, leaving space for the heating apparatus, pipes for gas and compressed air, and electric wires. Over this foot-wide space should be a shelf elevated 6 inches above the desk and closed in front. On this elevation it is advisable to put the necessary outlets at convenient points. The shelf is also useful for holding microscope cases, bottles, and other articles. The desk top and shelf should be stained black by the aniline hydrochloride process so that they will not be marred by spilled fluids.

The floor is best covered with linoleum, which can be strongly recommended as it is easy on the feet and saves many a dropped slide or piece of glassware from breaking.

Rooms furnished with desks in front of the windows need to be heated with care. The heat should escape in front of the windows, between them and the desks, so as to counteract the cold coming through the glass, not below the desks to come around the feet or up in the face, an intolerable condition. The heating unit itself

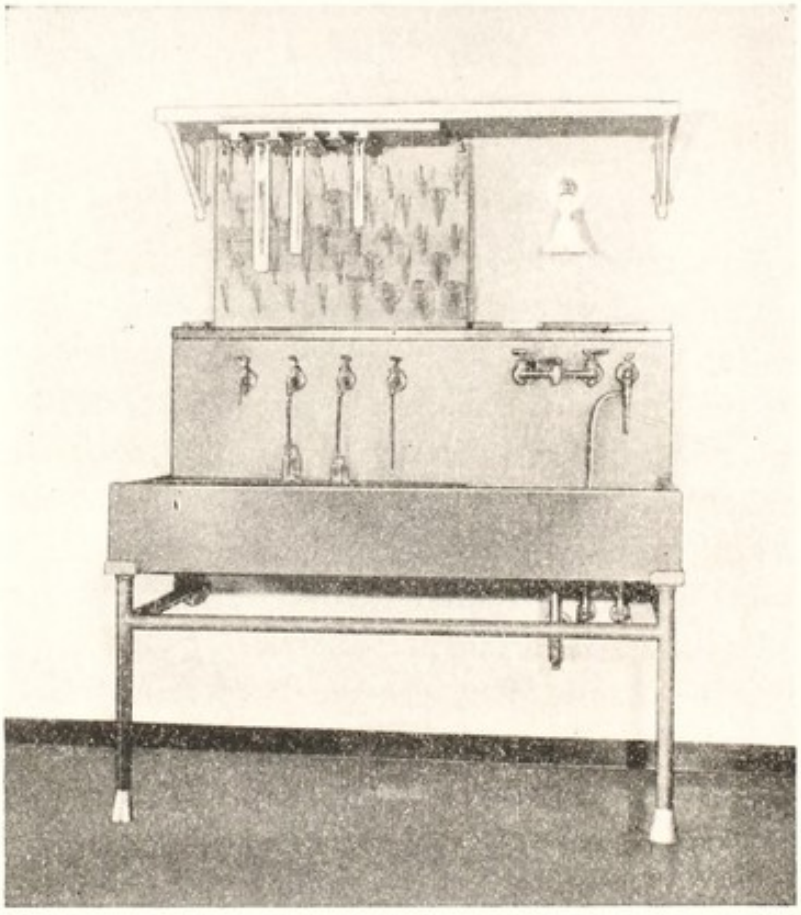

Fig. 1.—Laboratory sink showing arrangement of water outlets; also overhead light, shelf with support for inverted graduates and peg board for glassware.

should be enclosed so as to prevent the escape of more than a moderate amount of heat.

The sink should be made of soapstone (Fig. 1). One of convenient size extends 3 feet above the floor and measures 5 by 2¼ feet, with a depth of 8 inches. The back wall extends up 2 feet higher and has a 6 inch shelf at the top. The sink itself measures inside 2 feet square and should be provided with an overflow outlet 6 inches above the bottom. The rest of the space is occupied by a

sloping, grooved drain shelf. The back wall over the sink is provided with a faucet in which hot and cold water can be mixed. Running water for washing specimens, which have been fixed in Zenker's or other solutions, is most easily supplied by a water pipe furnished with numerous cocks 5 to 10 cm. apart placed horizontally above the draining shelf adjoining the sink. Attached to each cock is a rubber tube with a glass tube at the end long enough to reach to the bottom of a jar. By this arrangement the amount of water supplied to each specimen can be easily regulated. Above the sink is an electric light and above this a shelf, on the under surface of which is a holder for inverted graduates.

Other furnishings required are wall shelves 10 inches deep with shelves spaced 12 inches apart, stools and chairs (those intended for microscopic work revolving, adjustable as regards height and inclination, and cushioned), tables, writing desk, cabinets for books, slide boxes, filing cabinets, and so on.

2. THE MICROSCOPE

The most important laboratory instrument is the microscope. It should be, so far as means will permit, the best that skill can produce. Excellent microscopes are manufactured in this country as well as abroad.

The standard microscope of today is regularly equipped with all the necessary essentials, namely coarse and fine adjustment, quadruple nosepiece, Abbé illuminating apparatus and iris diaphragm. The best objectives and eyepieces are expensive. The achromatic lenses are perfectly serviceable and all that are necessary for the beginner. The apochromatic lenses are preferable, especially for the expert, if they can be afforded, and for microphotography they are practically indispensable. The powers required are low ($\times$ 10), medium ($\times$ 20), and high ($\times$ 40) in the dry series, and much higher in the oil immersion lens ($\times$ 90). These various objectives will give with the use of a low eyepiece magnifications of approximately 50, 100, 200 and 450 diameters. The magnifications can be increased by using higher eyepieces, of which a series of two or more is advisable. Beginners as a rule tend to use too high magnifications.

The oil immersion lens should always be cleaned after using by wiping off the oil with the fine lens paper manufactured for that

purpose. If the lens is sticky moisten the paper with xylol or benzol. The same process can be used, if necessary, for the dry lenses. Ordinarily, breathing on the lens and wiping with lens paper is sufficient.

A mechanical stage can be obtained for almost any type of microscope and can be readily attached. It is exceedingly useful for blood counting and for searching carefully the whole area of a stained coverslip or section.

For drawing, the Abbé camera lucida will be found extremely useful and convenient. Much use is also made of a vertical projection apparatus for the same purpose, especially when only outline drawings are required. For fine details it is not so useful.

The best illumination for microscopic work is that obtained from a white cloud, although for some purposes the light that filters through a white curtain on which the sun is shining is very useful, especially with the higher powers of the microscope. The use of artificial illumination is steadily increasing. Its advantages are that it is always available, constant in amount, and the intensity can be regulated. Electric lamps of various design are used almost exclusively for the source of illumination and new forms are constantly appearing. It is advisable to examine the latest designs before making a selection. Artificial illumination requires light filters, either globes filled with colored liquids, or sheets of colored glass. In addition a rheostat to control the degree of illumination is recommended.

In using the Abbé illuminating apparatus it is important to bear in mind that the best results are obtained by employing the plane mirror, for the condenser is designed for parallel rays of light. The concave mirror is to be used only when some near object, such as the window frame, is reflected into the field of vision, or when artificial light is employed.

Dark-field illumination is used in a pathological laboratory chiefly for the examination of secretions and tissues for spirilla and spirochetes, especially the *Treponema pallidum*. The instruments required are a good microscope, strong artificial illumination, and a dark-field condenser which is slipped into the microscope in place of the Abbé condenser. It is very convenient and time-saving to have a microscope set up and used for this purpose only.

A dark-field condenser blocks out the center rays of light coming

to it while the peripheral rays are directed from the side against any object present so that it appears bright on a dark background.

Light rays vibrate in all directions. Polarization is the process of excluding all rays excepting those vibrating in one plane. This is accomplished by the use of Nicol prisms. These are cleavage rhombohedrons of transparent calcite (Iceland spar) which are cut diagonally, the cut surfaces polished, and then cemented together with Canada balsam. Light rays entering the prism below are for the most part refracted by the Canada balsam. The only rays that can penetrate the balsam and emerge at the upper surface are those vibrating in a single plane.

The polariscopic microscope is fitted with a Nicol prism below the condenser (the polarizer) and a second (the analyzer) in the eyepiece. They are so arranged that the plane of vibration of light emerging from the analyzer is at right angles to the plane of vibration of light emerging from the polarizer. Thus when the two Nicols are crossed in the path of light, the field is dark.

Crystalline substances other than iso-axial crystals are doubly refractive (anisotropic). When introduced between the Nicol prisms, *i. e.*, on the stage of the microscope, they break the polarization and are visible as bright white bodies in the dark field. Substances with a single index of refraction (isotropic) do not interfere with the polarization and are invisible in the dark field.

Differences in the degree of birefringence of crystals may be determined by exacting and rather complex measures. From the practical medical standpoint the relatively simple procedure of identifying certain characteristic, doubly refractive substances usually suffices. The most important of these substances are cholesterol and its esters, and silica and its compounds.

Cholesterol in the pure state appears in the typical flat plates with notched edges. Crystals tend to occur in fused masses in most locations in the body, *e. g.*, arteries in atherosclerosis, organizing exudates, and so on. The ester forms are met with constantly in the cells of the adrenal cortex, and frequently in fatty liver cells (particularly in cirrhosis), in monocytes in xanthomas, xanthomatoses, Schüller-Christian's disease, the lesions of atherosclerosis and others. They occur in droplets and are seen as bright, tetrad-like spherical bodies with Maltese cross markings—the so-called fluid crystals of Lehmann.

Significant silica deposits in the tissues occur in the form of pure silica or of silicates. In addition to pure silica, serecite (a silicate) is accused of the production of silicosis. The silica particles responsible for the fibrosis characteristic of this disease are those that are less than 10 μ in diameter and particularly those less than 5 μ. The crystals are visible in lung sections as minute bright points or rodlike structures in the dark field.

In the disease asbestosis, elongated needle-like crystals of asbestos (a silicate) of varying lengths are found in the lung lesions. They are usually encrusted with brownish degeneration products of the silicate and are relatively so large and characteristic ("asbestosis bodies") that resort to polariscopy is not necessary for their identification.

Quantitation of the silica in lung tissue is determined by ashing random samples of the lung and by making a chemical examination of the residue.

Estimating the silica content of dusts is a complex problem, requiring identification and exclusion of other anisotropic substances which may be present even in granite dust. For this purpose the degrees of birefringence of the contaminating crystals are ascertained by suspending samples of the dust in fluids of known indices of refraction. This procedure requires a technical knowledge of petrography, and is beyond the purposes of the present publication.

A mica diaphragm in the substage above the polarizer makes it possible to study color production which depends on the amount of separation of light rays as they pass through anisotropic substances. The greater the birefringence the brighter the colors. Observation of color changes is of value in corroboration of other evidence of the degree of birefringence of mineral substances.

3. THE MICROTOME

Three different types of microtome are required in laboratory work. They are known as the rotary, sliding and freezing microtomes. Each has its own special field of usefulness.

Rotary Microtome.—Although paraffin sections can be cut on a sliding microtome, especially on the precision model designed by Minot, it is ordinarily preferable to have an instrument intended primarily for this purpose. The Minot rotary microtome is the model preferred in this country and several different models are

available. The main qualifications to be sought are simplicity of design, rigid construction, heavy base to give stability, and universal ball-and-socket specimen clamp with one screw only.

Sliding Microtome.—There are two types of sliding microtome, one in which the object can be raised by a screw, and another in which the object is raised by being moved up an inclined plane. The first type of machine is the better for two reasons: the screw affords greater accuracy in the even elevation of the object than is possible with an inclined plane, and the object remains at all times in the same relative position with regard to the knife, so that an equally long sweep of the blade can be obtained for every section. Several models of this type of instrument are available and can be highly recommended for practical laboratory work. More elaborate machines are manufactured and are useful for special purposes, such as cutting large sections of the brain or other organs.

Another type of sliding microtome, the precision, in which the knife remains fixed and is clamped at both ends, while the object holder, which is raised by a screw, moves back and forth beneath the knife, was designed by Dr. C. S. Minot and is manufactured in this country. It is intended for both celloidin and paraffin work and is very useful for certain purposes. The knife can be set obliquely, or at right angles to the long axis of the machine.

A dropping-bottle on an elevated stand, with screw arrangement for regulating the amount of alcohol, is the most convenient method for keeping the object and the knife wet while cutting celloidin sections. For this purpose 80 per cent alcohol should be used.

Freezing Microtome.—Several types of freezing microtome are obtainable. The simplest makes use of a short, broad, chisel-like blade for cutting sections but is rarely used nowadays. The more complicated and satisfactory models employ a short microtome knife moved by a handle obliquely over the surface of the freezing box which is automatically elevated after each section is cut.

Freezing by means of the evaporation of ether, more rarely of rhigolene, was originally the method in general use. The process was both expensive and slow. A much cheaper and more rapid method of freezing was originated many years ago in the Pathological Laboratory of the Harvard Medical School by Dr. S. J. Mixter, and has since been universally adopted. This method consists in the employment of compressed carbon dioxide which is

obtained commercially in iron cylinders, each ordinarily containing about 20 pounds of liquefied gas. It is commonly used for charging beer and soda water. As a rule the cylinders are loaned so that it is necessary to pay for the contents only.

The cylinder should be securely fastened in an upright position near the microtome, with its valve end below, and with its escape tube on a level with the entrance tube into the freezing box. In this position fluid carbon dioxide escapes through the outlet and acts much more quickly and effectively than when the container is placed in a horizontal position, or with the outlet at the top, and simply compressed gaseous carbon dioxide escapes. The cylinder is connected with the freezing microtome by means of a flexible metallic tube provided with a valve. The first time the cylinder is used for freezing, a little water may escape, causing considerable sputtering. In freezing, the valve should be turned carefully so that the gas may escape slowly and evenly. Tissues fixed in alcohol or any other reagent, with the exception of formalin, must be washed in running water for several hours before they can be frozen. Even for tissues fixed in formalin, washing in water for 10 to 30 minutes is advisable as better sections can then be obtained.

Small cylinders containing about 5 pounds of compressed carbon dioxide are obtainable and are convenient for use at operations outside the hospital when an immediate diagnosis by means of frozen sections is to be made. Each tank provides enough gas for two or three freezings.

Microtome Knives.—Various sorts are required for the three different types of microtome in general use. The knives for both the sliding and the rotary microtome should be heavy and not too long, so as to afford as great rigidity as possible, and should be biconcave. A knife that is only slightly or not at all biconcave requires a honing back, a slotted steel tube which is slipped over the back while it is being honed. Elaborate automatic knife sharpeners are available but are expensive and not really necessary. It is important that everyone who does much work in a pathological laboratory should learn to sharpen his own knives. The requisite skill is not difficult to acquire and the time spent in learning is fully compensated for by having a sharp knife in good condition when it is needed.

For honing (Fig. 2) a knife, either a fine yellow Belgian water

stone or a glass plate with diamantine and Vienna chalk may be used. A very fine carborundum "60 seconds" stone that is now made is advisable for removing nicks. In honing, the edge of the knife should be forward and the motion from heel to toe. The knife should always be turned on its back and the pressure should at all times be rather light.

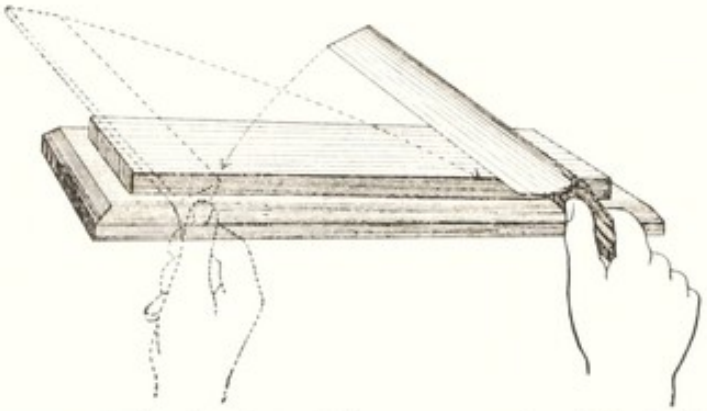

Fig. 2.—Diagram of the direction of the movements of the knife in honing.

In stropping (Fig. 3), the movement is reversed. The back of the knife necessarily precedes the edge and the motion is from toe to heel. The direction of the movements in honing and stropping is best illustrated by diagrams. The condition of the cutting edge can

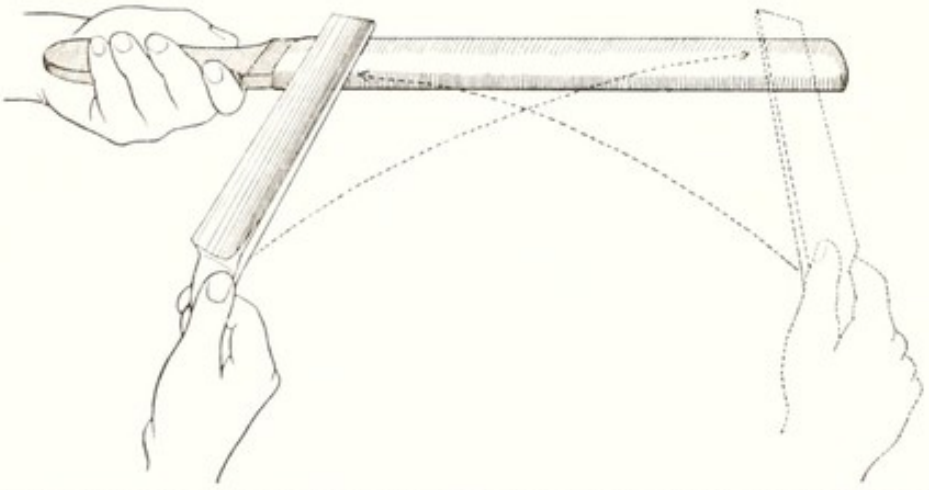

Fig. 3.—Diagram of the direction of the movements of the knife in stropping.

be examined by carefully drawing the knife flatwise across the low power field of the microscope. When the knife is properly sharpened the edge is smooth and even, without nicks. A razor strop paste greatly facilitates the smoothing of the knife edge in stropping.

Razor blades for cutting paraffin sections are invaluable for routine work in a pathological laboratory as calcified foci are com-

mon in tissues. If the blade becomes dull or is nicked it is easily replaced by a new one. All that is required is a rigid razor blade holder that can be shifted sufficiently laterally so that use can be made of the whole of the cutting edge of the blade, not of the center only.

Microtome knives are advisable for the best work. They should be restricted, however, to soft tissues that have been proved to be free of deposits of lime salts.

4. OTHER APPARATUS

Paraffin Oven.—The best oven for keeping paraffin at a constant temperature is one of suitable size with a hot water jacket, such as that used for growing cultures of bacteria. The paraffin is kept in it on shelves in glass dishes of various sizes. The temperature should be from 52° to 54° C. for ordinary use. The advantages of this method over the old way of using copper cups set into the top of a water bath are that the paraffin is kept free from dust, each worker can have his own set of dishes, and the smallest bits of tissue can be readily found in them because they are transparent.

Various other utensils and instruments are required in a laboratory and will be described briefly or only mentioned.

Centrifuge.—Its chief use in pathology is to centrifuge fluids (exudates and transudates) in order to concentrate any cellular components that may be present. The sediment thus obtained can then be cut, stained, and examined for the presence or absence of tumor cells. For this purpose a centrifuge capable of taking centrifuge tubes of 250 to 500 cc. capacity is best. It should have a speed of about 2000 to 3000 revolutions per minute.

Other Instruments.—Amputation knives are useful for cutting up tissues and trimming specimens. The biconcave type is more easily sharpened. Thin, double-edged brain knives of different sizes have many uses and one great advantage. With them a flat, even cut surface is readily obtainable. The same is true for the band-saw in cutting bones or calcified tumors. Small blocks of these tissues intended for decalcifying and embedding are best cut into small pieces with a jeweler's saw which uses very thin blades. The tissue adjoining the cut is less lacerated than with a coarse saw of any type.

Scalpels, cartilage knives, scissors and forceps of different sizes find many uses.

Section lifters (commonly called "spatulas") of various sizes are needed. They should be thin, smooth, flexible and large enough so that a section will not curl over the edge.

The best instrument for transferring sections under all circumstances is a piece of platinum wire mounted in an ordinary screw needle-holder. It is pliable and can be bent to any shape, will not break like a glass rod when dropped, and is not affected by acids. For ordinary use curved and straight steel needles mounted in wooden handles are very convenient.

Glassware.—Glass utensils of various sorts are required. Slides should be of colorless glass with ground edges and square, not rounded corners. The English form, measuring 1 by 3 inches (26 by 76 mm.) is to be preferred for ordinary use. Occasionally wider slides are needed. Moderately thick slides are preferable to too thin ones; the latter are so light that they are easily lifted by the oil immersion lens and they sometimes seem to warp when heated to attach paraffin sections. They also break readily if too much pressure is applied to them in wiping or rubbing.

Coverslips should be square or oblong, according to the shape of the specimen. Most dry lenses are adjusted for coverslips measuring 16 or 17μ in thickness, so that if possible no coverslip ranging outside of 15 to 18 μ should be used. With an oil immersion lens it is important only that they be thin enough.

Slides and coverslips are cleaned by treating first with dilute acid in water, thorough washing, then by dipping in alcohol and wiping dry with a soft linen towel. Coverslips, after they are clean, should be kept dry in covered dishes.

Staining Dishes.—Paraffin sections require one set of staining dishes; celloidin and frozen sections another.

The McJunkin staining dish (Fig. 4, D) is designed to hold one slide with attached sections in a horizontal position. It can be covered by a small plate of glass slightly larger than the ordinary slide. The dish is useful and convenient.

Larger dishes with covers are designed to hold 5 to 10 slides either on end (Fig. 4, C) or on the side (Fig. 4, E). They are indispensable when many slides are to be stained.

For celloidin or frozen sections, small (25 cc.; Fig. 4, B) and

large (150 cc.; Fig. 4, A), low, flat-bottomed glass dishes are required. They are known respectively as glass ash trays and as nappies or finger bowls, and can be bought cheaply. The small dishes are used for staining solutions and the larger ones for holding sections before and after staining. The great advantage of the larger dish is that a slide can be dipped under a frozen section and then lifted, spreading the section out evenly and flat on its surface.

Circular Stender dishes with glass covers are useful as staining dishes and to hold reagents for clearing sections.

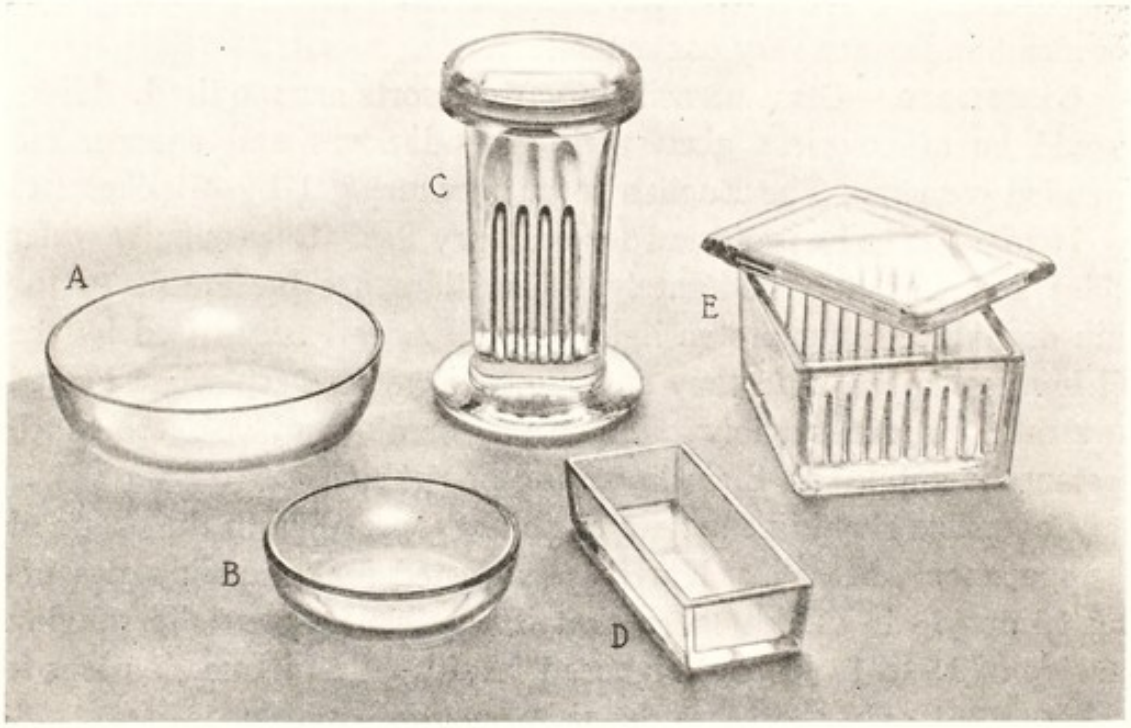

Fig. 4.—Different types of glass dishes. A, 150 cc. flat-bottomed dish for holding sections; B, 25 cc. staining dish; C, Coplin staining jar; D, McJunkin oblong staining dish; E, staining dish to hold 10 slides on side.

Large flat-bottomed glass dishes known as crystallizing dishes, holding 1 to 3 liters, are excellent for fixing tissues as they allow thin slices to lie flat. If several sizes are obtained the large dishes serve as covers for the smaller ones.

Bottles.—For coverslip smears and for staining on the slide dropping-bottles will be found extremely convenient. The pattern of 50 cc. capacity, with flat top, is probably the best form and size, but occasionally bottles holding 100 cc. or more are useful.

For stains and reagents bottles with glass or cork stoppers of various sizes are required. The sizes most used are those containing 125, 250, 500 and 1000 cc.

Preserving jars of half-pint or pint capacity can be highly recommended for holding pathological tissues after fixation. Wide-mouthed 60 and 120 cc. bottles with cork stoppers are useful for holding small amounts of tissue but there is always danger of evaporation.

Vulcanized Fiber.—This is a valuable material, when cut into blocks of suitable size, on which to mount pieces of tissue embedded in celloidin. It is compact, brown or gray in color, and can be bought in sheets or strips and cut up as desired. A useful thickness is 1.5 cm. Blocks (Fig. 5) measuring 2 cm. square and 2 by 3 cm. are convenient sizes, but larger ones are often needed. Parallel incisions 1 to 1.5 mm. wide and 2 to 3 mm. deep should be sawed in the upper surface to afford a firm foundation for the celloidin to grip. Cross incisions can be made if desired but are unnecessary and often cause difficulty when the celloidin, after drying, is removed from the blocks. Prepared blocks can be bought but are usually too thick and the incisions are too wide and too deep, thus wasting both fiber and celloidin.

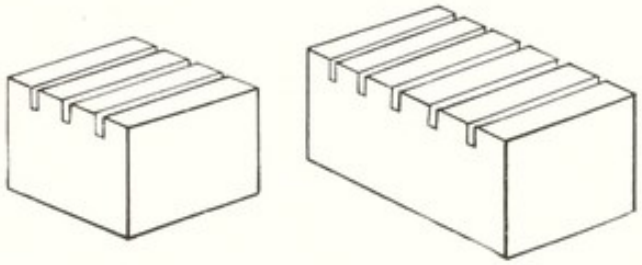

Fig. 5.—Blocks of vulcanized fiber prepared for mounting tissues embedded in celloidin.

Containers for Slides.—Mounted slides, when first received after being stained, are usually kept in pressed cardboard trays holding 20 and provided with lids to protect the preparations from light and dust while the balsam is hardening. In these containers the sections are readily available for examination. It is important that the compartments in the trays be large enough so that the slides do not bind in them and that they be provided with a curved cutout at each end so that the fingers of either hand can be used in removing them.

Later the slides can be kept in plain wooden boxes holding 12 to 25, or in larger boxes covered with colored paper and holding 100. In these larger boxes they can be grouped as desired and kept on shelves or in cabinets. The lateral incisions in the boxes into which

the slides fit should not be too narrow. They should admit easily both thin and thick slides.

In laboratories where the number of slides often runs into thousands they can be kept in large filing cabinets provided with drawers. The drawers are 1¼ inches deep and are divided into longitudinal compartments 3¼ inches wide. Strips of tin measuring 2 by 4 inches and bent up 1 inch at each end provide simple holders for convenient handling of the slides and for keeping them in place.

CHAPTER II

PREPARATION AND EXAMINATION OF UNFIXED MATERIAL

1. TEASED AND CENTRIFUGED SPECIMENS

UNFIXED tissues may be examined in gross, in teased preparations or by means of frozen sections.

Gross material yields much information to the sight and touch of the trained observer. Differences in color and consistence mean much to him. The iron reaction performed on the surface of a slice of a brown liver may demonstrate the presence of iron in slight or large amount. Iodine will show the presence of amyloid and scarlet red will bring out fat if abundant.

Teased preparations are made by cutting out a very small bit of the tissue in question and dividing it as finely as possible by means of two sharp clean needles on a slide in a drop or two of some indifferent fluid such as physiological saline solution. Teased preparations are often made, for instance, of the heart muscle when fatty degeneration is suspected. If the tissue is soft the cells are easily obtained by simply scraping the cut surface with the edge of the knife.

Unfixed preparations are sometimes treated with chemicals for various purposes. Of these chemicals acetic acid is the most generally useful in pathological histology. It shrinks the nuclei and renders their outlines more distinct. It swells connective tissue, making it more transparent, so that the elastic fibers which are unaffected stand out distinctly. It precipitates mucin and dissolves or renders invisible the albuminous granules. Its main use as a reagent for unfixed tissues is to demonstrate fat and to differentiate that substance from albuminous granules. It is ordinarily used in 1 to 2 per cent aqueous solution, a few drops of which are placed at one edge of the coverslip preparation and then drawn beneath it by placing a piece of filter paper on the opposite side. If in a hurry, however, stronger solutions, or even glacial acetic acid, may be used.

Hydrochloric acid in a 3 to 5 per cent solution is used to demon-

strate calcification. Calcium phosphate is simply dissolved, while from calcium carbonate bubbles of carbon dioxide (CO_2) are set free.

Unfixed tissues are usually examined in an isotonic salt solution —a 0.9 per cent aqueous solution of sodium chloride. It has the advantage over water of preventing excessive swelling of tissues, blood corpuscles are unaffected, and the finer structures are better preserved. A drop or two of Gram's iodine solution added to the stock bottle of salt solution will be found useful in preventing the growth of mold.

Macerating fluids are little used in pathology. Occasionally, however, when tissues are so tough that they cannot be teased apart readily, they are macerated in certain fluids that dissolve the substances holding the different elements together. The reagents most commonly used are Ranvier's one-third alcohol, which is made by taking 1 part of 95 per cent alcohol and 2 parts of water. Tissues should be left 24 hours in this solution. Chromic acid in very dilute aqueous solutions (1:10,000–1:30,000) is also recommended. A 20 per cent aqueous solution of potassium hydroxide is also used for macerating. Tissues should be left in this solution a few minutes to 1 hour; they must be examined in the same fluid as the cells are destroyed if the solution is weakened. This solution is used especially by dermatologists to demonstrate the mycelia of ringworm.

Fluids of various types, but especially those from the serous cavities, often require microscopic examination of the cells they contain, either in the unfixed condition or after fixation in various ways. If the amount of fluid is small, centrifuge at once. If it is large, add 2 per cent by volume of glacial acetic acid. This prevents coagulation and lakes the erythrocytes. Allow the cellular elements to settle somewhat, decant the supernatant fluid and centrifuge the residue until a small button forms at the bottom of the tube. Bits of this may be examined unfixed or in fixed and stained smears. More often the supernatant fluid is poured off and a fixative added, such as the usual formalin-alcohol mixture, 10 per cent formalin, Zenker's fluid or any other fixative desired. They cause the button to shrink and separate from the glass. It can then be treated like a piece of tissue, embedded in paraffin or celloidin, and sections cut and stained in the usual way. Foot recommends Masson's trichrome light green method.

2. FROZEN SECTIONS

Frozen sections are of great value to the pathologist. They often enable him to make a diagnosis in a few minutes instead of having to wait many hours to several days. They may be made either of unfixed or fixed tissues.

Cutting.—The piece of tissue from which frozen sections are to be cut should not be thicker than 5 mm., and a little water should be placed under it on the freezing box so as to aid in attaching it securely.

The consistence of the frozen tissue is important. Immediately after freezing it will usually be too hard to cut without yielding sections that break over the edge of the knife and are, therefore, to be rejected. If this happens, wait a few seconds and cut a section or two at short intervals until the specimen is found to have a consistence yielding satisfactory sections, whereupon a number of sections should be cut in quick succession. They are placed as cut in water in a glass dish.

Attaching Frozen Sections to the Slide.—Frozen sections of unfixed material for immediate diagnosis are usually stained before they are mounted on a slide. However, they may be attached to the slide by means of blotting or by one of the two methods used for fixed tissues given below. Float the section onto a slide and spread it out evenly. Then cover the section carefully by means of a dropping-bottle with 95 per cent or absolute alcohol, which acts as a fixative, so as to avoid wrinkling. After 30 seconds drain off the alcohol and blot with fine filter paper. The section may then be stained in any way that seems desirable.

Aniline and Oil of Cloves Method.—Coat the slide with a thick layer of Mayer's albumin-glycerin mixture and float the section onto it, spreading it out smoothly. Next wipe away most of the fluid from around the section and press the section onto the slide with smooth filter or blotting paper. Then without allowing the section to dry cover it with a mixture of equal parts of aniline and oil of cloves, and immediately rinse off the mixture with 95 per cent alcohol. After immersing in water to remove the alcohol, the section thus attached to the slide is ready for staining and mounting.

In spreading the section on the slide too long immersion of the slide in water may wash off the albumin-glycerin mixture and the section will not stick. This very rarely happens after a little practice.

Celloidin Method.—Float the section onto the slide from water, spread it out smoothly and press or blot it on the slide with fine filter or blotting paper. Next cover it with 95 per cent alcohol for about half a minute and blot it again. Then pour over the section and adjacent part of the slide a very dilute solution of celloidin, in equal parts of absolute alcohol and ether, which should be sufficiently dilute to flow readily and not to form too thick a film. Drain off the excess fluid at once, blow briskly on the section and immediately immerse the slide in water for a few seconds to harden the celloidin. The section is thus attached to the slide by a thin film of celloidin and may be stained by any of the usual methods, for the celloidin does not prevent the penetration of stains and does not interfere with the visibility of the section.

Drying of the section at any stage should be avoided by proceeding rapidly.

Wright's Gelatin Embedding Method for Frozen Sections.—This method permits the making, staining and permanent mounting of frozen sections of fragmented tissue, such as curettings, and of loose textured tissue, with minimum time and manipulation.

Prepare a 10 per cent solution of gelatin in distilled water and while still warm and fluid add 0.5 per cent of carbolic acid. The gelatin should not be heated more than is necessary for thorough solution. It keeps well in a stoppered bottle. The tissue may be unfixed or already fixed, preferably in 10 per cent formalin, for gelatin embedding.

To embed the tissue, the gelatin is liquefied by heat, without boiling, a small "pool" poured on an ordinary glass slide or similar object which may be conveniently handled, and the tissue, after being dried, is immersed and arranged therein. Then the "pool" is allowed to solidify in a cool place or in the ice-box for 2 hours or longer, after which a "block" is cut and trimmed from it. From this block frozen sections are made as from a single piece of tissue.

The gelatin enclosing the tissue should be kept from drying out, if it is not sectioned within 2 hours. This may be done by placing it and the slide on which it has solidified in a closed bottle with as much of a 10 per cent solution of formalin as will reach up to the tissue without covering it.

After cutting sections transfer them from the knife to water and attach them to the slide. The attachment to the slide is desirable

for facility in handling, and is necessary to prevent curling and shriveling during dehydration owing to shrinking of the gelatin. Sections are attached to the slide by the following procedure: Coat the slide with a thick layer of albumin-glycerin and float the section onto it, spreading smoothly. Remove excess of fluid from around the section, cover with a piece of thin cigarette paper and blot with a fine filter paper until the cigarette paper is partly dry. Then cover the cigarette paper with a mixture of equal parts of aniline and oil of cloves for a few seconds; drain off the oil and peel off the cigarette paper from the slide. The section adheres to the slide, which is then washed with 95 per cent alcohol to remove the oil, and immersed in water. The section, thus attached to the slide, is then ready for staining, dehydrating, clearing and mounting.

Staining Frozen Sections.—Frozen sections of unfixed material may be stained with a 0.5 per cent solution of thionin in 20 per cent alcohol. Stain for 30 seconds to 1 minute, wash in water and mount in water. The result is a brilliant differential stain; nuclei blue to purple, collagen reddish, and elastin light green. A more intense nuclear stain can be obtained by using in the same way a 1 per cent solution of toluidine blue in water or in 20 per cent alcohol. This stain is particularly useful at surgical operations when an immediate diagnosis is required.

For stains for amyloid see pages 131–135. Stains for fat are given on pages 116–125.

Alum hematoxylin followed by phloxine, or other stains desired, may be used on frozen sections of fixed tissues.

In addition to its use in cutting unfixed material the frozen section method may also be applied to fixed material. The fixative commonly used is formalin. If an immediate diagnosis is desired the tissue may be dropped in boiling 10 per cent formalin for 1 to 2 minutes and may be frozen, cut and stained with alum hematoxylin and phloxine or eosin immediately without washing out the formalin. Ordinarily, it is advisable to wash out the formalin for 1 to 2 hours before freezing tissues. In making sections for silver and gold impregnations of the central nervous system and for the study of the presence of fat in tissues, the frozen section method is often used.

CHAPTER III

FIXATION

THE various reagents used for fixing fresh tissues possess the properties of penetrating, killing and hardening in different degrees. A good fixative is a reagent that penetrates and kills tissues quickly, preserves the tissue elements, particularly the nuclei, in the condition in which they are at the moment when the reagent acts on them, and hardens or so affects them that they will not be altered by the various processes of dehydrating, embedding, staining, clearing and mounting. Most fixatives are mixtures of different reagents so combined that all the desirable properties may be present in as great a degree as possible.

The choice of the proper fixing reagent for a given tissue is often difficult and must depend largely on the nature of the pathological lesions present or suspected and on the purposes for which the tissue is preserved. The best general fixative yet devised for all kinds of tissues is, in my opinion, Zenker's fluid. It is recommended above all others after over 40 years of constant trial. Helly's modification is preferred by some and is indispensable for the preservation of certain cytoplasmic granules that are dissolved by the acetic acid in Zenker's fluid. Orth's fluid, perhaps, ranks next, but does not permit nearly so great a variety of stains to be used after it as Zenker's fluid does. It has the advantage of costing much less. As a general fixative for all sorts of tissues for diagnostic purposes formalin has, to a large extent, replaced alcohol. It permits about all the chemical reactions to be performed that are possible after alcohol fixation and has the additional advantage of preserving fat of all kinds, especially myelin in the sheaths of nerve fibers.

It is strongly advised that in all important cases tissues be fixed both in Zenker's fluid and in formalin; in Zenker's fluid for general histological study and for the preservation of nuclear figures, bacteria and fibrils of all kinds; and in formalin for the preservation of fat, myelin and various substances, such as amyloid and hemosiderin, to which it may be desirable to apply chemical tests. For certain specific purposes other fixatives, such as alcohol, are some-

times required for the preservation of mucus, glycogen, pigments and sodium urate crystals.

Tissues fixed in formalin or alcohol may remain as long as desired in those fluids. Tissues fixed in most of the other fixatives must be transferred, after thorough washing in water, to alcohol for preservation. It is usually recommended that specimens be passed through graded alcohols, either through 30, 60, 90 and 95 per cent, or through 50, 70 and 95 per cent, allowing them to remain for from a few hours to a day in each strength. For most purposes it will be found sufficient to transfer the specimens directly from water to 70 to 80 per cent alcohol, in which they may remain until it is desired to embed them.

Alcohol extracts chrome salts from tissues fixed in solutions containing them. As these salts are precipitated in the alcohol by the action of light it is desirable, although by no means necessary, to keep all such specimens in the dark.

It is strongly urged by some that distilled water be used in making all fixing solutions, and also that all fixatives be employed at body temperature as they will then penetrate more quickly and the tissues will, therefore, be better preserved. For most purposes, however, tap water is perfectly satisfactory and often even advisable as distilled water is almost always faintly acid.

Fixatives are generally used at room temperature. Heat at body temperature favors penetration of the fixative but also hastens post-mortem changes within the tissues. Cold, even to the freezing point, preserves the tissues better and does not greatly slow up the penetration of the fixative.

Alcohol.—Alcohol is a fair general fixative which both hardens and dehydrates tissues at the same time. As a fixing reagent formerly in much use its place is largely taken nowadays by formalin. In its favor, however, are several points. Bacteria, fibrin, mucus, various pigments, elastic fibers and certain cytoplasmic granules stain well after it, and it is the only fixative that preserves glycogen and allows it to be stained differentially. Its disadvantages are that it removes hemoglobin from the red blood corpuscles, shrinks tissues more or less, and does not give them so good a consistence as some of the other fixatives. Its greatest use is as a preservative of tissues after they have been fixed by other reagents. The strength of the stock alcohol ordinarily used in laboratories is 95 per cent.

Absolute alcohol is much more expensive. Tissues fixed in either of these percentages shrink a great deal. The exposed surface becomes extremely hard and the outer layers of the cells of tissues are as shrunken and flattened as though dried in the air. It is only inside of this hard casing, where the alcohol has penetrated more slowly and has been somewhat diluted by the fluid of the tissues, that the cells are better preserved. Moreover, this extreme hardening of the surface hinders the penetration of the alcohol into the deeper parts.

Tissue which is to be fixed in absolute or in 95 per cent alcohol should be cut into thin slices, preferably not over 2 to 5 mm. thick. The volume of alcohol used for fixing should be 15 to 20 times as great as the specimen and should be changed after 3 to 4 hours. The tissue should be kept in the upper part of the alcohol by means of absorbent cotton, or the jar may be inverted frequently and the alcohol thus kept of even strength.

The advantages of strong alcohol, 95 per cent and absolute, are that the tissue is more quickly fixed than with a weaker strength, and at the same time hardened. Tissues so fixed should later be transferred to 80 per cent alcohol for preservation, or the staining properties will gradually become impaired.

For general purposes it will be found better to place tissues at first in 80 per cent alcohol, which should be replaced in 2 to 4 hours by 95 per cent alcohol. In this way less shrinkage is caused and the surface of the tissues is not made so hard.

Carnoy's Fluid (1887).—This is one of the most penetrating and quickly acting fixatives known and is much used by the French. It preserves both nuclei and cytoplasm well. It is made up as follows:

Alcohol, absolute	60 cc.
Chloroform	30 cc.
Acetic acid, glacial	10 cc.

Fix tissues in the fluid for 1½ to 3 hours and then transfer to absolute alcohol.

Formalin.—The gas formaldehyde (HCOH) is soluble in water to the extent of 40 per cent. Solutions of this strength are manufactured by different commercial houses under the names of formalin, formol and formalose. The best strength of formalin to use for

fixing tissues is a 10 per cent solution, that is, 10 parts of the aqueous 40 per cent solution, no matter what name is given to it, to 90 parts of water. Unfortunately formic acid gradually develops in formalin, rendering it acid and exerting an injurious action on tissue preserved in it. On this account it is advisable for most purposes to neutralize the 10 per cent solution of formalin by adding calcium carbonate, or lead oxide or carbonate in excess.

Calcium carbonate cannot neutralize full strength formalin but readily renders the usual 10 per cent solution faintly alkaline. This slight alkalinity can hardly be regarded as injurious because post-mortem tissue is always acid in reaction and almost immediately renders the solution acid, as can readily be shown by having an indicator (phenolphthalein or phenol red) in it.

For certain purposes, however, it is sometimes advisable to add 5 per cent by volume of glacial acetic acid to the usual 10 per cent solution in order to improve its fixing properties, but tissues cannot be left in the mixture. They must be transferred after 24 hours to the neutral formalin solution.

Formalin penetrates very quickly. Its hardening action is not understood. It does not precipitate albuminous bodies but makes them quite firm. It also hardens nerve sheaths, acting toward them and red blood corpuscles in a manner similar to chrome salts. Formalin is very useful for preserving gross specimens as it gives them a rather tough elastic consistence and preserves the normal color better than other fixing fluids, and also the transparency of many parts, such as the cornea. In general histological work formalin is largely used nowadays as a fixative in place of alcohol.

As a fixative for specimens that are to be embedded in paraffin it is not recommended unless combined with other reagents, such as potassium bichromate in Orth's fluid, as it does not seem to harden the tissue elements sufficiently to enable them to resist the shrinking effects of prolonged exposure to alcohol and heat in the process of embedding. In frozen or celloidin sections, however, prepared by methods described elsewhere, this shrinkage of the tissue elements is not apparent, probably because prolonged exposure to heat in the paraffin embedding process is avoided.

The advantages of formalin are that it is comparatively cheap, can be obtained commercially in compact form, and keeps well. It fixes and hardens tissues, including red blood corpuscles, quickly

and well even in large slices and gives a firm consistence so that they can be cut easily on the freezing microtome or after embedding in celloidin. It permits the use of a large variety of staining methods. It also fixes and preserves fat so that this substance can be easily stained in frozen sections. In addition, it preserves myelin, and on this account is the best preliminary fixative of the central nervous system that we have.

The disadvantages of formalin are that it dissolves glycogen, uric acid and sodium biurate crystals, often changes the bile concretions from a yellow to a green color, does not preserve iron and other pigments as well as alcohol does, and frequently gives rise in the tissues to a fine, dark brown or black crystalline precipitate derived from laked hemoglobin. Two methods are recommended for removing the precipitate:

Schridde's Method (1906).—Place sections in the following mixture for 30 minutes:

Ammonia water (25–28 per cent)	1 cc.
Alcohol, 75 per cent	200 cc.

Wash thoroughly in water and preserve in 80 per cent alcohol, or stain as desired and mount.

Verocay's Method (1908).—Place sections in the following mixture for 10 minutes:

Potassium hydroxide, 1 per cent aqueous solution	1 cc.
Alcohol, 80 per cent	100 cc.

Wash thoroughly in at least 2 changes of water for 5 minutes and then place in 80 per cent alcohol for 5 minutes. Return again to water and stain as desired and mount.

Alcohol and Formalin.—The combination of alcohol and formalin is a most useful mixture, especially for rapid diagnosis of routine surgical specimens, as it fixes and dehydrates at the same time. It is made up as follows:

Formalin	10 cc.
Alcohol, 95 per cent	90 cc.

Corrosive Sublimate.—Corrosive sublimate is a useful fixing reagent but is best employed in combination with a chrome salt, as in Zenker's and Helly's fluids. Its great disadvantage when used

alone is that it causes serious shrinkage of the cells. A second disadvantage, which attends its use under all conditions, is that it gives rise to a crystalline precipitate of mercuric oxide. This precipitate can be removed from the tissues by means of iodine.

Do not add iodine to the alcohol in which the tissues are preserved as prolonged treatment with iodine exerts an injurious effect on the staining properties of the cells. Embed the tissues and cut sections without removing the precipitate, and then treat the sections, just before staining, with Lugol's solution of iodine for 10 to 20 minutes, followed by alcohol to remove the iodine.

Inasmuch as prolonged action with alcohol is frequently necessary in order to remove the iodine, it is often better to use a 0.5 per cent aqueous solution of sodium thiosulfate ("hypo") for this purpose instead of alcohol as it acts almost instantaneously and is easily removed by thorough washing in water.

The directions for the use of corrosive sublimate are as follows:

Use a saturated aqueous solution (about 6.9 per cent) made with the aid of heat. The addition of 5 per cent glacial acetic acid is usually advisable. Fix thin slices of tissue (2–5 mm.) for 6 to 24 hours. Do not wash in water but soak in repeated changes of 70 per cent alcohol and then preserve in 80 per cent alcohol.

Tissues fixed in corrosive sublimate stain quickly and brilliantly in nearly all staining solutions. It is the only fixative after which the Heidenhain-Biondi triple stain gives good results.

Giemsa's Corrosive Sublimate-Alcohol Fixative (1909).—This fixative is the one usually recommended for tissues that are to be stained by Giemsa's method, but Wolbach has shown that excellent results, but with a reversal of the color effect, may be obtained after fixation in Zenker's fluid, and the tissue preservation in general is much better. Giemsa's fixative is made up as follows:

Corrosive sublimate, saturated aqueous solution (about 6.9 per cent)	2 parts
Alcohol, absolute	1 part

Fixation requires at least 48 hours and the fixing fluid is to be renewed after 24 hours. The tissue may remain as long as 3 months in the solution without disadvantage if evaporation is prevented.

Chrome Salts.—Chromic acid is rarely used nowadays except in Flemming's solution. Chrome salts are employed instead, espe-

cially potassium bichromate, which is used in several well known fixing solutions. This salt penetrates slowly and is a poor fixative of nuclear material but is the best of all known hardening reagents. On this account tissues fixed in solutions containing it stand paraffin embedding with little or no shrinkage. Potassium bichromate has been used so long in the solution known as Müller's fluid that the latter solution is regarded as practically synonymous with it.

Müller's Fluid (1872).—

Potassium bichromate	2–2.5 gm.
Sodium sulfate	1 gm.
Water, distilled	100 cc.

Harden tissues 6 to 8 weeks. Change the fluid daily during the first week; once a week thereafter. Ordinary tissues are then washed in running water overnight before being placed in alcohol.

This famous hardening solution is rapidly giving way to better fixatives. It hardens tissues slowly, evenly, and with little or no shrinkage, but it is a poor nuclear fixative and does not permit any great variety of stains. The sodium sulfate seems to serve absolutely no function. For ordinary tissues Müller's fluid is being replaced by Zenker's, Helly's or Orth's fluids, all of which fix very quickly. For tissues from the central nervous system formalin, followed by other solutions of the chrome salts, is a great deal quicker and better.

Tellyesniczky (1898) has recommended the following solution, which has met with considerable favor, and which may be regarded as an improved Müller's fluid:

Potassium bichromate	3 parts
Water, distilled	100 parts
Acetic acid, glacial	5 parts

Fix thin pieces of tissue for 1 to 2 days; thicker pieces longer. Wash thoroughly in running water and dehydrate in graded alcohols.

Orth's Fluid (1896).—This is a general fixative consisting of the well known Müller's fluid plus 10 per cent formalin. It is made up as follows:

Potassium bichromate	2–2.5 gm.
Water, distilled	100 cc.
Formalin	10 cc.

The formalin should be added only at the time of using, for in 2 days the mixed solution becomes darker, and by 4 days a crystalline deposit begins to form. As fixation is ordinarily complete in 3 to 4 days this deposit is immaterial. The tissue should not be over 1 cm. in thickness. Small slices, 3 to 5 mm. in thickness, can readily be fixed in the incubator in 3 hours. The specimens should be washed thoroughly in running water 6 to 24 hours before placing in 80 per cent alcohol.

The method is particularly recommended for mitotic figures, red blood corpuscles, bone, and colloid material as it gives a firm consistence to the tissues, but the histological detail is not so perfect as after Zenker's fluid.

Regaud's Fluid (1910).—This solution is recommended for fixation of tissues containing Rickettsiae and for mitochondria. It must be made fresh each time for use as it does not keep.

Potassium bichromate, 3 per cent aqueous solution	80 cc.
Formalin	20 cc.

Fix tissues for 4 days, changing to fresh fluid every day. Chromatize tissues for 8 days longer in 3 per cent potassium bichromate. Wash in running water for 24 hours.

Zenker's Fluid (1894).—Zenker's fluid was originally Müller's fluid plus 5 per cent corrosive sublimate and 5 per cent glacial acetic acid, but the sodium sulfate is usually omitted nowadays as it seems to be of no particular use. The solution is made up as follows:

Potassium bichromate	2.5 gm.
Corrosive sublimate	5–8 gm.
Water, distilled	100 cc.
Acetic acid, glacial	5 cc.

Dissolve the corrosive sublimate and the potassium bichromate in the water with the aid of heat. Do not add the acetic acid to the stock solution but only in the proper proportion to the amount to be used for fixing pieces of tissue as the acetic acid evaporates readily and also produces changes in the chrome salts. Tissues float at first in this solution, which penetrates fairly quickly.

Fix tissues in the solution 12 to 24 hours. Wash in running water 12 to 24 hours and then preserve in 80 per cent alcohol until used.

Zenker-fixed tissues stain slowly but beautifully in alum hematoxylin. The most brilliant results, however, are obtained by staining with phloxine or eosin, followed by an alkaline methylene blue solution. Excellent results are also obtained by staining in phosphotungstic acid hematoxylin, and by the aniline blue method. These methods bring out fibrin and various kinds of fibrils in addition to nuclear details.

When Zenker-fixed tissues which have been kept for a long time are embedded and stained with alum hematoxylin, the places where the mercuric bichloride deposit was present stain a deep blue and thus disfigure the specimen. The only way found so far to prevent this staining is to soak the sections first for several weeks in acid alcohol (1 per cent hydrochloric acid in 70 per cent alcohol). The method is applicable only to celloidin sections as the acid dissolves the egg albumin used in attaching paraffin sections. This treatment causes no injury to the tissues but does, as a rule, prevent the disfiguring stains from appearing. On the other hand, the method will also remove certain pigments from the sections and therefore cannot always be used.

Helly's Fluid (1903).—This is a slight modification of Zenker's fluid; the glacial acetic acid is replaced by 5, occasionally 10, per cent of formalin added just before the mixture is used. For certain purposes, such as fixing the cytoplasmic granules in the islet cells of the pancreas, the formalin should be carefully neutralized. The solution is made as follows:

Potassium bichromate	2.5	gm.
Corrosive sublimate	5–8	gm.
Water, distilled	100	cc.
Formalin	5–10	cc.

Fix tissues in the fluid for 12 to 24 hours. Wash in running water 12 to 24 hours and then transfer to 80 per cent alcohol.

Osmic Acid.—Osmium tetroxide, commonly known as osmic acid, is a fixing reagent of considerable value, particularly for the demonstration of fat, but it penetrates tissues poorly. On this account it is generally used in combination with other reagents, some of which seem to increase its power of penetration. The solutions most in favor are as follows:

Flemming's Solution (1884).—Slices of tissue for fixation in

Flemming's solution should not be over 2 mm. in thickness as it has very slight penetrating properties. The solution is made as follows:

Osmic acid, 2 per cent aqueous solution	4 parts
Chromic acid, 1 per cent aqueous solution	15 parts
Acetic acid, glacial	1 part

It is best to keep the osmic acid in a 2 per cent and the chromic acid in a 1 per cent solution. The mixture can then be made up fresh quickly at the time it is needed.

Fix in the solution 1 to 3 days. Wash in running water 6 to 24 hours and then place in 80 per cent alcohol.

Hermann's Solution (1889).—This modification of Flemming's solution is perhaps an even better fixative but is more expensive. It should be employed in the same manner.

Osmic acid, 2 per cent aqueous solution	4 parts
Platinic chloride, 1 per cent aqueous solution	15 parts
Acetic acid, glacial	1 part

Marchi's Fluid (1885).—

Müller's fluid (p. 42)	2 parts
Osmic acid, 1 per cent aqueous solution	1 part

Place small pieces of tissue in the mixture for 5 to 8 days. Wash thoroughly in running water and then place in 80 per cent alcohol.

For its application to degenerated nerve fibers see page 237.

Bouin's Fluid (1897).—Picric acid is rarely used by itself as a fixative but is often combined with other reagents. Of the various solutions suggested, that formulated by Bouin has proved the most successful. It is one of the best fixing fluids and is highly recommended both for general purposes and for special study. It has been much used for fixing embryos. Its composition is as follows:

Picric acid, saturated aqueous solution (about 1.22 per cent)	75 cc.
Formalin	25 cc.
Acetic acid, glacial	5 cc.

Bolles Lee in his "Microtomist's Vade-Mecum" advised fixation up to 18 hours followed by washing in 50 per cent and then in 70 per cent alcohol until the picric acid is practically removed.

Masson fixes up to 3 days but no longer; after that time the nuclei fail to stain properly with alum hematoxylin. When fixation is complete he pours off the solution and covers the tissues with water but does not wash them with it. In this condition they will keep indefinitely until wanted. For embedding in paraffin he recommends the following method:

1. Alcohol, 80 per cent, 95 per cent and absolute, in each solution	3 hrs.
2. Amyl acetate and alcohol, absolute (equal parts)	3 hrs.
3. Amyl acetate	3 hrs.
4. Amyl acetate and paraffin (equal parts at 55° C.)	3 hrs.
5. Embed in paraffin, several changes	4 hrs.

The difficulty with paraffin sections of tissues fixed in Bouin's fluid lies in using water that is too hot and for too long a time in flattening sections. The water should not be above 45° C. and should not be applied longer than 45 seconds. Masson uses gelatin rather than Mayer's albumin-glycerin, flattens the sections on the slide rather than in a pan, drains off the excess water and blots firmly with smooth filter paper, and then dries in the incubator at 37° C.

Boiling.—Boiling precipitates the soluble albumin in tissues as a granular material which can be readily recognized. The method is used particularly for the demonstration of albumin in renal diseases and in edema of the lungs. By means of boiling the quickest permanent specimens can be obtained. The method is not advocated on account of the shrinkage caused by the heat, but will sometimes be found useful. Occasionally 10 per cent, or even undiluted formalin is employed instead of water.

Small pieces of tissue not over 5 mm. thick should be dropped into the boiling water for 30 seconds to 2 minutes; cool quickly in cold water and make frozen sections, or put into 80 per cent alcohol. Any stain may be used; methylene blue will be found excellent.

Washing.—Most tissues after fixation, especially in chrome salts, have to be washed thoroughly in running tap water to remove all acids and reagents. The simplest way is to stick one of the glass tubes extending down from the water pipe over the draining

board of the sink into the jar or bottle containing the tissue through a hole in a cap of wire netting placed over the top of the jar or bottle. This cap prevents any specimens from floating away. If necessary, tissues can be washed in a jar or dish without the use of running water by changing the water occasionally and moving the material about.

Tissues fixed in certain fluids, such as picric acid and corrosive sublimate, have to be washed in alcohol and it is done in the same way, by changing the alcohol occasionally and moving the specimens about. For small bits of tissue the Fairchild swimming cup of perforated unglazed porcelain, cork-stoppered, will be found convenient. The cup can be easily labeled with a lead pencil. A simple but useful method, especially when there is much tissue, as from an autopsy, is to use the common soapshaker found in most kitchens. It has a convenient handle to which a tag can be attached and any number of the shakers can be stuck into a pan of running water.

Dehydration.—The reagent ordinarily used for dehydration of fixed tissues is alcohol. The ideal method is to start with a strength of 50 per cent and to change to 60, 70 and 80 per cent, where they may be kept indefinitely. For practical purposes it has been found sufficient to transfer material fixed in Zenker's fluid directly from the water used for washing to 80 per cent alcohol. Tissues treated this way still stain perfectly as long as 40 years after preservation.

Isopropyl alcohol can be used in place of ethyl alcohol for dehydration and preservation of fixed tissues but not for the celloidin embedding process.

CHAPTER IV

DECALCIFICATION

Bone and calcified tissue from which later the lime salts will have to be removed, should, like other tissues, be cut or sawed into slices 2 to 5 mm. thick before fixation. For sawing, as thin a blade as is practicable should be used. A jeweler's saw with fine hair blades is recommended as it does less damage to the soft parts.

While tissues are being decalcified they should be suspended in the upper part of the fluid in a piece of gauze or in a perforated porcelain dish, so that the salts dissolved out may sink to the bottom of the jar. This step in the process has been found useful. Occasional stirring or agitation of the fluid hastens the process of decalcification.

In cutting sections after decalcifying and embedding it is necessary to discard the first half dozen sections or so, because the tissue is so lacerated to a slight depth by the movement of small fragments of bone in the process of sawing as to be useless for microscopic purposes. The extent of the decalcification may be tested at any time by thrusting a needle into the tissues, but it is best to avoid such a test as it tends to produce artefacts.

The following steps in the decalcification of tissues must be carefully borne in mind.

1. The tissues must first be thoroughly fixed and hardened. The most useful reagents for this purpose are alcohol, the alcohol-formalin mixture, and Zenker's and Orth's fluids. After fixing in the two latter reagents the tissues must be washed thoroughly overnight in water and placed in alcohol for at least 24 hours. They will then be ready for decalcification.

2. The decalcifying fluid must be used in large amounts and, if necessary, be frequently changed. Decalcification should never be prolonged beyond 4 days if possible; 24 to 48 hours are better.

3. After decalcification the tissues must be thoroughly washed in running tap water or soaked in several changes of alcohol for 24 hours to get rid of every trace of acid.

4. The tissues finally must be hardened again in alcohol.

5. Embedding in celloidin is generally preferable to paraffin except for bone marrow and tumors containing only delicate trabeculae of bone. As a rule these tissues are perfectly decalcified by fixation in Zenker's fluid, owing to the action of the acetic acid present, and require no further treatment. This procedure can be highly recommended as all the staining methods used on sections of tissues fixed in Zenker's fluid are available and the results are better than can be obtained by other decalcifying methods.

Of the various agents used for decalcifying bone—nitric, hydrochloric, chromic, picric, trichloracetic acids, and so on—the most important is nitric acid. It acts quickly without causing swelling of the tissues or attacking injuriously the tissue elements, and does not interfere to any marked degree with any subsequent staining process. Red blood corpuscles will be found uninjured in tissues fixed in Zenker's fluid even after remaining 4 days in 5 per cent nitric acid. This acid is used in dilute solutions either alone or in combination with other reagents.

Nitric Acid.—1. Decalcify in large quantities of a 5 per cent aqueous solution of nitric acid, changing the solution every day for 1 to 4 days.

2. Wash 24 hours in running water to remove every trace of acid.

3. Preserve in 80 per cent alcohol.

4. Embed in celloidin or paraffin, depending on the size of the block of tissue and the density of the bone.

According to Schaffer (1902), it is best to transfer the tissue directly from nitric acid to a 5 per cent solution of alum or of lithium or sodium sulfate for 24 hours before placing in running water so as to avoid any possibility of the tissue swelling, but this step hardly seems necessary.

Three different reagents have been recommended for addition to nitric acid in order to counteract any injurious effect—phloroglucin, formalin and alcohol. Of these, alcohol seems to be the best. Romeis advises 5 per cent nitric acid in 60 per cent alcohol and washes out the acid in 60 per cent alcohol. This is especially useful for bone marrow, where dense areas of bone are present, but the decalcifying process takes several days, depending on the size and thickness of the specimen. Tissues decalcified by this method must be transferred immediately to alcohol for removing the acid. Water

must not be used as the blood cells will dissolve instantly in the water and leave only the trabeculae of bone.

Sulfurous Acid.—A saturated solution (6–7 per cent) of sulfur dioxide (SO_2) in water is used. It works fairly quickly and causes but slight swelling. The tissues should be carefully washed in running water, as after nitric acid. The stock solution rapidly weakens through evaporation if the bottle is not kept tightly corked. Primary fixation in formalin is favored.

Trichloracetic Acid.—A 5 per cent solution of this acid is recommended for the decalcification of bone and teeth. It may be added directly to the 10 per cent formalin used for fixation. It acts more slowly than nitric acid and seems to possess no particular advantage over it. Tissues must be washed in 90 to 95 per cent alcohol afterward to remove the acid.

Picric Acid.—Picric acid is used in a saturated aqueous solution (about 1.22 per cent). It decalcifies very slowly (1 week to months) and is therefore used chiefly for embryonic tissues. It must be washed out in 70 to 80 per cent alcohol.

Sulfosalicylic Acid.—Gömöri (1933) recommends this acid highly as a decalcifying agent. Use a 6 to 8 per cent aqueous solution for 1 to 3 days, changing the solution once or twice. Wash thoroughly in running water for 24 hours and preserve in 80 per cent alcohol.

Ebner's Decalcifying Method (1875).—

Sodium chloride, cold saturated solution (about 35.7 per cent)	100 cc.
Water, distilled	100 cc.
Hydrochloric acid	4 cc.
(For teeth use 10–20 cc. HCl)	

This method takes a long time to decalcify but gives excellent results. Each day add 1 to 2 cc. of hydrochloric acid until the bone is soft.

Formic Acid.—This is used for softening large masses of bone. It may be used in an aqueous solution, but a 1 to 5 per cent solution in 70 per cent alcohol is preferable. Tissues should be fixed in 10 per cent formalin and decalcified 4 to 5 days in the 5 per cent alcoholic solution. When decalcification is completed tissues are washed in 70 per cent alcohol and not in water.

CHAPTER V

EMBEDDING PROCESSES

FIXED tissues are not firm and cohesive enough to permit perfect thin sections to be cut without being infiltrated with some supporting medium to furnish stability and to hold the cells and intercellular structures in proper relation to each other. For this purpose three different substances have been found available—paraffin, celloidin and gelatin. Each has its particular field of usefulness and its own advantages, and a thorough practical knowledge of each should be acquired.

Paraffin embedding affords the thinnest sections but the blocks of tissue must usually be comparatively small (not over 2 cm. square) if the best results are desired, and the sections cannot be properly handled except when attached to the slide. Hard tissues, such as muscle, and tissues of varying consistence, such as skin, cut with considerable difficulty. One great advantage possessed by the paraffin embedding method is that sections can be cut with safety razor blades when rigidly fixed in a holder. This means much in a pathological laboratory where tissues often contain unsuspected lime salts. It is much simpler to insert a new blade than to grind out nicks in the cutting edge of a microtome knife.

Celloidin embedding has certain advantages of its own. Tissues of almost any consistence or size can be cut and very thin sections are possible. The cut sections are available for use at once while paraffin sections always have to be put through a preliminary process to free them of paraffin and get them into water. Serial sections are possible but are not so easy to prepare.

While celloidin embedding is generally considered a much slower process than paraffin embedding, years of practical experience have shown that routine surgical material can be fixed in the formalin-alcohol mixture, embedded in celloidin, and cut and stained in 48 hours. Moreover, tissues so treated do not show the shrunken appearance so often apparent in paraffin sections embedded under like conditions.

Gelatin embedding is of minor importance but useful on occasion, as when it is necessary to keep a number of small fragments

of tissue together while making frozen sections of them. The gelatin embedding method is given in detail on pages 34–35.

1. PARAFFIN METHOD

Embedding in Paraffin.—Pure paraffin, melting at 125° F. (51.6° C.), can be obtained cheaply when bought in large quantities from the regular dealers in paraffin and can be used at all seasons of the year. Cakes of refined and of white filtered paraffin are carried by dealers in laboratory supplies but the cost is much higher. Paraffin of higher melting point (55°–56° C.) is sometimes preferred.

Paraffin embedding can be particularly recommended for tissues fixed in chrome salts, as for example Zenker's fluid, because the potassium bichromate gives to the tissues a denseness or solidity that prevents any marked shrinking, which is so evident after fixation in formalin and in alcohol. On this account it is sometimes advisable, when only formalin-fixed tissue is available, to mordant it for a week in 2.5 per cent potassium bichromate in the incubator at 37.5° C. before dehydrating and embedding.

Paraffin embedding is especially useful when very thin sections are desired. To obtain the best results the pieces of tissue should be small, soft, and of uniform consistence. In pathological work it is much better to cut the sections and to stain them after they are fastened to the slide than to stain in the mass beforehand, because then a variety of stains may be used. A complete or perfect series is not so important as in embryology, but with a little care can be obtained.

The first step in the preparation of fixed and hardened tissues for the paraffin bath is to cut them into thin, square or rectangular pieces, not over 2 cm. perhaps for the best results, and not over 2 to 3 mm. thick if they are not so cut before fixation. It should be stated, however, that with proper skill, a heavy sharp knife and a rigid microtome, very thin paraffin sections can be obtained with tissues measuring 4 by 3 cm., and even more. The pieces of tissue are then thoroughly dehydrated by soaking first in 95 per cent and then in absolute alcohol. From alcohol they are put into some medium that has the property of mixing with alcohol and of dissolving paraffin.

A variety of reagents can be used for this purpose. Bolles Lee preferred oil of cedarwood to all others. We used chloroform, a

general favorite, for many years and then shifted to oil of cedarwood. It has proved very satisfactory. Romeis prefers methylbenzoate and recommends it highly, especially when it contains an admixture of celloidin. Other reagents often used are benzol, xylol and toluol. The two latter tend to make the tissues hard and are not so easily removed from the paraffin bath as the more volatile benzol.

Method No. 1 (Oil of Cedarwood)

1. Alcohol, 95 per cent (2 changes)	6–24 hrs.
2. Alcohol, absolute (2 changes)	6–24 hrs.
3. Alcohol, absolute, and xylol (equal parts)	6–12 hrs.
4. Oil of cedarwood (2 changes)	6–24 hrs.
5. Xylol, to remove oil (2 changes)	30 min.
6. Paraffin bath (4 changes, until no odor of oil of cedarwood)	2– 8 hrs.
7. Block and cool quickly in cold water	

The following modification of Method No. 1 is recommended for certain brittle tissues, such as animal tissues, which are difficult to section after fixation in Zenker's fluid:

Method No. 1A

1. Alcohol, 95 per cent (2 changes)	2 hrs.
2. Alcohol, absolute (2 changes)	2– 4 hrs.
3. Alcohol, absolute, and xylol or benzol (equal parts)	1– 2 hrs.
4. Oil of cedarwood (2 changes)	12–24 hrs.
5. Benzol or xylol to remove oil (2 changes)	10–30 min.
6. Paraffin bath (4 changes)	2– 4 hrs.
7. Block and cool quickly in cold water	

Method No. 2 (Chloroform)

1. Alcohol, 95 per cent (2 changes)	6–24 hrs.
2. Alcohol, absolute (2 changes)	6–24 hrs.
3. Chloroform	6–24 hrs.
4. Chloroform saturated with paraffin	6–24 hrs.
5. Paraffin bath (4 changes)	2– 4 hrs.
6. Block and cool quickly in cold water	

Method No. 3 (Acetone-Benzol)

1. Acetone	½– 2 hrs.
2. Benzol	15–30 min.
3. Paraffin bath (3 changes)	30–90 min.
4. Block and cool quickly in cold water.	

This method is recommended when there is great haste but tissues shrink considerably, more so than in other methods.

Romeis prefers the following method of embedding with benzol:

Method No. 4 (Benzol)

1. Alcohol, 95 per cent (2 changes)	6–24 hrs.
2. Alcohol, absolute (2 changes)	6–24 hrs.
3. Benzol	6–24 hrs.
4. Paraffin bath (4 changes)	3– 6 hrs.
5. Block and cool quickly in cold water	

Method No. 5 (Methylbenzoate, Péterfi's Celloidin Method [1921])

1. Alcohol, 95 per cent (2 changes)	6–12 hrs.
2. Alcohol, absolute (2 changes)	6–12 hrs.
3. Methylbenzoate, containing 1 per cent celloidin (2 changes)	24–48 hrs.
4. Benzol (2 changes)	20–30 min.
5. Benzol saturated with paraffin (place on top of paraffin oven)	2 hrs.
6. Paraffin bath (3 changes)	2– 4 hrs.
7. Block and cool quickly in cold water	

The celloidin adds a certain amount of support to delicate tissues but for many it is unnecessary. It dissolves very slowly in the methylbenzoate. For this reason Romeis advises that in Step 5 sections be placed on top of the paraffin oven. Too high a temperature is to be avoided as it breaks down the celloidin and causes the formation of injurious acids.

Method No. 6 (Graupner's and Weissberger's Dioxane Method [1931])

The tissues are fixed in any desired fixative. After Bouin's fluid or formalin no washing is necessary. Dehydrate and embed as follows:

1. Dioxane, 100 per cent	1 hr.
2. Dioxane, 100 per cent	1 hr.
3. Dioxane, 100 per cent	2 hrs.
4. Paraffin bath	15 min.
5. Paraffin bath	45 min.
6. Paraffin bath	2 hrs.
7. Block and cool quickly in cold water	

If the tissue is very delicate it may first be put in a mixture consisting of 50 per cent fixative, and 50 per cent dioxane, or even started with only 25 per cent dioxane, then 50 per cent, 75 per cent and 100 per cent. When the tissue is spongy (containing air bubbles) fixation and dehydration should be done in a partial vacuum (use a chemical vacuum desiccating jar attached to an ordinary water vacuum pump).

Dioxane is heavier than paraffin. Because of this it will settle to the bottom of the paraffin cup and care should be taken to shake the cup well before emptying it. The dioxane may be reclaimed for further use in the first changes by keeping it in a flask in the bottom of which are a few lumps of unslaked lime (CaO_2). Calcium oxide is more satisfactory for this purpose than calcium chloride, which was recommended earlier for the purpose, as dioxane itself reacts with the latter.

This reagent dioxane (1:4 diethylene dioxide) was introduced in 1931 by Graupner and Weissberger and has recently been increasing in importance as a means of dehydrating tissues. It has the property of being miscible with water, ethyl alcohol, clearing oils and melted paraffin. It dissolves balsam, gum dammar and, with some difficulty, cold paraffin. Dioxane is about as inflammable as ,95 per cent alcohol but has a somewhat lower volatility. The vapor in a concentration of 1:1000 is definitely poisonous. Several cases of fatal poisoning have been reported among industrial workers following exposure to excessive amounts of vapor, but such an exposure is not likely to occur in an ordinary technical laboratory.

Dioxane has been used chiefly as a means of dehydration in paraffin embedding. Various techniques have been recommended. The above method is that given by Mossman (1937).

As yet no satisfactory method of using dioxane in the celloidin technique has been found. It can be used to replace alcohol in the staining of paraffin sections.

One important advantage of dioxane is that tissues may be stored in it for long periods of time (at least 2–3 years) without injuring their ability to stain or causing them to harden.

For blocking the specimens infiltrated with paraffin metallic boxes can be used, or forms made round or square from strips of sheet lead or tin. Many prefer paper boxes which we have always used and which can be made easily of any size desired from stiff writing paper.

Melted paraffin is poured into the paper box to the depth of about 1.5 cm. or more. The pieces of tissue are then placed in the box with the side down from which sections are to be cut. When all the pieces are arranged in order with a space about 0.5 cm. or more between them, the box is placed on the surface of a large dish of cold water, on which it floats, so that the paraffin may cool quickly without crystallizing. Sometimes before hardening the paraffin it is advisable to set the paper box with the specimens in it in the paraffin oven for a short while to get rid of any bubbles carried in by the specimens.

Cutting of Paraffin Sections.—After the paraffin has hardened the paper is removed and the tissues are cut apart with a scalpel and each block is fastened to a microtome disk by heating the latter in a flame until it will just melt the paraffin when the block is held in proper position against it. The holder is then quickly cooled in cold water.

The upper surface of the paraffin should now be shaved down to the specimen. The four sides are to be carefully trimmed; the upper and lower surfaces should be parallel and not cut too close to the specimen, otherwise the sections will not adhere to each other in cutting ribbons; the lateral surfaces should, as a rule, be cut close to the tissue, especially if very thin sections are desired, because if a rim of paraffin is left it is likely to cause wrinkling of the section. The holder is finally carefully adjusted in the paraffin microtome.

Paraffin sections should be cut not over 7 μ in thickness, preferably thinner if possible. To get good sections that will adhere to each other and form a ribbon the temperature of the room must be

regulated to suit the degree of hardness of the paraffin used. An open window will often make all the difference needed to obtain good results. The harder the paraffin the warmer the room must be. The temperature can be raised by burning a Bunsen flame near the microtome, or lowered by the presence of a lump of ice. It will often be found advantageous to dip the holder and paraffin block into ice water or to apply ice to the specimen, when fixed in the microtome, for a few minutes just before cutting.

The ribbons of sections as cut, usually a slideful, are laid on the surface of a large dish of warm water (about 44° C.) and if necessary gently stretched so as to remove all wrinkles. Place a drop or two of Mayer's albumin-glycerin mixture on a slide and spread evenly with a towel until only a faint layer is left. Dip the slide under the sections, arrange them in order, lift the slide and drain off the water. The slide is then placed in a slanting position until dry, when it is put into an open slide box and placed in the paraffin oven for 2 to 12 hours at a temperature of about 54° C. This process attaches the sections firmly on the slide.

To remove the paraffin from sections treat with 2 or 3 changes of xylol, and then with absolute followed by 95 per cent alcohol.

If for any reason the celloidin and oil of cloves mixture is used for attaching sections to the slide, the paraffin is removed by means of xylol, followed by oil of origanum or bergamot, and finally by 95 per cent alcohol as absolute alcohol will dissolve the celloidin.

Attachment of Paraffin Sections to the Slide.—The most reliable method is by means of Mayer's (1883) albumin-glycerin mixture, which is composed of equal parts of white of egg and of glycerin. The mixture should be thoroughly beaten and then filtered through absorbent cotton or filter paper. The process is hastened by filtering in the paraffin oven (54° C.). A small lump of camphor or of thymol should be added to prevent decomposition due to growth of microorganisms. Although it is stated that egg albumin is dissolved by alkalies and acids we have never experienced any trouble with the borax methylene blue solution or the acids used in decolorizing sections stained for the tubercle bacillus—to cite two examples.

Another method of attaching paraffin sections to the slide is by means of capillary attraction. It is the ideal method but is not so

reliable as when the slide is coated with albumin-glycerin. Use is made of distilled or tap water, or better still perhaps of 30 per cent alcohol. The slide must be absolutely clean. The strip of paraffin sections is flattened out on the surface of warmed water, or 30 per cent alcohol, in the usual way, the slide is slipped under it, lifted up and the fluid drained off. Evaporation of the fluid causes the sections to stick fast to the slide. The process is hastened by putting the slide into a drying oven with a temperature of 40° to 45° C. The incubator at 37° C. is not warm enough and the paraffin oven at 54° C. is too hot.

A third method recommended is to use Schällibaum's (1883) solution of celloidin 1 part in 3 to 4 parts of oil of cloves. Cover the slide with a thin layer of the solution. Arrange the sections in order on the slide and place it in the paraffin oven at 54° to 60° C. for several hours, or heat for a few seconds to half a minute over the flame until the oil of cloves runs together in drops. After cooling, remove the paraffin with xylol. As absolute alcohol must be avoided, pass through oil of origanum to 95 per cent alcohol and proceed as with other paraffin sections.

Masson's (1928) gelatin method of attaching paraffin sections to slides has been found useful where warm alkaline silver solutions are employed. Sections so treated seldom float off the slide. Dissolve 0.05 gm. of gelatin (ordinary sheet gelatin 5 mm. square) in 20 cc. of distilled water, warming it over the flame. Place slides on a warm plate, filter a large drop of the gelatin solution on each slide and float the paraffin section on it. As soon as the section spreads out, stand slide up to drain, holding the section in place with a brush or needle. When the excess gelatin has drained off (do not allow the section to dry), blot and place at once in the oven at 40° to 50° C. in formaldehyde vapor. The formaldehyde vapor is secured by placing an open dish of formalin together with a staining dish containing the slides inside a large covered culture dish. For staining with hematoxylin and aniline dyes 20 minutes in the hot formaldehyde vapor suffice; for silver staining the slides should be left for several hours or overnight.

Serial Sections by the Paraffin Method.—To obtain serial sections by the paraffin method it is necessary only to avoid losing any of the sections from the ribbon as ordinarily cut. Perhaps the easiest and safest way is to cut long ribbons and to place them on

sheets of paper in the proper order. They can then be divided readily by means of a scalpel into a short series of any desired number of sections and fastened to numbered slides by means of Mayer's albumin-glycerin mixture.

2. CELLOIDIN METHOD

Embedding in Celloidin.—Perfectly satisfactory celloidin of various makes is obtainable in jars, containing each about 28 gm., in the form of dry strips which can be put immediately into a mixture of equal parts of absolute alcohol and ether. One jar of celloidin to 350 cc. of absolute alcohol and ether will yield approximately an 8 per cent solution. This is the so-called "thick celloidin" and can be diluted to make a 4 per cent solution (thin celloidin) as desired. A preserving jar makes an excellent container and the celloidin goes into solution fairly readily if the jar is inverted from time to time or if the contents is stirred with a glass rod.

The steps of the celloidin embedding process are as follows: Pieces of tissue which have been fixed and finally preserved in 80 per cent alcohol are first to be cut up intelligently. They should rarely be over 2 to 4 mm. thick; for most purposes 2 mm. will be sufficient. Pieces of this thickness will furnish a hundred sections or more, will embed more quickly than larger pieces, and will be more rigid when mounted on a block. They should never be broader or longer than is necessary to show the whole process to be studied. Very thin celloidin sections cannot usually be obtained with tissues over 1.5 to 2 cm. square, and smaller dimensions are preferable. Beginners usually embed larger pieces than necessary.

The trimmed pieces of tissue are first hardened and dehydrated for 24 hours in 95 per cent alcohol, followed by 24 hours in absolute alcohol; then soaked in equal parts of absolute alcohol and ether for the same length of time to prepare them for the thin celloidin. In the latter they remain at least 24 hours, preferably for a number of days, if at all thick, for in this solution occurs most of the infiltration with celloidin. Finally, the tissues are soaked 24 hours or more in thick celloidin. They are then mounted on blocks of vulcanized fiber, placed in chloroform for 1 to 2 hours, and then transferred to 80 per cent alcohol.

Briefly summed up, the steps of embedding in celloidin are as follows:

1. Alcohol, 95 per cent (2 changes)	24 hrs.
2. Alcohol, absolute (2 changes)	24 hrs.
3. Alcohol, absolute, and ether (equal parts)	24 hrs.
4. Celloidin, thin	24 hrs. to 1 or more weeks
5. Celloidin, thick	
6. Mount on blocks of vulcanized fiber	
7. Harden celloidin in chloroform for 1 to 2 hours, followed by 80 per cent alcohol	

The quick embedding method used for surgical and other tissues shortens the various steps to a minimum. The solutions must be changed frequently owing to absorption of fat which softens the celloidin.

1. Fix thin pieces of tissue in the formalin-alcohol mixture	12–18 hrs.
2. Alcohol, 95 per cent (2 changes)	2 hrs.
3. Alcohol, absolute (2 changes)	3 hrs.
4. Alcohol, absolute, and ether (equal parts)	3 hrs.
5. Celloidin, thick	12–15 hrs.
6. Mount on blocks of vulcanized fiber and harden in chloroform for 1 hour followed by 80 per cent alcohol	

Cut and stain sections with alum hematoxylin and dilute eosin or phloxine, and mount as usual.

Instead of mounting directly from the thick celloidin it is sometimes advisable to allow the celloidin to evaporate until a firm mass is obtained. This is particularly true when very loose tissues are to be embedded.

The simplest method is to place the pieces of tissue, which have been soaking in thick celloidin, in proper position in a glass dish and pour thick celloidin over them. The dish is then covered, but not too tightly, and the ether is allowed to evaporate for 2 or more days until the proper consistence of celloidin is reached, so that it can be cut out in blocks enclosing the specimens. If the ether evaporates too rapidly place a large dish or a bell-jar over the

covered dish. Mount the blocks, after they have been cut out and trimmed, by dipping the bases in thick celloidin and then pressing them onto blocks of vulcanized fiber. Place in chloroform for 1 to 2 hours and then transfer to 80 per cent alcohol.

Cutting of Celloidin Sections.—After the celloidin mounts have been in 80 per cent alcohol for from 1 to several hours, the celloidin is of the proper consistence for cutting. It is best to take a sharp knife and trim the top of the celloidin down to where the first good section of the specimen can be cut; this will save considerable wear on the microtome knife.

In cutting, the microtome knife should be fastened obliquely, so that as much of the edge of the knife as possible shall be used in making each section. The surface of the knife should be kept wet with 80 or 95 per cent alcohol, preferably from an overhanging dropping-bottle. Celloidin sections should be cut as thin as possible, from 10 to 16 μ for tissues in general. Sections of bone often have to be cut as thick as 20 to 22 μ in order to get sections of the whole block of tissue.

If the sections curl, as often happens when they are thin, they are best flattened by unrolling them on the surface of the knife with a camel's hair brush just before the last edge of celloidin is cut through, as this serves to keep them fixed in place during the process. This method can be used when the simple transferring of sections from alcohol to water is not sufficient to uncurl them.

Celloidin sections can be stained by nearly all methods, without the necessity of removing the celloidin. When necessary, however, the celloidin is readily removed by transferring the sections from absolute alcohol into oil of cloves or into a solution of equal parts of absolute alcohol and ether for 5 to 10 minutes, and then passing them back through absolute into 95 per cent alcohol.

Serial Sections by the Celloidin Method.—1. With a little care perfect serial sections can be made by the following method, and each slide of sections can be stained in whatever way seems best. The specimen is embedded, mounted on vulcanized fiber, and hardened in chloroform followed by 80 per cent alcohol in the usual way. In cutting, moisten the microtome knife with 95 per cent alcohol. As the sections are cut they are drawn up on the surface of the knife and arranged in regular order by means of a camel's hair brush until a slideful is ready. They are then drawn on a clean

and numbered slide held against the back of the knife. After being carefully arranged the sections are fastened to the slide by means of ether vapor poured over them from a half-full bottle. Care must be taken that the entire edge of the celloidin is fully softened down. The slides are then placed in a jar of 80 per cent alcohol to be stained at leisure.

2. Rubaschkin (1907), Dantschakoff (1908) and Maximow (1909) recommend the following procedure: Slide the sections and as little of the 70 per cent alcohol as possible from the cutting knife to a slide on which has been spread a small drop of Mayer's albumin-glycerin mixture. Then press the sections down with smooth folded filter paper and cover with pure oil of cloves which will clear the sections in 5 to 20 minutes. Drain off the oil and pass the sections slowly through 3 changes of absolute alcohol (5–10 minutes). Place in alcohol and ether, equal parts, to complete the removal of celloidin and then transfer to 70 per cent alcohol before staining.

3. Another method, often convenient where the stain is of little importance, is as follows: The tissue is stained in bulk in alum cochineal, or some other staining fluid that will penetrate, and then embedded in celloidin in the usual way. After being mounted on vulcanized fiber the specimen is hardened in chloroform. From the chloroform it is transferred to oil of thyme. After it is thoroughly penetrated by the latter it is ready to be cut. The knife is to be moistened with oil of thyme. The sections as cut are arranged on the knife. The slides covered with sections can be placed under a bell-jar as fast as they are ready until all are cut, because the oil of thyme evaporates slowly. Balsam and coverslips can be added after the cutting is finished.

4. Darkshevitch's method is to prepare a series of circles of filter paper cut to fit a wide-mouthed bottle or jar and to number the papers consecutively. As each section is cut it is placed first in water to flatten it out and then in alcohol. It is then transferred by means of a spatula to its proper place on a numbered paper. The papers are piled up in numerical order in alcohol in the jar and can be kept indefinitely. It is comparatively easy to trace a lesion if desired and numbered sections can be removed and stained individually in flat dishes. Frequently every tenth or twentieth section is stained first and in this way much time can be saved.

5. Weigert's (1885) method for a series of celloidin sections was designed especially for the nervous system and is rather complicated. The process depends on transferring the sections to narrow strips of tissue paper. To do this each section as cut is arranged in proper position close to the edge of the knife. Then a strip of tissue paper twice as wide as the section is gently placed on it and the sections withdrawn from the knife. The success of the process depends on having but little alcohol on the knife, otherwise the sections will not stick. Each section is placed on the paper to the right of the last one. The strips of paper when full are kept moist by being placed with the sections uppermost on a moist surface composed of a layer of blotting paper wet with alcohol, covered with a sheet of tissue paper, and lying in a shallow dish.

When all the sections have been cut each strip of them is taken in turn and coated on both sides with a thin film of celloidin in the following way: A strip with the sections below is first pressed gently down on the surface of a slide covered with a thin layer of celloidin. This fastens the sections and the paper can be removed. Then a thin coat of celloidin is poured over the sections and the slide is placed on its edge to drain. When the surface of the celloidin is dry the strips can be marked by a fine brush dipped in methylene blue. As soon as the slides are placed in the staining solution the celloidin peels off, taking the sections with it. Later the strips of sections can be divided as desired. On account of their thickness they should be cleared, after dehydrating in 95 per cent alcohol, in a mixture of xylol 3 parts, and carbolic acid crystals 1 part.

6. Verhoeff (1907) recommends that in cutting the sections the knife should not be carried entirely through the celloidin block and an uncut edge, about 3 mm. wide, should be left each time. After 20 or more sections are cut in this way the knife is carried all the way through, thus producing a little book of sections. It is probably most convenient to keep each book in a separate bottle, but no difficulty is usually experienced in determining the proper order after the sections are mounted. Another way to keep them in order is to string them on a silk thread through their uncut margins. In beginning a new book a wider margin should be left for the first one or two sections, as otherwise the sections may not adhere, or the first section may be cut at double thickness. Each book is stained

in the same manner as a single section, except that it is best to use slowly acting stains so that the staining will be uniform throughout. The individual sections are not separated until the book is in alcohol preliminary to clearing. Then each section is either torn off with forceps, or the book is taken up on cigarette paper and the uncut margin removed with scissors. Each section in order is then removed, cleared quickly in oil of origanum, and placed on a slide.

7. Suzuki (1909) recommends spreading the sections out on a slide or glass plate, blotting the celloidin at one corner of the section, and marking the number of the section on it with India ink by means of a fine pointed brush. The sections are placed in 80 per cent alcohol after marking.

Attachment of Celloidin Sections to the Slide.—A celloidin section can be fairly well attached to a slide by transferring it from water to a slide freshly washed in alcohol and dried with a cloth. The section should then be blotted firmly with filter paper so as to apply it closely to the slide and to remove all wrinkles. It should not be allowed to dry. A section treated in this way will ordinarily stand considerable manipulation without becoming loose.

Celloidin sections can be more securely attached by transferring them from 95 per cent alcohol to clean slides and pouring over them ether vapor from a bottle half full of ether. With a little practice sections can be fastened in a few seconds. Follow slowly along the edge of the celloidin and the frills in it will soften down. Then dip the specimen in 80 per cent alcohol to harden the celloidin.

Another excellent method is that described for fixing frozen sections to the slide.

CHAPTER VI

DYES, SOLUTIONS AND NUCLEAR STAINING METHODS

1. GENERAL DISCUSSION

Dyes and staining methods are of the greatest importance in histological work, and yet most of what is known about them has been obtained empirically. Accidental observations have been the starting point of much of our information and have opened the way for the application of knowledge and training and for experimental work in many directions.

Dyes are ordinarily divided into two classes—the natural and the artificial. The natural dyes were used first and are few in number, the most important being hematoxylin, carmine and orcein. The artificial or coal tar dyes came into use later, are already very numerous and are steadily increasing in number. It is doubtful, however, if they ever entirely replace the natural stains because the latter have certain valuable qualities of their own.

The staining of tissue elements and pathological products depends on a variety of conditions which are mostly chemical or physical in nature. The staining of iron by the Berlin blue method is purely chemical, as is also the staining of iron, copper and lead by hematoxylin. On the other hand, the staining of fat by Sudan III and IV is due entirely to a physical property. Fat absorbs these dyes. The staining of nuclei by the dye hematoxylin is probably chemical in nature, due to the presence in them of metals (iron, aluminum, lead). Staining with alum hematoxylin, on the other hand, is probably not a chemical process.

The purpose of staining is to render prominent the different tissue elements so that they may be readily recognized and studied. The constant tendency nowadays is toward selective or differential staining methods, by which but one tissue element will be colored to the exclusion of all others, or at least of any element that might be confused with it morphologically. These selective stains enable us to differentiate from each other with ease and accuracy cellular and intercellular elements or pathological products which otherwise look alike.

The list given below does not pretend to be either complete or perfect in arrangement, but will give some idea of the various elements we wish to stain.

- **Cell**
  - **Nucleus**
    - Chromatin (chromosomes)
    - Nucleolus
    - Linin (spindle)
  - **Cytoplasm**
    - Centriole
    - Cilia
    - Golgi apparatus
    - Mitochondria (plastosomes)
    - Myelin
    - Dendritic processes of ganglion cells
    - Neurofibrils
    - Axis cylinders and terminal processes
    - Myoglia fibrils (smooth muscle)
    - Myofibrils (striated muscle; anisotropic and isotropic disks)
    - Fibroglia fibrils
    - Neuroglia fibrils
    - Epithelial fibrils
    - Erythrocytes
    - Blood platelets and megakaryocytes
    - **Granules**
      - Leukocytes, 5 different types
      - Tissue eosinophiles
      - Mast cells
      - Plasma cells
      - Parietal cells (stomach)
      - A, B and C islet cells (pancreas)
      - Zymogen granules (gastro-intestinal tract, pancreas)
      - Basophilic, acidophilic and chromophobic cells (pituitary gland)
      - Nissl bodies (ganglion cells)
- **Intercellular substances**
  - Cement substance of epithelial and endothelial cells
  - Ground substance of connective tissue
  - Collagen fibrils and reticulum
  - Mucous connective tissue, mucin
  - Elastic fibrils
  - Intercellular substance of cartilage
  - Ground substance of bone
- **Normal and pathological tissue constituents**
  - Glycogen
  - Mucin
  - Keratohyalin
  - Eleidin
  - Colloid
  - Amyloid
  - Hyalin
  - Fat
  - Fibrin
  - Melanin
  - Hemoglobin
  - Hemofuscin and copper hemofuscin
  - Hemosiderin
  - Hematoidin
  - Bile
  - Uric acid
  - Calcium

The simplest stain is, of course, that for nuclei, and it can be obtained with a great variety of staining reagents. The most diffi-

cult element heretofore to stain differentially has probably been the axis cylinder, but it is now easily stained in properly fixed and mordanted tissues by means of the lead chloride hematoxylin method.

Tissue elements and pathological products differ from each other not only in form and consistence but also in chemical properties. While perfect preservation of form is sufficient to distinguish certain cells or elements from each other, for instance, polymorphonuclear leukocytes from lymphoid cells, differentiation based on distinctive staining methods is always to be preferred when possible. A few of the tests employed are colorless, such as the precipitation of mucin by acetic acid. Certain tests, for instance the methylene blue stain for axis cylinders, can be applied to fresh tissues only. Others, such as various amyloid reactions, can be obtained with fresh or hardened tissues. Most of the differential staining methods, however, can be employed only with tissues that have been properly preserved. It is exceedingly important, therefore, that a tissue element should be so fixed and hardened that its peculiar chemical or physical properties are preserved intact, otherwise a differential stain for it is impossible. Each tissue element is a law unto itself. For example, certain peculiar chemical properties of red blood corpuscles depend on the presence in them of hemoglobin. As a differential stain of the red blood corpuscles depends on fixing this substance in them, it is necessary to find out the chemical properties of hemoglobin, such as the fact that it is soluble in water or dilute alcohol but not in salt solution, and that it is fixed in the red blood corpuscles by heat, absolute alcohol and ether (equal parts), corrosive sublimate, formalin, potassium bichromate, and so on.

While differential stains depend in part on the chemical and physical properties of the tissue elements, they depend to a certain extent on the chemical properties of the staining reagents and the decolorizers used.

Some of the tissue elements can be stained differentially in a number of ways, sometimes after one fixing agent, sometimes after another. The simplest differential stains are those where certain tissue elements stain directly in a given solution after they have been properly fixed. Excellent examples are Ehrlich's triple stain for certain cytoplasmic granules in leukocytes, and the direct stain for elastic fibers with an acid alcohol solution of orcein.

Other differential stains depend on the property of certain ele-

ments to retain colors they have once taken up when treated with decolorizers. The best example of this is the tubercle bacillus, which holds certain stains through various acids followed by alcohol and, if necessary, through a contrast stain.

Still another varied group of elements (certain bacteria, fibrin, neuroglia fibers) depend for a differential stain in part on changes produced in crystal violet by iodine and in part on the decolorizer employed for extracting the coloring reagent.

Although the steps of the various staining methods differ considerably, they may be roughly arranged in the following order:

1. Staining.
2. Differentiating.
3. Decolorizing.
4. Dehydrating.
5. Clearing.
6. Mounting.

Very often two or more of the steps are combined in one, as when aniline is used for decolorizing, dehydrating and clearing sections stained for certain bacteria. Sometimes the staining process occupies more than one step, as in Weigert's myelin sheath stain. In alum hematoxylin the differentiating reagent, the excess of alum, is combined with the stain; in Gram's method the differentiating reagent, iodine, forms a step by itself.

In order to avoid repetition, staining methods of general application are given under the important dye used, but methods of special application only will be found under the tissue element or pathological product for which they are designed.

The following steps are essential before staining cut sections of paraffin embedded tissues.

1. Pass slides through several changes of xylol to remove the paraffin. At least 2 changes are essential.

2. Absolute alcohol, 2 changes to remove xylol.

3. Place sections in 1 or 2 changes of 95 per cent alcohol.

4. If sections have been fixed in Zenker's fluid, or any other fixative containing mercuric chloride, place in a 0.5 per cent solution of iodine in 95 per cent alcohol for 5 to 10 minutes to remove the mercuric deposit.

5. Wash in water.

6. Remove iodine by several changes of 95 per cent alcohol or by treating with a 0.5 per cent aqueous solution of sodium thiosulfate ("hypo") for 5 minutes. The latter method is quicker and also cheaper.

7. Wash slides thoroughly in water.

The sections are now ready for any stain that may be desired. For stains made up with alcohol instead of water, it is sometimes desirable to place sections in 95 per cent alcohol before pouring on the stain.

2. NATURAL DYES

(1) Hematoxylin and Hematein

Hematoxylin is one of the most important and valuable staining reagents we have in histological work owing to the fact that it and its oxidized derivative hematein have the property of combining with various metals to form colored compounds. Some of these are soluble in water or other fluids and some are not. A few are valuable as staining reagents and have long been in use in the laboratory—for example, the combinations of hematein with aluminum, iron, chromium, copper and tungsten. The staining properties of the compounds depend both on the metals and on the salts of them employed.

The active coloring agent in most hematoxylin stains is hematein, which is gradually formed in the ordinary solutions from hematoxylin by oxidation, a process occupying a number of days or weeks and spoken of as "ripening." This ripening can be done almost instantaneously by the use of certain oxidizing reagents such as hydrogen peroxide, potassium permanganate, sodium iodate, sodium perborate and mercuric oxide, or, according to Neild (1934), by exposure in an open dish to a Cooper-Hewitt burner.

The various tissue elements and pathological products which hematoxylin and hematein in combination with certain metals can stain and the metals needed are as follows:

Nuclei	aluminum, iron, tungsten
Myelin sheaths	chromium, copper, iron
Elastic fibers	iron
Collagen	molybdenum
Fibroglia, myoglia, neuroglia and epithelial fibrils	tungsten
Axis cylinders	lead
Mucin	iron
Fibrin	tungsten

The oldest and most commonly used staining compound of hematoxylin is that with aluminum and it is as useful today as when first discovered. It is made up as follows:

Aqueous Alum Hematoxylin.—

Hematoxylin	1 gm.
Ammonium or potassium alum	20 gm.
Water, distilled	400 cc.
Thymol	1 gm.

The hematoxylin crystals are dissolved in 100 cc. of water by the aid of gentle heat and then added to the alum dissolved in the rest of the water. After the addition of thymol to prevent the growth of mold, the combined solution is exposed to air and light in a flask or bottle lightly stoppered with a plug of cotton. The solution will be ripened sufficiently for use in about 10 days, after which time it should be kept in a tightly stoppered bottle. The solution is very easily prepared, gives excellent results, and will keep at its best for 2 to 3 months.

More alum than is needed to combine chemically with the hematoxylin is always added to the solution for the reason that it acts as a differential decolorizer, limiting the stain largely to the nuclei of the cells. As alum hematoxylin solutions become older they stain more quickly but also more diffusely. The diffuseness of staining can be counteracted by adding enough alum water (5 per cent solution) to make the stain precise again. A good alum hematoxylin solution ought not to stain the celloidin in which a section is embedded. If the celloidin stains more or less deeply it shows that the solution requires more alum. The above solution can be ripened at once by adding to it 0.177 gm. of potassium permanganate or 17.7 cc. of a 1 per cent aqueous solution.

The simple aqueous alum hematoxylin solution is not very stable. In a large laboratory where much of it is used it is convenient to make up a supply every month or so, let it ripen spontaneously and use it while it is at its best. The hematoxylin present keeps ripening into hematein and then into a higher oxidized product which is precipitated, in consequence of which it is advisable always to filter the solution just before using it.

Various reagents have been added to alum hematoxylin solu-

tions in order to improve their keeping qualities but without great success. The best seem to be alcohol, glycerin and certain acids. The simple solution gives the richer staining, the acid solutions the sharper nuclei but little else. Some of them, such as the simple aqueous solution, require an antiseptic to prevent the growth of mold in the alum solution.

Method of Staining.—1. Stain formalin- or alcohol-fixed tissues in one of the hematoxylin solutions 1 to 5 minutes or sometimes even longer. Sections of Zenker-fixed tissue usually require at least 1 hour or longer.

2. Wash in several changes of tap water and then leave sections, if possible, for several hours or overnight in several changes of water, or better still, wash in running tap water for 10 to 30 minutes.

3. If a contrast stain is desired use a 0.1 to 0.5 per cent solution of phloxine or eosin in 25 per cent alcohol for 1 to 5 minutes.

4. Wash in water.

5. Differentiate in several changes of 95 per cent alcohol to remove excess of contrast stain.

6. Dehydrate in absolute alcohol if paraffin sections are used.

7. Clear in xylol and mount in balsam.

If celloidin sections are used transfer directly from 95 per cent alcohol to terpineol or oil of origanum and after blotting section on slide mount in balsam.

If overstaining of the tissue has occurred decolorize in 1 per cent alum water, 1 per cent acetic acid, or in acid alcohol, followed by thorough washing in tap water.

Results.—Nuclei bright blue; cartilage dark blue. Mucin tends to stain blue, especially after fixation in absolute alcohol. All other structures are stained a bright pink.

Several other standard solutions which have been much used and liked are as follows:

Delafield's Alum Hematoxylin (1885).—

Hematoxylin	4 gm.
Alcohol, 95 per cent	25 cc.
Ammonium alum, saturated aqueous solution (about 15 per cent)	400 cc.

Add the hematoxylin dissolved in the alcohol to the alum solution and expose the mixture in an unstoppered bottle to light and air for 3 to 4 days. Filter and add:

Glycerin	100 cc.
Alcohol, 95 per cent	100 cc.

Allow the solution to stand in the light until the color is sufficiently dark, then filter and keep in a tightly stoppered bottle. The solution keeps well and is extremely powerful. As it deteriorates it acquires a reddish tint. It would seem advisable, both in this solution and in Ehrlich's, to combine the alum, hematoxylin and water, and to ripen the solution for 2 to 3 weeks before adding the other ingredients which have a tendency to prevent oxidation. A fully ripened solution would then be obtained more quickly and surely.

The more customary method of using Delafield's alum hematoxylin solution is to filter a few drops of it into a dish of tap water and to stain sections for a long time, even overnight, with the very dilute solution thus obtained. It is sometimes advisable to use the aqueous alum solution in the same way.

Harris' Alum Hematoxylin (1900).—

Hematoxylin	1 gm.
Alcohol, absolute	10 cc.
Ammonium or potassium alum	20 gm.
Water, distilled	200 cc.
Mercuric oxide	0.5 gm.

Dissolve the hematoxylin in the alcohol, the alum in the water by the aid of heat, and mix the two solutions together. Bring the mixture to a boil as rapidly as possible and then add the mercuric oxide. The solution at once assumes a dark purple color. As soon as this occurs remove the vessel containing the solution from the flame and cool by plunging at once into a basin of cold water. As soon as it is cool the solution is ready for staining. This solution keeps for years in a tightly stoppered bottle, according to Harris. The addition of 4 per cent of glacial acetic acid increases the precision of the nuclear staining.

Ehrlich's Acid Alum Hematoxylin (1886).—

Hematoxylin	2 gm.
Alcohol, 95 per cent	100 cc.
Water, distilled	100 cc.
Glycerin	100 cc.
Ammonium or potassium alum	3 gm.
Acetic acid, glacial	10 cc.

Dissolve the hematoxylin in the alcohol and then add the other ingredients. Ripen by exposure to the air in a paper-capped vessel for 2 weeks or longer, agitating frequently. Tightly corked in a bottle it will keep for a long time. It may be ripened at once by the addition of 0.4 gm. of sodium iodate ($NaIO_3$).

The solution gives a sharp nuclear stain but sections must be washed thoroughly in tap water to remove all traces of acetic acid and to bring out the desired clear blue color.

Mayer's Acid Alum Hematoxylin (1891).—

Hematoxylin	1 gm.
Water, distilled	1000 cc.
Sodium iodate	0.2 gm.
Ammonium or potassium alum	50 gm.
Citric acid	1 gm.
Chloral hydrate	50 gm.

Dissolve the hematoxylin in the water, using gentle heat if necessary, then add the sodium iodate and the alum. Shake occasionally until the alum is dissolved, then add the citric acid and the chloral hydrate. The color turns a reddish violet. The solution keeps a long time without overripening. This is Mayer's latest alum hematoxylin solution. He prefers it to his hemalum solution, made with hematein, because hematoxylin is more dependable than its ripened derivative.

The principle of the staining method with the various solutions of alum hematoxylin is the same, but the time required depends on the ripeness of the solution and on the fixative used. It varies from 1 to 2 minutes or longer after alcohol or formalin to 1 hour or longer after Zenker's fluid. The washing in tap water must be thorough, especially if the staining solution contained an acid.

Alum hematoxylin is used chiefly as a nuclear stain, either alone,

or followed by phloxine, eosin or van Gieson's solution. It is also useful for staining nuclei in contrast with fat and tubercle bacillus stains.

Weigert's Iron Hematoxylin (1904).—*Fixation.*—Any fixative may be used.

Staining Solution.—

Solution A

Hematoxylin	1 gm.
Alcohol, 95 per cent	100 cc.

Solution B

Iron chloride (29 per cent aqueous solution)	4 cc.
Water, distilled	95 cc.
Hydrochloric acid	1 cc.

For use mix equal parts of A and B. The mixture will turn a deep black and is best prepared fresh each time, although it will keep and can be used for several days.

Method of Staining.—1. Stain sections for several minutes or longer.

2. Wash in water.

3. If a counterstain is desired, place sections for a few seconds in van Gieson's solution (p. 92).

4. Wash in water.

5. Differentiate in 95 per cent and dehydrate in absolute alcohol.

6. Clear in xylol and mount in balsam.

If celloidin sections are used transfer to terpineol or oil of origanum directly after 95 per cent alcohol and after blotting section on slide mount in balsam.

Results.—Nuclei black; connective tissue red; cytoplasm yellowish.

Heidenhain's Iron Hematoxylin (1896).—*Fixation.*—Corrosive sublimate, Zenker's fluid, alcohol or other fixatives. Embed in paraffin. Sections should not be cut over 5 μ thick.

Staining Solutions.—

Ammonio-Ferric Alum

Ammonio-ferric alum	2.5 gm.
Water, distilled	100 cc.

Alcoholic Hematoxylin

Hematoxylin	0.5	gm.
Alcohol, 95 per cent	10	cc.
Water, distilled	90	cc.

Dissolve the hematoxylin in the alcohol and add the water. Place in a bottle with a cotton plug and allow the solution to ripen for 4 to 5 weeks. For use dilute with equal parts of distilled water. The solution may be used over and over again.

Method of Staining.—1. Mordant the sections in the ammonio-ferric alum solution for 3 to 12 hours. The sections should be placed vertically in the solution so that no precipitate will fall on them.

2. Wash quickly in water.

3. Stain for 1 to 36 hours in the alcoholic hematoxylin solution.

4. Wash in water.

5. Differentiate in the ammonio-ferric alum solution, controlling the results with the microscope. The section should be rinsed before each examination in tap water, which immediately stops the decolorization.

6. Wash in running water for 15 to 60 minutes.

7. If desired, sections may be counterstained in a 0.1 per cent aqueous solution of rubin S (acid fuchsin) or of orange G or of light green for 5 to 15 minutes. Van Gieson's stain (p. 92) or a simple aqueous solution of eosin may also be used.

8. Rinse in 50 per cent alcohol.

9. Differentiate and dehydrate in 95 per cent alcohol, followed by absolute alcohol.

10. Clear in xylol and mount in balsam.

Results.—Chromatin, nucleoli, mitochondria, centrioles and certain parts of striated muscle fibers are stained black. Other tissue elements are colored by the contrast stain used.

Mallory's Iron Chloride (Ferric) Hematoxylin (1900).—*Fixation.*—Any fixative may be used. Embed in paraffin or celloidin.

Staining Solutions.—

Iron Chloride

Iron chloride (ferric)	5	gm.
Water, distilled	100	cc.

Hematoxylin

Hematoxylin	0.5 gm.
Water, distilled	100 cc.

The solution must be prepared fresh each time.

Method of Staining.—1. Mordant sections for 1 hour or longer in the iron chloride solution.

2. Rinse in 2 to 3 changes of tap water.

3. Stain for 1 hour or longer in the hematoxylin solution, until the sections are a deep blue-black.

4. Rinse in water and decolorize in a 0.25 per cent aqueous solution of iron chloride (ferric) moving the sections constantly and controlling the process with the microscope.

5. Wash thoroughly in tap water and counterstain if desired.

6. Dehydrate in 95 per cent alcohol followed by absolute alcohol for paraffin sections.

7. Clear in xylol and mount in balsam.

If celloidin sections are used clear in terpineol or oil of origanum directly after 95 per cent alcohol and after blotting on the slide mount in balsam.

Results.—Nuclei deep blue; other tissue elements colorless or stained by the counterstain used. In the common type of cirrhosis the hyalin in the liver will stain blue-black, if not too old.

Mallory's Phosphotungstic Acid Hematoxylin (1900).—*Fixation.*—Zenker's fluid is preferred for general use. Formalin- or alcohol-fixed tissues can also be used for certain purposes.

Staining Solution.—

Hematoxylin	1 gm.
Phosphotungstic acid	20 gm.
Water, distilled	1000 cc.

Dissolve the solid ingredients in separate portions of the water, the hematoxylin with the aid of gentle heat. When cool, combine. No preservative is necessary. Spontaneous ripening requires several weeks but can be accomplished at once by adding 0.177 gm. of potassium permanganate.

Method of Staining.—If Zenker-fixed tissues are used treat sections with iodine in the usual way in order to remove the mercury

precipitate and then extract the iodine by means of alcohol or a 0.5 per cent solution of sodium thiosulfate for several minutes. Rinse thoroughly in water.

1. Place sections in a 0.25 per cent aqueous solution of potassium permanganate for 5 to 10 minutes.
2. Wash in water.
3. Oxalic acid, 5 per cent aqueous solution, 10 to 20 minutes.
4. Wash thoroughly in several changes of water.
5. Stain in phosphotungstic acid hematoxylin for 12 to 24 hours.
6. Transfer sections directly to 95 per cent followed by absolute alcohol for paraffin sections. Dehydrate quickly because alcohol readily extracts the red part of the stain.
7. Clear in xylol and mount in balsam.

If celloidin sections are used, clear with xylol from 95 per cent alcohol by means of the filter paper blotting method. Origanum and other oils cause the blue stain to fade.

Results.—Nuclei, centrioles, achromatic spindles, and fibroglia, myoglia and neuroglia fibrils, fibrin and the contractile elements of striated muscle are stained blue; collagen (including reticulum) and the ground substances of cartilage and bone stain varying shades of yellowish to brownish red. Coarse elastic fibrils are sometimes colored a purplish tint.

(2) Cochineal, Carmine and Carminic Acid

Cochineal, a deep red dye, is extracted from the dried bodies of female cochineal insects. From it is manufactured carmine, which consists of the dye combined with aluminum, calcium and protein. The active staining principle of cochineal and of carmine is carminic acid which can be obtained in pure condition and which forms readily soluble salts with alkali metals, and insoluble salts with the heavy metals, especially aluminum. Staining solutions with somewhat different properties can be formed from all three dyes, but nowadays carmine is used chiefly.

Carmine solutions are used generally for four purposes: (1) as a stain for nuclei in bulk (a method of much less value to the pathologist than to the embryologist); (2) to stain nuclei red in contrast with blue stains for iron; (3) to stain glycogen; and (4) as a stain for mucin.

Carmine solutions give good nuclear stains, but of the finer de-

tails in a specimen they bring out distinctly less than a direct alum hematoxylin stain. They are much less used now than formerly for nuclear stains, except as a contrast for bacteria and fibrin in the methods of Gram and Weigert, for which purpose lithium carmine will usually give the best results, and as a contrast with the elastic tissue stain of Weigert.

Many formulas for carmine stains have been devised. The following seem sufficient to satisfy a pathologist's needs. Some of them stain progressively and sharply; others overstain and have to be differentiated in an alum solution or in a weak acid.

Grenacher's Alum Cochineal.—

Powdered cochineal	6 gm.
Ammonium alum	6 gm.
Water, distilled	100 cc.
Thymol	1 gm.

Boil for 30 minutes and then add water to make up for that lost by evaporation. Filter and add the thymol.

Grenacher's Alum Carmine (1879).—

Carmine	2 gm.
Ammonium alum	3–5 gm.
Water, distilled	100 cc.
Thymol	1 gm.

Combine and boil steadily for 1 hour, adding enough water to make up for that lost by evaporation. When cool, filter and add the thymol to prevent the growth of mold.

This stain is useful both for sections and for tissues in bulk.

Mayer's Carmalum (1892).—

Carminic acid	1 gm.
Ammonium or potassium alum	10 gm.
Water, distilled	200 cc.
Salicylic acid	0.2 gm.

Combine and dissolve, using heat if necessary. After cooling add the salicylic acid to prevent the growth of mold.

Stain as below. If the nuclei are not sharp enough differentiate in a 0.5 to 1 per cent aqueous solution of alum, a few seconds to minutes for sections, 12 to 24 hours for tissue in bulk, followed by thorough washing in water.

Any of the above solutions may be used in the method of staining given below.

Fixation.—Tissues should be fixed in alcohol or formalin.

Method of Staining.—1. Stain celloidin or paraffin sections for 5 to 20 minutes.

2. Wash thoroughly in water.

3. Dehydrate in 95 per cent and absolute alcohol for paraffin sections.

4. Clear in xylol and mount in balsam.

If celloidin sections are used clear in terpineol or in oil of origanum after 95 per cent alcohol.

Results.—Nuclei are stained a brilliant red. Overstaining does not occur.

These solutions cannot be recommended for tissues that stain with difficulty. When used for staining in bulk 24 to 48 hours are required.

Grenacher's Alcoholic Borax Carmine (1879).—

Carmine	2–3 gm.
Borax	4 gm.
Water, distilled	100 cc.
Alcohol, 70 per cent	100 cc.

Grind up the carmine and the borax in a mortar and then boil in the water for 30 minutes until dissolved. After cooling add the alcohol. Shake occasionally during a period of several weeks, then decant and filter.

This solution is useful for staining in bulk. The tissues, fixed, washed and dehydrated, are transferred from 80 per cent alcohol into the solution for 1 to 3 days, then transferred directly to 70 per cent alcohol containing 0.25 to 0.5 per cent hydrochloric acid until no more clouds of color are given off (shaking occasionally during the 1–3 days), and are then washed out in acid-free 70 per cent alcohol. The tissues are then ready for embedding.

Mayer's Alcoholic Carmine (Paracarmine) (1892).—

Carminic acid	1 gm.
Aluminum chloride	0.5 gm.
Calcium chloride	4 gm.
Alcohol, 70 per cent	100 cc.

Dissolve cold or with gentle heat; allow the solution to settle and then filter.

Stain for 15 to 30 minutes and without washing in water differentiate in 70 per cent alcohol, to which is added 2.5 per cent glacial acetic acid if a more purely nuclear stain is desired.

Staining in bulk requires 24 to 48 hours.

Orth's Lithium Carmine (1900).—*Fixation.*—Formalin or alcohol.

Staining Solution.—

Carmine	2.5–5 gm.
Lithium carbonate, saturated aqueous solution (1.25 gm.)	100 cc.
Thymol	1 gm.

Dissolve the carmine in the lithium carbonate solution and boil for 10 to 15 minutes. When cool add the thymol to prevent the growth of mold. When used as a counterstain for bacteria in the Gram-Weigert method this solution should be carefully filtered.

Method of Staining.—1. Stain sections 2 to 5 minutes.

2. Transfer directly to acid alcohol (hydrochloric acid 1 cc.; alcohol, 70 per cent, 99 cc.), 1 or more changes for several minutes or more to fix the dye in the nuclei and to differentiate the sections.

3. Wash in water.

4. Dehydrate in 95 per cent followed by absolute alcohol.

5. Clear in xylol and mount in balsam.

If celloidin sections are used clear in terpineol or in oil of origanum after 95 per cent alcohol.

Results.—This method gives an intense and permanent bright red nuclear stain. Overstaining is impossible. A trace of picric acid added to the alcohol used for dehydration affords a fine contrast stain.

It is not recommended for staining tissues in bulk as it macerates strongly.

Best's Carmine Stain for Glycogen (1906).—

Carmine	2 gm.
Potassium carbonate	1 gm.
Potassium chloride	5 gm.
Water, distilled	60 cc.

Boil gently and cautiously for several minutes. When cool add:

Ammonia water, 26 to 28 per cent	20 cc.

The solution should be tightly stoppered and kept in the ice-box as it deteriorates rapidly if left at room temperature.

For method of staining see pages 126–128.

Mayer's Mucicarmine Stain (1896).—

Carmine	1 gm.
Aluminum chloride, anhydrous	0.5 gm.
Water, distilled	2 cc.

Combine and heat over a low flame for 2 minutes, stirring constantly, until the reddish mixture becomes a dark color. Remove from the flame and gradually add, stirring constantly, 100 cc. of 50 per cent alcohol. Let the solution stand for 24 hours and then filter.

For method of staining see page 130.

(3) Orcein

Orcein, a vegetable dye obtained from certain tinctorial lichens, is used mainly for staining elastic fibers. It is soluble in alcohol and is employed either in a neutral or acid (HCl) alcoholic solution.

(4) Iodine

Iodine is the oldest of the histological stains, but is now little used for that purpose, except as a stain for amyloid. In a 0.5 per cent solution in 95 per cent alcohol it is used for the removal of the precipitate of mercury formed in tissues fixed in corrosive sublimate or in Zenker's fluid.

Lugol's solution, a solution of iodine in water containing potassium iodide, is of varying strength. Iodine in this form is used as a test for starch, amyloid, glycogen and corpora amylacea. In Gram's stain and its modifications iodine produces some chemical change in the coloring material employed, in consequence of which, when appropriate decolorizers are used, the stain remains fast in certain structures, while from others it is easily and entirely extracted.

The strength originally employed by Gram (1884) for his staining method was as follows:

Gram's Iodine Solution

Iodine	1 gm.
Potassium iodide	2 gm.
Water, distilled	300 cc.

Weigert (1887) in his modification of this method employed a stronger solution:

Iodine	1 gm.
Potassium iodide	2 gm.
Water, distilled	100 cc.

Later he recommended the following strength both for fibrin and for neuroglia fibers:

Potassium iodide	5 gm.	saturate with iodine
Water, distilled	100 cc.	

The only difference in the action of the various solutions probably is that the strong solution acts practically instantaneously, while the weaker solutions require some little time.

3. ARTIFICIAL DYES (ANILINE DYES)

Aniline dyes are indispensable for certain purposes. A list of tissue elements and pathological products which can be stained more or less specifically by them and of the dyes capable of doing so is given here.

Bacteria	methylene blue, crystal violet, fuchsin, and others
Nuclei	methylene blue, fuchsin, safranine, thionin, and others
Fibroglia and other fibrils	acid fuchsin, phloxine, eosin
Collagen	aniline blue, acid fuchsin
Elastic fibrils	fuchsin
Nerve fibrils	methylene blue
Amyloid	crystal violet, Congo red
Fibrin	crystal violet
Mucin	methylene blue, and others

We have been dependent in the past on Germany for practically all our aniline dyes. Now, as the result of the World War, we are manufacturing our own. Some of them are of the highest quality, others are not so reliable. They are constantly being tested by the

Commission on the Standardization of Biological Stains, founded in 1922. Dyes that meet certain specified requirements are certified. The important point is for the purchaser to insist on certified dyes, if they are obtainable, when buying from the retail dealer as many poor brands of dyes imported or manufactured by incompetents during and since the war are still for sale.

Because aniline dyes are not chemically pure reagents and often contain adulterants, especially dextrin, Dr. H. J. Conn, Chairman of the Commission, strongly advises the use of definite quantities of saturated alcoholic or aqueous solutions in all formulas when possible, instead of so many grams, because in this way the same quantity of dye will be obtained each time.

Aniline dyes come in the form of a powder or as crystals, and most of them keep well in that condition. Methylene blue for one, however, seems to be an exception. After the original package has been opened for a short while the dye is said to lose in intensity of staining power. It is well to keep on hand saturated alcoholic solutions of certain dyes because they keep well in that form and are ready for use when needed. This is particularly true of methylene blue, fuchsin (basic) and crystal violet.

Aniline dyes are coal tar dyes and may be considered as derivatives of the hydrocarbon, benzene, C_6H_6. They act as salts having basic or acid properties. The basic colors stain cell nuclei, including bacteria, for which they show a marked affinity. The acid colors stain diffusely. The basic dyes most commonly employed in pathological histology are methylene blue, fuchsin, crystal violet and safranine. Of the acid colors, eosin, phloxine, picric acid and acid fuchsin are most in use.

As a rule, every aniline dye has one or more standard solutions that are used largely to the exclusion of others for the reason that, being required for certain purposes, they are kept in stock. As they are thus always at hand they are used when simple solutions would suffice. For instance, Loeffler's methylene blue solution is often used, because ready and convenient, when a simple aqueous solution would do as well.

In the following pages are arranged under each dye the solutions most in use.

Any of the basic aniline dyes may be used as a nuclear stain after the following general method:

1. Stain paraffin sections in a strong solution of the dye preferred in water or in dilute alcohol for 5 to 30 minutes.
2. Wash in water.
3. Differentiate in 95 per cent alcohol.
4. Dehydrate in absolute alcohol.
5. Clear in xylol.
6. Mount in balsam.

If celloidin sections are used differentiate in 95 per cent alcohol, blot with filter paper and clear in xylol.

As a matter of fact, however, certain dyes and solutions are used generally in preference to others. Most of the colors are more or less affected by all clearing reagents except xylol. With paraffin sections and those from which the celloidin has been removed it is very easy to dehydrate in absolute alcohol and to clear in xylol. With celloidin sections, however, this is impossible because the absolute alcohol will dissolve the celloidin and this is usually not desirable. For celloidin sections, therefore, blot with filter paper and then pour on xylol; repeat the blotting, followed by xylol, two or three times until the specimen is perfectly clear and then mount in balsam.

In washing out the excess of color it is sometimes found advantageous to acidulate very slightly either the water or the first alcohol with acetic or hydrochloric acid. This process, if not carried too far, tends to make the nuclear stain sharper.

Methylene Blue.—Solubility at 26° C.: in water 3.55 per cent; in alcohol 1.48 per cent.

1. Saturated solution (about 1.48 per cent) in 95 per cent or absolute alcohol. A stock solution for making other solutions. It can be used as a stain by adding 1 part to 9 parts of water.

2. Aqueous solutions of various strengths can be made up as needed.

3. **Loeffler's Methylene Blue Solution** (1884).—

Methylene blue, saturated solution in 95 per cent alcohol (about 1.48 per cent)	30 cc.
Sodium hydroxide, 1:10,000 aqueous solution	100 cc.

This is one of the most useful of the aniline staining solutions and will keep for a long time without losing much in staining power.

4. **Kühne's Methylene Blue Solution.**—

Methylene blue, saturated alcoholic solution (about 1.48 per cent)	10 cc.
Carbolic acid water, 5 per cent	90 cc.

This is a stronger staining solution than Loeffler's but the resulting stain does not seem so sharp and clear.

5. **Gabbett's Methylene Blue Solution** (1887).—

Methylene blue	2 gm.
Sulfuric acid	25 cc.
Water, distilled	75 cc.

This stain is used as a decolorizer and as a contrast for tubercle bacilli.

6. **Unna's Alkaline Methylene Blue Solution** (1891).—

Methylene blue	1 gm.
Potassium carbonate	1 gm.
Water, distilled	100 cc.

This strongly alkaline solution of methylene blue, recommended by Unna for staining plasma cells, has been found extremely valuable as a general stain in combination with phloxine or eosin, which should be used first. The solution stains better after ripening for a week or two and should be diluted 1:10 or 1:5 for staining.

7. **Unna's Polychrome Methylene Blue Solution** (1891).—The polychrome methylene blue solution, much used by Unna in various staining methods, is an old alkaline solution of methylene blue, of which the one given above is the original formula and in which, in consequence of oxidation, methyl violet and methylene red have formed. Months are required for the process of oxidation to take place, at normal temperature. It may be greatly shortened by means of heat.

8. **Goodpasture's Acid Polychrome Methylene Blue Solution** (1917).—

Methylene blue	1 gm.
Potassium carbonate	1 gm.
Water, distilled	400 cc.
Acetic acid, glacial	3 cc.

Dissolve the ingredients thoroughly and boil in a flask for 30 minutes. The methylene blue will be polychromed and most of it

precipitated. When the solution is cool add the acetic acid. Shake thoroughly until the precipitate is dissolved and then boil gently for 5 minutes or until the solution is concentrated to a volume of 200 cc. Cool it in tap water. It is ready for use immediately, may be used over and over, does not precipitate and keeps indefinitely.

9. **Sahli's Borax Methylene Blue Solution** (1885).—

Methylene blue, saturated aqueous solution (about 3.55 per cent)	24 cc.
Borax, 5 per cent aqueous solution	16 cc.
Water, distilled	40 cc.

Mix, let stand a day and filter.

Mallory's Phloxine and Methylene Blue Stain.—The principle of this staining method was evolved over 40 years ago, *e. g.*, to stain the various structures in a section as deeply as possible with eosin and then to stain and differentiate in an alkaline solution of methylene blue. The method was applied to paraffin sections of tissues fixed in Zenker's fluid and for many years Unna's alkaline methylene blue solution was used. Two changes have been made in the course of time. Phloxine was substituted for eosin soluble in water at the suggestion of Dr. H. J. Conn and was found to give a more brilliant color. Secondly, a borax solution of methylene blue was decided on as giving more reliable results and to it was added later azure II. In this way there was obtained at once a ripened solution that could always be depended on.

Fixation.—Zenker's fluid. Embed in paraffin.

Staining Solutions.—

Phloxine

Phloxine	2.5 gm.
Water, distilled	100 cc.

Methylene Blue

Methylene blue	1 gm.
Borax	1 gm.
Water, distilled	100 cc.

Azure II

Azure II	1 gm.
Water, distilled	100 cc.

Method of Staining.—Deparaffinize in the usual way and then remove the mercuric deposit with a 0.5 per cent solution of iodine in 95 per cent alcohol, acting for 5 to 10 minutes. Remove the iodine by treating the sections with a 0.5 per cent solution of sodium thiosulfate ("hypo") for 5 minutes. Wash thoroughly in water.

1. Stain sections in phloxine for 1 hour or longer in the paraffin oven at 52° to 55° C.

2. Allow the solution to cool before pouring it off and then rinse carefully in water.

3. Stain in the methylene blue and azure II solution for 5 to 20 minutes.

For use take 5 cc. of the methylene blue solution, 5 cc. of the azure II solution, and add 90 cc. of water. Filter onto sections and pour on and off slides several times to obtain an even stain. After sections have remained in the solution the required time, place in water and decolorize each slide individually.

4. Differentiate in a dish containing 100 cc. of 95 per cent alcohol to which have been added 2 to 5 cc. of a 10 per cent solution of colophony (rosin) dissolved in absolute alcohol. Keep the slide in constant motion so that decolorization will be uniform. Control the result with the microscope.

5. When the pink color has returned to the sections and the nuclei are still a deep blue dehydrate with several changes of absolute alcohol.

6. Clear in xylol and mount in balsam. It is advisable to use at least two changes of xylol in order to remove the alcohol completely.

Results.—Nuclei and bacteria blue; collagen and other tissue elements bright rose color.

It is important to get a deep stain with phloxine because the methylene blue dissolves or washes it out to a considerable extent. The phloxine must be used first because methylene blue is readily soluble in an aqueous solution of phloxine and therefore is quickly extracted if the phloxine is used after it, while on the other hand phloxine is but slightly soluble in an aqueous solution of methylene blue.

The success of this staining method has been found by Wolbach to depend on the presence of colophony (rosin) in the alcohol used for differentiation. This is present in alcohol obtained from the barrel where it is used to seal the seams, but not in alcohol obtained

from glass or metal containers. It must, therefore, be added. Wolbach has also shown that sections fixed in formalin may be stained by this method, provided the amount of colophony in the alcohol be increased to from 3 to 10 per cent.

Fuchsin (Basic).—Solubility at 26° C.: in water 0.26 per cent; in alcohol 5.95 per cent.

1. Saturated alcoholic solution (about 5.95 per cent) to be kept in stock.

2. **Ziehl-Neelsen's Carbol Fuchsin.**—

Fuchsin (basic), saturated alcoholic solution (about 5.95 per cent)	10 cc.
Carbolic acid water, 5 per cent	90 cc.

Carbolic acid water is made by shaking together 5 cc. of melted carbolic acid crystals and 95 cc. of water.

The solution should be filtered. It is very powerful, stains quickly, keeps well, and can be employed for a variety of purposes. It is used chiefly for staining tubercle bacilli.

3. **Verhoeff's Carbol Fuchsin Solution** (1912).—

Fuchsin (basic)	2 gm.
Alcohol, absolute	50 cc.
Carbolic acid crystals, melted	25 cc.

Combine the ingredients and place overnight in an incubator to ensure complete solution, then cool and filter. This stock solution of carbol fuchsin, unlike the dilute aqueous solution which slowly deteriorates, is permanent and does not even require to be filtered again. It can be highly recommended.

For staining coverslip preparations add 2 drops of this stock solution to 8 drops of distilled water. When larger quantities of staining solution are required, the dilution is made in the proportion of 1 cc. of the stock solution to 6 cc. of distilled water.

Methyl Violet.—Solubility at 26° C.: in water 2.93 per cent; in alcohol 15.21 per cent.

1. Aqueous solutions of various strengths, 0.5 to 2 per cent, keep well and are used for staining nuclei, bacteria and amyloid.

2. Weigert (1887) recommends two permanent stock solutions by means of which the aniline methyl violet solution can be made up easily when wanted.

Solution A

Alcohol, absolute	33 cc.
Aniline	9 cc.
Methyl violet	in excess

Solution B

Methyl violet, saturated aqueous solution

The staining solution consists of Solution A, 1 part; and Solution B, 9 parts. This mixture will keep at the most for 14 days.

3. For staining neuroglia fibers Weigert employs a saturated solution made with the aid of heat in 80 per cent alcohol.

Crystal Violet.—Solubility at 26° C.: in water 1.68 per cent; in alcohol 13.87 per cent.

Crystal violet, because it is a definite chemical compound, is now substituted for both methyl violet and gentian violet in all bacteriological and histological staining methods. In all the formulas for stains given under gentian violet, crystal violet should replace the older, less reliable gentian violet dye.

Gentian Violet.—This dye is not a definite chemical substance but a mixture of crystal violet, methyl violet and dextrin. It is better to discard it entirely and to use crystal violet instead in the staining solutions given; they are cited here as originally given only because they are classical.

1. Saturated alcoholic solution to be kept in stock.

2. **Erhlich's Aniline Gentian Violet.**—

Gentian violet (crystal violet), saturated alcoholic solution (about 1.68 per cent)	16 cc.
Aniline water	84 cc.

Aniline water (aniline oil water) is made by shaking together 5 parts of aniline with 95 parts of water and filtering the resulting milky fluid. It should filter through perfectly clear.

During the first few hours after the solution is made up considerable precipitation takes place so that it is best not to use it for 24 hours. After about 10 days it begins to lose its staining power.

3. Zenker recommends a solution without alcohol. Dissolve the gentian violet (crystal violet) directly in the aniline water. The color is said to be less easily removed from tissues when this solution is used.

4. **Stirling's Gentian Violet** (1890).—

Gentian violet (crystal violet)	5 gm.
Alcohol, absolute	10 cc.
Aniline	2 cc.
Water	88 cc.

This solution keeps remarkably well.

5. **Carbol Gentian Violet.**—

Gentian violet (crystal violet), saturated alcoholic solution (about 1.68 per cent)	10 cc.
Carbolic acid water, 5 per cent	90 cc.

Safranine O.—Solubility at 26° C.: in water 5.45 per cent; in alcohol 3.41 per cent.

Safranine is one of the best of the nuclear staining aniline dyes. Tissues may be fixed in alcohol, corrosive sublimate, Flemming's, Hermann's or Zenker's fluids. The two following solutions of safranine can be thoroughly recommended.

1. Saturated aqueous solution (about 5.45 per cent) of safranine O (to be made with the aid of heat).

2. **Babes' Aniline Safranine** (1887).—

Aniline water, 2 per cent	100 cc.
Safranine O	in excess

Saturate the solution by heating it in a flask set in hot water at 60° to 80° C. and when cool filter.

This solution is extremely powerful, stains almost instantly, and will keep about 2 months.

Pyronine.—Solubility at 26° C.: in water 8.96 per cent; in alcohol 0.60 per cent.

1. A 1 per cent solution in water is used as a counterstain for the Gram method, for which purpose it is more reliable than fuchsin or safranine because it is much less likely to overpower the Gram-positive organisms and render part or all of them negative.

2. The dye is used also in combination with methyl green for staining gonococci and plasma cells.

Thionin.—Solubility at 26° C.: in water 0.25 per cent; in alcohol 0.25 per cent.

It is generally used in an aqueous solution but this does not keep

well. We have found a 0.5 per cent solution in 20 per cent alcohol very satisfactory because it obviates this drawback. The dye is used especially for staining frozen sections of unfixed tissue.

Toluidine Blue O.—Solubility at 26° C.: in water 3.82 per cent; in alcohol 0.57 per cent.

This is a valuable nuclear stain in a 0.3 to 1 per cent aqueous solution; excellent for frozen sections of unfixed tissue for which purpose a 0.5 per cent solution in 20 per cent alcohol is recommended because it keeps better.

Bismarck Brown.—Solubility at 26° C.: in water 1.36 per cent; in alcohol 1.08 per cent.

The most common solutions are the following:

1. A 1 per cent aqueous solution.
2. A saturated aqueous solution made by boiling (3–4 per cent).
3. A saturated solution in 40 per cent alcohol (2–2.5 per cent).

Unlike other aniline colors, Bismarck brown will keep in glycerin mounts and can be fixed in nuclei by acid alcohol. The stain is not used so much as formerly, except as a contrast stain in Gram's method and for photographic purposes.

Other basic stains less frequently used, and then generally in aqueous solutions, are dahlia, methyl green and iodine green.

Diffuse or contrast stains are useful to stain various tissue elements left uncolored by the nuclear stain. A greater richness of detail is obtained with diffuse stains if, after rather deep staining, the sections are washed out for some time in alcohol, because some structures possess a greater affinity than others for certain diffuse stains and by holding them are brought out sharply.

Of the diffuse stains, eosin, phloxine, picric acid and acid fuchsin in van Gieson's mixture are the ones most frequently employed.

Eosin Y (Water-Soluble Eosin).—Solubility at 26° C.: in water 44.20 per cent; in alcohol 2.18 per cent.

Keep on hand a saturated aqueous solution to which a crystal of thymol has been added and dilute with water as needed. The strength of solution to be used varies somewhat with the tissue and the reagent in which it has been fixed, but generally lies between 0.1 and 0.5 per cent when the eosin is used after a hematoxylin stain. These dilute solutions should contain 25 per cent of alcohol, otherwise they will not keep well. When eosin is employed before an aniline dye, such as methylene blue, a 2.5 to 5 per cent aqueous

solution should be used. Solutions of eosin should always be filtered immediately before use.

Phloxine B.—This dye has been found superior to eosin Y in Mallory's phloxine-methylene blue stain and at least as valuable as a contrast with alum hematoxylin stains. It gives a richer, brighter rose-red color. The solutions and methods of use are the same as for eosin Y.

Congo Red.—Slightly soluble in cold water; easily soluble in warm water.

It is used as an indicator for the presence of free acid in tissues, as a counterstain after Delafield's alum hematoxylin, and as a stain for amyloid.

Picric Acid.—Solubility at 26° C.: in water 1.22 per cent; in alcohol 8.96 per cent.

1. Saturated alcoholic (about 8.96 per cent) and aqueous (about 1.22 per cent) solutions should be kept in stock, to be diluted as needed.

2. Picric acid is used for contrast with the carmine stains, more rarely with alum hematoxylin. Striated muscle fibers and cornified epithelium are rendered especially prominent by it. To stain with picric acid it is only necessary to add a few drops of a saturated aqueous solution (about 1.22 per cent) to a dish of water, or of a saturated alcoholic solution (about 8.96 per cent) to a little alcohol, and allow sections to remain in the solution for a few seconds.

Acid Fuchsin.—Solubility not determined as yet.

1. Aqueous solutions of various strengths are used. It is advisable to keep on hand a 5 per cent solution and to dilute it to the strength required. A crystal of thymol should be kept in the solution to prevent the growth of mold.

2. **Altmann's Aniline Acid Fuchsin Solution** (1890).—

Acid fuchsin	20 gm.
Aniline water, 5 per cent	100 cc.

3. **Van Gieson's Picro-Acid Fuchsin Solution** (1889).—Van Gieson's stain is excellent as a contrast with alum hematoxylin, especially when it is desirable to render prominent connective tissue fibrils or certain pathological products. The nuclear stain with alum hematoxylin must be rather deep because the picric acid to some extent extracts or overpowers it.

Fixation.—Any fixative may be used.

Staining Solution.—This valuable solution was originally made by adding to a saturated aqueous solution of picric acid enough of a saturated aqueous solution of acid fuchsin to give to the fluid a deep garnet red color, and for certain purposes, as in staining after fixation in Zenker's fluid, this strong solution is to be preferred. Freeborn has given more precise directions for making up the solution according to the purpose for which it is to be used.

For Connective Tissue

Acid fuchsin, 1 per cent aqueous solution	5 cc.
Picric acid, saturated aqueous solution (about 1.22 per cent)	100 cc.

For the Nervous System

Acid fuchsin, 1 per cent aqueous solution	15 cc.
Picric acid, saturated aqueous solution (about 1.22 per cent)	50 cc.
Water, distilled	50 cc.

Method of Staining.—1. Stain deeply in alum hematoxylin (p. 70).

2. Wash in water.
3. Stain in van Gieson's solution 3 to 5 minutes.
4. Wash quickly in water.
5. Differentiate in 95 per cent and dehydrate in absolute alcohol.
6. Clear in xylol and mount in balsam.

If celloidin sections are used omit absolute alcohol and clear in terpineol or in oil of origanum.

Results.—Collagen red; smooth and striated muscle, cornified epithelium and some forms of hyalin yellow.

The Biondi-Heidenhain Stain (1892).—*Fixation.*—Tissues must be fixed in corrosive sublimate.

Staining Solution.—

Orange G, saturated aqueous solution (about 10.86 per cent)	50 cc.
Acid fuchsin, saturated aqueous solution (about 20 per cent)	10 cc.
Methyl green, saturated aqueous solution (about 8 per cent)	25 cc.

Make up the separate solutions and let them stand for several days with excess of coloring matter (shaking the bottles occasionally) until they are saturated. Then mix the solutions. For staining, dilute the combined solution with water 1:60 or 1:100.

The following tests are used for finding out if the proper combination has been obtained. The addition of acetic acid should make the solution redder; a drop of the solution on filter paper should make a blue spot with green in the center and orange at the periphery. If a red zone appears outside of the orange then too much acid fuchsin is present.

Method of Staining.—1. Stain paraffin sections 6 to 24 hours with the dilute solution.

2. Do not rinse in water but decolorize a little in 95 per cent alcohol. It is important to place the sections directly from the staining fluid into the alcohol as water washes out the methyl green almost instantly.

3. Dehydrate in absolute alcohol.

4. Clear in xylol and mount in balsam.

Results.—Chromatin blue-green; nucleoli red; cytoplasm and connective tissue elements different shades of red; red blood cells orange; mucin green.

CHAPTER VII

METALLIC STAINS AND IMPREGNATIONS

EXPERIMENTAL investigation has shown that a few metals can be used for staining certain tissue elements, either because they are directly reduced from solutions of appropriate salts or because they are taken up and retained by these tissue elements which are rendered prominent when the metallic salt is reduced later. The most valuable metals for this purpose are silver, gold and osmium, but especially the former and the latter.

Both silver and gold may demonstrate tissue elements by a transparent stain or by a fine precipitation (so-called impregnation). Methylene blue is capable of doing the same. Solutions of salts of these metals were applied at first to fresh tissues. As they penetrate but slightly they fix poorly and stain practically only surface elements. Many methods of using them have been devised, but the results obtainable are fickle and unreliable. The tendency nowadays is to use almost exclusively staining methods applicable to tissues first fixed in formalin, and several excellent and reliable methods have been developed and are in constant use.

Silver.—Silver, usually in the form of silver nitrate, is used to stain a variety of structures: (1) the cement substance between mesothelial and endothelial cells; (2) the reticulum of connective tissue; (3) the *Treponema pallidum* of syphilis; (4) a variety of nerve structures—ganglion cells and their processes, axis cylinders, neurofibrils and nerve endings; and (5) neuroglia cells and fibrils.

Gold.—Gold solutions have but a limited use nowadays, except for toning silver stains and improving their appearance. Gold is still of value, however, for demonstrating connective tissue fibrils, astrocytes and neurofibrils and nerve endings.

Osmium.—This metal is used in the form of the tetroxide, commonly called osmic acid. It is a much better fixative than any salt of silver or gold and is often used for that purpose alone, but its penetrating power is slight. It has the property of staining fatty substances brown to black and formerly was much used for that pur-

pose. Scarlet red and a few other dyes, however, are more reliable and are tending to replace it almost entirely except in the Marchi method for degenerated myelin sheaths. Osmic acid is combined more frequently with other substances in fixing solutions used for the demonstration of mitochondria, the Golgi apparatus and ganglion cells.

CHAPTER VIII

CLEARING AND MOUNTING REAGENTS

SECTIONS of tissue after being stained have to be dehydrated, and then passed through a medium that will mix both with the dehydrating fluid and with the reagent in which the sections are to be mounted. These intermediary fluids are called clearing reagents because they have a high index of refraction and thus render the sections more or less transparent so that the stained structures are distinctly visible and can be examined in such fluids if desired. One reagent, glycerin, commonly performs all three functions; it dehydrates, clears, and serves as a mounting medium. Some of the others perform two of the three functions.

Under clearing and mounting reagents substances for three types of sections have to be considered, namely, for frozen, celloidin and paraffin sections, as well as the question whether the mount is to be fluid or solid. Frozen sections which are to be mounted in a fluid ordinarily require no dehydration beyond that furnished by the fluid in which they are mounted. For celloidin sections a variety of clearing reagents is used; for paraffin sections usually only two—xylol and toluol.

1. DEHYDRATION REAGENTS

For celloidin and paraffin sections the dehydrating reagent generally used is alcohol. It is advisable to pass sections through a graded series of 60, 80 and 95 per cent for celloidin sections, followed by absolute for those that were embedded in paraffin. Ordinarily, stained sections of tissues are transferred directly from water to 95 per cent alcohol. Moving the sections about in the alcohol greatly hastens the process of dehydration. Acetone can be used instead of alcohol for paraffin sections, but if they are to be cleared in xylol they must pass through a graded series of mixtures of acetone and xylol 3:1, 1:1, 1:3 and finally pure xylol.

2. CLEARING REAGENTS

Xylol.—For aniline stains on paraffin sections the best clearing reagent is xylol, which, however, clears directly only from absolute alcohol. It can be used, however, for celloidin or other sections de-

hydrated in 95 per cent alcohol by a simple method originated by Welch and later brought into notice by Weigert. Blot the section on the slide with smooth fine filter paper and then pour on a few drops of xylol; repeat the blotting followed by xylol two or three times, and the section will be found to be perfectly clear.

Three other reagents sometimes used instead of xylol for clearing sections to be mounted in balsam are toluol, benzol and chloroform. The last named is especially useful for preparations stained with osmium as it does not cause the color to fade.

Terpineol (Lilacine).—This clearing reagent was introduced by P. Mayer in 1910 and is highly recommended by Romeis. It is colorless, has an agreeable odor, and clears from 90 per cent alcohol (from 80 per cent if necessary). It does not dissolve celloidin and is much cheaper than oil of origanum. Even delicate stains are as a rule not injured by this reagent, but it is said to cause fading of silver stains.

Oleum Origani Cretici.—This will be referred to as oil of origanum in directions given for its use. It is colorless to light brown, but grows darker on exposure to light and air. It is generally used for celloidin sections and clears readily from 95 per cent alcohol without dissolving the celloidin. Aniline colors are affected slowly. Unfortunately it is expensive. Ordinary oil of origanum is impure oil of thyme and should not be used.

Weigert's Carbol-Xylol.—This mixture is recommended especially for clearing thick celloidin sections of the central nervous system after carmine and hematoxylin stains. It is made up as follows:

Carbolic acid crystals	1 part
Xylol	3 parts

Rinse in xylol before mounting in balsam.

Weigert's Aniline-Xylol (1891).—This mixture is often used nowadays instead of carbol-xylol.

Aniline	2 parts
Xylol	1 part

Rinse in xylol before mounting in balsam.

Oil of Cedarwood.—This reagent has a pale straw color. It clears from 95 per cent alcohol but, unfortunately, clears celloidin sections very slowly. It does not affect aniline colors.

Aniline (Aniline Oil).—This is colorless when perfectly pure and fresh, but soon oxidizes and turns brown. It does not dissolve celloidin and clears readily from 70 per cent alcohol. It will clear from water by Weigert's filter paper blotting method. Aniline colors are extracted slowly. Rinse sections with xylol before mounting in balsam.

3. MOUNTING REAGENTS

(1) Fluid Mounts

Several reagents are available, of which the most important is glycerin. Two others, less often used, are potassium acetate (a saturated aqueous solution, about 25.3 per cent) and levulose (30 gm. of levulose are dissolved in 20 cc. of water by heating to 37° C. for 24 hours).

The disadvantage of fluid mounts is that they have to be ringed or sealed in by some solid medium so as to fix the coverslip in position and keep it from being displaced. Two methods of doing this are given below.

Du Noyer's Lanolin-Colophony Mixture (1918).—This is highly recommended for sealing fluid mounts and is prepared in the following manner:

Heat gently 20 parts of water-free lanolin in a porcelain evaporating dish for 15 to 30 minutes to drive off any trace of moisture. Then add 80 parts of crushed colophony (rosin) and beat the mixture until it becomes homogeneous, clear, and yellowish brown in color. (Caution: The mixture is inflammable!) For preservation pour the mixture into small paper boxes and let it harden.

For sealing mounts heat the lanolin-colophony mixture and take a glass rod or old scalpel and pick up some of the melted mass. With it fasten first the corners and then seal along the edges of the coverslip.

Duco Cement.—This cement has been found useful for sealing glycerin mounts. It comes in collapsible tubes, is colorless, and requires no preparation beyond squeezing into a small bottle so that it can be diluted with an equal volume of acetone. Use as follows:

Transfer the stained section from water to glycerin in a dish. After the section is cleared change to a fresh dish of glycerin. With a section lifter transfer the section to a slide, drain and wipe away excess of glycerin. Do not blot as bubbles of air are almost sure to be caught in the mount. Drop on a coverslip and press gently

to force out the excess of glycerin. Weight with a lead slug to flatten the section. Remove any excess of glycerin with a towel or with filter paper. Clean the corners with water and wipe dry. Put a drop or two of diluted Duco cement on the corners. In a few minutes they will be stuck tight. The edges of the coverslip can then be cleaned and sealed in with the cement in the same way. The whole process occupies less than 10 minutes and the mounts are as secure as though put up in balsam, provided all the glycerin has been removed from the surface of the glass. They can be handled and cleaned as readily as balsam mounts because Duco cement is insoluble in water, alcohol, xylol or oil of cedarwood, the only reagents with which it is likely to come in contact.

Glycerin yields solid mounts by combining it with gelatin. The preparations are practically permanent because of the solidity of the mounting medium. This medium is very useful for the preservation of scarlet red stains for fat in frozen sections of formalin-fixed tissues.

Kaiser's Glycerin Jelly (1880).—This is made up as follows:

Gelatin	40 gm.
Water, distilled	210 cc.
Glycerin	250 cc.
Carbolic acid crystals	5 gm.

Soak the gelatin in the water for 2 hours. Add the glycerin and the carbolic acid and heat gently for 10 to 15 minutes, stirring all the while until the mixture is smooth. Keep in the ice-box and melt when needed. The carbolic acid has unfortunately a somewhat deleterious action on alum hematoxylin stains.

(2) Solid Mounts

Several reagents are available. With one exception (oil of cedarwood) they have to be dissolved in a fluid medium which later evaporates. The sections are ordinarily dehydrated in alcohol and then cleared in fluids that will remove the alcohol and mix with the mounting medium.

Canada Balsam.—This reagent is the one most generally used for solid mounts. It is a yellowish, transparent, viscid, acid liquid and should not be used in this form. It must be heated and stirred with an admixture of calcium carbonate until a drop of it on a

slide will solidify on cooling. This procedure renders the balsam neutral. It is simpler to buy this form already prepared and to dissolve it as needed in neutral xylol to a rather thick syrupy consistence. In this condition it is often spoken of as xylol or neutral balsam, or simply as balsam.

Canada balsam has such a high index of refraction that tissues mounted in it become very transparent, and only those parts are visible that are stained. Other solvents of Canada balsam, such as chloroform and benzol, may be used but cannot be recommended for sections stained with aniline dyes. For tissues stained with osmic acid, however, chloroform balsam, prepared in the same way as xylol balsam, should always be used, otherwise the osmic acid stain will fade rapidly.

Oil of Cedarwood.—This is recommended as the best mounting reagent after Giemsa's stains. The thick evaporated form prepared for use with oil immersion lenses should be employed. It evaporates slowly to hardness.

Dammar.—This resin is sometimes used for mounting purposes but cannot be recommended because of its marked tendency to crystallize, in consequence of which specimens mounted in it are often ruined.

Colophony (Rosin).—This is an oleoresin obtained from different varieties of pine and like balsam is dissolved in xylol. It is not ordinarily used nowadays for mounting purposes. It consists chiefly (80–90 per cent) of abietic acid, or its anhydride, and also of pinic and sylvic acids.

Terpineol Balsam.—Terpineol dissolves neutral Canada balsam readily when placed in a paraffin oven. As it evaporates slowly, several days are required to harden the balsam. It is especially recommended for mounting sections stained with lead chloride hematoxylin after clearing in terpineol. Other clearing and mounting reagents, especially xylol, fade the color quickly.

CHAPTER IX

MICRO-INCINERATION

THIS is a method designed to reveal the mineral salts in sections of tissues. It was first successfully developed by Policard and his co-workers and later improved by Scott (1932), whose technique is given here.

1. Fix thin pieces of tissue for 12 to 24 hours in a mixture of 9 parts of absolute alcohol to 1 part of neutral formalin.

2. Complete dehydration in several changes of absolute alcohol followed by xylol.

3. Embed in paraffin.

4. Cut sections 3 to 5 μ thick.

5. Avoid contact with water. Flatten sections out on slide by means of a drop of absolute alcohol or of liquid petrolatum. Allow the sections mounted with alcohol to dry in the incubator at 37° C. for 1 to 12 hours. Drain those mounted with liquid petrolatum. Use every other section for incineration. The remaining sections are to be mounted in the usual way with Mayer's albumin-glycerin mixture and later stained by any of the usual histological methods. They are valuable as controls.

6. Place slides on quartz slides of equal size and insert them into the special quartz tube oven. Take care that the slide does not touch the walls of the quartz tube. Incinerate for 35 minutes. Raise the temperature gradually, using the first 10 minutes to reach a temperature of 100° C. and the other 25 minutes to attain a final temperature of about 650° C. Be sure that the oven is red hot during the last step, otherwise leave the slides in longer. The time must be varied, depending on the nature of the tissue. Some tissues ash readily, others only with the greatest difficulty. Special attention must be paid to the rise of temperature to 100° C. as most of the shrinkage occurs between 60° and 70° C. and this range should be passed through slowly.

7. Remove the slides and quartz slides from the incinerator with forceps, which should be heated first as otherwise the slides will crack. Remove the glass slides quickly from the quartz slides and

place on an asbestos plate. Cover the dry incinerated section with a thin coverslip by dropping it down at the site of the section. Let the slide cool and then seal the coverslip around the edge with sealing wax, paraffin or Duco cement. The coverslip serves as a mechanical protection and permits the use of an oil immersion lens. It also prevents excessive absorption of moisture and efflorescence of the ash.

The ash left on the slide may be examined under low power by oblique illumination from a parabolic mirror at an angle of approximately 30°. For oil immersion study the cardioid condenser of Zeiss is recommended. The residue is essentially a mineral skeleton of the cells free from organic materials.

The amount and distribution of the minerals in the cells and tissues are easily seen, but recognition and identification of the various substances present in the ash, aside from silica, iron and calcium, are extremely difficult at the present time.

CHAPTER X

INJECTIONS

THE injection of fluid substances, often called masses, into blood and lymph vessels is a procedure not much used in pathology. The process is an art that requires much patience and considerable experience. The purpose of an injection is to render vessels and vessel walls more prominent and visible than under ordinary conditions. Fluid mixtures that are usually colored and that may be solidified later are used.

Most injection fluids contain gelatin and on this account must as a rule be used warm. The best coloring materials are carmine and Berlin blue.

The instruments required are glass cannulas of various sizes and a syringe, or better still, a constant pressure apparatus.

When a warm injection fluid is used the bottle containing it must be placed in a water bath and kept at a temperature of about 45° C. The organ or animal to be injected must likewise be placed in a water bath of the same temperature.

It is very important that in connecting the end of the tube carrying the injection fluid with the cannula inserted in the vessel no air bubbles shall enter. When blood vessels are to be injected it is advisable to wash them out first with normal salt solution.

1. COLD INJECTION FLUIDS

Two are given here; milk is the simpler, the other contains gelatin.

Milk.—Fischer (1902) has obtained good results by washing out the vessels in the usual way with physiological salt solution or, better still, with a fibrin-dissolving fluid such as a freshly filtered 8 per cent aqueous solution of sodium nitrate or sulfate and then injecting fresh milk.

Fix the tissues after injection for at least 24 hours in a 1 per cent solution of formalin plus 1.5 per cent acetic acid. The acid is needed to coagulate the milk. Preserve in neutral 10 per cent formalin.

Cut frozen sections and stain with scarlet red (p. 117). Counterstain in alum hematoxylin (p. 70). Mount in glycerin or glycerin

jelly. The fat can also be stained with osmium tetroxide. The capillaries are outlined by the fat emulsion stained red or black.

Tandler's Fluid Gelatin (1901).—Dissolve 5 gm. of gelatin in 100 cc. of distilled water with the aid of gentle heat. Add enough of a saturated water-soluble Berlin blue solution to color it light or dark as desired. Then while constantly stirring the solution add 5 gm. of potassium iodide. It keeps the gelatin from solidifying down to 17° C. After the injection fix tissue in 5 per cent formalin, which prevents solution or decolorization of the gelatin while the sections are being stained as desired.

2. WARM INJECTION FLUIDS

Carmine Gelatin.—This is by all means the best injection material to use because the color is permanent, but it is very difficult to prepare properly (Hoyer, 1910).

Dissolve 1 gm. of carmine in 1 to 2 cc. ammonia water and 6 to 8 cc. distilled water and heat in a flask on a sand bath until the excess ammonia has been volatilized. Cool and filter.

Allow 50 gm. of sheet gelatin to swell in distilled water for 24 hours. Press out excess water by hand and dissolve the gelatin by heating on a water bath at a temperature not exceeding 60° C.

Add enough of the filtered carmine solution with continual stirring to the gelatin solution to obtain an intense red color.

Next, add 5 to 10 per cent by volume of glycerin and 2 to 3 per cent by weight of chloral hydrate. Filter through flannel in a hot water funnel. If the carmine is precipitated, the material must be discarded and a new lot prepared.

After injection the organ or animal is placed first in cold water for from 1 to 2 hours to hasten the solidification of the gelatin and then is transferred to 80 per cent alcohol or to 10 per cent formalin.

Berlin Blue Gelatin.—Two solutions are needed, a saturated aqueous solution of water-soluble Berlin blue and a solution of gelatin prepared as directed for the carmine gelatin. Warm both solutions in a water bath to a temperature of 60° C., and then add gradually the Berlin blue solution to the gelatin, stirring continually. When the mass is thoroughly mixed add 5 to 10 per cent by volume of glycerin and at least 2 per cent by weight of chloral hydrate. Filter through flannel in a hot water funnel.

Fix tissue after injection in 80 per cent alcohol or 10 per cent formalin. Fixatives containing chrome salts may also be used.

PART II. SPECIAL HISTOLOGICAL METHODS

CHAPTER XI

THE CELL—ITS COMPONENT PARTS, CONSTITUENTS AND PRODUCTS

CELLS in general have a certain structure and pass through certain changes in order to divide. Every cell at least at some stage of its development is composed of a nucleus surrounded by cytoplasm. Some cells develop special structures within their cytoplasm, others outside of it, to perform some special function. Various methods have been devised in order to demonstrate clearly, as far as possible, every variation in structure and function. In disease, cells undergo retrograde changes that require special staining methods for their demonstration. It is the function of this part of the book to set forth both the general and the special methods that will best accomplish these objects.

1. THE NUCLEUS

(1) GENERAL METHODS

For both general and special histological work no stain is more useful or can be more highly recommended than the phloxine- (or eosin-) methylene blue stain after fixation in Zenker's fluid. It brings out nuclei and nuclear figures with great sharpness, and at the same time stains the cytoplasm of certain cells so that they are well defined.

Next in point of general usefulness is the phosphotungstic acid hematoxylin stain, owing to the sharpness with which it stains nuclei, centrioles, and especially nuclear figures, including the spindle. It also demonstrates certain fibrils that other methods fail to show.

For routine laboratory work, namely autopsy and surgical material, when a diagnosis is wanted quickly alum hematoxylin followed by phloxine or eosin, or by van Gieson's picro-acid fuchsin stain for contrast, is still considered the best general stain for celloidin and paraffin sections after a variety of fixatives, including Zenker's fluid.

Of the carmine stains, lithium carmine, followed by picric acid, will be found the most brilliant, generally useful, and permanent, but it is practically useless after fixation in Zenker's fluid.

Safranine gives, perhaps, the most permanent stain of any of the basic aniline dyes, and confines itself exclusively to the nuclei. It is used after certain fixing reagents such as Flemming's and Hermann's solutions. The Heidenhain-Biondi triple stain is useful after fixation in corrosive sublimate, but cannot be used for celloidin sections, so that its field is limited. Other aniline dyes are used on occasion, or for some definite purpose, but not so generally as those mentioned above. For special study of nuclei in their various phases and of certain other structures Heidenhain's iron hematoxylin deservedly holds a very high place.

(2) Mitosis

For the study of mitosis it is important that the tissue be perfectly fresh and that it be fixed in a suitable reagent as quickly as possible. The best results cannot be obtained with tissues placed in a fixing fluid more than half an hour after removal. On the other hand, mitotic figures can be demonstrated in tissues for some time after death (24 hours or longer), especially if kept at a low temperature, before being put into a fixing reagent. However, the details of such figures are not so perfect as those in absolutely fresh tissues and the figures are not so numerous because some of them have completed their changes and can no longer be recognized. It is evident, therefore, that mitosis can be studied much better in tissues from animals, or in tissues obtained by operation from the human, than in organs and tissues removed postmortem.

The choice of fixing reagents for the study of mitotic figures is important. They can often be demonstrated after fixation in alcohol, or even in Müller's fluid, but for careful study quicker and more perfect fixing reagents must be used. The most important fixing reagents are Zenker's, Flemming's, Hermann's and Orth's fluids, and corrosive sublimate. Nearly all the reagents employed penetrate slowly, so that it is absolutely necessary for the best results that tissues to be fixed be cut into very thin slices, rarely over 4 mm. in thickness and preferably not over 2 mm. Flemming's and Hermann's solutions penetrate with great difficulty, so that tissues placed in them should be especially thin. The amount of

fixing reagent used should always be at least 10 to 20 times greater than the volume of the tissue and should be changed if it becomes cloudy.

The most generally useful stain for the study of mitosis is probably safranine. The time of staining varies with the solution used. Babes' is the quickest. The mitotic figures should be stained deeply; then, when treated with alcohol slightly acidulated with hydrochloric acid, they will retain the color while the resting nuclei will become very pale or even decolorized. In consequence of this intense stain mitotic figures can then be very readily found. Fixation in Zenker's fluid and staining in phosphotungstic acid hematoxylin can be highly recommended. Centrioles and spindles are brought out with great distinctness. Heidenhain's iron stain is useful for bringing out the details of the centriole and spindle. Other useful aniline stains are carbol fuchsin and aniline crystal violet, used in the same way as safranine. After fixation in corrosive sublimate mitotic figures can also be demonstrated by the Biondi-Heidenhain solution which stains resting nuclei blue-violet and mitotic figures green.

Safranine Stain for Mitotic Figures.—*Fixation.*—Flemming's or Hermann's solution. Embed in paraffin or celloidin.

Method of Staining.—1. Stain sections 5 minutes to 24 hours in one of the safranine solutions (p. 90). Babes' solution stains most rapidly, the others require 24 hours.

2. Rinse in tap water.

3. Differentiate in 95 per cent alcohol to which a few drops of acid alcohol have been added.

4. Dehydrate in 95 per cent followed by absolute alcohol.

5. Clear in xylol and mount in balsam.

For celloidin sections dehydrate in 95 per cent alcohol, blot and pour on xylol; repeat the last two steps until the specimen is clear.

Results.—Mitotic figures intense red; resting nuclei light red or colorless.

2. CELLULAR ELEMENTS OTHER THAN NUCLEI

Aside from the structures intimately connected with the nucleus (centriole, spindle) the cytoplasm of the different cells in the body contains a variety of structures formerly called cytoplasmic granules, but now recognized and classified under separate headings according to their morphology and staining reactions. They will be con-

sidered separately, most of them in the following pages, a few under bone marrow and blood.

(1) Mitochondria (Plastosomes)

Mitochondria is a term applied to certain bodies that occur in the cytoplasm of many cells and that have received various names. They may be round or oval, rod-shaped, or in the form of filaments. They disappear quickly after death and are destroyed by fixing solutions containing acetic acid, such as Zenker's fluid for example, if the acid content is too high. Special fixatives and staining methods are required for their demonstration. They may be seen and studied in the living cell. Janus green B and several other dyes stain them specifically supravitally. In order to demonstrate them in permanent mounts tissues must be obtained as fresh as possible, cut into thin slices not over 2 to 3 mm. thick and fixed as recommended below.

Mitochondria are acquiring increasing importance in pathological histology and should be studied carefully. The methods that seem to be the simplest and most reliable for their demonstration are the following:

Regaud's Method for Mitochondria (1910).—*Fixation.*—1. Fix for 4 days in the following mixture, freshly prepared, and change to a fresh solution each day:

Potassium bichromate, 3 per cent aqueous solution	80 cc.
Formalin	20 cc.

2. Mordant for 8 days longer in 3 per cent aqueous potassium bichromate, changing the fluid every other day.

3. Wash in running water for 24 hours.

4. Dehydrate and embed in paraffin in the usual manner.

Staining Solution.—Dissolve 1 gm. of hematoxylin in 10 cc. of absolute alcohol, and then add 10 cc. of glycerin and 80 cc. of distilled water. The solution requires several weeks for ripening.

Method of Staining.—1. Mordant section, not over 2 to 5 μ thick, for 8 to 10 days at room temperature (or 1–4 days at 35° C.) in a 5 to 15 per cent aqueous solution of ammonio-ferric alum.

2. Wash for a few minutes in water.

3. Stain for 24 hours with the hematoxylin solution given above.

4. Differentiate in a 5 per cent aqueous solution of ammonio-ferric alum, controlling the result with the microscope.

5. Wash in tap water for 30 minutes.

6. Dehydrate in 95 per cent followed by absolute alcohol.

7. Clear in xylol and mount in balsam.

Results.—Mitchondria stand out sharply stained black.

The Altmann-Kull Method for Mitochondria (1913).—*Fixation.*—Fix in Altmann's fluid (equal parts of 2 per cent osmic acid, and 5 per cent aqueous potassium bichromate) for 24 hours. Wash in running water 24 hours, preserve in 80 per cent alcohol, and embed in paraffin as usual.

Staining Solution.—Dissolve 20 gm. of acid fuchsin in 100 cc. of aniline water (5–10 cc. of aniline thoroughly shaken up in 100 cc. of distilled water and then filtered).

Method of Staining.—1. Pour the stain on the sections and heat gently over a flame until it steams.

2. Cool and wash off the stain with distilled water.

3. Counterstain in a 0.5 per cent aqueous solution of toluidine blue for 1 to 2 minutes.

4. Wash in distilled water.

5. Differentiate in a 0.5 per cent solution of aurantia in 70 per cent alcohol for 20 to 40 seconds, controlling the result with the microscope.

6. Dehydrate in 95 per cent followed by absolute alcohol.

7. Clear in xylol and mount in balsam.

Results.—Mitochondria intensely bluish red; chromatin blue; cytoplasm yellowish brown; albuminous granules bluish red.

Phosphotungstic Acid Hematoxylin Stain for Mitochondria.—Phosphotungstic acid hematoxylin has been used by several workers to demonstrate mitochondria after fixation in Regaud's or Helly's fluids. It stains them to some extent even after fixation in formalin and in alcohol. I have obtained much better results by the following simple method.

Fixation.—1. Fix in neutral 10 per cent formalin for 24 hours or longer.

2. Mordant in a 5 per cent aqueous solution of iron chloride (ferric) at room temperature for 2 to 5 days, changing the solution 2 or 3 times.

3. Wash off in tap water and transfer to 80 per cent alcohol for

24 to 48 hours, changing the solution 2 to 3 times as it becomes yellow.

4. Embed in paraffin and cut sections in the usual way.

Method of Staining.—1. Treat sections with a 0.25 per cent aqueous solution of potassium permanganate for 5 to 10 minutes.

2. Wash in water and place in a 5 per cent aqueous solution of oxalic acid for 3 to 5 minutes.

3. Wash thoroughly in tap water.

4. Stain in phosphotungstic acid hematoxylin (p. 76) for 24 to 48 hours.

5. Wash quickly in tap water.

6. Differentiate and dehydrate in 95 per cent followed by absolute alcohol.

7. Clear in xylol and mount in balsam.

Results.—Nuclei and mitochondria deep blue; collagen and elastic fibrils reddish; myoglia and other fibrils blue.

(2) Golgi Apparatus

Although the Golgi apparatus has not been shown as yet to have any particular significance pathologically, it is important to be able to demonstrate it histologically. The methods so far devised are frequently fickle and unreliable, but excellent results can be obtained with them sometimes. The three best methods available at present are given below.

Da Fano's Cobalt Nitrate Method for the Golgi Apparatus (1920).—*Fixation.*—Fix pieces of tissue 3 mm. in thickness for 6 to 8 hours at room temperature in the following cobalt nitrate solution:

Cobalt nitrate	1 gm.
Water, distilled	100 cc.
Formalin	15 cc.

This solution keeps indefinitely. The formalin need not be neutralized unless it is strongly acid. For embryonic organs and delicate tissues the amount of formalin may be reduced to 10, 8, or 6 cc. for every 100 cc. of distilled water during the first 1 to 2 hours. After that time the solution given above is used. Very small pieces of tissue are fixed in 2 to 4 hours, while pieces of adult spinal cord, cerebrum and cerebellum require 10 to 18 hours for fixation.

Method of Staining.—1. Wash the tissue quickly in distilled water.

2. Place in a 1.5 per cent aqueous silver nitrate solution in the dark at room temperature for 36 to 48 hours.

3. Wash quickly in distilled water and trim to a thickness of not over 2 mm.

4. Reduce in Cajal's reducer for 8 to 24 hours in the dark. For most soft tissues 2 to 8 hours are enough.

Cajal's Reducer

Hydroquinone	2	gm.
Formalin, neutral	15	cc.
Water, distilled	100	cc.
Sodium sulfite, anhydrous	0.3–0.5	gm.

This solution must be made up fresh each time.

5. Wash well in several changes of distilled water, dehydrate and embed in paraffin or celloidin. Cut sections 4 to 8 μ. Thicker ones may sometimes be useful.

6. Tone sections on the slide in a 0.2 per cent aqueous solution of gold chloride for 5 to 10 minutes.

7. Rinse quickly in distilled water.

8. Fix in a 5 per cent aqueous solution of sodium thiosulfate for 10 to 15 minutes.

9. Wash thoroughly in distilled water.

10. Counterstain with Mayer's paracarmine (p. 79).

11. Dehydrate in 95 per cent followed by absolute alcohol.

12. Clear in xylol and mount in balsam.

Results.—Golgi apparatus black; cells pink to red.

For tissues requiring decalcification Weatherford has worked out a slight modification of the Da Fano method which has given good results with teeth.

1. Fix as usual, 6 to 8 hours, in the cobalt nitrate solution.

2. Wash quickly in distilled water.

3. Place in a 5 per cent aqueous solution of trichloracetic acid, changing every other day to fresh acid. The acid is tested for calcium with a 5 per cent aqueous solution of ammonium oxalate. When, on addition of the ammonium oxalate, a flocculent precipitate is no longer formed, decalcification is prolonged overnight in a fresh trichloracetic acid solution as an added precaution.

4. Transfer tissue directly to 80 per cent alcohol for 24 hours, changing the alcohol several times to ensure the removal of the acid.

5. Remove the alcohol by repeated changes of distilled water.

6. Place in the cobalt nitrate fixative for at least 1 hour.

7. Wash the tissue quickly in distilled water.

8. Place in a 1.5 per cent aqueous silver nitrate solution in the dark at room temperature for 36 to 48 hours.

9. Proceed as in the original Da Fano method beginning with Step 3.

Kolatschew and Nassonov's Modification of Kopsch's Osmic Acid Method (1923–1924).—*Fixation.*—Fix very small pieces of tissue in a modification of Champy's fluid for 24 hours at room temperature.

Potassium bichromate, 3 per cent aqueous solution	4 parts
Chromic acid, 1 per cent aqueous solution	4 parts
Osmic acid, 2 per cent aqueous solution	2 parts

Fixation and impregnation must be carried out in glass-stoppered bottles (8 cc. vials are convenient).

Method of Staining.—1. Wash in running water 6 to 24 hours, then rinse in several changes of distilled water.

2. Place in a 1 per cent aqueous solution of osmic acid at 35° C. for 3 to 9 days. If the fluid turns black within the first 4 days renew it.

3. When the reduction of the osmic acid is sufficiently advanced wash the tissue well in running water, usually overnight.

4. Pass rapidly through the alcohol series and oil of cedarwood.

5. Embed in paraffin and cut sections 2 to 5 μ thick.

If the cut sections are too dark the excess osmium may be removed by turpentine. Old oxidized turpentine removes the osmium quicker than new.

Sections may be mounted directly after the removal of paraffin or counterstained first. Kull's method with Altmann's aniline acid fuchsin gives a good counterstain.

6. Stain sections in Altmann's aniline acid fuchsin (p. 92) in the paraffin oven for 30 minutes and cool.

7. Rinse in distilled water.

8. Differentiate with 0.5 per cent aurantia in 70 per cent alcohol for 20 to 40 seconds, controlling with the microscope.

9. Dehydrate in 95 per cent followed by absolute alcohol.

10. Clear in xylol and mount in balsam.

Results.—In sections that are not counterstained the Golgi apparatus appears black on a yellow to gray background. In sections treated by Kull's method the mitochondria are red on a yellow background in contrast with the black Golgi apparatus.

Cajal's Uranium Nitrate Method for the Golgi Apparatus (1912).—*Fixation.*—Fix perfectly fresh pieces of tissue for 10 to 24 hours in Solution A. For nerve cells it is better to fix in Solution B for 10 to 24 hours. Agitate gently once in a while.

Solution A	
Uranium nitrate	1 gm.
Water, distilled	85 cc.
Formalin, neutral	15 cc.
Solution B	
Uranium nitrate	1 gm.
Water, distilled	80 cc.
Formalin, neutral	15–20 cc.
Alcohol, absolute	30 cc.

Method of Staining.—1. Wash quickly in distilled water for a few seconds.

2. Place in 1 to 1.5 per cent aqueous solution of silver nitrate in the dark at room temperature for 36 to 48 hours, according to the size of the piece.

3. Wash quickly in distilled water.

4. Place in the following reducing solution for 8 to 24 hours.

Hydroquinone	1–2 gm.
Formalin, neutral	15 cc.
Water, distilled	100 cc.
Sodium sulfite, anhydrous	0.1–0.5 gm.

Make up the solution just before using it each time. The amount of sodium sulfite used should be just sufficient to produce a yellow color in the solution.

5. Wash in tap water.

6. Dehydrate in graded alcohols and embed in paraffin or celloidin.

Results.—A successful impregnation shows the Golgi apparatus black on a clear yellow background.

3. FATS AND FAT-LIKE SUBSTANCES

Under this heading are included a large number of substances that are chemically and physically related to the fats. Many of them occur in the human body under normal and under pathological conditions united with the protoplasm of the cell in a combined or invisible form and also deposited in adipose tissue subcutaneously, in the omentum, and elsewhere.

These fat-like substances are usually divided into two main groups:

1. Neutral fats, which are esters of fatty acids (chiefly oleic, palmitic and stearic acids) and glycerol.

2. Lipoids, which are substances of a fat-like nature. The most important lipoids are the phosphatides (lecithin, cephalin and sphingomyelin), the cerebrosides (phrenosin and kerasin), and cholesterol. Lecithin is a compound of two fatty acid molecules, phosphoric acid, glycerol, and choline (trimethyl oxyethyl ammonium hydroxide). Cephalin is similar except that the nitrogenous base is amino ethyl alcohol. Sphingomyelin contains a fatty acid, phosphoric acid and two bases (choline and sphingosine), but no glycerol. The cerebrosides contain a carbohydrate (galactose), a fatty acid, and a base (sphingosine), but no phosphorus. Cholesterol is a sterol which occurs free or as an ester in the body.

Lecithin is soluble in alcohol, ether, chloroform and benzene. Cephalin differs in that it is almost insoluble in alcohol. Sphingomyelin and the cerebrosides are relatively insoluble in ether, but soluble in hot alcohol, benzene or pyridine.

These different fats and fat-like substances can be recognized positively only by chemical methods, but many of them possess more or less definite morphological or physical properties or staining reactions that distinguish them from other substances and to some extent from each other. Whenever possible, chemical analyses should be carried out together with the histological examination.

The more or less common properties by which microscopic differentiation of fats and lipoids is made are as follows:

Solubility in alcohol, ether, chloroform and benzene; insolubility in water, dilute acids and alkalies.

Refractiveness, which causes small drops to appear dark in water, while large drops have dark contours and light centers; some lipoids are doubly refractive when viewed under a polarizing microscope.

Reduction of osmic acid as a result of which fats are stained black.

Staining with certain aniline dyes which are soluble in fats.

Examination for fat can be made in the fresh state, either in teased preparations or in frozen sections, in smears, or after fixation. Demonstration of fat is most successful in unfixed tissues. If formalin-fixed material is used it should preferably not remain in the formalin longer than a few days. Fat-dissolving substances must be avoided.

The most important of the general fat stains is scarlet red or Sudan IV. The other general fat stain, formerly used, is osmic acid. It does not react with all fats and fat-like substances and does react with many non-fatty substances, so that it is of limited value as a fat stain.

The supposedly specific fat stains are Nile blue sulfate for separating neutral fats from other fats, Fischler's method for demonstrating fatty acids, and Ciaccio's and Smith-Dietrich's methods for differentiating lipoids from neutral fats. Weigert's method stains normal myelin of the central nervous system and Marchi's method brings out degenerating myelin. Kaufmann and Lehmann (1926, 1928, 1929) have thoroughly examined the specificity of these stains and have come to the conclusion that it is impossible to distinguish between various kinds of fat and fat-like substances by means of stains.

Polarized light is also used for differentiating neutral fats (isotropic) from lipoids (frequently anisotropic). It is impossible, however, to distinguish between neutral fats and fatty acids or between cholesterol esters, phosphatides and cerebrosides.

Scarlet Red (Sudan IV) Stain for Fat.—Almost all fats occurring in the body stain with scarlet red. Because the dye is more soluble in fat than in alcohol, the fat by a purely physical staining process takes up the scarlet red from the alcoholic solution.

Originally Sudan III (Daddi) was used, but it stains less brilliantly. A saturated solution of scarlet red in 70 per cent alcohol

was the solution formerly used. It requires staining overnight in order that the best results may be obtained. Herxheimer's (1901) scarlet red solution stains more rapidly and intensely. It can be highly recommended and is used extensively.

Fixation.—Fix in 10 per cent formalin. Cut frozen sections.

Coverslip preparations fixed in formaldehyde vapor for 5 to 10 minutes can be stained by scarlet red by the same method used for frozen sections.

Staining Solution.—

Herxheimer's Scarlet Red Solution

Scarlet red	1 gm.
Alcohol, 70 per cent	50 cc.
Acetone, C.P.	50 cc.

Keep in a tightly stoppered bottle.

Method of Staining.—A tightly stoppered bottle should be used for staining, as any evaporation of the alcohol and acetone causes a precipitation of the stain.

1. Dip for an instant in 70 per cent alcohol.
2. Stain in the scarlet red solution 2 to 5 minutes.
3. Wash quickly in 70 per cent alcohol.
4. Wash in water.
5. Stain nuclei in alum hematoxylin (p. 70).
6. Wash thoroughly in water.
7. Mount in glycerin or glycerin jelly.

If, after staining with alum hematoxylin, the sections are put into a 1 per cent aqueous solution of acetic acid for 3 to 5 minutes and then washed thoroughly in tap water, the color of the nuclei is a clearer blue, in better contrast with the red color of the fat, and the staining is sharper.

Results.—Nuclei blue; fat orange to red; cholesterol less brilliantly red; normal myelin unstained; and fatty acids unstained.

Osmic Acid Stain for Fat.—Oleic acid and its compounds (also certain non-fatty substances, such as tannic acid and eleidin) are blackened by the reduction of osmium tetroxide to osmium dioxide acting on them. Palmitic and stearic compounds are stained only when exposure to osmic acid is followed by treatment with 60 to 70 per cent alcohol, which changes it to osmium hydroxide—a process called secondary staining of fat.

Fat can be stained by fixing tissues in solutions, such as Flemming's or Marchi's, that contain osmic acid, but there is always danger of reduction of the osmium by embedding fluids dissolving the fat. In embedding in paraffin avoid xylol; use oil of cedarwood instead. Thus, the blocks of tissue are removed from absolute alcohol and transferred to oil of cedarwood. Change the oil of cedarwood several times until the tissue is cleared, and then embed directly in paraffin. To remove the paraffin after cutting place the sections in warm oil of cedarwood in the paraffin oven, or treat quickly with chloroform. For mounting use thickened oil of cedarwood (immersion oil); place 1 drop on the section and cover with a coverslip, or mount in chloroform balsam. The best contrast stain is safranine, as used for mitotic figures, but treatment with acid alcohol must be omitted. Embedding in celloidin is also possible; the alcohol probably protects the osmium from the injurious action of the ether. For a mounting reagent use oil of origanum. The best method, however, and the one recommended, is to stain the fat in frozen sections of formalin-fixed tissue.

Fixation.—Fix in 10 per cent formalin for 24 hours. Cut frozen sections and place in distilled water.

Method of Staining.—1. Place sections in a 1 per cent solution of osmic acid for 24 hours. (Flemming's or Marchi's solution can be used instead.)

2. Wash thoroughly in running water for 6 to 12 hours.

3. Place in absolute alcohol for several hours to obtain the secondary staining of fat.

4. Wash in distilled water.

5. Mount in glycerin jelly.

Results.—Fat black; background yellow-brown.

Nile Blue Sulfate Stain for Separating Neutral Fats from Other Fats.—This dye was introduced by Lorrain Smith (1907–08) as a stain for fat. Boiled with dilute sulfuric acid it is hydrolyzed and produces a new dye of the class known as oxazones. This new dye is red and soluble in neutral fat. Nile blue sulfate itself is not fat soluble but combines readily with fatty acids.

The dye, as bought in powder form, contains a certain amount of red oxazone. This is easily tested for by dissolving a little of the dye in water in a test tube and then adding 1 cc. or more of xylol and shaking thoroughly. If the red oxazone is present the xylol will

assume a fluorescent red color. If not, or if present in too small an amount, it can be developed by boiling the dye in a 0.5 per cent solution of sulfuric acid for 1 to 2 hours under a condenser.

While this dye is not so good or intense a stain for fat in general as scarlet red, it has certain valuable properties. For example, it gives a double stain, coloring neutral fats red, lipoids varying shades of blue. Often there are shades between red and blue owing to the presence of mixtures of various lipoids. There is no danger of loss of fat because the dye is used in an aqueous solution.

Fixation.—Fix in 10 per cent formalin. Cut frozen sections and place them in distilled water.

Method of Staining.—1. Stain in a saturated (less than 2 per cent) aqueous solution of Nile blue sulfate for 20 minutes.

2. Rinse in water.

3. Differentiate in 1 per cent aqueous solution of acetic acid 10 to 20 minutes, or until the colors are clear, which may happen in 1 to 2 minutes.

4. Wash thoroughly in distilled water, which is changed several times, for 1 to 2 hours.

5. Place section on slide, remove excess of water and mount in glycerin or glycerin jelly.

Results.—Neutral fats pink; fatty acids and other fatty substances blue to violet; nuclei and elastic tissue dark blue.

Fischler's Method for Staining Fatty Acid Crystals and Soaps (1904).—This is a modification of Benda's stain for fat necrosis and is based on the observation that fatty acid crystals and their calcium salts, after mordanting in copper acetate, will form with hematoxylin a black compound that is almost insoluble in Weigert's differentiating mixture.

Inasmuch as the sodium and potassium salts of the fatty acids (soaps) are soluble in formalin they must be converted into the insoluble calcium soap by saturating the fixing solution (10 per cent formalin) with calcium salicylate. By comparing stained sections of material fixed each way it is possible to judge how much, if any, soap was present in addition to the fatty acids.

Calcium soaps are not soluble in a mixture of equal parts of alcohol and ether, or in hydrochloric acid, but are in a combination of the two. Consequently they can be differentiated from fatty

acids, which are soluble in the alcohol-ether mixture, or from calcium, which is soluble in hydrochloric acid.

Fixation.—Fix in 10 per cent formalin. Cut frozen sections.

Staining Solutions.—

Weigert's Hematoxylin Solution

Solution A

Hematoxylin	1 gm.
Alcohol, absolute	10 cc.

Solution B

Lithium carbonate, saturated aqueous solution (about 1.25 per cent)	1 cc.
Water, distilled	90 cc.

Mix A and B and let the combined solution ripen for several days before use.

Weigert's Borax-Potassium Ferricyanide

Potassium ferricyanide	2.5 gm.
Borax	2 gm.
Water, distilled	100 cc.

Method of Staining.—1. Mordant the sections in a saturated aqueous solution (12.5 per cent) of copper acetate for 2 to 24 hours at room temperature.

2. Wash in distilled water.

3. Stain for at least 20 minutes in Weigert's hematoxylin.

4. Differentiate in Weigert's borax-potassium ferricyanide mixture, greatly diluted, until the red blood corpuscles are decolorized.

5. Wash thoroughly in distilled water.

6. Mount in glycerin or glycerin jelly.

Results.—Fatty acids deep blue-black. Iron, hemoglobin and calcium may stain also.

If the neutral fats are to be stained by scarlet red proceed as follows after Step 5:

6. Stain in the scarlet red solution (p. 118) several minutes.

7. Pass quickly through 70 per cent alcohol.

8. Wash in distilled water.

9. Mount in glycerin jelly.

Ciaccio's Method of Staining Lipoids (1909–10).—The

claims of Ciaccio that the method stains lipoids specifically have not been substantiated.

Fixation.—1. Place very thin slices of fresh or formalin-fixed tissue in the following solution for 2 days:

Potassium bichromate, 5 per cent aqueous solution	80 cc.
Formalin	20 cc.
Acetic acid, glacial	5 cc.

2. Transfer to a 3 per cent aqueous solution of potassium bichromate for 5 to 8 days.

3. Wash in running water 24 hours.

4. Dehydrate in graded alcohols up through 95 per cent for 24 hours. Absolute alcohol 1 to 2 hours. Embed in paraffin after passing through benzol. Cut sections and return them to 70 per cent alcohol.

Staining Solution.—

Alcohol, ethyl, 80 per cent	95 cc.
Acetone	5 cc.
Sudan III to saturation at 50° C.	

Cool and filter.

Method of Staining.—1. Stain in the above solution in the incubator at 37° C. for ½ to 1 hour.

2. Wash quickly in 50 per cent alcohol.

3. Wash well in distilled water.

4. Stain nuclei in alum hematoxylin (p. 70).

5. Wash in water.

6. Mount in the Apáthy-Kasarinoff gum solution made as follows:

Gum arabic	50 gm.
Cane sugar	20 gm.
Water, distilled	50 cc.
Thymol	0.05 gm.

Filter in oven at 55° C.

Results.—The lipoids that stain are colored orange-yellow; the medullary sheaths of nerves stain bright red.

If Nile blue sulfate is used instead of Sudan III, the lipoids are

stained blue-violet. After Step 2 (under *Fixation*) it is possible to stain the neutral fats with osmic acid which makes a good contrast.

The Lorrain Smith-Dietrich Stain for Lipoids (1910).—This method is based on Weigert's stain for the medullary sheath which consists chiefly of glycolipides (cerebrosides), phospholipides and cholesterol. The method is supposedly positive for phosphatides (sphingomyelin, cephalin), cholesterol fatty acid mixtures, cerebrosides and to some extent for fatty acids and soaps. However, iron, hemoglobin and hematogenous pigment may stain also.

Fixation.—Fix in 10 per cent formalin. Cut frozen sections.

Staining Solution.—

Hematoxylin, 10 per cent in absolute alcohol	10 cc.
Acetic acid, 2 per cent aqueous solution	90 cc.

The alcoholic hematoxylin solution should have ripened at least 6 months.

Method of Staining.—1. Mordant sections in a 5 per cent aqueous solution of potassium bichromate for 24 to 48 hours at 37° C.

2. Wash in distilled water.

3. Stain for 4 to 5 hours in the hematoxylin solution at 37° C.

4. Wash in distilled water.

5. Differentiate in Weigert's borax-potassium ferricyanide solution (p. 121) overnight.

6. Wash thoroughly in distilled water.

7. Mount in levulose syrup made up as follows:

Levulose	30 gm.
Water, distilled	20 cc.

Mix and let thicken for 24 hours at 37° C.

Results.—Lipoid substances blue-black.

Cholesterol and Cholesterol Compounds.—The polarizing microscope is frequently used for the demonstration of the presence of cholesterol and its compounds. Cholesterol compounds usually appear as doubly refractive (anisotropic) spheres with a dark axial cross. However, any fat-like substance except neutral fats and fatty acids may appear as anisotropic spheres with a dark axial cross. It is, therefore, impossible to distinguish with the polarizing

microscope between cholesterol esters, phosphatides and cerebrosides.

Method for Demonstrating Cholesterol after A. Schultz (1924–25).—This method is an application of the Liebermann-Burchardt cholesterol test. It is specific for cholesterol whether free or as an ester. The reaction is never positive if cholesterol is absent, but it sometimes may be negative when cholesterol is present.

Fixation.—Fix in 10 per cent formalin. Cut frozen sections.

Staining Solutions.—

Stock Iron Alum Solution.—Dissolve 100 gm. of ammonio-ferric alum in 1000 cc. of distilled water at room temperature to make a 10 per cent solution.

Dilute Iron Alum Solution.—Dilute 1 volume of the stock solution to 4 times its volume with distilled water, making a 2.5 per cent solution.

Acid Mixture.—Add slowly with external cooling concentrated sulfuric acid (98 per cent H_2SO_4) to an equal volume of glacial acetic acid.

Method of Staining.—1. Place frozen sections for 3 days at 37° C. in the 2.5 per cent ammonio-ferric alum solution.

2. Rinse in distilled water.

3. Float on a slide which has a film of albumin-glycerin.

4. Blot dry with filter paper.

5. Treat with a few drops of the acid mixture dropped on with a glass rod.

6. Cover with a coverslip.

Results.—Within a few seconds a blue-green color appears after the acid mixture has been added, if cholesterol is present. The stain is not permanent; it becomes a grayish brown within ½ hour.

Windaus' Digitonin Reaction for Cholesterol (1909, 1910).—This method is a reaction for free sterols. More work needs still to be done on it. It has the disadvantages that the digitonin is very expensive and that coverslips and slides must be absolutely clean, since any fingerprints give a positive reaction, because of the cholesterol present in perspiration.

Method of Staining.—Mount a frozen section on a slide and cover with a coverslip. Then place 1 drop of a 0.5 per cent solution

of digitonin in 85 per cent alcohol at the edge of the coverslip and let it run under.

Results.—If cholesterol is present digitonin cholesteride crystals (pointed needles) are formed immediately. The digitonin cholesteride crystals may be identified by their solubility. They are insoluble in water, acetone, ether; almost insoluble in cold 85 to 95 per cent alcohol; slightly soluble in boiling absolute alcohol and in methyl alcohol; soluble in glacial acetic acid; and very soluble in pyridine and chloral hydrate.

Golodetz (1908) recommends applying to the frozen sections a mixture of 5 parts of sulfuric acid to 2 parts of 30 per cent formalin. After 1 to 2 minutes cholesterol-containing tissue stains a deep brownish red.

Lipochrome and Lipofuscin.—These are terms applied to fats colored by carotene dissolved in them. They occur in liver and nerve cells and in cardiac muscle fibers under a variety of pathological conditions. The yellow pigment carotene is derived from ingested plant foods, such as carrots, squash or other yellow vegetables.

Myelin.—Morphologists apply the term myelin to the fatty substances forming the medullary sheaths of nerves. The two best methods for its demonstration are Weigert's stain for normal myelin (p. 231) and Marchi's method for degenerating myelin (p. 237).

4. HYALINE SUBSTANCES

A number of hyaline substances occur in the body in normal, and especially in pathological conditions. Three of them, glycogen, mucus and amyloid, give fairly characteristic physical or chemical reactions. The others do not. Some stain with basic, others with acid dyes or not at all. The three definite hyaline substances will be taken up here, the others under the organ or tissue in which they are found.

(1) Glycogen

Glycogen is a carbohydrate of slightly varying composition occurring in cells and nuclei, more rarely in the intercellular tissue, either diffusely or more commonly in the form of larger or smaller masses and droplets of a transparent homogeneous appearance. Like amyloid it stains brown with iodine but can easily be differentiated from it by the fact that, with the exception of the glycogen

from certain sources such as cartilage cells, it is readily soluble in water and does not give the iodine-sulfuric acid reaction.

Fixation.—Two methods are available for fixing and retaining glycogen in tissues, the formalin and the alcohol method. The first is useful for frozen sections. The second is more convenient and is generally regarded as better. Either may be used for celloidin embedding.

Formalin.—Formalin is used either in full strength or diluted, and is saturated with dextrose as described by Neukirch (1909). For frozen or celloidin sections fix 6 to 24 hours and then transfer frozen sections to a saturated aqueous solution of dextrose, and celloidin sections to 80 per cent alcohol saturated with dextrose. Frozen sections can be kept in the dextrose solution or transferred to 95 per cent alcohol. Water must be avoided both before and after fixation. The alum hematoxylin used for staining the nuclei in frozen sections and the water used for washing out the stain must be saturated with dextrose.

Alcohol.—Absolute or 95 per cent alcohol, also Carnoy's fluid and the alcohol-formalin mixture are useful. One point to be borne in mind is that alcohol, especially if absolute, in penetrating the tissue drives the glycogen ahead of it to the inner side of the cells; at least this occurs in the cells near the surface of the fixed tissue.

Embedding.—This should be done in celloidin as it fixes the glycogen in position and thus permits the sections to be treated with water.

Staining.—For staining, two methods are available: staining with iodine or with carmine.

Langhans' Iodine Stain for Glycogen (1890).—*Fixation.*—Formalin saturated with dextrose (see above) or alcohol. Cut frozen sections or embed in celloidin.

Method of Staining.—1. Stain sections in Gram's iodine solution (p. 82) 5 to 10 minutes.

2. Dehydrate in a mixture of 1 part of a 10 per cent solution of iodine in 95 per cent alcohol and 3 or 4 parts of absolute alcohol.

3. Clear and mount in oil of origanum. The sections are mounted in the same oil, as other oils are not so satisfactory. Seal with Duco cement. Preparations keep only a few months.

Results.—Glycogen brown.

Best's Carmine Stain (1906).—This stain affords by all odds

the most brilliant, permanent and satisfactory method of demonstrating glycogen in tissues and has practically superseded the use of iodine.

Fixation.—Fix tissues in absolute alcohol. Embed in celloidin, which prevents the glycogen from dissolving in water. Paraffin and frozen sections should not be used.

Staining Solution.—The stock carmine solution is made as follows:

Carmine	2 gm.
Potassium carbonate	1 gm.
Potassium chloride	5 gm.
Water, distilled	60 cc.

Boil gently and cautiously for several minutes. After cooling add:

Ammonia water, 28 per cent	20 cc.

It is advisable to keep the stock solution in the ice-box.

Differentiating Solution

Alcohol, absolute	80 cc.
Alcohol, methyl	40 cc.
Water, distilled	100 cc.

Method of Staining.—1. Stain sections deeply with alum hematoxylin (p. 70) and wash in water.

2. Decolorize with acid alcohol (1 per cent hydrochloric acid in 70 per cent alcohol), if necessary, after which wash thoroughly in running water.

3. Stain for 5 to 30 minutes in the following solution:

Stock carmine solution, freshly filtered	20 cc.
Ammonia water, 28 per cent	30 cc.
Alcohol, methyl	30 cc.

4. Transfer sections from stain with a glass rod or platinum needle without touching water into the differentiating solution for 1 to 3 minutes, changing the fluid occasionally until it remains clear.

5. Rinse quickly in 80 per cent alcohol.

6. Dehydrate in 95 per cent alcohol.

7. Clear in oil of origanum and mount in balsam.

Results.—Glycogen red; nuclei blue. The method also stains the chief cells of the stomach, the corpora amylacea of the nervous system, and occasionally mucin in goblet cells and the granules of mast cells.

(2) Mucin

Mucus is produced by fibroblasts and by epithelial cells. It is characterized by the presence of mucin, a term used chemically to include a series of closely related nitrogenous substances.

Mucin swells but is not soluble in water. It is precipitated by dilute acetic acid (pseudomucin is not) and by alcohol. If placed in water the precipitate caused by alcohol disappears, that caused by acetic acid does not. Mucin is easily soluble in dilute alkaline fluids.

The commonest and most reliable test for mucin is precipitation in the fresh condition by dilute acetic acid. Place material or tissue (teased or frozen sections) on a slide in water and draw a drop of glacial acetic acid under the coverslip by placing a piece of filter paper on the opposite side.

Fixation.—The best fixative is absolute alcohol, which precipitates the mucin in the form of fine granules, droplets and threads. After 24 hours the tissue may be preserved in 80 per cent alcohol. Formalin can also be used, provided it is not alkaline, without injuring the staining properties and it solidifies the mucus without precipitation. The addition of 5 per cent of acetic acid is advised. After fixation for 12 to 24 hours, preserve in 80 per cent alcohol. Fixation in a saturated solution of corrosive sublimate for 2 to 8 hours followed by several changes of 80 per cent alcohol has been much advocated in the past but does not give nearly so good staining results.

Staining.—Several staining methods are available for demonstrating mucin in sections after fixation. Although not specific they are useful and fairly satisfactory.

Alum hematoxylin stains mucin well under certain conditions after various fixatives, especially alcohol, formalin, or a combination of the two. The best results are obtained by diluting the alum hematoxylin used, taking 1 part of stock solution (p. 70) to 9 parts of 10 per cent alcohol made up with tap water, which is faintly alkaline and will counteract somewhat the acidity of the alum. The alcohol keeps the mucin from swelling. Fixation in absolute alcohol

is strongly advised in spite of its tendency to cause shrinkage. Stain paraffin or celloidin sections for 30 minutes to 1 hour or longer, wash for 5 to 10 minutes in several changes of tap water and dehydrate and mount in the usual way. The mucin derived from fibroblasts stains intensely blue. The same is true of fresh mucin of epithelial origin but not of old mucin. A light contrast stain with phloxine is sometimes desirable. The sections should not touch water at any stage until after they are stained.

The two following staining methods can be highly recommended and the results are almost specific.

Mayer's Muchematein Stain (1896).—This stain is especially recommended for mucin of fibroblastic origin. Mucin produced by epithelial cells sometimes stains intensely (bronchus), or poorly (colon), or not at all (stomach), unless the mucus is being actively formed.

Fixation.—Absolute alcohol. Embed in paraffin or celloidin.

Staining Solutions.—Two different solutions are recommended. The alcoholic solution is considered preferable.

Aqueous Solution

Hematein	0.2	gm.
Aluminum chloride	0.1	gm.
Glycerin	40	cc.
Water, distilled	60	cc.

Mix the hematein with a few drops of glycerin and add the aluminum chloride, then add the rest of the glycerin and water.

Alcoholic Solution

Hematein	0.2	gm.
Aluminum chloride	0.1	gm.
Alcohol, 70 per cent	100	cc.
Nitric acid	1	drop

Method of Staining.—1. Stain sections for 10 to 15 minutes, sometimes for 1 hour or longer.

2. Wash for 5 to 10 minutes in several changes of distilled water.

3. Dehydrate in 95 per cent followed by absolute alcohol.

4. Clear in xylol and mount in balsam.

Celloidin sections should be cleared in terpineol or oil of origanum from 95 per cent alcohol.

Results.—Only mucin stains blue.

Mayer's Mucicarmine Stain (1896).—This stain is particularly valuable for mucin derived from epithelial cells (bronchi and colon for example). Mucin derived from fibroblasts stains poorly.

Fixation.—Absolute alcohol. Embed in paraffin or celloidin.

Staining Solution.—

Carmine	1 gm.
Aluminum chloride, anhydrous	0.5 gm.
Water, distilled	2 cc.

Combine and heat over a small flame for 2 minutes, stirring constantly, until the reddish mixture becomes a dark color. Then gradually add carefully, while stirring constantly, 100 cc. of 50 per cent alcohol. Let the solution stand for 24 hours, then filter. This stock solution keeps well.

For use dilute 1 part of stock solution with 10 parts of distilled water or of 50 to 70 per cent alcohol. Again, the alcoholic solution is preferable.

Method of Staining.—1. Stain sections for 10 to 15 minutes or longer with the diluted mucicarmine stain.

2. Wash quickly in water.

3. Dehydrate in 95 per cent followed by absolute alcohol.

4. Clear in xylol and mount in balsam.

Celloidin sections should be cleared in terpineol or oil of origanum from 95 per cent alcohol.

Results.—Only mucin stains red.

Thionin Stain for Mucin.—Mucin tends to stain more or less intensely with certain basic aniline dyes. The most useful is thionin because it gives a metachromatic stain.

Fixation.—Absolute alcohol. Embed in paraffin or celloidin.

Method of Staining.—1. Stain sections in a 1 per cent solution of thionin in 25 per cent alcohol for 15 minutes to 1 hour or longer.

2. Differentiate in 95 per cent followed by absolute alcohol.

3. Clear in xylol and mount in balsam.

Clear celloidin sections after 95 per cent alcohol in terpineol or oil of origanum.

Results.—The mucin appears light to dark red or purple, or even blue, depending on the intensity of the stain.

Fuchsin Stain for Mucin.—The following simple stain, although less specific, is sometimes useful.

Fixation.—Absolute alcohol. Embed in paraffin or celloidin.

Method of Staining.—1. Stain sections in dilute alum hematoxylin as already recommended above for 5 to 15 minutes.

2. Wash in several changes of water.

3. Stain 5 to 10 minutes in a 0.1 per cent aqueous solution of basic fuchsin.

4. Decolorize and dehydrate in 95 per cent followed by absolute alcohol.

5. Clear in xylol and mount in balsam.

Celloidin sections are cleared in terpineol or in oil of origanum, after 95 per cent alcohol.

Results.—Epithelial mucin is stained intensely red but so also are mast cells, hyaline droplets in plasma cells and zymogen granules in the glands of the stomach. The mucus in the stomach does not stain by this method or by any other unless freshly produced.

Two staining methods in common use give an excellent idea of the general distribution of epithelial mucin. The phloxine-methylene blue stain after Zenker fixation stains the mucin pale blue, even in the stomach. The second method, Mallory's aniline blue collagen stain, also stains mucin blue, but of a deeper shade. For staining the mucin in the stomach it seems to be the most satisfactory stain we have.

(3) Amyloid

Amyloid is a combination of an albuminous substance with chondroitin sulfuric acid. It is insoluble in water, alcohol, ether and dilute acids, and is not digested by pepsin and hydrochloric acid. It is distinguished from the other homogeneous substances, except glycogen, by the fact that iodine stains it mahogany brown. The reaction is particularly useful as a test on fresh gross material. If a section containing amyloid be quickly and lightly stained in Gram's iodine solution and then transferred to sulfuric acid, the color of the amyloid will usually change at once or in a few minutes from red to violet to blue. Sometimes the color turns merely a deeper brown. Several of the aniline dyes, especially crystal violet, give almost as perfect characteristic color reactions (metachromatic) for amyloid as iodine, and are perhaps to be preferred for the pur-

poses of histological study. Any of these differential stains may be used with fresh or fixed tissues. Alcohol as a fixing reagent gives the best results, but the other fixatives, especially formalin, may be employed. Mayer's method for amyloid gives a metachromatic stain that keeps well for years. Another useful, simple and permanent method is that of Bennhold, using Congo red. The nuclei can be stained with alum hematoxylin, thus affording a bright contrast of color. The ordinary double stains of alum hematoxylin with phloxine or van Gieson's mixture will often be found of value in studying the distribution of amyloid. The aniline blue connective tissue stain can also be highly recommended as it stains amyloid light blue so that it stands out in marked contrast to the red of the tissue cells, or the bright blue of collagen fibrils.

Iodine Reaction for Amyloid.—*Fixation.*—Fix in absolute alcohol or 10 per cent formalin. Cut paraffin or frozen sections.

Method of Staining.—1. Stain sections in a weak solution of iodine (Gram's solution [p. 82] diluted until of a clear yellow color) for 3 minutes.

2. Wash in water.

3. Mount and examine in water or glycerin.

Results.—Amyloid mahogany brown. If the reaction of the tissue is strongly alkaline, a condition that may result from postmortem decomposition, the color reaction with iodine will not take place. In such cases the tissue or sections should be treated with dilute acetic acid before applying the test. The reaction of amyloid with iodine may be increased by treating the section after staining with dilute acetic acid.

Langhans' Method for Obtaining Permanent Mounts with Iodine (1890).—*Fixation.*—Fix in alcohol. Embed in celloidin.

Method of Staining.—1. Stain in Mayer's alcoholic carmine solution (p. 79) for 10 to 15 minutes or longer.

2. Place sections in Gram's iodine solution (p. 82) 5 to 10 minutes.

3. Dehydrate quickly in 10 per cent tincture of iodine 1 part, absolute alcohol 3 or 4 parts.

4. Clear and mount in oil of origanum.

Results.—Amyloid brown. The color is said to keep remarkably well. Other oils or balsam cause it to fade quickly. Staining

in Gram's iodine solution may be omitted as the tincture of iodine usually stains the amyloid sufficiently deeply.

Iodine and Sulfuric Acid Reaction.—*Method of Staining.*—1. Stain frozen sections of fresh unfixed tissue quickly and lightly in Gram's iodine solution (p. 82).

2. Treat with sulfuric acid, either concentrated or dilute (1–5 per cent) on a slide or in a staining dish. Strong hydrochloric acid may be used in the same way.

3. Examine sections in water or glycerin.

Results.—Amyloid stains mahogany brown with iodine. On the addition of sulfuric acid the change of color from red to violet to blue usually occurs within a few minutes, but occasionally does not take place at all.

The following substances give reactions with the above iodine tests:

1. Cholesterol crystals are stained rather deeply brown with dilute iodine solution, and turn a brilliant blue color at the edges on the application of strong sulfuric acid.

2. Corpora amylacea in the prostate and central nervous system stain brown with the dilute iodine solution.

3. Starch granules stain blue with dilute iodine solution.

4. Cellulose stains yellow with iodine. If washed and treated with strong sulfuric acid it turns blue where the acid touches it.

Bennhold's Congo Red Stain for Amyloid (1922).—This method will be found simple and reliable, and the results are excellent. It stains amyloid vitally if injected intravenously.

Fixation.—Absolute alcohol or 10 per cent formalin. Embed in paraffin or celloidin, or cut frozen sections.

Method of Staining.—1. Stain sections in a 1 per cent aqueous solution of Congo red for 10 to 20 minutes, or longer if a deep color is desired.

2. Dip in a saturated aqueous solution of lithium carbonate (1.25 per cent) for 15 seconds.

3. Decolorize in 80 per cent alcohol until clouds of stain no longer come from the section. If the section is not uniformly decolorized Steps 2 and 3 can be repeated.

4. Wash in water for 15 minutes.

5. Counterstain with alum hematoxylin (p. 70).

6. Wash in water.

7. Dehydrate in 95 per cent and absolute alcohol.

8. Clear in xylol and mount in balsam.

Clear celloidin and frozen sections in terpineol or in oil of origanum after 95 per cent alcohol.

Results.—Amyloid red; nuclei blue; hyalin and colloid unstained.

Crystal Violet Stain for Amyloid.—*Fixation.*—Absolute alcohol or 10 per cent formalin. Cut frozen sections.

Method of Staining.—1. Stain sections of unfixed or of fixed tissue in 1 per cent aqueous solution of crystal violet 3 to 5 minutes.

2. Wash in a 1 per cent aqueous solution of acetic acid.

3. Wash thoroughly in water to remove all trace of acid.

4. Examine in water or in glycerin. The stain will keep for some time if the sections are mounted in a saturated solution of potassium acetate (about 200 per cent) or in levulose (p. 123).

Other methods are to stain in aniline crystal violet (p. 89) and to wash out in 1 per cent hydrochloric acid, or to stain in a strong solution of crystal violet to which acetic acid is added, and to differentiate in water.

Results.—The amyloid is stained violet-red, the tissue blue. The color reaction shows best when the light for the microscope is taken from a white cloud, not from the blue sky.

Iodine Green Stain for Amyloid.—*Fixation.*—Absolute alcohol or 10 per cent formalin. Fresh unfixed material may be used also. Cut frozen sections.

Method of Staining.—1. Stain in a 0.33 per cent aqueous solution of iodine green for 24 hours.

2. Wash in water.

3. Mount in water or glycerin.

Results.—Amyloid violet-red; other tissue elements green. Stilling claims that the reaction is surer than that with crystal violet.

Mayer's Stain for Amyloid.—*Fixation.*—Absolute alcohol or 10 per cent formalin. Embed in paraffin.

Method of Staining.—1. Transfer paraffin sections without previous treatment directly from the knife to a warmed (40° C.) 0.5 per cent aqueous solution of methyl violet (crystal violet) for 5 to 10 minutes.

2. Wash in water and differentiate in a 1 per cent solution of acetic acid for 10 to 15 minutes.

3. Wash thoroughly in water.

4. Transfer to a saturated aqueous solution of ammonium or potassium alum (about 15 per cent) diluted one-half with water and wash in water.

5. Transfer sections to slide and allow the water to evaporate.

6. Remove paraffin, clear with xylol and mount in balsam.

Results.—Nuclei blue; amyloid reddish violet.

5. PIGMENTS

The various pigments found in the human body under normal and pathological conditions are commonly divided into three groups: (1) hematogenous pigments, derived from the coloring matter of the blood; (2) autogenous pigments, formed by cells from colorless elements of nutrition; and (3) exogenous pigments, introduced into the body from without.

(1) Hematogenous Pigments

These occur in a considerable variety. Some, such as hemoglobin, contain iron but it is too intimately bound up to the protein group in the molecule to react to the ordinary tests. From the pathological point of view hemosiderin is the most important of these pigments, which are listed below with their characteristics and staining reactions.

(*a*) Hemoglobin and Methemoglobin.—Soluble in water and in dilute alcohol. They occur as yellowish to yellowish brown granules and droplets, which in hemoglobinuria may be compacted into casts, and in the fresh condition but not after fixation stain bright red on treatment with potassium hydroxide, sodium carbonate or prussic acid. For fixation use the same solutions as for the preservation of red blood corpuscles, namely Zenker's fluid, formalin or corrosive sublimate. The granules will stain intensely with eosin or phloxine.

(*b*) Parhemoglobin.—A form of hemoglobin, crystallizes like it but is insoluble in alcohol.

(*c*) Sulfmethemoglobin (Sulfhemoglobin).—A greenish compound of sulfur and methemoglobin to which the greenish color frequently seen in the abdominal wall of cadavers is due. It may occur in the blood during life (sulfhemoglobinemia). The diagnosis is made by spectroscopic examination of the blood, but it must be distinguished from methemoglobin.

(*d*) HEMATIN.—Occurs as an amorphous, dark brown or bluish black material in old extravasations of blood.

(*e*) HEMOFUSCIN.—Light yellow granules which stain intensely with certain aniline dyes, especially basic fuchsin and methylene blue. They do not give the iron reaction, are insoluble in hydrogen peroxide, are found in the liver and certain other tissues in hemochromatosis, and are well preserved in all ordinary fixatives.

Mallory's Fuchsin Stain for Hemofuscin.—*Fixation.*—Zenker's fluid, alcohol or 10 per cent formalin. Embed in paraffin or celloidin.

Method of Staining.—1. Stain in alum hematoxylin (p. 70) until the nuclei stand out sharply defined.

2. Wash thoroughly in water.

3. Stain for 5 to 20 minutes in the following solution:

Fuchsin, basic	0.5 gm.
Alcohol, 95 per cent	50 cc.
Water, distilled	50 cc.

4. Wash in water.

5. Differentiate and dehydrate in 95 per cent followed by absolute alcohol.

6. Clear in xylol and mount in balsam.

Celloidin sections are cleared in terpineol or oil of origanum after 95 per cent alcohol.

Results.—Nuclei blue; hemofuscin granules bright red; hemosiderin unstained. The stain sharply differentiates the granules of hemofuscin from those of hemosiderin but no other claim is made for it except that it does not stain melanin.

(*f*) MALARIAL PIGMENT (Hematin, According to W. H. Brown).—A black pigment occurring as granules. It is formed only by the action of the malarial parasites living on or in the red blood corpuscles and can be distinguished from carbon by its solubility in concentrated sulfuric acid, but it does not give iron reactions.

(*g*) HEMOSIDERIN.—Occurs as bright colored, yellowish brown to brown granules and masses. It is insoluble in water, alcohol, ether and alkalies, but is soluble in acids. It does not stain with fat stains and gives reactions for iron both in the fresh state and after fixation. The best fixative for tissues containing hemosiderin is alcohol, the alcohol-formalin mixture comes next, formalin third

The formalin used should be neutral. Fixatives containing chrome salts are generally advised against and yet good results can be obtained after fixation in Zenker's fluid. Hemosiderin tends to diffuse in the tissues as the result of postmortem changes. Hence it is important to obtain tissues as fresh as possible and to cut them into thin slices for fixation.

There are two diagnostic chemical reactions commonly used in histological work for the demonstration of iron—the Berlin blue method and the Turnbull blue method of Tirmann and Schmelzer. The latter is based on the iron sulfide reaction of Quincke. In the Berlin blue method the sections are treated with hydrochloric acid and potassium ferrocyanide, either combined or used separately. In the Turnbull blue method the sections are first treated with ammonium sulfide, which converts the iron compounds into iron sulfide, and then with an acidulated solution of potassium ferricyanide which transforms the ferrous sulfide into Turnbull's blue. In handling frozen and celloidin sections it is necessary to use glass or platinum needles as the iron solutions cause corrosion of ordinary metal instruments.

It has long been a question which of these two diagnostic iron reactions demonstrates the greatest amount of iron present and which gives the truest picture. As a rule preference has been given to the Turnbull blue method of Tirmann and Schmelzer (1898).

Recently (1936) Gömöri has studied critically the various iron reactions and reached certain definite conclusions which seem well substantiated. The first and most important one is that the best microchemical reagent for iron is a mixture of equal parts of 20 per cent hydrochloric acid and a 10 per cent solution of potassium ferrocyanide. The exposure should be for 30 minutes. The two solutions should be freshly prepared with distilled water and filtered. After staining, the sections should be washed thoroughly in distilled water and then counterstained by the method given for hemofuscin. The results obtained by this method are clean-cut and all that could be asked for.

One of the numerous modifications of the original Berlin blue method, however, seems worth while adding, that by Liesegang, as it excludes any chance of iron contained in the acid staining the section. Soak the section for 5 minutes or longer in a 2 per cent aqueous solution of potassium ferrocyanide and then transfer it to a

slide, invert it over a small dish containing a few drops of concentrated hydrochloric acid, and expose it to the vapor for 30 minutes or longer. Set slide and dish inside of a larger covered dish to prevent evaporation. Wash and counterstain with Mallory's fuchsin stain for hemofuscin.

Turnbull's Blue Method for Hemosiderin (1898).—*Fixation.*—Absolute alcohol or 10 per cent formalin. Embed in paraffin or celloidin.

Method of Staining.—1. Transfer sections from distilled water into a saturated, somewhat yellowish aqueous solution of ammonium sulfide (ammonium sulfide is very soluble in water) for 1 to 24 hours. Personally I prefer to use a mixture of 1 part of strong yellow ammonium sulfide to 3 parts of 95 per cent alcohol. Use a glass staining dish with tightly fitting cover to prevent evaporation of the ammonia. The alcohol serves two purposes; it prevents paraffin sections from dissolving off the slide, and protects celloidin sections from wrinkling.

2. Wash thoroughly in distilled water.

3. Transfer to a freshly prepared mixture of equal parts of 1 per cent hydrochloric acid and 20 per cent solution of potassium ferricyanide for 10 to 20 minutes to convert the ferrosulfide which is formed into Turnbull's blue.

4. Wash thoroughly in distilled water.

5. Counterstain in a 0.5 per cent solution of basic fuchsin in 50 per cent alcohol for 5 to 20 minutes.

The counterstains ordinarily used on sections after the iron reaction to bring out the nuclei are paracarmine (p. 79) and Kernechtrot. Lithium carmine should not be used because of the injurious effect of the alkali on the iron stain. I can strongly recommend basic fuchsin. It not only stains the nuclei sharply but also the hemofuscin granules sometimes present, especially in pigment cirrhosis.

6. Wash off in water.

7. Differentiate and dehydrate in 95 per cent followed by absolute alcohol.

8. Clear in xylol and mount in balsam.

Clear celloidin sections in terpineol or in oil of origanum after 95 per cent alcohol.

Results.—Nuclei and hemofuscin granules bright red; hemosiderin blue.

The specific stains with Berlin blue and Turnbull's blue are not permanent. They soon fade, often within a few months, sometimes within a few weeks. The same is true of the iron sulfide reaction. The fading invariably starts in the center and spreads peripherally. The color can be restored quickly by exposing the section to air or, even better, to a dilute hydrogen peroxide solution. According to Gömöri the decolorization is due to reduction. The Canada balsam takes up the oxygen and reduces the stains to colorless compounds. Therefore, he advises the use of a mounting medium rich in oxygen. The best he could devise was almost dry Canada balsam diluted with old oxidized oil of turpentine to a thick syrup-like consistence.

Although not ordinarily used for that purpose, hematoxylin is probably the most delicate of all stains for iron; unfortunately it is not specific. It is equal, however, to Quincke's iron sulfide test in the sharp picture it gives and has the great advantage over it that it is permanent. The stain is of great value in the study of pigment cirrhosis because only two metals are present, iron and copper, and it distinguishes between them by the color reactions, iron brownish black to black and copper clear blue. The stain is also useful for microphotographic purposes.

Mallory's Hematoxylin Stain for Iron and for Copper.—*Fixation.*—Absolute or 95 per cent alcohol. After formalin fixation the iron stains only yellow to brown. Embed in paraffin or celloidin.

Staining Solution.—Dissolve 5 to 10 mg. of hematoxylin in a few drops of 95 per cent or absolute alcohol and add to it 10 cc. of freshly boiled distilled water. Boiling for 5 minutes is sufficient to drive off the contained carbon dioxide and render the water neutral.

Method of Staining.—1. Stain sections in the hematoxylin solution for 1 hour or longer.

2. Wash in several changes of tap water for 1 hour.

3. Dehydrate in 95 per cent and absolute alcohol.

4. Clear in xylol and mount in balsam.

Clear celloidin sections in terpineol or in oil of origanum after 95 per cent alcohol.

Results.—Nuclei bluish gray; hemosiderin intense black; copper light to dark clear blue.

Stain for Gross Specimens Containing Hemosiderin.—The

potassium ferrocyanide and hydrochloric acid reaction can easily be applied to slices of tissue in the fresh condition for diagnostic purposes or after fixation to make attractive and instructive preparations for use in teaching or for preservation in a medical museum.

Method of Staining.—1. Fix large slices of liver and other organs containing iron in Kaiserling's or Jores' Solution No. I (pp. 380, 381) for several days

2. Wash thoroughly in running water for 24 hours.

3. Preserve in 80 per cent alcohol.

4. Cut into thin slices so as to obtain perfectly smooth surfaces.

5. Place the slices to be stained in a freshly prepared solution of equal parts of 20 per cent hydrochloric acid and 10 per cent aqueous solution of potassium ferrocyanide for 15 to 30 minutes, moving them about frequently.

6. Wash in running water for 24 hours or longer to remove all trace of chemicals.

7. Preserve and mount in 80 per cent alcohol (not in Kaiserling's Solution No. III, which causes the blue color to diffuse) with unstained slices of the same specimens for contrast.

Results.—Hemosiderin blue.

(*h*) HEMATOIDIN (BILIRUBIN ITS ISOMER).—Occurs as orange colored or red rhombic plates, or as radiating bunches of yellow needles, contains no iron, is insoluble in water, ether, and nearly so in alcohol, but dissolves in chloroform. It is found in old hemorrhages, especially in infarcts of the spleen and brain.

(*i*) BILE PIGMENT (BILIRUBIN-HEMATOIDIN).—Insoluble in water or ether and nearly so in alcohol. It occurs as yellowish granules and masses, and by oxidation is converted into the green biliverdin (this reaction takes place also when tissues containing bile are fixed in solutions containing potassium bichromate). Bile pigment on the addition of nitric acid containing a trace of nitrous acid gives a succession of colors, green, red, then blue, which can be observed with the microscope (Gmelin's test).

(*j*) HEMATOPORPHYRIN (HEMATIN MINUS IRON).—Traces of it appear normally in the urine but the amount may be much increased in certain diseases and then may color the urine Burgundy red. It is decomposed by concentrated sulfuric or nitric acid, with the same color reaction (green, red, blue) as bile pigment, but it is not soluble in dilute acids or alkalies, and does not bleach.

(2) Autogenous Pigments

The pigments produced in the body, aside from those of hematogenous origin, are grouped under the terms waste pigments and melanin. Melanin occurs normally in hair and in the deeper layers of the epidermis (most abundantly in people of dark complexion), and in the melanophores of the corium and pia, and in the eye.

Pathologically, melanin is present abundantly in the melanoblasts and melanophores, forming freckles and pigmented nevi. The tumors arising from the pigment-forming cells are known as melanoblastomas. When the tumor growth is very extensive the pigment may be excreted in the urine, usually in the form of melanogen. In Addison's disease, which results from destruction of the adrenal glands, usually by tuberculosis, the deposit of melanin is greatly increased in the epidermis, liver, heart and brain.

Melanin is a dark brown amorphous substance of great coloring power which occurs in cells as brown to black granules. It contains no iron or fat and does not react to the iron tests but is tinged slightly by the dyes used in fat stains, such as scarlet red and Nile blue sulfate. It is insoluble in ordinary reagents except alkalies and is very slowly bleached by sunlight and by oxidizing agents such as hydrogen peroxide and ferric chloride. It is stained black by the silver nitrate methods of Bielschowsky and Levaditi.

(3) Exogenous Pigments

Various substances gain entrance to the body and are deposited there as pigments. The most important of these are carotene, carbon and silver.

(*a*) Carotene.—The principal one of the carotenoids is an orange-yellow pigment derived from carrots, squash and certain other vegetables. It is easily decolorized by the action of strong sunlight and by oxidizing agents such as hydrogen peroxide and ferric chloride, and does not stain by the silver nitrate methods. It is readily soluble in fats and fat solvents and is, therefore, on account of this latter property easily extracted from tissues by the action of alcohol, ether or chloroform. The yellow color of fats in the human body is due to it. If it is ingested in too large quantities it may color yellow the skin of infants or the conjunctivae of adults, suggesting jaundice.

Carotene exists in the carrot in granular form. It does not stain with Nile blue sulfate. In the human body carotene is always dissolved in fat which in consequence is colored by it. Hence, Nile blue sulfate is often used to differentiate this stained fat from waste pigment present in brown atrophy of the liver and heart. The important point to realize is that the Nile blue sulfate dye stains the fat but not the pigment.

Fat colored with carotene (lipochrome) often occurs in the same cells (liver, heart) with waste pigment and occasionally with hemosiderin; hence confusion arises if differential stains are not properly carried out and the results correctly interpreted.

If fixed tissues, in order to be embedded, are passed through alcohol, ether, chloroform, and so on, only hemosiderin, hemofuscin, melanin and waste pigment persist. The carotene is dissolved out with the fat; hence it must be studied in the unfixed state or in frozen sections of tissues preserved in formalin.

(*b*) CARBON.—Occurs commonly and most abundantly in the lungs and peribronchial lymph nodes but is sometimes transplanted to the spleen and liver where its recognition occasionally is important. It must be distinguished from malarial pigment, from hemoglobin-formalin precipitate, and possibly from iron. Its distinguishing characteristics are its black color and its insolubility in concentrated sulfuric acid in which all other pigments dissolve.

(*c*) SILVER.—Appears brown to black, is turned black by ammonium sulfide, and is removed by a mixture of potassium ferricyanide and sodium thiosulfate, or by Weigert's borax-potassium ferricyanide differentiating fluid. As a result of treatment therapeutically with silver nitrate, silver may be deposited in the skin, intestine, spleen, kidney, and so on, or in the kidney alone, as the result of injection of argyrol into the pelvis of the kidney.

6. INORGANIC SUBSTANCES

(1) IRON

To demonstrate iron in tissues the most commonly used microchemical method is the Turnbull blue method as given by Tirmann and Schmelzer (p. 138).

Iron can be removed from tissues by a 5 per cent solution of oxalic acid acting for 1 hour or more.

(2) Lead

Mallory's Hematoxylin Test for Lead.—Lead in tissues is stained bluish gray to black by freshly prepared, faintly alkaline solutions of hematoxylin. As soon as the hematoxylin in a solution ripens (minutes to hours), it colors the lead brownish and is useless for test purposes. Alcohol slows up considerably the process of ripening in alkaline solutions—for example, those containing ammonium hydroxide, lithium carbonate or sodium bicarbonate. The most satisfactory solution for test purposes yet discovered is that made with calcium carbonate.

Fixation.—Absolute alcohol or 10 per cent formalin. Embed in celloidin or paraffin.

Staining Solution.—Dissolve 5 to 10 mg. of hematoxylin in a few drops of absolute or 95 per cent alcohol. Add 10 cc. of a saturated aqueous solution of calcium carbonate (about 0.146 per cent).

Method of Staining.—1. Stain sections in the hematoxylin solution for 2 to 3 hours in the paraffin oven at a temperature of about 54° C.

2. Wash sections in several changes of tap water for 10 to 20 minutes.

3. Dehydrate in 95 per cent alcohol.

4. Clear in terpineol and mount in terpineol balsam.

Fresh neutral balsam fades the stain slightly, giving it a grayish tint, and old xylol balsam turns it brown at once; hence the method advised above.

Results.—Lead in tissues stains bluish gray to black.

(3) Calcium

Calcification, the more common form of petrifaction, is the term applied to the infiltration of tissues with calcium phosphate and calcium carbonate. The salts appear microscopically as small, very refractive granules which may be mistaken for fat, or as large masses formed by the fusion of granules. They are dissolved by hydrochloric or nitric acid (5 per cent solution). If calcium carbonate is present bubbles of carbon dioxide are set free. Calcium phosphate dissolves without effervescence. To differentiate between lime salts and other substances soluble in hydrochloric acid use concentrated sulfuric acid to form lime sulfate (gypsum), which appears as fine, short radiating needles. On dissolving out the lime

salts a matrix of dead tissue or of hyalin will usually be found left behind. As a rule this hyaline material is colored deep blue in alum hematoxylin or red in van Gieson's stain.

The deposits of calcium salts themselves also stain with hematoxylin, so that this dye can be used to demonstrate the masses and coarser granules of them. The tissue must, however, first be freed of certain iron combinations, which are often associated with deposits of lime and also stain with the hematoxylin.

Kóssa has shown that calcium phosphate can be demonstrated by means of silver nitrate, which forms silver phosphate on the surface of the granules and blackens in the presence of light. It gives an exaggerated picture of the amount of lime salts present. Klotz has shown that the silver nitrate acting for many hours affects calcium carbonate also; the granules become coated with silver carbonate, which in sunlight gives off carbon dioxide, leaving the black silver oxide. This process can be hastened by putting the sections, after staining and thorough washing, into a dilute soluble sulfide.

Kóssa's Silver Nitrate Method for Staining Calcium (1901). —*Fixation.*—Fix tissues in 80 to 95 per cent alcohol. Cut frozen sections of non-decalcified or not completely decalcified material, or embed in paraffin.

Method of Staining.—1. Rinse sections in distilled water.

2. Place in a 5 per cent aqueous solution of silver nitrate for 10 to 60 minutes and expose to strong light.

3. Wash thoroughly in distilled water.

4. Reduce for a few minutes in a 5 per cent solution of sodium thiosulfate to remove excess of silver nitrate.

5. Wash thoroughly in distilled water.

6. Counterstain in safranine (p. 90) or carmalum (p. 78).

7. Dehydrate in 95 per cent and absolute alcohol.

8. Clear in xylol and mount in balsam.

Results.—The calcium is stained a deep black where it occurs in masses. Finely dispersed granules do not stain deeply. Nuclei are red.

Gömöri's Silver Nitrate Stain for Insoluble Lime Salts (1933).—The principle of this method is to stain the lime salts in blocks of fixed tissue, before decalcification and sectioning, by means of silver nitrate and then to decalcify, embed and stain by routine methods.

Fixation.—Cut or saw thin blocks of fresh tissue 1 to 2 mm. thick and fix in 80 to 95 per cent alcohol for 2 to 4 days. The dilution of alcohol must be made with distilled water. Tissues may also be fixed by boiling in a 2 per cent potassium nitrate or a 5 per cent alum solution in 20 per cent alcohol for 5 minutes.

Method of Staining.—1. Wash tissues in distilled water for 3 to 4 hours.

2. Impregnate in 1.5 per cent aqueous solution of silver nitrate for 6 to 10 days at room temperature. Change silver solution once or twice.

3. Wash for 3 to 4 days in distilled water, changed daily 4 or 5 times, until the last washing water decanted does not show the slightest turbidity when mixed with hydrochloric acid.

4. Reduce in a 5 per cent aqueous solution of sodium thiosulfate. Before use add 4 to 5 drops of a N/10 sodium hydroxide solution to each 100 cc. of the reducer. Keep blocks in reducer for 4 to 8 days.

5. Wash in running water for 3 to 4 hours.

6. Fix in a 3 to 5 per cent aqueous solution of sodium thiosulfate for 2 days.

7. Wash in running water for at least 24 hours.

8. Decalcify in a 6 to 8 per cent aqueous solution of sulfosalicylic acid for 1 to 3 days, according to the thickness or the hardness of the blocks. Change the acid solution once or twice.

9. Wash in running water for at least 24 hours.

10. Embed and cut sections by routine methods.

11. Stain as desired.

Results.—Calcium is stained black.

Alum Hematoxylin Method for Staining Sodium Urate Crystals.—Another form of petrifaction is that found in gout, due to the infiltration of certain tissues with uric acid salts, of which sodium urate is the most common. The crystals are soluble with difficulty in cold water, insoluble in alcohol and ether.

Fixation.—Alcohol, 95 per cent or absolute. Embed tissues in celloidin.

Method of Staining.—1. Stain sections quickly in a cold solution of alum hematoxylin (p. 70).

2. Wash quickly in cold water.

3. Dehydrate in 95 per cent alcohol.

4. Clear in terpineol or in oil of origanum and mount in balsam.

Results.—Sodium urate crystals and nuclei are stained a deep blue.

The Schultz-Schmidt Stain for Monosodium Urate (1931). —*Fixation.*—Fix in absolute alcohol. Place thin slices of tissue in acetone for 4 to 5 hours, changing the acetone 3 times. Instead of embedding directly in paraffin it is better to place the tissue for ½ hour in a mixture of equal parts of acetone and of benzol, and then for ½ to 1 hour in benzol. Embed in paraffin.

Staining Solution.—

Carmine Solution

Carmine	1	gm.
Ammonium chloride	2	gm.
Lithium carbonate	0.5	gm.
Water, distilled	50	cc.

Boil and after cooling add:

Ammonia water, 26–28 per cent	20	cc.

For staining add to 3 cc. of the filtered carmine solution 1.5 cc. ammonia water and 2.5 cc. methyl alcohol.

Method of Staining.—1. Remove paraffin with xylol and place sections in absolute alcohol.

2. Without washing in water stain in the carmine solution for 5 minutes keeping the slide in motion.

3. Rinse in several changes of absolute alcohol.

4. Stain for 30 seconds in a saturated solution of methylene blue in 95 per cent alcohol (about 1.48 per cent) diluted one-half with absolute alcohol, keeping the slide in motion.

5. Rinse in absolute alcohol.

6. Stain about 15 seconds in the following filtered mixture: saturated aqueous solution of picric acid (about 1.22 per cent) 9 cc., and aqueous sodium sulfate solution, saturated by heat (about 42.7 per cent), 1 cc. Keep the slide in motion.

7. Dehydrate in several changes of absolute alcohol.

8. Clear in xylol and mount in balsam.

Results.—Nuclei gray-blue; cytoplasm yellowish; monosodium urate brilliant green; crystalline uric acid deep blue-green.

7. ORGANIC SUBSTANCES (THYMONUCLEIC ACID)

Feulgen's Stain for Thymonucleic Acid (1924).—Feulgen has devised a staining method for thymonucleic acid, a constituent of the chromatin of nuclei and occurring there only. The stain is therefore specific for nuclei. The principle of the method depends on removing the purine bodies from combination with nucleic acid by means of a mild acid hydrolysis. This process sets free reducing aldehyde groups which unite with fuchsin-sulfurous acid to give an intense reddish violet color (Schiff's reaction for aldehydes). The method thus permits the recognition of thymonucleic acid.

Fixation.—Feulgen employed a corrosive sublimate solution:

Corrosive sublimate, 6 per cent aqueous solution	98 parts
Acetic acid, glacial	2 parts

This fixative causes swelling and vacuolation of the chromatin. Therefore, Bauer recommends as preferable fixatives, Champy's solution, Flemming's fluid without acetic acid, Zenker's or Helly's fluids and several others. The embedding is done preferably in paraffin. Sections are cut 10 μ thick and are attached to the slide with albumin-glycerin. The paraffin is removed with xylol, followed by absolute alcohol. Leave sections in 95 per cent alcohol for 24 hours to remove the plasmals.

Solutions.—

Normal Hydrochloric Acid.—Add 82.5 cc. of concentrated hydrochloric acid with a specific gravity of 1.19 to distilled water to make up a volume of 1000 cc.

Fuchsin-Sulfurous Acid.—Add 1 gm. of powdered basic fuchsin to 200 cc. of boiling water in an Erlenmeyer flask and agitate frequently for 5 minutes to dissolve it. After cooling to 50° C., filter and add 20 cc. of normal hydrochloric acid. Cool further to 25° C. and add 1 gm. of anhydrous sodium sulfite. Let the solution stand at room temperature for at least 24 hours while the fluid becomes decolorized to a pale yellow. It will keep a long time in the dark if well corked. It must always contain a certain excess of sulfurous acid to prevent decomposition which is shown by its becoming reddish.

Sulfurous Acid Water for Washing Sections.—To 200 cc. of tap water add 10 cc. of a 10 per cent aqueous solution of anhydrous

sodium bisulfite and 10 cc. of the normal hydrochloric acid solution. The mixture must be made up fresh as needed. It smells strongly of sulfur dioxide. The 10 per cent solution of sodium bisulfite can be kept on hand if it is well corked.

Method.—1. Hydrolysis is carried out by removing sections from water and placing them in a warm normal hydrochloric acid solution at 60° C. for 4 minutes. The temperature can be kept most constant by placing a beaker containing the sections in a water bath. Exact temperature and time are very important.

2. Dip the section in water and wash out the acid rather quickly.

3. Place the section in fuchsin-sulfurous acid for 1 to 1½ hours in a glass-covered staining dish. As soon as the solution turns reddish it must be renewed.

4. Wash in sulfurous acid water 3 times, for 2 minutes in each, in 3 different beakers.

5. Wash in tap water 5 to 10 minutes.

6. Dehydrate in 95 per cent and absolute alcohol.

7. Clear in xylol and mount in balsam.

Results.—The thymonucleic acid-containing substances stain intensely red-violet.

8. FERMENTS

For the demonstration of the presence of oxidizing ferments in leukocytes and other cells several methods have been devised. Directions for carrying out these reactions are given in the sections on blood, bone marrow and skin:

(1) Oxidase reaction, pages 185–187 and 199–200.

(2) Peroxidase reaction, pages 188 and 200–202.

(3) Dopa reaction, pages 188–189 and 260–262.

9. DEGENERATION AND NECROSIS

(1) Cloudy Swelling

Cloudy swelling is a term applied to a condition, evident on gross examination, which is found postmortem in the liver, kidneys and heart in acute febrile diseases. It is characterized by increase in size of the organs and an appearance as though the tissue had been dipped into boiling water. It is due to increase of fluid and to changes in the mitochondria of the liver and kidneys and in the myofibrils of the heart muscle. Hydrops of the liver is common for example in lobar pneumonia; vacuoles appear in the cells and contain a single ball or threads or even networks of fibrin. In the

kidneys hyaline droplets of various sizes are formed. Fat may appear in addition in all three organs and is recognized in the fresh condition by not disappearing on the addition of acetic acid. In formalin-fixed tissue it may be demonstrated by staining with scarlet red.

Cloudy swelling may be studied after various fixatives and the usual routine stains but special study demands fixation and staining for mitochondria and fibrin and, in the heart, for the myofibrils.

(2) Necrosis

Necrosis in tissues is generally recognized by two features: either by the disappearance of the nuclei, although the cell outlines may be visible, so that the staining of the nuclei is no longer possible; or by the presence of irregular, larger or smaller masses, due to a fragmentation or breaking up of the chromatin, which stain intensely with nuclear stains. The disappearance of the nucleus is not synchronous with the death of the cell, but begins some 24 hours later, so that it is really evidence of changes following necrosis. It follows from the above that the microscopic evidence of necrosis is best studied in sections of tissues fixed in fluids which favor nuclear staining, such as Zenker's fluid, formalin and others. Teased preparations and frozen sections of fresh tissue are much less useful.

For the study of sections the stains with alum hematoxylin and phloxine or eosin, or, still better with phloxine followed by an alkaline methylene blue solution after Zenker's fixation, are very useful for the reason that the necrotic areas usually stain rather deeply with the diffuse stain and are thereby brought out sharply. This is particularly true of necroses of the liver.

For rendering fragmented nuclei prominent the staining methods for mitosis are best. A basic fuchsin stain washed out by picric acid in the alcohol will often give excellent results.

(3) Caseation

Caseation is a tissue change following local necrosis. Macroscopically and microscopically it appears as coarsely or finely granular masses which have more or less completely lost the original tissue structure. The chemical changes which have taken place have not been studied. Fibrin may or may not be present. Caseous tissue possesses no peculiar staining reactions. Fragmented nuclei are frequently present in it, especially in the peripheries of the areas.

CHAPTER XII

SPECIAL CELLS AND TISSUES

1. Epithelium

This term includes a great variety of cells of ectodermal, mesodermal and entodermal origin, which cover surfaces, line glands, ducts and tubes, and form more or less solid organs. They are of many different shapes and perform a great number of functions. Many produce characteristic secretions, some of which are external and others internal. Some of the cells contain granules with selective staining reactions, while others are provided with cilia, prickles or fibrils.

Epithelial cells can be studied in the fresh state in scrapings or teased preparations but the pathologist usually prefers sections made from unfixed or formalin-fixed material, or paraffin or celloidin sections of tissues fixed in Zenker's fluid or other reagents and stained in various ways.

The best fixatives and stains will be taken up in detail under the different tissues and organs where these cells occur.

2. Connective Tissue

(1) THE FIBROBLAST

The fibroblast is the most versatile cell in the body. It gives rise to all types of connective tissue and to cartilage and bone. It produces fibroglia fibrils in the cuticle of its cytoplasm and in addition various intercellular structures, such as collagenous and elastic fibrils, mucus, and, under certain conditions, the ground substances of cartilage and bone. Under pathological conditions it may produce amyloid. These different structures call for a great variety of differential methods in order to demonstrate them satisfactorily.

(2) FIBROGLIA FIBRILS

Fibroglia fibrils are intimately connected with the cytoplasm of the fibroblast, running over its surface and extending out along its processes. They are very delicate, straight or slightly curved, run

in parallel lines, and are definitely separated from one another. They seem to pass from cell to cell, thus aiding to form a syncytium.

Fibroglia fibrils are most evident when fibroblasts are multiplying, as in granulation tissue, in the organization of fibrin, in the stroma of carcinomas, and in fibrosarcomas if the latter are not growing too rapidly. In order to demonstrate them the tissue must be absolutely fresh, that is, removed surgically and placed immediately in a suitable fixative because the fibrils quickly lose their characteristic staining properties postmortem.

Two routine staining methods bring them out distinctly, phosphotungstic acid hematoxylin and Mallory's aniline blue collagen stain. By the first method they are stained blue, by the second red. Heidenhain's iron hematoxylin also gives excellent results but is more complicated. A special method that will sometimes be found useful is the following:

Mallory's Acid Fuchsin Stain.—*Fixation.*—Zenker's fluid. Embed in paraffin.

Method of Staining.—1. Stain sections in a 1 per cent aqueous solution of acid fuchsin overnight in the cold, or for 1 hour in the paraffin oven at 54° C.

2. Drain slides and differentiate in a 0.1 per cent aqueous solution of potassium permanganate for 40 to 60 seconds. This step must not be prolonged beyond the exact time needed or the section will be decolorized.

3. Dehydrate in 95 per cent followed by absolute alcohol.

4. Clear in xylol and mount in balsam.

Results.—Nuclei and fibroglia fibrils red; collagen pale reddish yellow; elastic fibrils bright lemon yellow; red blood corpuscles purplish red.

(3) THE LIPOBLAST (FAT CELL)

The fat cell is a specific mesenchymal cell which multiplies or atrophies and disappears according to the needs of the body. It is not a fibroblast which has taken up fat and it produces no fibrils. The fat cell of fetal type suggests a liver cell but with coarser granules. It is often present after birth in the retroperitoneal tissue. In one instance, when the cells were numerous or in clumps, in an emaciated child, they were mistaken at autopsy for a tumor. As the cells mature they accumulate fat which fuses into one large globule with the cytoplasm stretched around it.

In addition to fixation for general stains to demonstrate nuclei and cytoplasmic granules, tissue must also be fixed in formalin so that fat stains can be done.

(4) COLLAGEN AND RETICULUM

Collagen consists of bundles of exceedingly delicate fibrils which usually present a wavy appearance. They may be more or less widely separated from each other, united into thin or coarse strands, or fused into fibrillar or homogeneous masses. Reticulum is the term applied to collagen that stains by the silver method and this occurs only under certain physical conditions, *i. e.*, when individual or small strands of fibrils are separated from each other by cells, elastic fibrils or fluid, but not when they are fused together. The appearance of the deeply stained fibrils is so striking in contrast with the lightly staining, denser masses of collagen that they have very generally been assumed to be of a different chemical nature, without taking into consideration the results obtainable by the application of other staining methods for collagen.

Collagenous fibrils retain their characteristic staining properties for some time postmortem, but absolutely fresh tissue is preferable for their study whenever obtainable. Any fixative may be employed but Zenker's fluid is recommended. More or less specific stains in considerable variety are available for their demonstration. The oldest and most generally useful is van Gieson's solution as it stains well after all ordinary fixatives. However, it does not stain the fibrils so intensely as is desirable.

a. Staining Methods for Collagen and Reticulum

Van Gieson's Picric Acid and Acid Fuchsin Stain (1889). —*Fixation.*—Almost any fixing fluid may be used. Embed in paraffin or celloidin.

Staining Solution.—This solution was originally made by adding to a saturated aqueous solution of picric acid (about 1.22 per cent) enough of a saturated aqueous solution of acid fuchsin (about 20 per cent) to give to the fluid a deep garnet red color. Nowadays it is usual to employ a 1 per cent aqueous solution of acid fuchsin and to add 5 to 15 cc. of it to 100 cc. of a saturated aqueous solution (about 1.22 per cent) of picric acid, according to the intensity of stain desired, or to the nature of the fixative employed.

Method of Staining.—1. Stain sections rather deeply with alum hematoxylin (p. 70). For sections of nerve tissue Weigert's iron hematoxylin (p. 74) for 5 to 20 minutes is preferable.

2. Wash in water.

3. Stain in van Gieson's solution (p. 92) for 5 minutes or longer.

4. Transfer directly to 95 per cent alcohol followed by absolute.

5. Clear in xylol and mount in balsam.

For celloidin sections clear in terpineol or in oil of origanum after 95 per cent alcohol.

Results.—Collagen brilliant red; smooth and striated muscle yellow; cornified epithelium yellow; neuroglia fibrils yellow; and nuclei blue.

The two drawbacks to this method are that the red stain of the collagenous fibrils is not sufficiently intense and that it tends to fade.

Mallory's Aniline Blue Collagen Stain (1936).—*Fixation.*—Zenker's fluid. Embed in paraffin or celloidin.

Staining Solution.—

Aniline blue, soluble in water	0.5	gm.
Orange G	2	gm.
Phosphotungstic acid, 1 per cent aqueous solution	100	cc.

Phosphomolybdic acid was used in this method originally but the acid fuchsin stain tended to fade. Phosphotungstic acid causes no deterioration.

Method of Staining.—1. Stain sections, which have been treated with iodine as usual after Zenker's fluid, in a 0.5 per cent aqueous solution of acid fuchsin for 1 to 5 minutes or longer. If it is desirable to bring out the collagenous fibrils as sharply as possible, omit the staining with acid fuchsin.

2. Transfer directly to the aniline blue solution without washing in water and stain for 20 minutes to 1 hour or longer.

3. Transfer directly to 95 per cent alcohol, several changes to remove excess stain.

4. Dehydrate in absolute alcohol.

5. Clear in xylol and mount in balsam.

For celloidin sections shorten the staining time, decolorize and dehydrate in 95 per cent alcohol and clear by the blotting paper xylol method or in terpineol. Mount in balsam.

Results.—Collagenous fibrils intense blue; ground substances of cartilage and bone, mucus, amyloid and certain other hyaline substances varying shades of blue; nuclei, fibroglia, myoglia and neuroglia fibrils, axis cylinders and fibrin red; nucleoli, red blood corpuscles and myelin yellow; elastic fibrils pale pink or yellow.

The aniline blue stain as first published was not perfect and has been often modified with the desire of making the blue more intense and the red sharper. Of the various modifications, that of Heidenhain seems to be the best. He replaced the acid fuchsin with azocarmine B or G. The first dye is more readily soluble in water, but he preferred azocarmine G.

Heidenhain's Aniline Blue Stain (1915).—*Fixation.*—Zenker's, Helly's, Bouin's or Carnoy's fluids, and corrosive sublimate. Embed in paraffin.

Staining Solutions.—

Azocarmine

Azocarmine B	0.25–1 gm.
Water, distilled	100 cc.
Acetic acid, glacial	1 cc.

If azocarmine G is used instead of B add 0.1 gm. to the 100 cc. of water, bring it to a boil, cool to room temperature and filter through coarse filter paper in the paraffin oven at 51° to 55° C. so that fine particles of dye will also pass through. After cooling add the 1 cc. of glacial acetic acid.

Aniline Blue

Aniline blue	0.5 gm.
Orange G	2 gm.
Water, distilled	100 cc.
Acetic acid, glacial	8 cc.

Boil and filter after cooling. For staining, dilute the stock solution 1:3 with distilled water.

Method of Staining.—1. Stain in the azocarmine solution in a glass-covered dish in the paraffin oven at 51° to 55° C. for 45 to 60 minutes, then cool at room temperature 5 to 10 minutes.

2. Wash in distilled water.

3. Differentiate in an alcoholic solution of aniline made up as follows:

Aniline	1 cc.
Alcohol, 90 per cent	1000 cc.

4. Rinse in acetic acid alcohol made up as follows for 30 seconds to 1 minute.

Acetic acid, glacial	1 cc.
Alcohol, 95 per cent	100 cc.

5. Mordant in 5 per cent aqueous solution of phosphotungstic acid 1 to 3 hours.

6. Wash quickly in distilled water.

7. Stain in the aniline blue solution 1 to 3 hours.

8. Wash quickly in water.

9. Differentiate in 95 per cent alcohol followed by absolute.

10. Clear in xylol and mount in balsam.

Results.—Collagen and reticulum deep blue; chromatin red; muscle tissue reddish to orange; erythrocytes red; neuroglia reddish; and mucin blue.

Lee-Brown's Modification of Mallory's Aniline Blue Stain (1929).—This method is particularly valuable for staining sections of kidney.

Fixation.—Fix in Zenker's fluid. Embed in paraffin.

Staining Solution.—

Aniline blue	0.5 gm.
Orange G	2 gm.
Phosphomolybdic acid	2 gm.
Water, distilled	100 cc.

Method of Staining.—1. Place sections, which have been treated with iodine as usual after Zenker's fluid, in 1 per cent aqueous solution of phosphomolybdic acid for 30 seconds.

2. Wash in distilled water 1 to 2 minutes.

3. Stain in the aniline blue stain, preheated in the paraffin oven, for 30 minutes at 55° C.

4. Wash in distilled water for 2 to 5 minutes.

5. Place in 1 per cent aqueous solution of phosphomolybdic acid for 30 seconds.

6. Wash in distilled water for 1 to 2 minutes.

7. Dehydrate in 95 per cent followed by absolute alcohol.

8. Clear in xylol and mount in balsam.

Results.—Collagen blue; nuclei orange. The glomerular basement membrane of the kidney stains a deep blue.

Mallory's Phosphomolybdic Acid Hematoxylin Stain (1891).—*Fixation.*—Zenker's fluid. Embed in paraffin.

Staining Solution.—

Hematoxylin	1 gm.
Phosphomolybdic acid	2 gm.
Water, distilled	100 cc.

The solution requires several weeks in order to ripen but may be ripened at once by the addition of 5 cc. of a 1 per cent aqueous solution of potassium permanganate.

Method of Staining.—1. Stain sections in the hematoxylin solution for 2 to 3 hours or more.

2. Wash in water.

3. Decolorize and dehydrate in 95 per cent alcohol followed by absolute.

4. Clear in xylol and mount in balsam.

Results.—Single collagenous fibrils are brought out a deep blue color but are not so intensely delineated as by the silver methods.

Masson's Trichrome Stain for Connective Tissue (1928).—*Fixation.*—Fix in Bouin's fluid for 3 days, or in Regaud's for 24 hours. Cut paraffin sections at 5 μ and fix them to the slide by the gelatin method of Masson (p. 58).

Staining Solutions.—

Regaud's Hematoxylin

Hematoxylin	1 gm.
Alcohol, 95 per cent	10 cc.
Glycerin	10 cc.
Water, distilled	80 cc.

Picric Alcohol

Alcohol, 95 per cent saturated with picric acid (about 7 per cent)	2 parts
Alcohol, 95 per cent	1 part

Solution A

Acid fuchsin	0.3 gm.
Ponceau de xylidine	0.7 gm.
Water, distilled	100 cc.
Acetic acid, glacial	1 cc.

Solution B

Phosphomolybdic acid	1	gm.
Water, distilled	100	cc.

Solution C

Acetic acid, glacial	2	cc.
Water, distilled	100	cc.
Aniline blue	to saturation	

Method of Staining.—1. Free sections from paraffin by xylol, alcohol and water.

2. Mordant in 5 per cent ammonio-ferric alum, previously heated to 45° to 50° C. for 5 minutes.

3. Wash in water.

4. Stain in Regaud's hematoxylin for 5 minutes at 45° to 50° C.

5. Rinse in 95 per cent alcohol.

6. Differentiate in the picric alcohol.

7. Wash in running tap water.

8. Stain in Solution A for 5 minutes.

9. Rinse in distilled water.

10. Differentiate in Solution B for 5 minutes.

11. Without rinsing pour on 10 drops of Solution C and let stand for 5 minutes.

12. Rinse in distilled water.

13. Place in Solution B again for 5 minutes.

14. Place in 1 per cent acetic acid in distilled water for 5 minutes.

15. Dehydrate in 95 per cent followed by absolute alcohol.

16. Clear in xylol and mount in balsam.

Results.—Nuclei black; argentaffin granules black or red; cytoplasm and neuroglia fibrils vermilion red; collagen intense blue.

b. Silver Impregnation Methods for Collagen and Reticulum

Bielschowsky originally devised his silver impregnation method for the study of neurofibrils, but Maresch applied it to connective tissue for the study of the finer fibrils of collagen, the so-called reticulum. Reticulum fibrils are brought out by this method more clearly and beautifully than by any other and the study of such preparations is a revelation of their complexity and number. Bielschowsky's method depends on the reduction by formalin of the easily reducible soluble silver salt formed by dissolving with ammonia the precipitate caused by adding a solution of sodium

hydroxide to a solution of silver nitrate. Numerous modifications of his methods have been devised but the basic principles remain the same.

Certain precautions must be taken in carrying out these methods, for, as is well known, silver is easily affected by organic material. Some of the main points to be observed are given below:

1. All glassware must be chemically clean. This is best accomplished by soaking it in the sulfuric acid-potassium bichromate cleaning solution, followed by thorough washing with tap water and then with distilled water.

2. The distilled water must be free from organic material. This can be tested for by adding a few drops of a silver nitrate solution and watching for a color change or precipitate. Da Fano recommends using water redistilled over potassium permanganate.

3. All chemicals must be of the highest purity.

4. In handling frozen or celloidin sections, glass instruments, never metal, should be used.

5. Certain filter papers contain sufficient organic material to affect the silver solutions and such should be avoided.

6. Filter all solutions.

7. Use a graduated glass cylinder with a glass stopper for making up the silver solutions.

Of the numerous modifications of Bielschowsky's methods, we are giving only a few. These would seem to be sufficient for ordinary laboratory work. If further methods are desired, the reader should consult more specialized works. For laboratories where Zenker-fixed tissues and paraffin sections are the rule, we can recommend highly Foot's modifications.

The Bielschowsky-Maresch Method (1905).—*Fixation.*—Fixation in 10 per cent formalin is preferable. Maresch states that alcohol-fixed tissue also may be used but sections from such tissue should be put in 10 per cent formalin for several hours, followed by washing in distilled water before staining. Material fixed in corrosive sublimate, osmic acid or chrome salts is not satisfactory as impregnation is uneven and the silver precipitates out in fine discrete granules.

Frozen sections give the best results but celloidin sections can be used and it is not necessary to remove the celloidin. Paraffin sections may also be used.

Staining Solutions.—

Bielschowsky's Ammoniacal Silver Solution.—To 10 cc. of a 10 per cent aqueous solution of silver nitrate add first 5 drops of a 40 per cent aqueous solution of sodium hydroxide, then strong ammonia water (26–28 per cent) drop by drop, stirring constantly with a glass rod, until the brown to black precipitate is just dissolved; an excess of ammonia is to be avoided and this is best controlled by stopping the addition of the ammonia while there is a granule or two of the precipitate still undissolved. Dilute the amount of fluid to 25 cc. with distilled water and filter. This solution must be made fresh each time before use.

Formalin.—The reduction is carried out by a 20 per cent solution of neutral formalin in tap water as the free acid which often occurs in commercial formalin interferes with the reducing process.

Gold Chloride.—The gold chloride solution is made up of 10 cc. of distilled water to which have been added 5 drops of a 1 per cent aqueous solution of gold chloride and 1 drop of glacial acetic acid.

Method of Staining.—1. Wash formalin- or alcohol-fixed tissue several hours in running water before freezing.

2. Cut frozen sections and place in distilled water.

3. Place sections in a 2 per cent aqueous solution of silver nitrate in the dark for 24 hours.

4. Rinse quickly in distilled water, 2 to 3 seconds.

5. Transfer sections to the ammoniacal silver solution for 2 to 30 minutes, depending on their thickness. They turn yellowish brown.

6. Rinse quickly in 2 changes of distilled water, 2 to 3 seconds.

7. Reduce in a 20 per cent solution of neutral formalin in tap water for 5 to 30 minutes, until no more white clouds appear in the fluid. The preceding rinse in distilled water in Step 6 acts as a differentiator for this reducing process. If the rinsing in Step 6 is too short, the sections will be too dark; if too long, the sections will not be dark enough; as a general rule the time should be short.

8. Wash thoroughly in distilled water.

9. Tone in the acid gold solution until the background becomes red-violet; with sections 10 μ thick, 10 minutes is usually sufficient.

10. Wash in distilled water.

11. Place for 15 to 30 seconds in a 5 per cent aqueous solution of sodium thiosulfate.

12. Wash thoroughly in running water.

13. Dehydrate in 95 per cent followed by absolute alcohol.

14. Clear in xylol and mount in balsam.

Frozen sections are cleared preferably with carbol-xylol after 95 per cent alcohol.

Results.—The connective tissue fibrils, even the finest, are a deep black on a clear background. Thick collagen bundles are a reddish brown or violet color. Nuclei as a rule are not stained to any degree and aniline dyes may be used for their demonstration.

Perdrau's Modification of Bielschowsky's Method.—The method of procedure given below is that described by Bailey and Hiller (1924).

Fixation.—Fix in 10 per cent neutral formalin. Wash blocks of tissue 12 to 24 hours in running tap water, then 24 hours in distilled water, changing the water several times. Cut frozen sections 15 to 25 μ in thickness.

This method may also be used on paraffin sections if the slides are not agitated in the solutions, otherwise the sections will come off the slide.

Staining Solutions.—

Pal's Decolorizer.—Equal parts of a 1 per cent aqueous solution of oxalic acid and a 1 per cent aqueous solution of acid potassium sulfite are added together.

Bielschowsky's Ammoniacal Silver Solution.—To 5 cc. of a 20 per cent solution of silver nitrate add first 2 drops of a 40 per cent solution of sodium hydroxide, and then strong ammonia water (26–28 per cent) drop by drop until the precipitate is just dissolved; dilute to 50 cc. with distilled water and filter. This solution must be prepared each time just before use.

Method of Staining.—1. Wash sections in distilled water 24 hours.

2. Treat sections for 10 minutes with a 0.25 per cent aqueous solution of potassium permanganate.

3. Wash in distilled water.

4. Place in Pal's decolorizer until sections are white.

5. Wash thoroughly overnight in several changes of distilled water.

6. Place in a 2 per cent aqueous solution of silver nitrate in the dark for 24 hours.

7. Wash in distilled water for not more than 5 minutes.

8. Treat sections 40 to 60 minutes with Bielschowsky's ammoniacal silver solution.

9. Wash quickly in distilled water.

10. Reduce 30 minutes in a 20 per cent solution of formalin (not neutralized) made with tap water.

11. Wash in distilled water.

12. Tone in a 1:500 aqueous solution of gold chloride until sections are an even violet color.

13. Wash rapidly in distilled water.

14. Fix in a 5 per cent aqueous solution of sodium thiosulfate 2 minutes.

15. Wash thoroughly in distilled water.

16. Dehydrate in 70 and 95 per cent alcohol.

17. Clear in carbol-xylol-creosote (p. 249).

18. Rinse in xylol and mount in balsam.

Results.—The broader bands of collagen are stained reddish, and the finer strands, so-called reticulum, black; background almost colorless. The stain is fairly specific but may color neuroglia fibrils.

Foot's Modification of Bielschowsky's Method (1924).—*Fixation.*—This method was devised especially for Zenker-fixed tissue. Formalin-fixed tissue also may be used and in our experience has proved quite satisfactory. This method is intended for paraffin sections; we have had no experience with its use on frozen or celloidin sections.

Staining Solution.—

Ammoniacal Silver Hydroxide Solution.—To 20 cc. of a 10 per cent aqueous solution of silver nitrate add 20 drops of a 40 per cent aqueous solution of sodium hydroxide. The resulting brownish precipitate is dissolved in strong ammonia water (26–28 per cent), which is added slowly, shaking continually; about 2 cc. will be needed so it is well to add the ammonia drop by drop as this point is neared until the precipitate is almost dissolved. It is better to filter out a few undissolved granules than to run the risk of adding too much ammonia. The resulting solution is made up to 80 cc.

with distilled water and filtered before use. This solution must be made fresh each time as it does not keep well.

Method of Staining.—1. Remove paraffin from the sections in the usual manner.

2. Place in 0.5 per cent iodine in 95 per cent alcohol 5 minutes.

3. Wash in water and place in 0.5 per cent sodium thiosulfate for 5 minutes.

4. Wash in tap water.

5. Treat sections with a 0.25 per cent aqueous solution of potassium permanganate for 5 minutes.

6. Rinse in tap water.

7. Place sections in a 5 per cent aqueous solution of oxalic acid for 15 to 30 minutes.

8. Wash thoroughly in tap water.

9. Rinse in distilled water. Use distilled water for washing until after sections have been treated with formalin.

10. Leave sections for 48 hours in a 2 per cent aqueous solution of silver nitrate in subdued light, but not in the dark.

11. Wash a short time in distilled water.

12. Place in the ammoniacal silver solution for 30 minutes.

13. Wash quickly in distilled water.

14. Reduce in a 5 per cent neutral formalin solution for 30 minutes. It is well to change the solution after the first 10 to 15 minutes.

15. Rinse in tap water.

16. Tone in a 1 per cent aqueous solution of gold chloride for 1 hour.

17. Rinse in tap water.

18. Remove excess silver by treating sections with a 5 per cent aqueous solution of sodium thiosulfate for 2 minutes.

19. Wash thoroughly for several hours in running tap water.

20. Stain in alum hematoxylin (p. 70) 10 minutes, or Weigert's iron hematoxylin (p. 74) 1 minute.

21. Wash in tap water until blue.

22. Counterstain in van Gieson's solution (p. 92) for 30 seconds.

23. Dehydrate in 95 per cent followed by absolute alcohol.

24. Clear in xylol and mount in balsam.

Results.—Coarser collagenous fibrils stain red to rose, finer col-

lagenous fibrils, so-called reticulum, black to dark violet; nuclei black, blue or brownish; cytoplasm grayish yellow; muscle fibers and elastic fibers more brightly yellow.

The finest reticulum fibrils are brought out by this method for if they are not penetrated by the silver, the acid fuchsin will stain them so they sometimes appear beaded black on vermilion.

Instead of staining with alum hematoxylin and van Gieson's stain, Mallory's aniline blue collagen stain may be used, especially for the study of fibroglia and myoglia fibrils and the relation of these to collagen. Sometimes, especially for photographic purposes, it is of advantage to omit a counterstain.

Wilder's Silver Impregnation Method for Reticulum Fibers (1935).—This method was primarily designed for material fixed in formalin and embedded in paraffin.

Fixation.—Fix in 10 per cent formalin, Zenker's or Helly's fluid. Embed in paraffin or celloidin, or cut frozen sections. Paraffin sections are mounted on slides before staining, celloidin and frozen sections are stained before mounting.

Staining Solution.—

Ammoniacal Silver Hydroxide Solution.—To 5 cc. of 10.2 per cent aqueous solution of silver nitrate add 26 to 28 per cent ammonia water drop by drop until the precipitate which forms is dissolved. Add 5 cc. of 3.1 per cent sodium hydroxide and just dissolve the resulting precipitate with a few drops of ammonia water. Make the solution up to 50 cc. with distilled water.

Reducing Solution

Water, distilled	50 cc.
Formalin, neutralized with magnesium carbonate	0.5 cc.
Uranium nitrate, 1 per cent aqueous solution	1.5 cc.

Method of Staining.—1. Place sections from water into 0.25 per cent aqueous solution of potassium permanganate or in 10 per cent aqueous solution of phosphomolybdic acid for 1 minute.

2. Rinse in distilled water.

3. Place in hydrobromic acid (Merck's concentrated, 34 per cent, 1 part; distilled water, 3 parts) for 1 minute. Hydrobromic acid may be omitted if phosphomolybdic acid is used in Step 1.

4. Wash in tap water, then distilled water.

5. Dip in a 1 per cent aqueous solution of uranium nitrate (sodium-free) for 5 seconds or less.

6. Wash in distilled water 10 to 20 seconds.

7. Place in the ammoniacal silver solution for 1 minute.

8. Dip quickly in 95 per cent alcohol.

9. Reduce for 1 minute in the reducing solution.

10. Wash in distilled water.

11. Tone in 1:500 aqueous gold chloride (Merck's reagent) for 1 minute.

12. Rinse in distilled water.

13. Place in 5 per cent aqueous solution of sodium thiosulfate 1 to 2 minutes.

14. Wash in tap water.

15. Counterstain, if desired, with alum hematoxylin and van Gieson's stain or alum hematoxylin and phloxine.

16. Differentiate and dehydrate in 95 per cent followed by absolute alcohol.

17. Clear in xylol and mount in balsam.

Celloidin sections are cleared in oil of origanum following 95 per cent alcohol.

Results.—Fine reticulum fibers are stained black with great precision; the collagen is a rose color.

Gömöri's Silver Impregnation of Reticulum (1937).—*Fixation.*—Fix in 10 per cent formalin and embed in paraffin.

Staining Solution.—

Ammoniacal Silver Hydroxide Solution.—To a 10 per cent aqueous silver nitrate solution add ⅙ to ¼ its volume of a 10 per cent aqueous solution of potassium hydroxide. Add 26 to 28 per cent ammonia water drop by drop, while shaking the container continuously, until the precipitate is completely dissolved. Add again, cautiously, silver nitrate solution drop by drop until the resulting precipitate easily disappears on shaking the solution. Make up the solution with distilled water to twice its volume. It can be kept in a stoppered bottle for 2 days.

Method of Staining.—1. Remove paraffin with xylol, 2 changes of alcohol and wash in tap water.

2. Oxidize with a 0.5 to 1 per cent aqueous solution of potassium permanganate for 1 to 2 minutes.

3. Rinse in tap water.

4. Decolorize with a 1 to 3 per cent aqueous solution of potassium metabisulfite for 1 minute.

5. Wash in tap water for several minutes.

6. Sensitize in a 2 per cent solution of ammonio-ferric alum in distilled water for 1 minute.

7. Wash in tap water for a few minutes, followed by 2 changes of distilled water.

8. Impregnate in the silver solution for 1 minute.

9. Rinse quickly in distilled water for 5 to 10 seconds.

10. Reduce for 3 minutes in 10 to 20 per cent formalin.

11. Wash in tap water for a few minutes.

12. Tone in 0.1 to 0.2 per cent solution of gold chloride for 10 minutes.

13. Rinse in distilled water.

14. Reduce toning in a 1 to 3 per cent aqueous solution of potassium metabisulfite for 1 minute.

15. Fix in a 1 to 2 per cent aqueous solution of sodium thiosulfate for 1 minute.

16. Wash in tap water.

17. Dehydrate in 95 per cent alcohol, followed by absolute alcohol.

18. Clear in xylol and mount in balsam.

Results.—Finest connective tissue fibrils are sharply delineated in black.

Foot's Modification of Hortega's Silver Carbonate Method for Reticulum (1927).—*Fixation.*—Fix in Zenker's fluid, and embed in paraffin.

Staining Solution.—

Ammoniacal Silver Carbonate Solution.—To 10 cc. of a 10 per cent aqueous solution of silver nitrate add 10 cc. of a saturated (1.25 per cent) aqueous solution of lithium carbonate. Wash the white precipitate 3 times with distilled water, allowing the precipitate to settle each time and pouring off the supernatant fluid. Add 25 cc. of distilled water. Almost dissolve the precipitate with 26 to 28 per cent ammonia water added drop by drop (6–15 drops) while shaking the container vigorously. Avoid adding too much ammonia water; it is better to leave a few grains of precipitate than to add too much. The solution is then made up to 100 cc. with distilled water and heated to 50° C.

In order to prevent sections from floating off the slide in this ammoniacal silver solution we use 95 per cent alcohol instead of distilled water in making up the ammoniacal silver solution to 100 cc. Thus, after dissolving the precipitate with ammonia water, add 95 per cent alcohol to make the solution up to 100 cc. A precipitate is again formed which is dissolved by a few more drops of ammonia water. This alcoholic solution is filtered and warmed for 20 minutes in a paraffin oven at 50° C. and then the sections are placed in this solution for 20 minutes in the oven.

Method of Staining.—1. After removing the paraffin from the sections in the usual way rinse in water and place sections in a 0.5 per cent solution of iodine in 95 per cent alcohol for 5 minutes.

2. Wash in water.

3. Bleach in 0.5 per cent aqueous solution of sodium thiosulfate for 5 minutes.

4. Wash in tap water.

5. Place in 0.25 per cent aqueous solution of potassium permanganate for 5 minutes.

6. Rinse in tap water.

7. Place sections in a 5 per cent aqueous solution of oxalic acid for 10 minutes.

8. Wash well in tap water, followed by distilled water.

9. Place in the warm silver ammonium carbonate solution in the incubator at 37° C. for 10 to 15 minutes.

10. Rinse in distilled water.

11. Reduce in 20 per cent aqueous solution of neutral formalin for 5 minutes.

12. Wash well in tap water.

13. Tone in a 1:500 aqueous solution of gold chloride for 5 minutes.

14. Wash in tap water.

15. Fix in a 5 per cent aqueous solution of sodium thiosulfate for 2 minutes.

16. Wash well in tap water.

17. Counterstain, if desired, for nuclei and collagen by means of alum hematoxylin and van Gieson's solution.

18. Differentiate in 95 per cent alcohol and dehydrate in absolute alcohol.

19. Clear in xylol and mount in balsam.

Results.—Coarser collagen fibrils red to rose; reticulum black to dark violet; nuclei black, blue or brownish; cytoplasm grayish yellow; muscle fibers and elastic fibers lighter yellow.

Laidlaw's Silver Stain for Reticulum (1929).—*Fixation.*—Fix in Bouin's fluid for 3 days, or in 10 per cent neutral formalin for 3 days. Old formalin material may be improved by immersion in fresh neutral formalin for 3 days. Formalin-fixed tissue immersed in Bouin's fluid for 3 days will give nearly perfect Bouin pictures; a positive endothelial or smooth muscle nucleus here and there betrays the original formalin fixation. Embed in paraffin or celloidin, or cut frozen sections. Attach paraffin sections to the slide, using Masson's gelatin method (p. 58).

Staining Solution.—

Modified Lithium Silver Solution of Hortega.—In a 250 cc. glass-stoppered graduate dissolve 12 gm. of silver nitrate in 20 cc. of distilled water. Add 230 cc. of a saturated solution of lithium carbonate in distilled water (about 1.33 per cent); shake well; let settle to about 70 cc. of precipitate. Wash well with distilled water 3 or 4 times.

After settling again to about 70 cc. of precipitate, decant wash water, and add ammonia water (26–28 per cent), shaking constantly until the fluid is almost clear.

Add distilled water to a total of 120 cc.; shake and filter into stock bottle. The solution keeps for many months. Ordinary filter paper is apt to turn brown and discolor the solution while filtering; for that reason use Whatman filter paper No. 42 or No. 44.

Method of Staining.—1. After removal of the paraffin, wash sections fixed in Bouin's solution in running water for 20 minutes to remove the picric acid; wash formalin-fixed sections for 5 minutes.

2. Place sections in a 1 per cent alcoholic solution of iodine for 3 minutes.

3. Wash in tap water.

4. Treat with 5 per cent sodium thiosulfate for 3 minutes.

5. Rinse in tap water.

6. Place in 0.25 per cent potassium permanganate for 3 minutes.

7. Rinse in tap water.

8. Place in 5 per cent oxalic acid for 3 minutes.

9. Wash well in running tap water for 10 minutes.

10. Wash in 3 changes of distilled water for 5 to 10 minutes.

11. Stain in the lithium silver solution. Heat the stock solution in the paraffin oven to 50° C. and stain in the oven for 5 minutes. (The used solution can be filtered and used a dozen times or more.)

12. Rinse the slides by pouring distilled water over both sides.

13. Treat sections with 1 per cent formalin in tap water for 3 minutes, changing solution several times.

14. Rinse both sides of slide with distilled water.

15. Immerse slides in a 1:500 aqueous yellow gold chloride solution at room temperature for 10 minutes.

16. Rinse in distilled water.

17. Pour 5 per cent sodium thiosulfate onto slide, changing the solution as often as it becomes turbid, for 10 minutes.

18. Wash well in running water.

19. Counterstain if desired and mount in balsam.

Results.—Collagen stains reddish purple; the more delicate reticulum fibers appear as fine black threads. The other results vary with the fixative used. After Bouin's fluid only the cytoplasm of the epithelial cells is colored black, the nuclei are colorless; whereas with formalin fixation the nuclei are black, the cytoplasm colorless.

(5) ELASTIC FIBERS

Elastic fibers are not affected by dilute potassium or sodium hydroxide or by acids. These reagents are often used, therefore, to demonstrate elastic fibers in the fresh condition, for example in sputum, because they render them prominent by clearing or destroying the other tissues. The fibers show a marked affinity for osmic acid, staining with greater rapidity than most other tissue elements.

For demonstrating elastic fibers in sections of fixed tissues there are three excellent differential stains:

Weigert's Stain for Elastic Fibers (1898).—*Fixation.*—Alcohol or 10 per cent formalin is preferable, but other fixing reagents give excellent results. After fixation in Zenker's fluid sections stain slowly and there is a greater tendency, perhaps, to diffuse coloring of the collagen fibrils. Embed in celloidin or paraffin.

Staining Solution.—

Fuchsin, basic	2 gm.
Resorcin	4 gm.
Water, distilled	200 cc.

Bring the solution to a boil in an enamel dish and, when briskly boiling, add 25 cc. of a 29 per cent aqueous solution of ferric chloride. Stir and boil for 2 to 5 minutes more. A precipitate forms. Cool and filter. The filtrate is discarded. Leave the precipitate on the filter paper until it is thoroughly dry. Then return filter paper and precipitate to the enamel dish, which should be dry but still contain whatever part of the precipitate remains adherent to it. Add 200 cc. of 95 per cent alcohol and heat carefully. Stir constantly and remove the filter paper when the precipitate is dissolved off. Cool, filter and add 95 per cent alcohol to make up the 200 cc. Add 4 cc. of hydrochloric acid. The solution keeps well for months.

Method of Staining.—1. Stain sections in the fuchsin solution for 20 minutes to 1 hour or longer.

2. Wash off excess stain in 95 per cent alcohol. If the sections are stained diffusely differentiate in acid alcohol for several minutes and then wash thoroughly in tap water.

3. The nuclei may be stained with Orth's lithium carmine (p. 80) before the elastic tissue is stained, if no further counterstain is desired, or afterwards with alum hematoxylin (p. 70) followed by either dilute phloxine or van Gieson's solution (p. 92).

4. Differentiate and dehydrate in 95 per cent alcohol followed by absolute.

5. Clear in xylol and mount in balsam.

If celloidin sections are used clear in oil of origanum or in terpineol after 95 per cent alcohol.

Results.—The elastic fibers are dark blue to black; nuclei a brilliant red if carmine is used, or bluish to black if stained with alum hematoxylin; collagen pink to red; other tissue elements yellow if stained with van Gieson's solution.

Hart's Modification of Weigert's Elastic Tissue Stain (1908).—*Fixation.*—Zenker-fixed tissues can be stained satisfactorily by the following modification of Weigert's method. Embed in paraffin or celloidin.

Staining Solution.—

Weigert's resorcin fuchsin stain (p. 168)	5 cc.
Alcohol, 70 per cent	100 cc.
Hydrochloric acid, concentrated	1 cc.

Method of Staining.—1. Treat sections, which have been treated with iodine as usual after Zenker's fluid, with a 0.25 per cent aqueous solution of potassium permanganate for 10 minutes.

2. Wash in water.

3. Place in a 5 per cent aqueous solution of oxalic acid for 20 minutes.

4. Wash in water.

5. Stain overnight in the modified Weigert stain.

6. Rinse in 95 per cent alcohol.

7. Wash in water and counterstain as in Weigert's original method (Step 3).

Results.—Essentially the same as in Weigert's original method. Degenerated elastic fibers (elacin) also stain by this method.

Verhoeff's Elastic Tissue Stain (1908).—*Fixation.*—Formalin, 10 per cent, or Zenker's fluid is preferred. Embed in paraffin or celloidin.

Staining Solution.—

Hematoxylin	1 gm.
Alcohol, absolute	20 cc.

Dissolve in a test tube by the aid of heat, filter and add in order given:

Ferric chloride, 10 per cent aqueous solution	8 cc.
Iodine solution (iodine 2 gm., potassium iodide 4 gm., distilled water 100 cc.)	8 cc.

It is not necessary to treat tissues or sections with iodine before staining. Mercurial precipitates are removed by the staining solution. For the best results the solution should be used within 24 hours.

Method of Staining.—1. Immerse sections in the staining fluid for 15 minutes to 1 hour until perfectly black.

2. Differentiate in a 2 per cent aqueous solution of ferric chloride. The differentiation requires only a few minutes. To observe the stages in the differentiation the sections may be examined

in water under a low magnification. If the differentiation has been carried too far the sections may be restained, provided they have not been treated with alcohol.

3. Wash in water.

4. Place in 95 per cent alcohol to remove the stain of the iodine solution, and then leave sections in water 5 minutes or longer.

5. Counterstain in a 0.5 per cent aqueous solution of phloxine or in van Gieson's stain (p. 92) for 3 to 5 minutes.

6. Differentiate and dehydrate in 95 per cent followed by absolute alcohol.

7. Clear in xylol and mount in balsam.

Celloidin sections are cleared in terpineol or in oil of origanum directly after 95 per cent alcohol.

Results.—Elastic fibers intensely blue-black to black; nuclei blue to black; collagen red; other tissue elements yellow if van Gieson's stain is used. Degenerated elastic tissue (elacin) is also stained by this method.

The Taenzer-Unna Orcein Method for Elastic Fibers (1891).—*Fixation.*—Any fixing fluid may be used. Embed in paraffin.

Staining Solution.—

Orcein	1 gm.
Alcohol, 70 per cent	100 cc.
Hydrochloric acid, concentrated	1 cc.

Method of Staining.—1. Filter stain on sections and warm gently in an incubator or over a small flame for 10 to 15 minutes until the solution thickens, or leave in the solution at room temperature overnight.

2. Wash off thoroughly in 70 per cent alcohol.

3. Wash in water to remove all acid and to fix the color.

4. Nuclei may be stained by Unna's polychrome methylene blue (p. 85) or by alum hematoxylin (p. 70). If it is desirable to have only the elastic fibers stained, dip sections for a few seconds in acid alcohol before washing in water and omit the nuclear stain.

5. Differentiate and dehydrate in 95 per cent followed by absolute alcohol.

6. Clear in xylol and mount in balsam.

Results.—Elastic fibers are stained a deep rich brown color;

connective tissue a pale brown; nuclei bright blue or blue to black, depending on the nuclear stain.

(6) CARTILAGE AND BONE

Excellent work can be done after fixation in alcohol, alcohol-formalin and 10 per cent formalin. The histological structure is, however, better preserved in Zenker's or Orth's fluids. In decalcifying bone, after proper fixation, thin pieces should be taken, not more than 2 to 4 mm. thick, so that the decalcification may be completed as quickly as possible. While tubercle bacilli will stain readily after being 24 or even 48 hours in 5 per cent nitric acid, it is impossible to stain them after they have been subjected to the same strength of nitric acid for 4 days. For details in regard to decalcification see pages 48–50.

Celloidin is preferable to paraffin for embedding. In addition to a simple stain with alum hematoxylin, a counterstain with neutral carmine or phloxine is sometimes advantageous. The best results with carmine as the contrast stain are obtained by staining first in alum hematoxylin, washing 12 to 24 hours in tap water and then staining in neutral carmine. The carmine stains decalcified bone and osteoid tissue red. Phosphotungstic acid hematoxylin will sometimes be found useful, especially when cartilage is present, because it stains the intercellular substance, both of bone and of cartilage, pink to red, while the nuclei are stained blue. The ground substance of cartilage, especially in tumors, often stains so intensely with alum hematoxylin that the nuclei are quite obscured. For the same reason iron hematoxylin is often useful because it does not stain the ground substance.

Schmorl's Methods for Staining Lacunae and Canaliculi of Bone (1914).—**Method 1.**—*Fixation.*—Preferably in Müller's or Orth's fluid or 10 per cent formalin. Do not use a corrosive sublimate solution. Decalcify by one of the slower methods, namely in Ebner's fluid, or in Müller's fluid 100 cc. plus nitric acid 3 cc. Embed in celloidin.

Staining Solutions.—

Nicolle's Carbol Thionin Solution

Thionin, saturated solution (about 0.25 per cent) in 50 per cent alcohol	10 cc.
Carbolic acid water, 1 per cent	100 cc.

Thionin Solution

Thionin, saturated solution (about 0.25 per cent) in 50 per cent alcohol	2 cc.
Water, distilled	10 cc.

Method of Staining.—1. Place the sections for at least 10 minutes in water to remove any trace of alcohol.

2. Stain for 5 to 10 minutes or longer in Nicolle's carbol thionin solution or in the thionin solution.

3. Wash in water.

4. Place in a saturated aqueous solution of picric acid (about 1.22 per cent) for ½ to 1 minute.

5. Wash in water.

6. Place in 70 per cent alcohol for about 5 to 10 minutes until no more dense clouds of color are given off.

7. Dehydrate in 95 per cent alcohol.

8. Clear in terpineol or in oil of origanum and mount in balsam.

Results.—Bone substance yellow to yellowish brown; bone lacunae and canaliculi dark brown to black; cells red. Fat cells after fixation in Müller's fluid stain a reddish violet. Osseous tissue stains a deeper yellow than osteoid tissue. Canaliculi stain in osseous tissue, but not in osteoid tissue unless the thionin solution is made alkaline by the addition of 1 to 2 drops of ammonia water. (This solution cannot be recommended for general use.)

This method is not a true stain but resembles Golgi's method; a precipitation of coloring matter takes place in the lacunae and canaliculi; it also takes place to a considerable extent in other narrow spaces in the tissues. It can be eliminated to some extent without injury to the stain by leaving the sections in Step 5 for half an hour. The canaliculi are now usually brownish red to red, and the bone substance blue to colorless. In this case it is often best to stain the sections first in alum hematoxylin to bring out the nuclei.

Method 2.—This gives excellent results with the bones of children only.

Fixation.—1. Fix very thin pieces of tissue in Müller's fluid, or in Orth's fluid followed by hardening in Müller's fluid for 6 to 8 weeks at room temperature, or for 3 to 4 weeks at 37° C.

2. Wash in water and decalcify in Ebner's solution (p. 50).

3. Wash thoroughly again in running water and embed in celloidin. Cut sections very thin.

Method of Staining.—1. Stain in Nicolle's carbol thionin, or better in an alkaline thionin solution (add 1–2 drops of ammonia water to the thionin solution) for 3 minutes.

2. Transfer to a saturated aqueous solution of phosphotungstic or phosphomolybdic acid (they are both readily soluble in water) for a few seconds or longer (use a glass or platinum needle). The sections become blue, green or gray in color.

3. Wash in water 5 to 10 minutes until they acquire a sky blue color.

4. Place in dilute ammonia water (1 part ammonia water to 10 parts distilled water) for 3 to 5 minutes to fix the color.

5. Transfer directly to 90 per cent alcohol and change several times to get rid of the ammonia.

6. Dehydrate in 95 per cent alcohol.

7. Clear in carbol-xylol and mount in balsam.

If the ground substance is stained too deeply by the alkaline thionin solution, treat the sections with acid alcohol for 5 minutes, followed by water before dehydrating.

Results.—The borders of the lacunae and canaliculi stain bluish black; the ground substance of bone a clear to a greenish blue; cellular elements a diffuse blue color. In rachitic bones the canaliculi are brought out only in osseous tissue.

3. Mast and Plasma Cells

(1) Mast Cells

Mast cells are found in tissues under a variety of conditions, normal as well as pathological. They are often numerous in chronic inflammatory processes and occasionally occur abundantly in leiomyomas. Their cytoplasmic granules stain intensely with basic aniline dyes, especially after fixation in alcohol or formalin. In tissues fixed in Zenker's fluid they usually do not stand out prominently. Several methods of staining mast cell granules are given. Unna's polychrome methylene blue stain differentiates between the granules of the mast cell and the plasma cell.

Ehrlich's Stain for Mast Cell Granules in Sections (1891). —*Fixation.*—Fix in absolute alcohol. Embed in celloidin.

Staining Solution.—

Acetic acid, glacial	12.5 cc.
Alcohol, absolute	50 cc.
Water, distilled	100 cc.
Dahlia	to saturation

Method of Staining.—1. Stain sections in the dahlia solution 12 hours.

2. Differentiate in 95 per cent alcohol.

3. Clear in oil of origanum and mount in balsam.

Results.—Granules of the mast cells red-violet.

Unna's Stain for Mast Cell Granules in Sections (1910).—*Fixation.*—Fix in absolute alcohol. Embed in celloidin.

Method of Staining.—1. Stain sections for 3 hours to overnight in polychrome methylene blue (p. 175) to which is added a knife-pointful of alum to a watchglass of the stain.

2. Rinse in distilled water.

3. Dehydrate in 95 per cent alcohol.

4. Clear in oil of origanum and mount in balsam.

Results.—Nuclei blue; mast cell granules red.

(2) PLASMA CELLS

Plasma cells arise from lymphocytes. They are often abundant in subacute and chronic pathological processes and are characterized by a cytoplasm that stains quite deeply with alkaline methylene blue solutions. The phloxine-methylene blue stain, after fixation in Zenker's fluid, brings them out very sharply. The two methods best suited for their demonstration furnish at the same time a differential color stain for mast cells.

Unna's Differential Stain for Mast Cells and Plasma Cells in Sections (1910).—*Fixation.*—Fix in absolute alcohol or in formalin-alcohol (1 part formalin plus 2 parts 80 per cent alcohol). Embed in celloidin or paraffin.

Staining Solutions.—

Polychrome Methylene Blue

Methylene blue	1 gm.
Water, distilled	100 cc.
Alcohol, 95 per cent	20 cc.
Potassium carbonate	1 gm.

Boil down to 100 cc. on a water bath.

Glycerin-Ether

Glycerin	50 cc.
Calcium chloride, anhydrous	10 gm.

This mixture is prepared in the following way: Distil together and collect the fraction which goes over at 120° to 220° C. Mix the brown liquid with animal charcoal and distil again. Collect the light yellow distillate coming off at 100° C. Glycerin-ether may be purchased already prepared.

Method of Staining.—1. Stain sections in the polychrome methylene blue for 10 minutes.

2. Rinse in distilled water.

3. Differentiate in Unna's glycerin-ether mixture diluted 5 to 10 times with distilled water for ½ to 1 minute or until the section is a medium blue. Be careful not to differentiate too long!

4. Wash thoroughly in water 2 to 5 minutes and blot with filter paper.

5. Dehydrate rapidly in absolute alcohol.

6. Clear in xylol and mount in balsam.

Results.—Nuclei blue; granules of mast cells red; plasma cells blue.

The Unna-Pappenheim Methyl Green-Pyronine Stain for Plasma Cells (1899).—*Fixation.*—Fix in absolute alcohol or Carnoy's fluid. The method may also be successful after fixation in Helly's, Orth's or Regaud's fluids. Embed in paraffin or celloidin.

Staining Solution.—

Methyl green	0.15 gm.
Pyronine	0.25 gm.
Alcohol, 95 per cent	2.5 cc.
Glycerin	20 cc.
Carbolic acid water, 0.5 per cent	77.5 cc.

Method of Staining.—1. Stain sections in the above solution for 20 minutes.

2. Rinse rapidly in water.

3. Differentiate and dehydrate rapidly in absolute alcohol.

4. Clear in xylol and mount in balsam.

Celloidin sections are dehydrated rapidly in 95 per cent alcohol, cleared in terpineol or in oil of origanum and mounted in balsam.

Acetone may be used to advantage in place of alcohol to dehydrate, because it has less tendency to extract the pyronine from the cytoplasm of the cells.

Results.—Nuclei green; cytoplasm deep red.

4. Muscle Tissue

For the demonstration of muscle cells double stains, such as alum hematoxylin and phloxine, or phloxine and methylene blue, are sufficient.

For bringing out the finer details in the cytoplasm, however, phosphotungstic acid hematoxylin and Mallory's aniline blue stain for collagen are much to be preferred. It is imperative that the tissue be perfectly fresh, especially if the myoglia fibrils in smooth muscle cells are to be studied, because they undergo postmortem changes very rapidly. Thin sections of the tissues to be studied should be put into Zenker's fluid within 5 to 10 minutes after removal from the body if the best results are desired.

Autopsy material is practically useless. The most desirable tissues are those obtained directly at operations.

CHAPTER XIII

SPECIAL ORGANS

TISSUES that are to be preserved for study should be obtained as fresh as possible. For this reason autopsies rarely furnish such perfect material as can be obtained from surgical operations or from experimental lesions in animals. However, most of the pathological material is from autopsies, and it is encouraging to know that excellent results may be secured with tissues fixed 24 hours or even later after death. The most valuable autopsies are those that are performed immediately after death, and in which but one etiological factor has been concerned, so that the relation between the etiology and the lesion produced is uncomplicated and can be readily grasped and understood.

The choice of the proper fixing reagent varies with the tissue, the lesion, and the use to which the material is to be put. It is advised as a routine procedure to fix tissues in three different solutions: in Zenker's fluid for general histological study and for the demonstration of injurious agents of various kinds and the inflammatory reactions to them; in formalin for the preservation especially of fat and myelin; and in alcohol for the demonstration of glycogen, mucin, amyloid, sodium urate crystals, hemofuscin, iron and other metals. With these three fixatives it is possible to accomplish a great deal in pathological histology. Helly's and Orth's fluids may be substituted for Zenker's but are distinctly not so useful except for certain cells and tissues.

It is imperative that pieces of tissue for histological study should be placed in the proper fixative as soon after the removal of the organs or tissues from the body as possible, so that the surface will not dry or the blood and other fluids escape from the vessels. Do not wash off the surface with water. The tissues should almost invariably be cut into thin slices, not over 2 to 4 mm. thick.

In fixing tissues it is very important to use enough of the fixing reagent, that is, 10 to 20 times as much as there is tissue. It is advisable to use flat-bottomed glass dishes and to stir the tissues occasionally, so that they may come in contact with fresh fluid.

After Zenker's fixative the best stain to use for general histological study is the phloxine and methylene blue stain. For slides to be used for study by students the alum hematoxylin and phloxine stain makes a fairly satisfactory substitute, but it does not demonstrate any bacteria present. Other useful stains are phosphotungstic acid hematoxylin, the aniline blue method for collagen, Verhoeff's elastic tissue stain and the various bacteriological stains. After formalin fixation the presence of fat may be demonstrated by staining frozen sections with scarlet red and alum hematoxylin.

These methods of fixation and staining are applicable to most of the tissues listed below and constitute the routine stains for almost all organs with the exception of the central nervous system, and even for this purpose they are often useful. It is unnecessary to repeat them under each tissue and organ.

1. Circulatory System

(1) HEART

The routine fixatives and staining methods advised above are about all that is necessary for the study of the heart. A few stains, however, deserve special mention.

The presence of fat is often of importance and it is readily demonstrated by means of the scarlet red stain on formalin-fixed tissue.

Most infectious agents are demonstrated by the phloxine-methylene blue stain after fixation in Zenker's fluid and by the usual bacterial stains.

Demonstration of *Treponemata pallida* is often of importance, especially in congenital syphilis, and requires special staining methods. Tissues must be fixed in formalin and the treponema demonstrated by one of the special silver stains. Levaditi's stain for blocks of tissue and Heitzman's stain for frozen sections can be recommended.

Iron stains are sometimes necessary to distinguish between hemosiderin and the pigment of brown atrophy. For this purpose fixation in alcohol is advisable. Fixation in alcohol or formalin is best for amyloid, which sometimes occurs in the heart and nowhere else.

The Purkinje fibers contain much glycogen, for the demonstration of which Best's carmine stain is recommended after fixation in alcohol.

(2) BLOOD VESSELS

Routine staining methods, and in addition Verhoeff's elastic tissue stain, bring out clearly in tissues fixed in Zenker's fluid the various elements in the walls of the blood vessels.

In addition, certain pathological products of much importance require special consideration. The first of these is fat, which occurs in foci of degeneration and also in macrophages. It is readily demonstrated by the scarlet red stain on frozen sections of formalin-fixed material.

Another pathological product is calcium which is often deposited in large amounts in foci of degeneration. Kóssa's silver stain can be recommended for the demonstration of calcium after alcohol fixation.

2. BLOOD

(1) PREPARATION OF BLOOD SMEARS

Smears may be made on either coverslips or slides. Whichever are used must be thoroughly cleaned as described for the preparation of glassware in the section on supravital staining. When coverslips are used a small drop of blood is placed in the center of one coverslip and another coverslip is immediately dropped upon it in such a way that it is not completely superimposed but leaves the corners of each uncovered. As soon as the blood spreads the coverslips are pulled apart quickly with a sliding motion, are waved in the air, and are then allowed to dry at room temperature.

When smears are made on slides a small drop of blood is placed near the end of one slide. The end of another slide, held at an angle of 45° to the first, is then slid up to the drop. As soon as the drop spreads the second slide is pulled backward rapidly along the surface of the first slide, making a thin uniform smear. The smeared slide is then waved in the air and allowed to dry at room temperature.

(2) STAINING METHODS

Stains may be made in the laboratory or may be purchased, either as staining solutions ready for use, or in the form of dry stains which are to be dissolved in their proper solvents. In our experience reliable and satisfactory staining solutions are now available. However, since some workers desire to prepare their own stains the methods of doing so are given below:

Wright's Stain (1902).—*Fixation.*—No fixation necessary. Use air-dried smears.

Staining Solution.—To a 0.5 per cent aqueous solution of sodium bicarbonate add methylene blue in a proportion of 1 gm. of the dye to each 100 cc. of the solution. Heat the mixture in a steam sterilizer at 100° C. for 1 full hour, counting the time after the sterilizer has become thoroughly heated. The mixture is to be contained in a flask or flasks of such size and shape that it forms a layer not more than 6 cm. deep. After heating, the mixture is allowed to cool, placing the flask in cold water if desired, and is then filtered to remove the precipitate which has formed. It should, when cold, have a deep purple-red color when viewed in a thin layer by transmitted yellow artificial light. It does not show this color while it is warm.

To each 100 cc. of this filtrate add 500 cc. of a 0.1 per cent aqueous solution of yellowish water-soluble eosin and mix thoroughly. Collect on a filter paper the abundant precipitate which immediately appears. When the precipitate is dry dissolve it in methyl alcohol in a proportion of 0.1 gm. of the precipitate to 60 cc. of the alcohol. In order to facilitate solution the precipitate should be ground up in the alcohol in a porcelain dish or mortar with a spatula or pestle.

This alcoholic solution of the precipitate is the staining fluid. It should be kept in a well stoppered bottle because of the volatility of the alcohol. If it becomes too concentrated by evaporation and thus stains too deeply, or forms a precipitate on the blood smear, the addition of a suitable quantity of methyl alcohol will quickly correct such faults. It does not undergo any spontaneous change other than that of concentration by evaporation.

A most important fault of some solutions of this stain is that they fail to stain red blood corpuscles a yellow or orange color, staining them a blue tint which cannot readily be removed by washing with water. This fault is due to a defect in the specimen of eosin employed. It can be eliminated by using a proper yellowish water-soluble eosin.

In staining, either distilled water, tap water or a buffered solution is used as a diluent. The buffered solution should have a *p*H of approximately 6.4. The McJunkin-Haden buffer solution is made as follows:

Monopotassium acid phosphate	6.63	gm.
Disodium acid phosphate, anhydrous	2.56	gm.
Water, distilled	1000	cc.

Method of Staining.—1. Cover the smear with a noted quantity of the staining fluid by means of a pipette.

2. After 1 minute add to the staining fluid by means of a pipette the same quantity of distilled or tap water or buffered solution, or an amount sufficient to cause the formation of a film on the surface of the staining fluid. Allow it to remain for 2 to 3 minutes, according to the intensity of the staining desired. A longer period of staining may produce a precipitate.

The periods of time given for staining are only approximate. It is necessary to determine for each lot of stain the optimum times, for both the undiluted stain and the stain after the addition of the water. The use of distilled water is recommended as the *p*H is more constant but the tap water in many localities is perfectly satisfactory.

3. Wash the preparation in distilled water for 30 seconds, or until the thinner portions of the preparation become yellow or pink in color.

4. Blot, dry and mount in balsam.

Results.—Red cells are orange or pink in color. Polychromatophilia and punctate basophilia or granular degeneration are well brought out. The nucleated red cells have deep blue nuclei and the cytoplasm is usually of a bluish green tint.

The polymorphonuclear neutrophilic leukocytes have a dark blue or dark lilac colored nucleus, and the granules are usually of a reddish lilac color.

The eosinophilic leukocytes have blue or dark lilac colored nuclei. The granules have the color of eosin, while the cytoplasm in which they are embedded has a blue color.

The basophilic leukocytes have purplish or dark blue nuclei. The granules are of a dark purple color and may appear almost black.

Stem cells (myeloblasts) have blue or lilac colored nuclei with prominent nucleoli. Their cytoplasm is deep blue and contains no granules. Promyelocytes have blue or lilac colored nuclei and contain purplish red granules in their cytoplasm.

Myelocytes have dark blue or lilac colored nuclei and contain

the different specific types of granules described above for the granular leukocytes.

Monocytes have lilac colored nuclei with a reticular arrangement of the chromatin. Their cytoplasm is a grayish blue and contains fine light red granules.

Lymphocytes have dark purplish blue nuclei and robin's egg blue cytoplasm in which a few purplish granules are sometimes present.

The blood platelets are well stained. In the best preparations they generally appear as round or oval blue bodies with smooth or finely irregular margins, containing, chiefly in their central portions, many small violet to purplish granules. They are usually of a diameter of one-third to one-half that of the red blood corpuscles. They frequently occur in groups and masses. Occasionally elongated forms are seen which may have a length of six or more times their width. These are, according to Wright's observations, the detached larger pseudopods of the giant cells of the bone marrow, just as the smaller platelets are detached smaller pseudopods or fragments of the larger pseudopods of the same cells.

Giemsa's Stain (1902, 1904, 1905, 1909, 1922).—

Fixation.—

A. Methyl Alcohol.—Air-dried smears are fixed 1 to 3 minutes in methyl alcohol and are then allowed to dry.

B. May-Grünwald Method (1902).—Fix air-dried smears in the May-Grünwald solution for 3 minutes. This solution is prepared by adding 1 gm. of eosin to 1000 cc. of distilled water, and 1 gm. of methylene blue to 1000 cc. of distilled water. When the two solutions are mixed a precipitate forms. Allow the mixture to stand for several days and then filter. Wash the precipitate with distilled water until no more color comes through the filter. Allow the precipitate to dry. Make a saturated solution in methyl alcohol (0.25 gm. of precipitate to 100 cc. of methyl alcohol).

Add an equal amount of distilled water to the May-Grünwald solution and leave smears in this dilute solution 1 minute longer.

Staining Solution.—

Azure II eosin	0.6	gm.
Azure II	0.16	gm.
Glycerin	50	cc.
Alcohol, methyl	50	cc.

The dyes are dissolved in the glycerin, warmed to 60° C. Then the methyl alcohol, warmed to the same temperature, is added and the container shaken. When the solution is cool it is filtered and kept in tightly stoppered bottles.

An alternative method of preparation is to add to the dyes contained in a mortar the mixture of glycerin and methyl alcohol. This should be added slowly and the dyes thoroughly ground with a pestle.

Method of Staining.—In carrying out the staining it is advisable to float the coverslip smear side down, or if the smears are on slides to invert them, supporting one end of the slide in the staining dish with a piece of glass rod. This is done to avoid the deposition of precipitates.

For staining use 1 drop of stain to 1 cc. of distilled water or buffered solution made as follows:

Solution A

Monopotassium acid phosphate (KH_2PO_4)	9.078	gm.
Water, distilled	1000	cc.

Solution B

Disodium acid phosphate ($Na_2HPO_4 \cdot 2H_2O$)	11.876	gm.
Water, distilled	1000	cc.

For use take 4 parts of Solution A and 6 parts of Solution B and to this mixture add 10 to 20 volumes of distilled water. This dilution should be made just before use.

A. Smears Fixed in Methyl Alcohol.—1. Stain for 15 minutes in Giemsa's stain diluted as directed above.

2. Wash in distilled water until properly differentiated.

3. Blot, dry and mount in balsam.

B. Smears Fixed in the May-Grünwald Solution.—1. Without rinsing, place the coverslip or slide in the Giemsa stain, diluted as directed above, and stain for 15 minutes.

2. Wash in distilled water.

3. Blot, dry and mount in balsam.

Results.—The staining results are essentially similar to those obtained with Wright's stain, but in general the colors are more delicate.

Note: Giemsa's stain is very sensitive to changes in the hydrogen ion concentration. The more acid the solution, the more intense the red; the more alkaline, the deeper the blue shades. Advantage may be taken of this fact to bring out certain desired structures.

Ehrlich's Triacid Stain (Heidenhain, 1888).—*Fixation.*—Air-dried smears are fixed by heat at 110° C. This temperature is most easily attained by heating a brass plate at one end with a Bunsen burner. Then, at a point between where a drop of water boils and a drop sputters and rolls about, the temperature will be about 110° C. and at this place the coverslips, smear side up, are left for 5 to 30 seconds. The brass plate should be about 3 mm. thick and 38 to 45 cm. long.

Staining Solution.—The original formula was as follows:

Orange G, saturated aqueous solution (about 10.8 per cent)	10 cc.
Acid fuchsin, saturated aqueous solution (about 20 per cent)	1 cc.
Methyl green, saturated aqueous solution (about 8 per cent)	3 cc.

Various modifications have been suggested and the reader is referred to special textbooks for these. The preparation of the stain at best is difficult and it would seem wiser to purchase it in a prepared form.

Method of Staining.—1. Stain in the above solution 5 to 10 minutes.

2. Wash in distilled water until no more stain comes away.

3. Dry and mount in balsam.

Results.—Nuclei green (for the most part rather indistinct); neutrophilic granules violet-red; eosinophilic granules bright red; basophilic granules unstained, as is the cytoplasm of the lymphocytes; red blood cells orange.

The Winkler-Schultze Oxidase Reaction (1907, 1909, 1910, 1917).—*Fixation.*—Fix air-dried smears for 2 hours in alcohol-formalin (1 part formalin to 4 parts 95 per cent alcohol).

Staining Solution.—

Solution A.—Heat 1 gm. alpha-naphthol with 100 cc. of distilled water in an Erlenmeyer flask until the water begins to boil and the alpha-naphthol is melted. Then, shaking the flask gently,

add drop by drop a 25 per cent aqueous solution of potassium hydroxide until the melted alpha-naphthol is completely dissolved. When cool, sometimes a few crystals precipitate out. Use the supernatant light yellow fluid. This solution keeps for about 4 weeks and should be preserved in the dark.

Solution B.—Dissolve 0.5 gm. dimethyl-p-phenylenediamine base in 50 cc. of distilled water at room temperature. The solution is to be preserved in brown bottles and keeps for 2 to 3 weeks.

Method of Staining.—1. Stain 2 to 5 minutes in a mixture of equal parts of Solutions A and B.

2. Wash in distilled water.

3. Mount in glycerin.

Results.—Oxidase granules dark blue. Preparations keep for several months.

Graham's Alpha-Naphthol-Pyronine Stain for Oxidase Granules (1916).—*Fixation.*—Fix air-dried smears for 1 to 2 minutes in a freshly prepared mixture of 1 part formalin to 9 parts of 95 per cent alcohol.

Staining Solution.—

Solution A		
Alpha-naphthol	1	gm.
Alcohol, 40 per cent	100	cc.
Hydrogen peroxide	0.2	cc.
Solution B		
Pyronine	0.1	gm.
Aniline	4	cc.
Alcohol, 40 per cent	96	cc.

Dissolve the pyronine in the alcohol and add the aniline. This solution keeps well.

Method of Staining.—1. Wash in water.

2. Stain with Solution A for 4 to 5 minutes.

3. Wash in running water for about 15 minutes.

4. Stain with Solution B for 2 minutes.

5. Wash in water.

6. Stain with a 0.5 per cent aqueous solution of methylene blue ½ to 1 minute.

7. Wash in water.

8. Blot, dry and mount in balsam.

Results.—The resulting picture is much like that of the Romanowsky stains, except for the greater prominence of the granules. The neutrophilic granules are usually very abundant, so that the cytoplasmic substance of the cell appears almost completely filled with them. They are somewhat irregular in form and size and are purplish red in color. Occasional cells show fewer and more faintly stained granules. They may represent old degenerating forms of the cell. The eosinophilic granules are larger, somewhat lighter and more refractile, and have the appearance of spherical bodies with lighter staining centers. The basophilic granules take a more basic stain, so that they appear of a deep purple color. Myelocytes have granules of varying number and size. Erythrocytes are greenish yellow to pink; platelets blue; nuclei of all cells blue; cytoplasm light blue.

The best results are secured with fresh smears or with those not over a few days old. After 10 days to 2 weeks the reacting substances begin to disappear, so that in older specimens many of the granules fail to stain and some cells may appear entirely devoid of them. The eosinophilic granules retain their staining power much longer than the neutrophilic.

Graham's Benzidine Stain for Oxidase Granules (1918).—*Fixation.*—Fix fresh smears in a freshly prepared mixture of formalin 1 part, and 95 per cent alcohol 9 parts for 1 to 2 minutes.

Staining Solution.—Prepare a stock solution of 40 per cent alcohol containing 0.2 per cent hydrogen peroxide. At the time of staining saturate the required quantity of this fluid with benzidine.

Method of Staining.—1. Wash fixed smears with tap water.

2. Cover with the benzidine mixture for 5 to 10 minutes.

3. Wash with tap water.

4. Stain with Loeffler's alkaline methylene blue solution (p. 84) for 30 seconds.

5. Wash in water.

6. Blot, dry and mount in balsam.

Results.—The neutrophilic and eosinophilic granules are stained a yellow-brown. The nuclei of the leukocytes are dark blue, those of the lymphocytes a slight purplish tint. The red blood cells are greenish yellow to greenish blue; the blood platelets are blue.

Sato's Peroxidase Reaction (1927–28).—*Fixation.*—No fixation necessary. Use air-dried smears.

Staining Solution.—

Benzidine Solution.—To 0.1 gm. of benzidine in a mortar add slowly, grinding with a pestle all the time, 100 cc. of distilled water. Filter and add 2 to 4 drops of hydrogen peroxide to the filtrate. This solution keeps fairly well at room temperature for several weeks.

Method of Staining.—This is best done by floating coverslips on various solutions.

1. Mordant in 0.5 per cent aqueous solution of copper sulfate 1 minute.
2. Wash quickly in water. The time of washing is of prime importance. If too short, a crystalline precipitate will appear on the smears following Step 3; if too long, either no reaction or a very weak one will take place. It is our custom to dip the coverslip quickly in the water 3 times.
3. Place immediately in the benzidine solution for 2 minutes.
4. Wash in water.
5. Stain in 1 per cent aqueous solution of safranine 20 seconds.
6. Wash in water.
7. Blot, dry and mount in balsam.

Results.—Peroxidase granules deep blue; nuclei and cytoplasm of lymphocytes red; basophilic granules red.

Bloch and Peck's Modification of the Dopa Reaction for Leukocytes (1930).—*Fixation.*—Fix smears in hot formaldehyde vapor for 20 minutes.

Staining Solution.—

Stock Dopa Solution.—Dissolve 0.1 gm. 3,4-dioxyphenylalanine in 100 cc. of physiological salt solution. Keep tightly corked in the refrigerator where it will remain good for many weeks. The solution is usable as long as it remains colorless or shows no more than a slightly reddish tinge.

Method of Staining.—1. Immerse smears in the dopa solution.

2. Then add 0.2 cc. of N/10 sodium hydroxide solution for each 10 cc. of stock dopa solution. The mixture turns red, then brown.

3. Leave at room temperature for 1 to 2 hours, examining the coverslip or slide every 30 minutes.

4. When the leukocyte granules are stained (usually in 1–1½ hours) wash in running water, dry and mount, or

5. Wash in distilled water.

6. Immerse in 2 per cent aqueous solution of silver nitrate for 2 hours.

7. Wash in distilled water.

8. Treat for 10 minutes with a saturated solution of sodium thiosulfate (about 100 per cent).

9. Wash in distilled water.

10. Stain nuclei with alum hematoxylin (p. 70) for 15 to 20 minutes.

11. Wash in water.

12. Blot, dry and mount in balsam.

Results.—Dopa-positive granules yellow to yellowish brown; following treatment with silver nitrate yellowish brown to dark brown.

(3) SUPRAVITAL STAINING

Supravital staining is a method employed for the study and identification of living cells. All the dyes used are toxic and therefore the strength of the solution and the time of exposure of the cells to the dye must be taken into consideration for each stain employed. In brief, the method consists of exposing the cells suspended in a neutral medium such as blood serum or Locke's solution to the particular dye and observing the character, distribution, and so on, of the stained structures in the cells. For material from warm-blooded animals the use of a warm-box or a warm-stage kept at 37.5° C. is advisable.

The dyes used may be divided into two main groups: (1) dyes such as neutral red, Congo red, methylene blue, Nile blue sulfate, and others, which stain specific granules, phagocytosed material and certain other structures; and (2) those that stain mitochondria specifically; of these latter stains the best known is Janus green.

Preparation of Glassware.—The most satisfactory method of employing these stains is in the form of a thin film on coverslips or slides made by allowing an alcoholic solution to evaporate. (It has been our practice to utilize slides exclusively, but coverslips may be used.) In order to get a uniformly thin film it is essential

that the slides or coverslips be free from grease. This is ensured by the following procedures:

1. Place all glassware to be used in cleaning solution (sulfuric acid-bichromate) for several days.
2. Wash for several hours or overnight in running tap water.
3. Place in distilled water overnight.
4. Leave in 95 per cent alcohol for 2 to 3 days.
5. Wipe with gauze, removing each slide with a pair of forceps and taking care not to let the fingers come in direct contact with the slides.
6. Holding the slide in a pair of forceps, flame over a Bunsen burner. Keep in a closed box protected from dust.

Staining Solutions.—*Neutral Red.*—This is the most widely used of the supravital stains of the first group mentioned above. Its toxicity is relatively low and it has the added advantage of being an indicator—red in acid solutions and yellow in alkaline. A stock solution of neutral red is prepared by making a supersaturated solution in absolute alcohol (about 0.25 per cent) kept in a tightly stoppered bottle. For use, 20 to 50 drops of this solution are added to 10 cc. of absolute alcohol. This dilute solution is most conveniently kept in a bottle with a glass stopper that contains a capillary pipette with a nipple. Agitation of the stock solution when removing a sample must be avoided.

The slides are flooded with the dye, the excess being allowed to run back into the bottle. The slides are then placed upright until the films are dry, when they are ready for use. Such stained slides keep indefinitely but should be protected from dust. Films prepared on a humid day are apt to be somewhat uneven, due to the absorption of moisture by the alcohol.

Janus Green.—As stated above, Janus green is the most widely used stain for mitochondria. It may be used alone, but in the study of blood cells it is usually employed combined with neutral red. A supersaturated solution of Janus green in absolute alcohol (about 0.2 per cent) serves as a stock solution. When it is to be used alone 15 to 30 drops of this solution are added to 10 cc. of absolute alcohol and films are made as directed for neutral red. When used in combination with neutral red, 3 to 6 drops of Janus green solution are added to 2 cc. of dilute neutral red solution and films on slides are made immediately, since the combined solutions

precipitate on standing. The amount of Janus green to be used varies with the type of blood or tissue to be studied and has to be determined for each lot of dye. The amount necessary for satisfactory staining also depends somewhat on the number and character of the cells to be studied.

Methods of Staining.—*A.* Blood.—A drop of blood is placed on the stained slide and covered gently with a coverslip. The size of the drop is of prime importance. It should be such that it will just fill the space between the slide and the coverslip. If too large the leukocytes will float and be spherical in shape; if too small, a considerable number of the cells will be damaged or killed from the pressure of the coverslip. After the blood has spread out between the coverslip and the slide, the edges of the coverslip are sealed with melted vaselin. This may be applied with a syringe and needle or with a swab-stick or match-stick. The preparation is then placed in a warm-box or warm-stage and examined. It usually takes several minutes for the stain to penetrate the cells and also for the cells to attain their normal motility.

Results.—The granules of the leukocytes of different species of animals vary somewhat in their staining reactions. The descriptions given below apply to those of human blood.

Polymorphonuclear Neutrophiles.—The granules stain a brownish red. When a cell dies the granules become faintly yellow or colorless. The cells are actively motile and the granules can be seen streaming into the pseudopods.

Eosinophiles.—The granules are large and stain a brilliant yellow. The cells are motile.

Basophiles.—The granules are a deep red. The cells are motile.

Lymphocytes.—The granules are few in number and stain a lighter red than those of the basophiles. When Janus green is used in addition, numerous rod shaped mitochondria colored a bluish green can be distinguished. Lymphocytes are sluggishly motile.

Monocytes.—The granules are a salmon pink and are fairly numerous. When the cell is actively motile no definite arrangement can be made out. When they are moving slowly or are at rest the granules are arranged in a rosette around the centrioles in the neighborhood of the nucleus. With Janus green the mitochondria can be seen situated at the periphery of the rosette.

Myelocytes.—The granules of various types stain as do those

of the adult forms, but more intensely. The mitochondria vary in number according to the stage of maturation of the cell, being most numerous in the more immature forms. They are non-motile.

Stem Cells (Myeloblasts, Monoblasts, Lymphoblasts).—The granules are very small, are salmon pink in color and are arranged in a small rosette in each cell. Mitochondria are numerous. These cells are non-motile.

Macrophages (Clasmatocytes, Histiocytes, Endothelial Leukocytes).—Irregular in shape; they are larger than any other cell, and contain no specific granules, but often contain phagocytosed material which stains various shades of red or yellow.

Erythrocytes.—Red blood cells stain a faint yellow. Reticulated red cells show granules of red material.

Comment: When neutral red alone is used the cells remain viable for several hours. As time goes on the granules tend to stain more intensely and in monocytes an exaggerated form of rosette is often observed. When the cell dies it becomes spherical and the granules lose their specific stain, tending to collect around the nucleus. The nucleus tends to stain red.

Janus green is more toxic and the cells are usually killed within an hour.

When the combined neutral red and Janus green stain is used, staining by neutral red is often less intense than when it is used alone. Therefore, it is a good practice to use a preparation with neutral red alone, as well as with the double stain.

B. Tissues and Exudates.—The same technique as given for the blood may be applied here. In the case of exudates or transudates a drop of the fluid, diluted if necessary, is treated the same as a drop of blood. If tissue is being examined the surface may be scraped or with specimens such as lymph nodes, cellular tumors, and so on, a small piece of tissue may be placed in a drop of fluid and a coverslip gently pressed down. The best fluid in which to suspend tissue cells is blood serum, either human or animal. Locke's solution may be used but it is less satisfactory. Care must be taken not to let the tissue dry.

This method of study can be profitably applied, not only to normal tissues but also to tumors, and a diagnosis can often be rapidly made.

(4) STAINING OF FIBRIN

Fibrin usually appears as delicate, transparent, slightly refractive threads which are often closely matted together to form large masses. More rarely it appears as coherent masses of the finest granules, as homogeneous glassy lumps, or as thin sheets. The characteristic reaction for fresh fibrin is that it swells up quickly and optically dissolves in very dilute acetic acid.

Fibrin is well brought out in sections by the alum hematoxylin and phloxine stain, or by phloxine followed by an alkaline methylene blue solution, especially if the tissue has been fixed in Zenker's fluid. Two other stains that bring it out with great sharpness are phosphotungstic acid hematoxylin and Mallory's aniline blue method for collagen. As a specific method for fibrin Weigert devised a differential stain which is a modification of Gram's method for bacteria.

Weigert's Differential Stain for Fibrin (1887).—*Fixation.*—Fix in absolute alcohol, Carnoy's fluid or alcohol-formalin. Zenker's and Helly's fluids may also be used but after fixation in fluids containing chrome salts the sections must be treated with potassium permanganate (0.25 per cent for 10 minutes) and oxalic acid (5 per cent for 20 minutes) before being stained. Embed preferably in paraffin.

Staining Solution.—

Solution A

Alcohol, absolute	33 cc.
Aniline	9 cc.
Methyl violet (crystal violet)	in excess

Solution B

Methyl violet (crystal violet), saturated solution in distilled water (about 1.68 per cent)

Just before use mix 3 cc. of Solution A with 27 cc. of Solution B. The stock solutions will keep indefinitely, the combined stain for only 1 to 2 weeks.

Method of Staining.—1. Stain nuclei by the lithium carmine method (p. 80) in the usual way and wash thoroughly in water.

2. Stain sections for 5 to 10 minutes in the staining solution.

3. Drain and blot with filter paper.

4. Pour over the sections Gram's iodine solution (p. 82) and allow it to act for 5 to 10 minutes.

5. Drain off solution and blot with filter paper.

6. Differentiate in a mixture of equal parts of aniline and xylol from a dropping-bottle. Blot and pour on fresh aniline-xylol several times until the section is well differentiated and no more purple color is removed.

7. Blot and pour on xylol several times to remove the aniline.

8. Mount in balsam.

Results.—Fibrin and Gram-positive bacteria blue to blue-black; nuclei red.

3. Bone Marrow

Smears.—Smears may be made in a number of ways.

1. A slide or coverslip may be touched to the surface of the tissue.

2. A small piece of tissue, held in a pair of forceps, may be rubbed over the surface of a slide or coverslip.

3. A drop of serum of the size used for blood smears is placed in the center of a coverslip. A small piece of tissue is teased out in the drop with forceps or needles, and the larger bits of tissue are then removed and discarded, leaving a suspension of cells in the serum. Another coverslip is then dropped on the first, and a smear made in the same way as a blood smear.

Fixation and Staining.—The smears may be allowed to dry in the air and be fixed and stained by any of the methods used for blood smears, such as Wright's stain, Giemsa's stain, and so on.

However, better results are often obtained by fixation while the smears are still wet, followed by staining with a dilute solution of Wright's stain. This method is carried out as follows:

1. Fix smear while still wet in methyl alcohol for 1 minute.

2. Stain for 3 to 5 minutes with Wright's stain, diluted with an equal amount of distilled water. This dilution must be made immediately before use.

3. Wash in water.

4. Dehydrate in acetone.

5. Clear in oil of turpentine.

6. Mount in turpentine colophony.

Instead of Steps 5 and 6 may be substituted clearing with xylol and mounting in balsam.

Oxidase and peroxidase reactions are carried out as directed for blood smears.

Supravital preparations of bone marrow often yield information of considerable value and are made as directed under the section on supravital staining of blood.

Sections.—In our experience, the best preparations of bone marrow are paraffin sections of Zenker-fixed material stained with Mallory's phloxine-methylene blue stain or with Wolbach's modification of the Giemsa stain. This applies to both biopsy and autopsy material. The pieces of marrow are cut so thin before fixation that the lime salts are completely removed during the 24 hour period in Zenker's fluid. This obviates the necessity of any further decalcifying process and is highly desirable, since in our experience any treatment with other decalcifying agents interferes with satisfactory staining.

If further decalcification is necessary, we have found a 5 per cent solution of nitric acid in 95 per cent alcohol to be the most satisfactory agent. After decalcification is complete, the tissues are transferred directly to 95 per cent alcohol, not water. Change the alcohol repeatedly to remove the acid, and then embed as usual.

Wolbach's Modification of Giemsa's Stain (1919).—*Fixation.*—Fix in Zenker's fluid. Embed in paraffin and cut sections at 5 μ or less.

Staining Solution.—

Giemsa's Stain

Azure II eosin	3	gm.
Azure II	0.8	gm.
Alcohol, methyl (Merck's reagent)	375	cc.
Glycerin	125	cc.

Combine reagents and filter. For use take:

Giemsa's stain	2.5	cc.
Alcohol, methyl (Merck's reagent)	3	cc.
Water, distilled (to which have been added 2–4 drops of a 0.5 per cent aqueous solution of sodium bicarbonate)	100	cc.

Method of Staining.—Wolbach suggests that after sections have been deparaffinized and treated with iodine in the usual way, as

an extra precaution to ensure the complete removal of the corrosive sublimate crystals deposited in tissues by Zenker's fluid, sections should be treated with a 0.5 per cent solution of sodium thiosulfate for 10 to 15 minutes. Wash thoroughly in tap water and place in distilled water.

1. Pour stain over slides immediately after mixing. The stain should be changed twice during the first hour. Leave sections overnight in the third change of staining solution.
2. Pour off stain and place sections in distilled water.
3. Differentiate each slide individually in 95 per cent ethyl alcohol to which a few drops of 10 per cent solution of colophony (rosin) in absolute alcohol have been added.
4. Dehydrate sections in absolute alcohol.
5. Clear in xylol and mount in oil of cedarwood.

Since it has been possible to obtain neutral balsam sections mounted in this medium have not faded to any extent in several years. Neutral balsam gives a harder and more permanent mount.

Results.—Essentially similar to those obtained by the phloxine-methylene blue stain except that the color contrast is more delicate.

Maximow's Azure II Eosin and Alum Hematoxylin Stain (1909).—*Fixation.*—Fix in Maximow's modification of Helly's fluid. This is prepared by adding to 90 cc. of Zenker's fluid (without acetic acid) 10 cc. of formalin just before use. Fix for 24 hours, wash 24 hours in running water, and place in 80 per cent alcohol.

Staining Solution.—

Solution A

Eosin, water-soluble, yellowish	0.1 gm.
Water, distilled	100 cc.

Solution B

Azure II	0.1 gm.
Water, distilled	100 cc.

Directly before using, dilute 10 cc. of Solution A with 100 cc. of distilled water or buffered solution and add 10 cc. of Solution B. The combined solution is dark violet.

The stain is very sensitive to the hydrogen ion concentration so it is advisable to use either freshly redistilled water or a buffered solution with a *p*H of 6.8 to 7.0. This may be prepared as follows:

M/15 monopotassium acid phosphate	4 parts
M/15 disodium acid phosphate	6 parts
Water, distilled	10–20 parts

The monopotassium acid phosphate solution is made by dissolving 9.078 gm. KH_2PO_4 in 1000 cc. of distilled water, and the disodium acid phosphate solution by dissolving 11.876 gm. $Na_2HPO_4.2H_2O$ in 1000 cc. of distilled water.

Method of Staining.—Either paraffin or celloidin sections may be used.

1. Stain for 24 hours in dilute Delafield's alum hematoxylin (p. 71) made by adding 1 to 2 drops of the stain to 100 cc. of distilled water.
2. Wash in distilled water 24 hours.
3. Stain in the azure II eosin mixture 6 to 24 hours.
4. Differentiate the overstained sections in 95 per cent alcohol until the red blood cells and collagen are pink to red.
5. Dehydrate in absolute alcohol ½ to 1 minute.
6. Clear in xylol and mount in balsam.

Results.—The results are essentially the same as with the phloxine-methylene blue or Giemsa's stain.

Wright's Stain for Megakaryocytes and Blood Platelets (1910).—*Fixation.*—Fix in 10 per cent formalin or in a saturated solution of mercuric bichloride (about 6.9 per cent) in 0.9 per cent sodium chloride solution. Downey recommends a mixture of 10 cc. formalin and 90 cc. of 0.9 per cent sodium chloride solution saturated with mercuric bichloride.

Staining Solution.—The staining fluid is composed of 1 part of a modified methylene blue solution and 10 parts of a 0.1 per cent solution of water-soluble eosin in methyl alcohol.

The solution of methylene blue is prepared as follows: Dissolve 1 gm. of methylene blue as thoroughly as possible in 100 cc. of a 0.5 per cent aqueous solution of sodium bicarbonate in an Erlenmeyer flask. The flask and its contents are then placed in an ordinary steam sterilizer and kept at 100° C. for 1½ hours, counting the time after the steaming has become vigorous. When cool the mixture is filtered and the filtrate is the modified blue solution. It must be of a pronounced purple color when viewed in a thin layer by the transmitted yellow light of an ordinary incandescent electric bulb. This color appears only after cooling.

Variations in the solutions of the blue and of the eosin may require that the proportions given above be changed slightly. An excess of eosin delays the appearance of the scum on the surface of the diluted staining fluid and prolongs the time required for staining. On the other hand, an excess of the modified blue component hastens the appearance of the scum and may cause overstaining and a granular precipitate to form on the preparation.

Method of Staining.—1. Equal parts of the staining fluid and distilled water are mixed and immediately poured on the slide. At least 2 cc. of the freshly diluted staining fluid are spread out over the slide, which should be supported on some object in such a way as to prevent the fluid from running off. The spreading out of the fluid in a layer is important, because it facilitates the evaporation of the alcohol, whereby the staining elements slowly precipitate out of solution and, while doing so, stain the tissue elements. This precipitate appears as a yellowish metallic scum which slowly forms on the surface of the mixture. The diluted staining fluid is allowed to act for about 15 minutes, when the preparation is immediately washed in water. The exact time required for the best results has to be determined for each batch of staining fluid. Proper staining of the preparation may be judged by examining it under low power by artificial light after pouring back the diluted staining fluid into the container. When the cytoplasm of the giant cells has acquired a bright red color and the fibrils of the reticulum begin to take on a red color also, the staining is stopped by washing the preparation in water. If the staining is found not sufficiently intense the diluted staining fluid is poured back on the preparation and allowed to act longer. Overstaining and the formation of a black-red granular precipitate on the preparation occur if the diluted staining fluid is allowed to act longer than a certain time.

2. Dehydrate in pure acetone. On account of the great volatility of acetone some care is necessary to prevent the drying of the preparation.

3. Clear in xylol or oil of turpentine.

4. Mount in a thick solution of colophony in xylol or oil of turpentine.

Before mounting the preparation the superfluous turpentine should be removed, because this reagent rapidly takes up water

from the air and thus may cause clouding of the preparation or fading of the stain.

The solution of colophony is made by saturating a quantity of turpentine with powdered colophony and keeping the filtered solution in the paraffin oven until it has evaporated to the required consistence.

The use of acetone instead of alcohol for dehydrating is an important feature of the method, for the latter spoils the characteristic staining of the granules in the giant cells and platelets.

Results.—The blood platelets typically appear as rounded bodies, more or less jagged in outline and composed of a hyaline, blue-staining substance in which are embedded, chiefly in the central portions, fine red to purplish granules. The cytoplasm of the giant cells shows the same structure and staining peculiarities. The sections should be examined by electric light in order to bring out the colors to the best advantage. By this method all grades of transition can be shown between pseudopod-like processes of the giant cells or detached masses of giant cell cytoplasm and blood platelets.

The Winkler-Schultze Oxidase Reaction.—*Fixation.*—Fix in 10 per cent formalin or in Orth's fluid.

Method of Staining.—1. Cut frozen sections and stain in a mixture of alpha-naphthol and dimethyl-p-phenylenediamine base (as given for blood smears) for 2 to 5 minutes.

2. Wash quickly in distilled water and mount in glycerin, or

3. Wash for a short time in distilled water and place the sections for 2 to 3 minutes in dilute iodine solution (1 part Gram's iodine solution [p. 82] to 2 parts distilled water).

4. Transfer to distilled water to every 10 cc. of which 1 to 2 drops of a 0.5 per cent aqueous solution of lithium carbonate have been added. Leave for 10 minutes to 24 hours, until the granules become blue.

5. Counterstain with alum carmine (p. 78) or alum hematoxylin (p. 70).

6. Wash in water.

7. Embed in glycerin or glycerin jelly.

Results.—Oxidase granules dark blue.

Graham's Alpha-Naphthol-Pyronine Method.—*Fixation.*—Fix in 10 per cent formalin and use freshly cut frozen sections,

since after standing 24 to 28 hours in water the granules may fail to react.

Method of Staining.—1. Stain rather lightly in alum hematoxylin (p. 70). The solution must not be too acid.

2. Wash in water. Place in a saturated aqueous solution of lithium carbonate (about 1.33 per cent) for about 5 minutes, and then return sections to water for a few minutes.

3. Stain 10 minutes in a mixture made by adding a 2 per cent aqueous solution of pyronine to the alcoholic alpha-naphthol solution (prepared as described in the section on blood smears) in the proportion of 1 drop of pyronine to 2 cc. of alpha-naphthol solution. The mixture must be prepared immediately before use. Evaporation may be prevented by staining in a closed container such as a covered Stender dish. Shake gently from time to time to ensure even exposure.

4. Wash in water, then place for 15 to 20 minutes in a saturated aqueous solution of lithium carbonate.

5. Wash thoroughly in several changes of water.

6. Differentiate and dehydrate in 80 per cent followed by 95 per cent alcohol.

7. Transfer section to a slide, clear with xylol by the blotting method, and mount in balsam.

Results.—Granules intense red; nuclei greenish blue to blue.

Graham's Benzidine Stain for Oxidase Granules.—*Fixation.*—Fix in 10 per cent formalin.

Staining Solution.—Use the same benzidine solution that is used for blood smears.

Method of Staining.—1. Cut frozen sections and stain in the benzidine solution for 5 minutes.

2. Wash in water.

3. Counterstain rather deeply in alum hematoxylin (p. 70).

4. Wash in several changes of water.

5. Dehydrate in 80 per cent followed by 95 per cent alcohol.

6. Clear on the slide by the blotting paper xylol method and mount in balsam.

Results.—Essentially the same as in blood smears.

Sato's Method for Peroxidase Granules.—*Fixation.*—Fix in 10 per cent formalin or in Orth's fluid.

Method of Staining.—1. Cut frozen sections and stain them in

the same way as blood smears, but after the safranine stain wash in water, dehydrate in 95 per cent alcohol, clear in oil of origanum and mount in balsam.

Results.—Results are the same as in blood smears.

McJunkin's Benzidine Stain for Paraffin Sections (1922–23).—*Fixation.*—Fix in 10 per cent formalin.

Embedding.—1. Pieces of formalin-fixed tissue 1 mm. thick are placed in 70 per cent acetone for 1 hour.

2. Dehydrate in acetone for 30 minutes.

3. Clear in benzol for 20 minutes.

4. Embed in paraffin oven for 20 minutes.

Staining Solution.—

Benzidine Solution

Alcohol, 80 per cent methyl	25	cc.
Benzidine	0.1	gm.
Hydrogen peroxide	2	drops

Method of Staining.—Sections 3.5 to 5 μ in thickness, cut in the usual way, are attached to slides with Mayer's albumin-glycerin fixative and allowed to dry overnight at room temperature.

1. Remove paraffin with benzol (20 seconds) and acetone (10 seconds).

2. Wash in water for a few seconds. Remove the excess water and apply the benzidine solution diluted with 1 to 2 parts of distilled water for 5 minutes. Two parts give less intense staining.

3. Wash in water for 5 minutes.

4. Stain with alum hematoxylin (p. 72) for 2 minutes.

5. Wash in water 1 minute.

6. Stain with 0.1 per cent aqueous solution of eosin for 20 seconds.

7. Dehydrate with 95 per cent alcohol for 30 seconds and absolute alcohol for 5 seconds.

8. Clear in xylol and mount in balsam.

Results.—The reacting cytoplasm, which is arranged in the form of more or less definite granules, is colored yellow to brown. The initial color formed is blue, but this soon changes to brown.

Note: We have found that when tissues have been fixed for a considerable period of time in 10 per cent formalin, and as a result the peroxidase reaction has become weak or negative, this reaction

can be revived by treating the frozen sections for a few minutes with a 2.5 per cent aqueous solution of potassium bichromate. The sections are then washed in water and the staining is carried out as usual.

4. Lymph Nodes

Routine fixatives and stains are practically all that is necessary for the study of the lymph nodes. Mallory's aniline blue and the reticulum stains, as well as phosphotungstic acid hematoxylin, are valuable for bringing out sharply the finer collagen fibrils and their networks, the so-called reticulum. The various bacterial stains are used for infectious lesions.

For hematological studies sections of lymph nodes may be stained by Giemsa's method. In cases of leukemia the oxidase reaction may be of importance.

5. Spleen

Owing to the presence usually of a large amount of blood in this organ, sections of the spleen must be cut very thin, preferably not over 2 to 3 mm. thick, because the blood prevents the deep penetration of the fixing fluid. If the spleen is very soft it is sometimes advisable to cut the sections parallel with the surface so as to include the capsule, which tends to hold the tissue together.

Zenker's fluid is an excellent fixative for routine stains, but formalin is preferable for certain purposes, such as the amyloid reaction, for the preservation of fat, and for oxidase reactions in cases of leukemia, myelopoiesis, and so on.

Smears made of the splenic pulp suspended in serum may be stained supravitally (p. 189) or with the stains given for the blood (p. 180).

6. Respiratory System

In the preservation of the lungs it is important to save portions that have not been squeezed, so that the relations of the exudations will not be changed or the alveoli compressed. Thin slices are usually preferable to cubical pieces, and should be cut with a very sharp knife in order not to compress the tissue. They should be dropped immediately into the fixing fluid before the contents of the bronchi and small cavities have had time to run out. An emphysematous lung is so delicate that it is usually better to inject a

whole lung through the bronchi with the fixing fluid or to snip out small pieces with scissors. Some of the sections of the lungs should be taken through the base so as to include the larger bronchi.

The most common and important lesions of the lungs are of an infectious nature and include lobar and bronchopneumonia and abscesses. They are caused by a variety of organisms, of which the most common are the pneumococcus, the streptococcus, *Staphylococcus aureus* and the tubercle bacillus. The various bacterial stains are used for their demonstration.

Much fibrin is usually present in the lesions and is well brought out by routine stains, especially by phosphotungstic acid hematoxylin. Weigert's fibrin stain is sometimes useful. Organization of fibrin is especially well demonstrated by Mallory's aniline blue stain and is often found after pneumonia and in tuberculous lesions.

7. Digestive System

(1) TEETH

The enamel is produced by the ameloblasts, epithelial cells of ectodermal origin. These cells, of cylindrical shape, are characterized by having their nuclei situated at the ends away from the stroma and they produce enamel prisms which fuse into a homogeneous substance and become calcified to form the enamel covering the exposed end of the tooth. The mesenchymal cells adjacent to the layer of ameloblasts become stretched in a radial direction and are transformed into odontoblasts. These are important for the formation of dentine, another homogeneous substance, which also becomes calcified and which, like enamel, contains no cells. Later the odontoblasts form a layer of true bone, outside of the dentine, covering the root of the tooth, the so-called cementum. Epithelial fibrils penetrate delicate canals in the enamel and fibroglia fibrils in like manner penetrate the dentine.

Fixation.—The usual fixatives are satisfactory. Fetal tissue of various ages furnishes the best material for the study of the development and structure of the teeth. Extracted teeth are excellent for the study of degenerative and infectious processes. Necessarily decalcification must be thorough and must follow fixation. It can be hastened by first sawing the fixed tooth into very thin slices.

Staining.—Phosphotungstic acid hematoxylin after Zenker fixation is useful in bringing out clearly the epithelial and fibroglia

fibrils and showing their penetration into the enamel and dentine. It stains enamel blue-black and the dentine brownish. The routine stains give a good idea of the pathological changes present. For study of the infectious lesions, the phloxine-methylene blue, and the various bacterial stains should be used.

(2) SALIVARY GLANDS

The salivary glands are composed of two types of glands, the mucous and the serous, which may be grouped separately or in various combinations. At the base of some of the glands basket cells with epithelial fibrils occur. The epithelial cells may contain eosinophilic and zymogen granules, also mucigen droplets, and extrude serous and mucous secretions.

Fixation.—Zenker's fluid, 10 per cent formalin and alcohol suffice for most purposes.

Staining.—The routine stains show most of the structures present. Phosphotungstic acid hematoxylin stains the fibrils of the basket cells sharply after fixation in Zenker's fluid. Mucus can be demonstrated by Mayer's mucicarmine stain after fixation in alcohol.

(3) GASTRO-INTESTINAL TRACT

Portions of the stomach and intestine should be fixed as soon after death as possible for satisfactory study because the gastro-intestinal tract undergoes postmortem changes so rapidly. It has been suggested, in appropriate cases where an autopsy is allowable, to inject the stomach with the desired fixing solution by means of a rubber tube as soon after death as feasible. Under no circumstances should the mucous surface of the stomach or intestine be washed with water. Use either physiological salt solution or the fixing fluid. It is important to keep the tissue flat while hardening. This can usually be done by placing it, with the peritoneal surface down, on thick filter paper, to which it readily adheres. Sometimes it is necessary to pin the specimens down at the edges on flat pieces of cork and then invert them in the fixative. Do not let the surface dry before the specimen is placed in the fixing solution.

Fixation.—Zenker's fluid can be highly recommended but formalin and alcohol are sometimes useful. As a fixative for the various granules in the epithelial cells Maximow recommends Heidenhain's corrosive sublimate-osmic acid mixture (equal parts of a

1 per cent aqueous solution of osmic acid, and a saturated solution of corrosive sublimate [about 6.9 per cent]) followed by thorough washing in tap water.

Staining.—Four types of epithelial cells occur in the stomach: (1) Chief or zymogenic cells, containing zymogen granules (which probably give rise to the pepsin of the gastric juice) which are difficult to fix and stain; and prozymogen granules, which stain with basic dyes, especially iron hematoxylin and toluidine blue. (2) Parietal cells (which probably produce the hydrochloric acid of the gastric juice) which stain with acid dyes such as phloxine, eosin and Congo red. They also contain a basket-like network in their cytoplasm which is best brought out by the methods for the Golgi apparatus. (3) Mucous cells which contain granules of mucigen or of mucus of a peculiar kind, not precipitated by acetic acid. These granules can be stained by mucicarmine and by Mallory's aniline blue stain. (4) Argentaffin cells, which occur rarely in the stomach (see intestine).

Four types of epithelial cells also occur in the intestine: (1) epithelial cells, which are stained well by phloxine-methylene blue; (2) goblet cells, the mucus of which is shown up well by Mallory's aniline blue stain; (3) the cells of Paneth, which stain well with iron hematoxylin; and (4) argentaffin cells, or cells of Kultschitzsky, which are best stained according to the directions given by Masson.

Masson's Silver Method for Argentaffin Cells in Blocks (1928).—*Fixation.*—Fix blocks of tissue in Bouin's solution for 3 days. Cut slices 2 to 3 mm. thick and wash them in running water for 24 hours.

Staining Solution.—To 100 cc. of a 20 per cent aqueous solution of silver nitrate add ammonia water drop by drop, shaking well until the precipitate of silver oxide is just dissolved. Add a few drops of the 20 per cent silver nitrate solution until there is a persistent opalescence. The fluid should have no odor of ammonia. Add distilled water to 200 cc., the solution now containing 10 per cent silver nitrate. Keep in a clean glass bottle and filter just before use.

Method of Staining.—1. Place blocks for 24 hours in a solution of 2 drops of ammonia water to 100 cc. of distilled water.

2. Impregnate for 24 hours in the ammoniacal silver nitrate solution diluted with 3 volumes of distilled water.

3. Rinse in distilled water.
4. Tone in Cajal's mixture for 24 hours.

Ammonium sulfocyanide	3 gm.
Sodium thiosulfate	3 gm.
Water, distilled	100 cc.
Gold chloride, 1 per cent aqueous solution	1 cc.

5. Wash in water for several hours.
6. Embed in paraffin or celloidin.
7. Cut sections and mount without counterstaining.

Results.—All argentaffin granules opaque black; nuclei brownish.

The phloxine-methylene blue stain after Zenker's fixation gives the best general picture of the normal histology of the gastro-intestinal tract. The nuclei and the eosinophilic granules stain brilliantly. Mitochondrial stains are sometimes advisable.

In the study of the pathological lesions of the stomach and intestine the phloxine-methylene blue method holds the first place. It stains distinctly the nuclei, bacteria, and acidophilic granules and cytoplasm. For this reason it is particularly valuable in the study of the changes occurring in the lesions of typhoid fever.

In the lesions caused by *Endamoeba histolytica* and *Balantidium coli* the phosphotungstic acid hematoxylin stain is especially useful. It colors intensely the ectosarc of the *Endamoeba* and the cilia of the *Balantidium*. Another useful stain for the *Endamoebae* is Mallory's thionin oxalic acid method (p. 297) after alcohol or formalin fixation. It stains the nuclei of the *Endamoebae* differentially, red instead of blue.

Tuberculous lesions necessarily call for stains for the tubercle bacillus; other infectious lesions for the various bacterial stains.

(4) LIVER

Lesions of the liver are common, of considerably variety and of much importance. Some are uniformly distributed, others occur focally. Slices of tissue for fixation should be cut accordingly and should not be limited exclusively to the subcapsular regions. The routine stains advised will demonstrate well most of the lesions, but certain points require special mention.

The phloxine-methylene blue method reveals necrotic cells dis-

tinctly, staining them a deep reddish color. Nuclei and organisms, if present, are in sharp contrast colored blue. Organisms are best brought out by the bacterial stains.

Alcohol fixation and special staining methods are required for glycogen, amyloid and hemosiderin. The same fixation is needed in order to demonstrate copper in cases of hemochromatosis (p. 139).

In one type of cirrhosis, usually associated with the drinking of alcoholic beverages, but occurring occasionally in young children and therefore not due to alcohol *per se*, there is a peculiar type of lesion occurring in the liver cells and often spoken of as "alcoholic hyalin." It is diagnostic of that type of cirrhosis. This hyalin gives two special reactions. It stains by the methods devised for phosphates and it also gives the hematoxylin test for lead (p. 143). After Zenker fixation it is stained intensely red by the phloxine-methylene blue method and deep blue by phosphotungstic acid hematoxylin. It is also colored deep blue by Mallory's iron chloride hematoxylin stain after fixation in alcohol and in formalin. This intense staining by all three methods applies only to fresh, recently formed hyalin. Old hyalin stains poorly or not at all.

Mallory's Phloxine Stain for Alcoholic Hyalin.—This is a useful and simple stain.

Fixation.—Alcohol or 10 per cent formalin. Embed in celloidin or paraffin.

Method of Staining.—1. Stain nuclei with alum hematoxylin (p. 70).

2. Wash in tap water.

3. Stain deeply with a 0.5 per cent solution of phloxine in 20 per cent alcohol for 10 to 30 minutes or longer.

4. Wash in tap water.

5. Immerse in a 0.1 per cent aqueous solution of lithium carbonate for 30 to 60 seconds.

6. Wash in tap water.

7. Dehydrate in 95 per cent followed by absolute alcohol.

8. Clear in xylol and mount in balsam.

If celloidin sections are used clear in oil of origanum or terpineol after 95 per cent alcohol and mount in balsam.

Results.—Fresh hyalin is stained intensely red; older hyalin pink to colorless; nuclei blue.

Mallory's Thionin Stain for Alcoholic Hyalin.—The method

is simple and gives a very sharp stain of the early stages of hyalin formation.

Fixation.—Fix in 95 per cent alcohol or 10 per cent formalin. Embed in celloidin or paraffin.

Method of Staining.—1. Stain sections for 5 to 10 minutes in a 0.5 per cent solution of thionin in 20 per cent alcohol.

2. Differentiate and dehydrate for several minutes in 80 per cent followed by 95 per cent alcohol.

3. Clear in terpineol.

4. Mount in terpineol balsam.

Results.—Nuclei blue; fine and coarse granules and networks formed from them red to purple; old hyalin blue.

In addition to the aniline blue stain for collagen and reticulum, which is particularly useful for the study of chronic passive congestion, hemorrhagic central necrosis and cirrhosis, a silver stain for reticulum is sometimes advisable.

The bile capillaries may be demonstrated by the same method that is used for neuroglia fibrils, namely, fixation in Zenker's fluid, followed by staining in phosphotungstic acid hematoxylin. The treatment with potassium permanganate and oxalic acid must be more prolonged than usual, however, otherwise the albuminous granules in the cytoplasm will stain too deeply and obscure the capillaries.

(5) PANCREAS

Interesting and valuable work has been done on the normal histology of the pancreas, especially with reference to the cytoplasmic granules in the different kinds of cells of the ducts, glands and islets. Some attempt has been made to apply the methods to the lesions of the pancreas, more particularly to those associated with the syndrome known as diabetes mellitus. The results so far obtained are promising and encourage further study along the same lines. One point to be borne in mind is that postmortem changes take place very quickly in the pancreas.

For routine microscopic study of the pancreas, fixation in Zenker's fluid and staining by the phloxine-methylene blue method are recommended. The zymogen granules do not stain intensely after this procedure, as in the glands of the stomach and intestine, but require special methods for their demonstration. Staining with phosphotungstic acid hematoxylin after fixation in formalin is some-

times useful, especially for zymogen granules. Fixation in formalin is also advisable for certain other purposes, such as examination for fat, while alcohol is the best fixative for hemosiderin and amyloid.

For the specific granules of the alpha and beta cells in the islets of the pancreas, the best technique, in Bensley's opinion, is the neutral gentian (crystal violet) stain after fixation in Zenker's fluid minus acetic acid, as the latter dissolves both the mitochondria and the characteristic granules of the islet cells.

Bensley's Neutral Crystal Violet Stain for the Pancreas (1912).—*Fixation.*—Zenker's fluid without acetic acid for 24 hours. Embed in paraffin.

Staining Solution.—The stain used by Bensley is the neutral dye obtained when a solution of crystal violet is precipitated by its equivalent of a solution of orange G. The dye is prepared in the following way: 1 gm. of crystal violet is dissolved in 25 cc. of distilled water, and 1 gm. of orange G is dissolved in 25 cc. of distilled water. Add these two solutions together, shaking gently until practically complete precipitation has taken place. Filter and wash the precipitate with water at once. Drain and dry. Dissolve the residue in 25 cc. of absolute alcohol.

For staining, add the stock solution of this neutral compound to 20 per cent alcohol until a solution having the color of a good hemalum solution is obtained. Allow this solution to stand 24 hours to permit the excess of dye to separate out.

Method of Staining.—1. Stain sections in the neutral crystal violet solution for 24 hours.

2. Blot slide between several layers of filter paper.

3. Dehydrate in acetone.

4. Place sections in toluol or xylol.

5. Differentiate in a mixture of absolute alcohol 1 part and oil of cloves 3 parts.

6. Rinse with toluol or xylol and mount in balsam.

Results.—The alpha cells in the islands of Langerhans contain orange-red granules; the beta cells have violet granules; zymogen granules of the exocrine cells are violet; and the background is brown.

Goodpasture's Eosin-Methylene Blue Stain for the Pancreas (1917).—Goodpasture has found eosin and his acid poly-

chrome methylene blue solution a very useful method for staining differentially the zymogen and the alpha and beta granules after fixation in Helly's or in Orth's fluid made up with neutral formalin.

Fixation.—1. Fix thin pieces of fresh pancreas for 24 hours in:

Formalin, neutral	10 cc.
Zenker's fluid without acetic acid	90 cc.
or in:	
Formalin, neutral	10 cc.
Potassium bichromate, 2.5 per cent aqueous solution	90 cc.

2. Wash in running water 24 hours. Embed in paraffin.

Method of Staining.—1. After removing the paraffin in the usual way, place sections in potassium permanganate, 1 per cent aqueous solution, for 1 minute.

2. Transfer to oxalic acid, 5 per cent aqueous solution, for 1 minute.

3. Wash thoroughly in water.

4. Stain in an aqueous solution containing 1 per cent eosin and 1 per cent potassium bichromate for 1 to 5 minutes.

5. Rinse quickly in water.

6. Stain in acid polychrome methylene blue (p. 85) 1 to 5 minutes.

7. Rinse quickly in water.

8. Differentiate rapidly in 95 per cent alcohol and dehydrate in absolute alcohol.

9. Clear in xylol and mount in balsam.

Results.—In properly stained sections zymogen granules stain deep purple; cytoplasm light blue; nuclei light purple; alpha granules brick red, and beta granules dark blue.

Bensley's Modification of Mallory's Aniline Blue Stain for the Pancreas (Warren, 1930).—*Fixation.*—Fix in Helly's fluid for 4 to 24 hours. Fresh fixation, within 40 minutes postmortem, is necessary for satisfactory results.

Staining Solution.—

Aniline blue soluble in water	0.5 gm.
Orange G	2 gm.
Water, distilled	100 cc.

Any trace of ammonia in the distilled water interferes with the stain. By slight increase in the amount of orange G it is possible to stain the zymogen granules orange.

Method of Staining.—1. Stain in Altmann's aniline acid fuchsin stain (p. 92) for 10 minutes.

2. Wash rapidly in distilled water.

3. Place in a 1 per cent aqueous solution of phosphomolybdic acid for 10 minutes.

4. Stain in the aniline blue-orange G solution for 1 hour or less.

5. Differentiate in 95 per cent alcohol until color no longer comes out in clouds and dehydrate in absolute alcohol.

6. Clear in xylol and mount in balsam.

Results.—Alpha cells show rather scanty orange-red granules; beta cells show bluish granules; duct epithelium is a very light blue; acinar cells are bluish purple, occasionally with orange zymogen granules; red blood cells are red.

Bensley's Acid Fuchsin-Methyl Green Stain for the Pancreas (1912).—*Fixation.*—Fix for 24 hours in acetic-osmic-bichromate made up as follows:

Osmic acid, 4 per cent aqueous solution	2 cc.
Potassium bichromate, 2.5 per cent aqueous solution	8 cc.
Acetic acid, glacial	1 drop

Embed in paraffin and cut sections 4 μ thick. Tissues fixed in Zenker's fluid can also be stained by this method.

Method of Staining.—1. Attach sections to slides by the water method, free them from paraffin by toluol, pass them through absolute alcohol to water.

2. Treat with 1 per cent aqueous potassium permanganate for 1 minute.

3. Place in 5 per cent oxalic acid for 1 minute.

4. Wash thoroughly in water.

5. Stain for 5 minutes in Altmann's aniline acid fuchsin solution (p. 92) which has been previously warmed to 60° C.

6. Wash thoroughly in distilled water.

7. Dip for an instant into a 1 per cent aqueous solution of methyl green.

8. Wash in water.

9. Dehydrate rapidly in absolute alcohol (avoid dilute alcohols).

10. Clear in toluol and mount in balsam.

Results.—This stain after the acetic-osmic-bichromate fixation has the advantage that it differentiates all epithelial elements of the pancreas including granular contents and mitochondria in a single preparation. Acinus cells green with green nuclei; zymogen granules red; basal filaments and mitochondria red; granules of A cells deep red; granules of B cells green.

For demonstration of zymogen granules fixation in 10 per cent neutral formalin, followed by phosphotungstic acid hematoxylin, gives excellent results. Organisms in infectious lesions may be brought out by the various bacterial stains.

8. Excretory System

Sections of the kidney for microscopic examination should run vertically through both cortex and medulla. The latter is especially important in studying the deposit of glycogen. In cases of chronic nephritis the capsule should not be peeled from the parts of the kidney to be fixed. It is important that tissue be fresh, owing to the rapid postmortem changes that take place in this organ.

Fixation.—Zenker's fluid and formalin as fixatives answer most purposes, but alcohol (95 per cent or absolute) is required to preserve glycogen, hemosiderin and certain crystalline deposits. Formalin has to a large extent replaced Flemming's solution as a fixative for fat to be stained by the scarlet red method. Fixation by boiling is still used to demonstrate any albuminous exudate in the capsular space. Paraffin embedding is generally to be preferred, particularly when lesions of the glomeruli are present.

Staining.—The general staining methods already recommended will be found the most satisfactory, especially the phloxine-methylene blue stain for general purposes and particularly for infectious lesions, which are usually due to the Gram-negative colon bacillus. Mitochondrial stains are sometimes valuable.

Much of the intensive work on the kidney has been done with the aniline blue stain because it brings out sharply the collagenous walls of the capillaries in the glomerular tufts. It also colors the hyaline droplets often present in renal cells, staining them light

to deep blue. The procedure recommended is Lee-Brown's modification of Mallory's aniline blue stain for use on kidney tissue (p. 155)

Phosphotungstic acid hematoxylin stains the granules in the cytoplasm of the renal cells fairly deeply but otherwise is not particularly useful. Special methods needed on occasions are the various bacterial stains, Best's carmine stain for glycogen, scarlet red for fat, crystal violet for amyloid, and Kóssa's stain for calcium. Uric acid and sodium biurate crystals require a high content of alcohol in the staining and washing fluids and a minimum exposure to each. The alum hematoxylin stain for sodium urate (p. 145) under these conditions gives excellent results. For the demonstration of uric acid and urates in sections Schultz and Schmidt devised a satisfactory method (p. 146).

9. Reproductive System

(1) Male Genital System

Most of the histological work on the genital organs is done with the routine fixatives and stains. Few special methods are called for. Bacterial stains are of importance in infectious diseases.

Testes.—The cells peculiar to these organs are the seminiferous epithelial cells which give rise to the spermatozoa. The numerous mitotic figures in them and the heads of the spermatozoa are stained intensely by phosphotungstic acid hematoxylin. In the stroma are found certain interstitial cells of mesenchymal origin which often contain varying amounts of lipoid, pigment and crystalloid structures.

Seminal Vesicles.—The epithelium lining the lumen of the seminal vesicles contains numerous granules of a yellow pigment, which is fatty in nature and makes its first appearance at the time of puberty.

Prostate.—The smooth muscle cells present in great numbers are best brought out by phosphotungstic acid hematoxylin and by the aniline blue stains.

(2) Female Genital System

Interest for the pathologist centers chiefly in the lesions of the uterus, tubes and ovaries.

Fixation.—Zenker's fluid gives the best results but formalin and alcohol are sometimes necessary.

Staining.—The phloxine-methylene blue stain gives the best general view of any pathological changes that have taken place. The aniline blue stain and the bacterial stains are often useful.

Ovaries.—Two structures are peculiar to these organs and of much interest. The first are the ova which are well shown by the routine stains, and the second are the corpora haemorrhagica and lutea. The luteal cells are enlarged fibroblasts with numerous, somewhat coarse fibroglia fibrils which are rendered very prominent by phosphotungstic acid hematoxylin after Zenker fixation. The changes in the blood pigment from hemoglobin to hemofuscin and then to hemosiderin are best shown after alcohol fixation by the regular stain for iron followed by basic fuchsin.

Fallopian Tubes.—The lesions are chiefly of an inflammatory nature (gonorrheal infection) and are well shown by the routine stains. Tubal pregnancy sometimes occurs and the cell changes are similar to those in the uterus under the same conditions.

Uterus.—The commonest change is pregnancy and the formation of decidual cells in response to the stimulus furnished by the implanted ovum. The decidual cells are derived from fibroblasts, but unlike the luteal cells, although they enlarge characteristically, they produce no fibroglia fibrils. The fertilized ovum develops and produces the chorionic villi of the placenta. Trophoblasts derived from the epithelium covering the villi invade the uterine mucosa, pass between the decidual and smooth muscle cells and penetrate into the uterine blood vessels. Their cytoplasm becomes basophilic and develops numerous epithelial fibrils which sometimes have a corkscrew appearance.

10. Nervous System*

(1) The Central Nervous System

Fixation.—The choice of a method for the preservation of the central nervous system is governed by the kind of investigation to be attempted. For gross anatomical studies and the coarser microscopic work, *i. e.*, tract degeneration, fixation of the whole brain without sectioning is recommended. For this purpose 10 per cent formalin has almost entirely superseded the older method of fixation in chrome salt solutions. The brain has a slightly greater specific gravity than the formalin solution and hence will sink in

* This section is based in part on a former revision by Dr. Samuel T. Orton.

the fixing jar and suffer distortion by flattening. This difference in specific gravity is so slight, however, that a thread slipped under the basal vessels will suspend the brain without undue strain on the vessels and consequently with very little distortion, except in the extremely soft brains of very young children and of individuals dying from acute infections. The formalin should be changed after 3 to 4 days and again after 2 to 3 weeks. It requires 2 to 3 weeks of fixation to harden a brain so that gross slices can be cut of any desired thickness, preferably about 1 cm., for orientation of deep gross lesions, and these may then be cut for finer histological study. Material so fixed, however, is not suitable for some of the best methods for demonstrating special structures. Some of these methods will not give the best results after even a short fixation period in formalin, and there are several that are not satisfactory after the 2 to 3 weeks' fixation required for whole brains. When, therefore, the finer histological changes are to be studied, immediate sectioning of the fresh brain and direct fixation in several different fixatives are recommended.

Formalin.—In addition to its use as a preservative for whole brains, formalin is widely used for the fixation of small pieces. In some procedures the period of fixation is not important but in others the formalin serves only as a preliminary fixative and the material must be carried on into mordanting solutions within 24 to 48 hours after fixation starts. If only one fixative is to be employed formalin is unquestionably the best because of its wide range of usefulness. It is probably, however, the most variable in its results of any of the standard fixatives. During the early stages of fixation the nervous tissues swell considerably in formalin and then gradually undergo a shrinkage, leading often to a marked distortion of the cells and other delicate structures. The fixation produced by formalin is apparently not firm enough to withstand later dehydration with alcohol without marked distortion by shrinkage, so that material so fixed does not give good paraffin sections. Further mordanting, for example in preparing formalin material for the Weigert medullary sheath stain, will often overcome some of this difficulty. Certain methods, such as those of Bielschowsky and Weigert, can be successfully applied after several years' preservation in formalin if care is taken to wash the tissue thoroughly. Even with short fixation it is advisable to wash overnight in running tap water be-

fore further mordanting or embedding, and with very old tissues washing for 48 to 72 hours is recommended.

Alcohol.—For the study of the extranuclear chromophile particles of the nerve cells, the Nissl or tigroid bodies, the best fixative is 95 per cent alcohol. Even when relatively small pieces are preserved the alcohol should be in the proportion of about 10 to 20 times the volume of the tissue and should be changed daily for at least a week. If larger pieces are used the changes should be continued on alternate days through the second week and for 2 or 3 times during the third week. After such hardening blocks may be kept indefinitely in alcohol and embedded when wanted. Blocks so fixed give excellent pictures even after 8 to 10 years' preservation.

Zenker's Fluid.—Zenker's fluid is one of the most valuable fixatives for brain and cord material. Fix as directed on page 43, except that better results will be obtained with brain tissue by dehydrating in graded alcohols, instead of placing directly in 80 per cent alcohol after washing in water, as there advised. Material so fixed may be preserved for years and may be used for the study of neuroglia fibrils with the phosphotungstic acid hematoxylin stain, and for the study of blood vessels, meninges, inflammatory exudates and many tumors after the phloxine-methylene blue stain and Verhoeff's elastic tissue stain.

Weigert's Neuroglia Mordant (1896).—This mixture, used after preliminary fixation for 24 hours in formalin, or better used with formalin added during the first 24 hours, is valuable in conjunction with Alzheimer's modification of the Mann eosin-methyl blue stain for demonstrating neuroglia cells, especially the so-called "ameboid" forms of astrocytes. This material can also be used for Weigert's stain for neuroglia fibrils. The fixing fluid is the fluorchrome copper acetate and acetic acid mixture given as the secondary mordant under the Weigert method for medullary sheaths, and the formula and method of preparation are given there. For fixation of fresh tissues 1 part of formalin is added to 8 parts of neuroglia mordant and tissues are placed in this for 24 hours and then transferred to a fresh solution to which no formalin has been added. Daily changes of the mordant for the first week are recommended. The best results are obtained with tissues sectioned shortly after this mordanting period, but excellent stains can usually be obtained after several months to a year in the mordant.

Müller's Fluid.—Müller's fluid as a primary fixative is no longer used so freely as formerly. It is valuable, however, in the fixation of tissues for the Marchi method. This method is frequently employed after formalin fixation but the results are by no means so satisfactory. Müller's fluid hardens slowly and frequent renewal during the fixation period is necessary. Fresh changes daily during the first week, every other day during the second week, and twice during the third week are recommended. Material so fixed is excellent for Weigert's medullary sheath preparations, as well as for the Marchi method.

Formalin-Ammonium Bromide.—This fixing solution was first recommended by Cajal (1923) for the fixation of nervous tissue when neuroglia cells were to be demonstrated by the metallic impregnation methods. The duration of the fixation should be from 2 to 25 days at room temperature, protoplasmic astrocytes requiring a shorter, fibrous astrocytes a longer fixation time. The addition of ammonium bromide to formalin improves the vigor of the impregnation of the neuroglia cells and lessens the tendency to granular staining. Bromuration appears to mordant the neuroglia cells for silver salts; it improves the staining of the astrocytes but is not indispensable for them, whereas it seems to be necessary for impregnating oligodendroglia and microglia. At the same time the presence of ammonium bromide suppresses the impregnation of neurofibrils and affords a clearer picture of the neuroglia tissue. Formalin-ammonium bromide has the advantage of combining the processes of fixation and bromuration. Bromuration after simple formalin fixation can be carried out by Globus' method (1927), providing the tissue has not been in formalin too long. According to Globus' method frozen sections of formalin-fixed tissue are washed rapidly in several changes of distilled water, then placed in a 10 per cent solution of ammonia water for 24 hours at room temperature or for a shorter time in the incubator. Wash rapidly in 2 changes of distilled water and place in a 10 per cent solution of hydrobromic acid (pure 41 per cent) for 2 to 4 hours. Again wash in 2 changes of distilled water to which a few drops of ammonia water have been added and then impregnate with the gold or silver solutions as usual.

Staining.—The staining of the various histological elements of

the nervous system and the fixing reagents best suited for each of them will be considered under the following headings:

a. General stains.
b. Nerve cell stains.
  (*a*) Stains for special parts of the cell body.
    (1) Nissl substance.
    (2) Mitochondria.
    (3) Golgi apparatus.
    (4) Pigment.
    (5) Neurofibrils.
  (*b*) Stains for axis cylinders.
  (*c*) Stains for myelin sheaths.
    (1) Normal myelin.
    (2) Degenerating myelin.
c. Neuroglia stains.
  (*a*) Stains for astrocytes.
  (*b*) Stains for oligodendroglia.
d. Microglia stains.
e. Stains for connective tissue and blood vessels of the nervous system.

a. General Stains

General stains include the ordinary nuclear stains with or without a counterstain, and certain diffuse stains which color practically all elements of the nerve tissue, but with slight variations of intensity or shade so that individual structures can be identified. The best fixation for general stains is Zenker's fluid followed by the phloxine-methylene blue stain. After other fixatives, especially after fixation for a long time in formalin, alum hematoxylin or Weigert's iron hematoxylin as a nuclear stain, followed by either phloxine or preferably van Gieson's picro-acid fuchsin for the nervous system (p. 93) as a counterstain, give fair results. Mallory's aniline blue collagen stain can also be recommended. The phosphotungstic acid hematoxylin method is valuable as a general stain and also for rendering prominent the neuroglia fibrils. For various infections of the central nervous system and the meninges appropriate bacterial methods should be employed.

For the demonstration of the shape and relationship of neurons the Golgi silver methods are of importance. By these methods a black deposit of a reduced silver salt brings out a certain number

of neurons often in their entirety, leaving many neurons and neuroglia unstained. The picture thus obtained is incomplete but clearer than if all cells were equally impregnated. Irregular precipitates are sometimes formed, which are confusing, and structures other than neurons may be stained. The Golgi method occasionally still in use is the rapid method which is given here.

Golgi's Rapid Method for Impregnating Neurons (1894).—*Fixation.*—Place small pieces of fresh tissue 2 to 3 mm. thick in a mixture of 40 cc. of a 2.5 per cent aqueous potassium bichromate solution and 10 cc. of a 1 per cent aqueous osmic acid solution. Renew the solution if it becomes turbid. Cotton should be placed on the bottom of the container, so that the fixing fluid will have access to the tissue from all sides. The fixation time varies; take out a piece of tissue for impregnation every 12 hours from the second to the seventh day of fixation.

Method of Staining.—1. Dry tissue with filter paper and place in a 0.75 per cent aqueous silver nitrate solution for 24 to 48 hours. If the solution turns yellow renew it.

2. Wash in 40 per cent alcohol renewed several times for 1 to 2 hours.

3. Place in 80 per cent, then 90 per cent alcohol.

4. Embed rapidly in celloidin by dehydrating in absolute alcohol for 12 hours, ether-alcohol for 2 to 4 hours, and 4 per cent celloidin 1 to 2 days. Mount on blocks with thicker celloidin and harden in 80 per cent alcohol. Cut thick sections in 95 per cent alcohol.

5. Wash sections carefully in 80 per cent alcohol to remove excess silver.

6. Dehydrate in absolute alcohol.

7. Clear in creosote, then terpineol.

8. Place sections on a slide, remove the clearing fluid with filter paper carefully and add a drop of thick balsam. Let it dry, heating the balsam to drive off the solvent. A coverslip may be put on just before the balsam cools or the preparation may be kept without a coverslip.

In the original method the sections were placed on a slide after Step 4, the alcohol blotted off, and a mixture of sandarac used for mounting without a coverslip. This mixture consists of 75 gm. of sandarac, 15 gm. of camphor, 30 cc. of turpentine, 22.5 cc. of oil

of lavender, 75 cc. of absolute alcohol, and 5 to 10 drops of castor oil.

Results.—If successful the separate nerve cells and their processes are deep black on a light yellow background.

b. Nerve Cell Stains

(*a*) Stains for Special Parts of the Cell Body

(1) **Nissl or Tigroid Substance.**—The original Nissl method is used very little today because of the difficulty in cutting the unembedded blocks of even thickness and of handling the sections through the staining and mounting processes. It has been largely superseded by methods that permit embedding. The toluidine blue stain can be recommended most highly for dependability, simplicity and permanence.

Toluidine Blue Stain for the Nissl Substance (1894).—*Fixation.*—Fix in 95 per cent alcohol. Renew the alcohol every day. The duration of the fixation varies with the size of the block of tissue; about 5 days will be sufficient. Embed in celloidin.

Or formalin-fixed material may be stained by this method after embedding either in celloidin or in paraffin. Thionin and cresyl violet give slightly better results after formalin fixation than toluidine blue, but neither of them compares favorably with the alcohol-toluidine blue method. Alcohol seems to act as a better coagulant of the tigroid body material than either formalin or Zenker's fluid and the masses appear larger, more sharply defined and in a clearer matrix after such fixation. In formalin material which has been preserved for a long time the whole cell body is frequently intensely stained so that the details of the Nissl bodies cannot be well made out, and often the intercellular background also stains deeply so that in differentiation most of the cell detail is lost before the background clears up.

Staining Solution.—Make up a 1 per cent aqueous solution of toluidine blue. A 1 per cent aqueous solution of cresyl violet or thionin, or Nissl's soapy methylene blue (methylene blue B 3.75 gm., scraped venetian soap 1.75 gm., distilled water 1000 cc., ripened for 3 months) may be substituted for toluidine blue in this method, but they do not give quite so sharp a metachromatic differentiation as toluidine blue which keeps well and improves somewhat with age.

Method of Staining.—1. Transfer celloidin sections, 15 to 20 μ thick, from 80 per cent alcohol to the 1 per cent aqueous toluidine blue stain in a porcelain evaporating dish. Heat to steaming, withdraw the flame and leave for 10 to 15 minutes.

2. Rinse in distilled water, then in 70 per cent alcohol.

3. Decolorize in 95 per cent alcohol. Control decolorization with the microscope. The background should be colorless or very pale blue.

4. Clear in terpineol or oil of origanum, and after blotting rinse with xylol to remove oil and mount in balsam.

Results.—With a ripened solution the Nissl bodies are strikingly stained and show an intense blue or purplish color which is in sharp contrast with the dark blue of the nucleus. This same contrast is evident in plasma cells where the cytoplasm takes the purple color and the nucleus the blue. The apical process and the larger basal processes are faintly stained for a short distance but the axis cylinders, medullary sheaths and neuroglia fibers are unstained, giving a clear background. Neuroglia cell nuclei are stained a deep blue. The cytoplasm of neuroglia cells is pale blue.

(2) **Mitochondria.**—The methods given on pages 110–112 may be used for demonstrating mitochondria in the nervous system.

(3) **Golgi Apparatus.**—The Golgi network in nerve cells is best brought out by the methods given on pages 112–116.

(4) **Pigment.**—Some of the pigments contained in nerve cells are stained by both the scarlet red and the osmic acid methods, but resist solution in the embedding reagents and remain in alcohol-fixed tissues, for example, as pale yellowish globular accumulations in the infranuclear zone of the cytoplasm. Some of these are probably extrinsic pigments derived from foodstuffs and belong to the carotenoids. After fixation in formalin these may be stained more or less selectively either by overstaining in a saturated aqueous solution of Nile blue sulfate and differentiating in weak acetic acid, or by using the dye as a progressive stain, placing the sections in a 1:10,000 aqueous solution for about 30 minutes. Others, particularly the relatively insoluble pigments, are probably intrinsic, for example hemosiderin, which is sometimes present in abundance in the ganglion cells in cases of hemochromatosis or in connection with hemorrhages. The same reactions (pp. 136–140) are employed as with other organs.

(5) **Neurofibrils.**—These include all of the fibrillar mechanism of the cell body and its expansions, *i. e.*, that part of the neuron which is looked on as the transmitting mechanism—the intracellular neurofibrils, the dendrites, the axis cylinder and its arborization, and often its terminal structures. With few exceptions the methods employed for the demonstration of these structures depend on impregnation with a metal salt and later reduction or development within the tissues. All of the impregnation methods are strikingly variable in their results, especially when applied to human material with a varying postmortem period before fixation. In some structures it is also probable that the metallic deposit is in the nature of a surface crustation rather than of incorporation of the metal with the tissue element, and distortions both of size and of relation may occur because of this fact. The classical Golgi methods, while still in use occasionally, have been largely superseded by others. The method in most common use on pathological material is Bielschowsky's. Bielschowsky has published two methods, one for sections and one for blocks, but the many failures of infiltration and the great variability of results in the block method have brought it into more or less disfavor.

Bielschowsky's Method for Neurofibrils in Sections (1908–09).—*Fixation.*—Fix in 10 per cent formalin for at least 14 days. Wash freshly fixed material thoroughly in running water for 2 to 5 hours, old material 24 to 48 hours, then in distilled water 1 to 2 days. Cut frozen sections 5 to 10 μ thick.

Staining Solution.—Make up the ammoniacal silver solution in a glass-stoppered graduate which is kept only for silver solutions. Great care in the use of pure distilled water and pure chemicals and clean glassware is necessary. To 10 cc. of a 10 per cent aqueous silver nitrate solution add 5 drops of a freshly prepared 40 per cent aqueous sodium hydroxide solution. A black-brown precipitate of silver oxide is formed. Add ammonia water drop by drop until the precipitate is almost dissolved, shaking vigorously after the addition of each drop, and leaving a few grains of the precipitate. Add distilled water to make the solution up to 20 cc. The amount of ammonia water added to dissolve the precipitate should never exceed 18 to 20 drops. If more is needed the results are rarely satisfactory. Washing the precipitate 3 times by diluting with distilled water, shaking, letting it settle and then decanting, before dissolving

the precipitate with ammonia water, has been recommended by some authors.

Method of Staining.—1. Rinse sections in 2 or more changes of distilled water for 1 to 2 hours. Handle with glass needles from here on.

2. Transfer to pure pyridine for 24 hours and wash thoroughly again in distilled water. This step is an addition to the earlier formula and may be omitted.

3. Impregnate in a 2 per cent aqueous solution of silver nitrate for 24 hours in the dark.

4. Carry sections through distilled water rapidly.

5. Place in a freshly prepared ammoniacal silver solution for 10 to 20 minutes, or until the sections become a dark seal brown.

6. Wash rapidly in 2 or 3 changes of distilled water.

7. Reduce for 10 minutes in 20 per cent formalin.

8. Wash in tap water 15 minutes.

9. Rinse in distilled water and tone in the following solution for 10 to 20 minutes, or until the color is a reddish violet. Add 3 to 5 drops of a 1 per cent solution of yellow gold chloride to 10 cc. of distilled water and to this add 2 or 3 drops of glacial acetic acid.

10. Rinse in distilled water and fix 1 to 2 minutes in 5 per cent aqueous solution of sodium thiosulfate.

11. Rinse thoroughly in tap water 1 to 2 hours.

12. Dehydrate in 95 per cent and absolute alcohol.

13. Clear in carbol-xylol (1 part carbolic acid to 9 parts of xylol) and mount in balsam.

Results.—This method serves as an excellent one to stain axis cylinders and dendrites, and in well stained thin sections the intracellular neurofibril net will be seen as a delicate meshwork of dark brown or black lines. The axis cylinders and their processes and the muscle plate termini in striated muscle are stained intensely black. The neuroglia fibrils are very faintly stained and can be seen only with difficulty except where there has been marked proliferation, as in the neuroglia scars in tabes or multiple sclerosis. Here they can be seen as fine, usually closely packed wavy lines colored a delicate lavender or purple. Longitudinal sections of the cord in multiple sclerosis stained by this method show to excellent advantage the axis cylinders intact but with their medullary sheaths replaced by neuroglia fibrils.

Ranson's Pyridine-Silver Stain for Non-Myelinated Nerve Fibers (1914).—*Fixation.*—Fix for 48 hours in absolute alcohol to which 1 per cent ammonia water has been added.

Method of Staining.—1. Wash from ½ to 3 minutes, according to the size of the block of tissue, in distilled water.

2. Place in pyridine for 24 hours.

3. Wash in many changes of distilled water for 24 hours.

4. Impregnate for 3 days in the dark in a 2 per cent aqueous solution of silver nitrate at 35° C.

5. Rinse in distilled water.

6. Reduce in a 4 per cent solution of pyrogallic acid in 5 per cent formalin (4 gm. of pyrogallol in 100 cc. of 5 per cent formalin in distilled water) for 24 to 48 hours.

7. Rinse in distilled water, dehydrate, clear and embed in paraffin.

8. Cut sections are ready for examination after clearing in xylol and mounting in balsam.

Results.—This method is said to give excellent results with the peripheral nerves, to be applicable to larger pieces with uniform impregnation, and to be much more reliable than the other silver methods. It stains non-myelinated fibers black, myelinated fibers yellow, thus serving as a differential stain. Huber and Guild applied the method to decalcified material.

(*b*) Stains for Axis Cylinders

Ehrlich's Supravital Methylene Blue Stain for Nerve Fibers (1886).—This method originated with Ehrlich. It is used chiefly in the study of the peripheral nerves, particularly as regards their distribution in tissues and organs, although it is also applicable to the central nervous tissues. Its use has been largely restricted to the study of normal material. Many modifications of the original procedure have been suggested with a view to making the results surer or the specimens more permanent. Tissues can be stained either by injection or by immersion. The methylene blue used should be "rectified methylene blue for vital injections."

Method of Staining.—For injection in the blood or lymph vessels of live or dead animals a dilute solution of methylene blue in physiological salt solution, ¼ to ⅛ per cent, is recommended. A 1 per cent stock solution of methylene blue is made up in a 0.9 per

cent sodium chloride solution and this stock solution is diluted with physiological salt solution to make ½, ¼, ⅛, 1/16 per cent solutions of methylene blue for use. It is probably best to wash out the vessels first with warm (37° C.) physiological salt solution before injecting the filtered, dilute warm methylene blue solution. The injected organs are exposed to the air until a bluish tint is visible. As soon as the greatest intensity of stain is reached, varying from 5 minutes to 2 hours, the color in the preparation is fixed by placing small bits of the tissue in a freshly filtered, aqueous solution of ammonium picrate, saturated in the cold (about 1.1 per cent), for 2 to 24 hours, or better still in Bethe's fixing solution.

For immersion, the method employed for human tissues, very small or thin pieces of tissue are placed in a very dilute solution of methylene blue in physiological salt solution, ¼ to ⅛ per cent, at 37° C. Add more staining fluid when it evaporates. Examine the tissue with the microscope after 15 minutes and keep in the stain until the nerves are colored blue, optimum staining 1 to 2 hours. The stain is then fixed in a saturated aqueous solution of ammonium picrate for 2 to 24 hours, or in Bethe's fixing solution.

Fixation According to Bethe (1900).—1. Wash off excess of color with physiological salt solution.

2. Place in the following solution:

Ammonium molybdate	1 gm.
Water, distilled	10 cc.
Osmic acid, 0.5 per cent aqueous solution	10 cc.
Hydrochloric acid, concentrated	1 drop

A precipitate forms on making up the solution but disappears on shaking. The solution is best made up fresh each time. It should be used as cold as possible, preferably surrounded by a mixture of ice and salt. Leave tissue in the cold solution for 2 to 5 hours and then for a while longer at room temperature.

3. Wash in running water ½ to 2 hours.

4. Dehydrate as quickly as possible in 70, then 95 per cent alcohol in the ice-box 1 to 2 hours.

5. Clear in terpineol followed by benzol.

6. Embed in paraffin and cut sections.

7. The sections may be cleared and mounted without further

staining or may be stained with alum cochineal (p. 78) for contrast. Keep out of alcohol as much as possible.

Results.—Nerve cells and their processes and end arborizations are colored blue. Other tissues are almost colorless. Elastic fibers and connective tissue cells may at times stain blue also.

Alzheimer's Modification of Mann's Eosin-Methyl Blue Stain (1909–10).—This stain (p. 245) gives excellent pictures of the axis cylinders and affords a selective differentiation of the degenerating from the normal before changes are demonstrable by the Marchi method in the medullary sheaths. In cross-sections of the white matter of the cord the normal axis cylinders are deep purple or blue, while those undergoing degeneration are shining red. This change can be demonstrated in the experimental animal within 48 hours after damage to the cord.

Mallory's Lead Chloride Hematoxylin Stain for Axis Cylinders (1936).—Freshly dissolved hematoxylin in a slightly alkaline solution has the property of staining axis cylinders grayish blue to blue-black after the tissue containing them has been fixed in 10 per cent formalin and mordanted in a solution of lead chloride. Ripened hematoxylin will not do so.

This staining method is of especial value to the pathologist because it is reliable, in contrast with the silver methods which, although they often give brilliant results, are not always dependable.

Fixation.—1. Fix in 10 per cent neutral formalin for 24 hours or longer. The tissue should be as fresh as possible and slices should not be over 3 to 4 mm. thick.

2. Transfer directly to a saturated aqueous solution of lead chloride (slightly over 1 per cent) and mordant for 6 weeks at room temperature, or for 7 days in an incubator at 37° C. Change the fluid at the end of 24 hours and once or twice later.

3. Wash in running water for 24 hours to get rid of the unfixed lead chloride so as to prevent its precipitation by alcohol and preserve in 80 per cent alcohol.

4. Embed in celloidin or paraffin. Celloidin is as a rule preferable for the spinal cord; paraffin for sympathetic nerve ganglia.

Staining Solution.—Dissolve 5 to 10 mg. of hematoxylin in a few drops of 95 per cent or absolute alcohol and then add 10 cc. of a saturated solution of magnesium or calcium carbonate.

Method of Staining.—1. Stain sections ½ to 1 hour or longer.

A fresh solution should be made and used each hour because the hematoxylin ripens. The hematoxylin magnesium carbonate solution stains quicker and perhaps deeper. Staining with the hematoxylin calcium carbonate solution can be hastened by heat (paraffin oven at 54° C. for 1 hour or longer).

2. Wash sections 5 to 30 minutes or longer in distilled water. The slight acidity present sharpens the stain.

3. Dehydrate in 95 per cent alcohol.

4. Clear in terpineol.

5. Mount in terpineol balsam. Xylol balsam fades the blue color.

The stain of the axis cylinders can be intensified by placing the sections, before staining (Step 1), in a saturated solution of lead chloride for 1 hour or longer, followed by washing in 2 or 3 changes of tap water.

If the sections after Step 2 are again for a third time put into a saturated solution of lead chloride for 5 to 10 minutes and then washed in several changes of tap water the color is made a brighter blue.

Results.—Axis cylinders blue to black; nuclei blue; cytoplasm bluish; neuroglia fibrils dull bluish gray; collagen, elastin and myelin unstained.

The Gros-Schultze Silver Method for Axis Cylinders and Nerve Terminals.—*Fixation.*—Fix in 10 per cent neutral formalin. Cut frozen sections.

Staining Solution.—To 10 cc. of a 20 per cent silver nitrate solution add strong ammonia water drop by drop, shaking, until the brown precipitate is just dissolved. To 5 cc. of this solution add 3 drops of ammonia water. Different workers differ in the amount of ammonia water to be used. Romeis uses 5 drops to 5 cc. after solution of the precipitate. Barrera, working with Laidlaw, found it necessary to increase this added ammonia water to 12 drops to 5 cc. in order to obtain colorless collagen.

Method of Staining.—1. Place sections in distilled water containing a trace of formalin.

2. Transfer sections to a 20 per cent aqueous solution of silver nitrate in the dark for 5 minutes, 4 or 5 sections at once. The original directions were 1 hour, then 20 minutes. Both these periods were too long, according to Heringa.

3. Prepare 4 dishes of a 20 per cent aqueous solution of for-

malin. Pass sections quickly from one to another as fast as clouds are given off. Duration 10 minutes.

4. Immerse sections for a few seconds only in the ammoniacal silver solution to bleach the collagen. Control with the microscope until only axis cylinders are black on a colorless background.

5. Rinse quickly in a mixture of distilled water 8 cc. and ammonia water 2 cc., 1 minute.

6. Rinse quickly in 20 cc. of distilled water to which 2 drops of acetic acid have been added. Draw sections through rapidly.

7. Tone in 1 per cent aqueous yellow gold chloride for 1 hour.

8. Place in 5 per cent sodium thiosulfate for 1 minute.

9. Wash in water.

10. Dehydrate in 95 per cent followed by absolute alcohol.

11. Clear in xylol, float on slide and mount in balsam.

Laidlaw found that immersion in silver nitrate for 2 minutes, in gold chloride 1:500 for 10 minutes, and in 5 per cent sodium thiosulfate for 10 minutes gave good results. According to him it is unnecessary to stain with silver nitrate in the dark. If the axis cylinders are not stained after 5 minutes, immersion in formalin again may succeed; 5 minutes in Step 4 may give better results than the shorter time. Paraffin sections of formalin-fixed material also stain perfectly.

Results.—Axis cylinders are stained black. Heringa says that the above method stains sympathetic nerve fibers better than the method of Bielschowsky, which often fails here, but it stains the elastin of vessels also, which is confusing.

Bodian's Method for Nerve Fibers and Nerve Endings in Paraffin Sections (1936).—*Fixation.*—Fix in 10 per cent formalin or in alcohol-formalin (9 parts of alcohol to 1 part of 10 per cent formalin). Embed in paraffin.

Method of Staining.—1. Remove paraffin with xylol, followed by absolute and 95 per cent alcohol and water.

2. Place sections in a 1 per cent aqueous solution of protargol containing 4 to 6 gm. of metallic copper per 100 cc. of solution for 12 to 48 hours at 37° C.

3. Wash in distilled water.

4. Reduce in hydroquinone 1 gm., sodium sulfite 5 gm., distilled water 100 cc. for 10 minutes.

5. Wash in distilled water thoroughly.

6. Tone in a 1 per cent gold chloride solution containing 3 drops of glacial acetic acid per 100 cc. of solution for 5 to 10 minutes.

7. Wash in distilled water.

8. If sections do not have a light purple color, place in 2 per cent oxalic acid until sections have a definite purplish tinge (5–10 minutes). Then wash in distilled water.

9. Remove the residual silver salts with a 5 per cent aqueous sodium thiosulfate solution for 5 to 10 minutes.

10. Wash thoroughly in distilled water.

11. Dehydrate in 95 per cent followed by absolute alcohol.

12. Clear in xylol and mount in balsam.

Results.—Myelinated fibers, the finest non-myelinated fibers of the central and peripheral nervous system, the end feet of Held and the neurofibrils are stained black.

(*c*) Stains for Myelin Sheaths

(1) **Normal Myelin.**—The medullary sheaths are probably complex mixtures of lipoids. These lipoids will reduce osmic acid to metallic osmium and the earlier methods of staining the sheaths depended on this reaction. These have been discarded, however, because of their expense and the fact that osmic acid does not penetrate well.

Weigert originated the differential hematoxylin stain which in some of its modifications is universally used as a sheath stain today. These stains depend on mordanting with a chrome salt which combines with the lipoids of the sheaths in such a way as to render them insoluble in the embedding reagents and also to prepare them for the stain or for further mordanting. This combination of a chrome salt with the medullary sheaths also alters them so that they will no longer reduce osmic acid, while the fats produced by their degeneration are not so changed, and this serves as a basis of the Marchi method for the demonstration of degenerating medullary sheaths. Weigert further mordanted this chromated material with a copper salt which served to bind the copper to the chromated sheaths, with the resultant formation in the staining fluid of a copper compound of hematoxylin which is resistant to the decolorizing action of certain differentiating fluids. Weigert later recommended an iron hematoxylin which gives a stain somewhat more resistant to the differentiating solutions. In the earlier methods the mordanting

process was slow, the background remained yellow or yellow-brown in finished preparations, and the differentiating fluid acted so vigorously that there was danger of completely decolorizing some of the finer sheaths of the cortex. Many modifications of the original method were suggested. Weigert himself offered a mordant containing two chrome salts (Schnellbeize) which reduces very materially the time required in the original procedure for mordanting the sheaths. Of the others, the most commonly used are Pal's modification, which aims at clearing the background by bleaching with potassium permanganate and oxalic acid, Kultschitzky's method, which gives a very slow differentiation and hence with less danger of decolorization of the finer fibers, and Wolters' modification of Kultschitzky's method which combines with the latter Pal's method of bleaching the background.

The choice between these various methods depends on the purpose for which the material is to be used. The Weigert or the Weigert-Pal method is advised where the coarser mass degenerations, as, for example, the tabetic degeneration of the posterior columns, are to be demonstrated and where the finer, more delicate sheaths of the gray matter are not considered of consequence. Weigert-Pal sections give clear-cut pictures, permit the use of counterstains such as van Gieson's, and are much to be preferred for projection and naked eye demonstration specimens. Their decolorization is so drastic, however, that many of the finer sheaths are bleached and they are not advised for more careful study. For this purpose the Kultschitzky stain is most highly recommended. Wolters' method gives clearer pictures but partakes of the dangers of Pal's method. In the order of their dependability for staining the more delicate fiber sheaths these four methods probably rank as follows: Kultschitzky's, Weigert's original, Wolters', and Pal's. In the Kultschitzky method the staining is done in an acid hematoxylin solution, and the formation of the colored compound is carried out at the same time as the differentiation by combining the lithium carbonate with the potassium ferricyanide.

Embedding should be in celloidin. Thin sections are not desirable except for detailed studies of the cortex. The medullary sheath stains give negative pictures of defects, *i. e.*, it is the normal sheaths that are stained and the loss of an occasional fiber cannot be detected. The method is, therefore, most applicable to coarse loss of

groups of fibers, as in tract degenerations, and thick sections give much better contrast with these areas of defect than thinner ones. The usual thickness for cortex sections is 30 μ. Cord sections are often better at 20 μ.

The sheaths stain for some time after degeneration begins and it is not uncommon to find masses of degenerated sheath material, which still take the characteristic stain, engulfed in phagocytes. Because of this fact and the very slow absorption of degenerated sheaths, the Weigert method and its modifications do not give true pictures of the amount of fiber loss until 2 or 3 months after the occurrence of the lesion.

For total brain sections the Kultschitzky method is recommended. The brain is first fixed *in toto* in 10 per cent formalin for 3 weeks. Slices not over 2 cm. thick are then cut and put into Müller's fluid for 10 weeks or more, with frequent changes, or in Weigert's second mordant. The use of Weigert's double chrome mordant, the first mordant, is not recommended. It acts more quickly and gives more consistent pictures of the finer sheaths, but it does not penetrate so well and renders the tissues more brittle, and as these total sections are used chiefly for the topographical survey of coarse lesions the minor losses in fine fibrils are not a detriment. After careful dehydration the slices are carried through absolute alcohol and ether as usual, into thin celloidin, and are left in this for 6 to 8 weeks in closed vessels and then for about a week in thick celloidin. After this the celloidin is allowed to harden by slow evaporation of the solvents and the block mounted and cut. Sections are carried through the stain between sheets of tissue paper.

Two methods are given for staining the medullary sheaths in frozen sections. In both of these the stain is an iron hematoxylin and the mordanting is done on sections and is comparatively very brief. These methods have the advantage of rapidity and are accurate but they have also the disadvantages of the frozen section method.

Weigert's Method for Myelin Sheaths (1884, 1896).—*Fixation.*—Fix in 10 per cent formalin 2 to 3 days or in formalin followed by Müller's fluid (p. 42). The latter method gives excellent pictures but as the material ages it gets progressively more brittle. Formalin material of almost any age gives good coarse pictures but in old material the finer sheaths often fail to stain.

Staining Solutions.—

Weigert's First Mordant (Schnellbeize)

Potassium bichromate	5	gm.
Fluorchrome	2.5	gm.
Water, distilled	100	cc.

Boil, cool and filter.

Weigert's Second Mordant (Neurogliabeize)

Copper acetate, neutral, normal, cupric	5	gm.
Fluorchrome	2.5	gm.
Water, distilled	100	cc.

Boil and then add:

Acetic acid, glacial	5	cc.

The fluorchrome is dissolved by boiling the water, the flame is withdrawn and the acetic acid added as soon as boiling has ceased, and then finely powdered copper acetate is added and stirred vigorously until dissolved. This mordant may be applied to either blocks or sections.

Iron Hematoxylin Solution

A

Hematoxylin, ripened, 10 per cent solution in absolute alcohol	10	cc.
Alcohol, 95 per cent	90	cc.

B

Ferric chloride, 29 per cent aqueous solution	4	cc.
Water, distilled	96	cc.

Equal parts of A and B are mixed for use; this mixture blackens immediately through the formation of iron hematoxylin and forms the staining fluid. Keep in stock a 10 per cent solution of hematoxylin in absolute alcohol. At least 10 days of exposure to light are required to ripen this solution and it does not reach its greatest staining strength for several months.

Differentiating Solution

Borax	2	gm.
Potassium ferricyanide	2.5	gm.
Water, distilled	100	cc.

Method of Staining.—1. Place small blocks of tissue, not over 1 cm. thick, in the first mordant for 4 to 6 days at room temperature or for 2 to 3 days at 37° C.

2. Without washing dehydrate in the dark in graded alcohols, beginning with 50 per cent and with no steps greater than 10 per cent to avoid shrinkage. If desired the embedding process may be carried out after the second mordanting.

3. Embed in celloidin; let celloidin harden in the air.

4. Place in the second mordant for 1 day at 37° C.

5. Put in 80 per cent alcohol.

6. Cut sections 20 to 30 μ thick.

7. Stain sections in the iron hematoxylin solution for 24 hours.

8. Wash in tap water ½ to 1 hour.

9. Differentiate in the borax-potassium ferricyanide solution, controlling with the microscope, until the white matter stands out black against the light yellow to brown gray matter. Differentiation is usually complete in 15 to 30 minutes, sometimes within a few minutes. If it takes place too rapidly the fluid may be diluted one-half.

10. Wash thoroughly in tap water for 24 hours to stop the decolorization.

11. Dehydrate in 95 per cent alcohol.

12. Clear in aniline-xylol or carbol-xylol, rinse in xylol and mount in balsam.

Results.—Myelin sheaths blue-black; background pale yellow or almost colorless.

Weigert-Pal Method for Myelin Sheaths (1886).—*Fixation.*—Fix in Müller's fluid for 2 weeks or more or in 10 per cent formalin followed by Müller's fluid. Dehydrate in alcohol, embed in celloidin and cut sections 20 to 30 μ thick.

Staining Solutions.—

Weigert's Lithium Hematoxylin Solution

Hematoxylin	1 gm.
Water, distilled	90 cc.
Alcohol, absolute	10 cc.
Lithium carbonate, saturated aqueous solution (about 1.33 per cent)	1 cc.

Or, *Pal's Hematoxylin Solution.*—Make up fresh a 0.75 per cent aqueous hematoxylin solution, by heating it, and when cool add a little alcohol. Just before use add 2 cc. of a saturated aqueous solution of lithium carbonate (about 1.33 per cent) to 100 cc. of the hematoxylin solution.

Decolorizing Solution

Oxalic acid	1 gm.
Potassium sulfite	1 gm.
Water, distilled	200 cc.

Make this solution up cold and keep in a well stoppered bottle.

Method of Staining.—1. Place sections either at once in the staining solution, or if they are greenish instead of brown, place them for a few hours in 0.5 per cent chromic acid or in 2 to 3 per cent potassium bichromate for longer.

2. Otherwise, pass sections from alcohol into Weigert's hematoxylin stain, to which 2 to 3 cc. of a solution of lithium carbonate saturated in the cold (about 1.33 per cent) have been added, for 24 to 48 hours at room temperature or for 2 hours in the oven; or place them in Pal's hematoxylin solution for 5 to 6 hours.

3. Wash in water to which 2 to 3 drops of saturated lithium carbonate (1.33 per cent) solution are added.

4. Place in 0.25 per cent solution of potassium permanganate for 15 to 20 seconds.

5. Wash in water.

6. Decolorize a few seconds in the decolorizing solution until only the myelinated nerve fibers remain blue.

7. Wash thoroughly in tap water.

8. Dehydrate in 95 per cent alcohol.

9. Clear in carbol-xylol followed by xylol and mount in balsam.

Results.—Myelin sheaths are deep black; the background is unstained. This method gives excellent pictures for low power examination and projections but is not trustworthy for finer studies.

Kultschitzky's Modification of the Weigert Method (1890).—This is the recommended method for finer studies of cortical architecture and for total brain sections.

Fixation.—Fix in 10 per cent formalin and mordant for 4 to 5 days in Weigert's second mordant (p. 232). Dehydrate in alcohol and embed in celloidin.

Staining Solution.—

Hematoxylin, ripened 10 per cent solution in absolute alcohol	10 cc.
Acetic acid, 2 per cent aqueous solution	90 cc.

Differentiating Solution

Lithium carbonate, saturated aqueous solution (1.33 per cent)	100 cc.
Potassium ferricyanide, 1 per cent aqueous solution	10 cc.

Method of Staining.—1. Stain sections for 12 to 24 hours in the hematoxylin solution.

2. Carry over directly into the differentiating fluid. Differentiation is very slow by this method and can be readily controlled with the microscope. It may take 4 to 12 hours. Change fluid. Further, the fact that the formation of the hematoxylin compound takes place in the differentiating fluid minimizes the danger of decolorizing the finer sheaths.

3. Wash thoroughly in water.

4. Dehydrate in 95 per cent alcohol.

5. Clear in terpineol or in oil of origanum and after blotting rinse in xylol to remove oil and mount in balsam.

Results.—Finest myelin sheaths deep black.

Wolters' Modification of the Weigert Method for Myelin Sheaths (1890).—*Fixation.*—Fix in Müller's fluid or 10 per cent formalin followed by Müller's fluid. Wash, dehydrate in alcohol, embed in celloidin and cut sections.

Staining Solution.—

Kultschitzky's Hematoxylin Solution

Hematoxylin	2 gm.
Alcohol, absolute, sufficient to dissolve the hematoxylin	
Acetic acid, 2 per cent aqueous solution	100 cc.

Decolorizing Solution

Oxalic acid	1 gm.
Potassium sulfite	1 gm.
Water, distilled	200 cc.

Method of Staining.—1. Place sections at once in Kultschitzky's hematoxylin solution for 24 hours on top of a paraffin oven which has a temperature of 45° C.

2. Dip sections in Müller's fluid (p. 42).

3. Place in 0.25 per cent potassium permanganate 20 to 30 seconds.

4. Wash in water.

5. Differentiate in a freshly prepared decolorizing solution for ½ to 3 minutes.

6. Wash thoroughly in water.

7. Dehydrate in 95 per cent alcohol.

8. Clear in terpineol or in oil of origanum and after blotting rinse in xylol and mount in balsam.

Results.—Myelin sheaths stain intensely blue-black; nerve fibers blue-black; background light; ganglion cells yellow to yellowish brown.

Wright's Method for Myelin Sheaths in Frozen Sections.—*Fixation.*—Fix in 10 per cent formalin. Cut frozen sections and attach the sections to the slide by the aniline-oil of clove method (p. 33). The attachment of the section to the slide by the method specified is important, because it prevents distortion of the section and because this treatment of the section facilitates the staining, apparently by the removal of interfering fatty material.

Method of Staining.—1. Cover the sections with a 10 per cent aqueous solution of ferric chloride for 5 minutes.

2. Remove excess of ferric chloride solution from around the section, leaving some on the section, and pour on the slide as much as it will hold of a freshly prepared aqueous solution of hematoxylin. The hematoxylin solution is prepared by heating a few crystals of hematoxylin in about 15 cc. of distilled water. The hematoxylin must be of good quality. Stain for 30 minutes.

3. Wash quickly in water.

4. Differentiate by moving the slide about in a 10 per cent aqueous solution of ferric chloride until the gray substance is defined and the pia appears white.

5. Wash thoroughly in several changes of water.

6. Dehydrate in 95 per cent alcohol.

7. Clear in terpineol or in oil of origanum and mount in balsam.

Results.—Myelin sheaths blue-black.

Spielmeyer's Method for Myelin Sheaths in Frozen Sections (1930).—*Fixation.*—Fix in 10 per cent formalin 3 days at least and wash in running water 1 hour. Cut frozen sections 20 to 30 μ thick.

Staining Solution.—

Hematoxylin, 10 per cent solution in absolute alcohol, well ripened	5 cc.
Water, distilled	100 cc.

The hematoxylin solution must be old and can be used over and over again as its staining power improves with age.

Method of Staining.—1. Place for 6 hours or longer in 2.5 per cent aqueous solution of ammonio-ferric alum.

2. Rinse in water and then place for 10 minutes in 70 per cent alcohol. The washing in 70 per cent alcohol is to remove fat substances which might interfere with even staining.

3. Stain 10 to 24 hours in the hematoxylin solution. Occasional irregular staining indicates that the fat has not been entirely removed. Such sections may be carried back through water to 70 per cent alcohol and again stained and differentiated.

4. Rinse in distilled water.

5. Differentiate in the ammonio-ferric alum solution given above. Control results with the microscope.

6. Wash thoroughly in 2 changes of distilled water, then 1 to 2 hours in tap water.

7. Dehydrate in 95 per cent alcohol.

8. Blot, clear in xylol, repeat blotting followed by xylol twice and mount in balsam.

Results.—Myelin sheaths black.

(2) **Degenerating Myelin.**—The majority of the products of degeneration in the nervous system are fatty in nature and the most important methods for their demonstration are the scarlet red and osmic acid procedures. Scarlet red stains not only the neutral fats and fatty acids produced by degeneration and autolysis of nerve cells but also many lipoid pigments. There are, however, a number of other degenerative products.

Marchi's Method for Fatty Degeneration (1885).—This method is the classical one for the demonstration of secondary degeneration of the myelin sheath following death of the axis cylinder, but can also be used to demonstrate fatty changes within the nerve

cells, and elsewhere. In contrast with Weigert's stains it gives a positive picture of the sheaths that are actually undergoing degeneration and has been the chief method for following the course of individual fiber groups and tracts. It is applicable, however, only to medullated fibers and the effect of a lesion is not demonstrable until a week after its occurrence. Normal sheaths reduce osmic acid to metallic osmium but previous treatment with a chrome salt so alters them that this reduction does not take place. The neutral fats, fatty acids and most lipochromes are not so altered, however, and hence blacken on exposure to osmic acid. Osmium which has been reduced by fat is soluble in ether, turpentine and xylol but much less so in alcohol, chloroform, oil of cloves and oil of origanum.

Fixation.—Fix in Müller's fluid. For fiber tract study preliminary hardening in formalin is possible but is not recommended. For the finer stippling of early fatty degeneration of the cells fixation should always be in Müller's fluid direct. Small pieces should be fixed for 8 days to 5 weeks with frequent changes of the fixing solution.

Method of Staining.—1. Transfer directly to the following freshly prepared solution for 4 to 30 days in the dark with changes of the fluid when it blackens:

Müller's fluid (p. 42)	2 parts
Osmic acid, 1 per cent aqueous solution	1 part

Brain tissue must remain in this mixture longer (20–30 days) than the spinal cord (8–12 days).

2. Wash 24 hours in running water.

3. Dehydrate and embed rapidly, in paraffin for cellular study or in celloidin for tract preparations. The steps of the embedding methods should be shortened as much as is consistent with proper impregnation. With small thin pieces paraffin embedding (by the chloroform, not the xylol, method) may be accomplished satisfactorily in 12 to 16 hours.

4. Cut paraffin sections as thin as possible, 4 to 6 μ, and counterstain lightly, if desired, with van Gieson's stain (p. 92). Celloidin preparations for tract studies may be much thicker, 20 μ.

5. Dehydrate rapidly in 95 per cent followed by absolute alcohol.

6. Clear in chloroform and mount in chloroform balsam.

Celloidin sections are cleared in oil of origanum after 95 per cent alcohol and mounted in balsam.

Results.—Foci of degeneration are stained black. Degenerating myelin appears as black dots on a yellowish background of normal myelinated fibers, neuroglia and nerve cell bodies.

Very thin pieces of tissue fixed for 24 hours in formalin, then washed out and left for 1 to 2 weeks in the dark in Flemming's fluid (p. 44) with changes as the fluid blackens, give excellent pictures of the fats in phagocytes and in the vascular endothelium but they are by no means so accurate as those stained by the Marchi method.

c. Neuroglia Stains

Under this heading are included two different types of cells. (1) The astrocyte, which occurs in two forms, the protoplasmic and the fibrous or fibrillary. The latter produces the neuroglia fibrils. (2) The oligodendroglia cells.

(a) Stains for Astrocytes

The older conception of the neuroglia was gained largely through the application of the Golgi silver methods and the pictures obtained of the relation of the neuroglia fibrils to the cell bodies were misleading in that the incrustation led directly over from the fiber to the cytoplasmic extensions of the angular cell body and gave a picture of simple elongation of the cytoplasm into fibrillar expansions. The later methods, particularly Mallory's phosphotungstic acid hematoxylin and Weigert's stain, color the fibrils selectively and have led to the present conception of the fiber as a differentiated structure which, in the mature state, lies in contact with the parent cell. For demonstration of the neuroglia fibrils fresh tissue is of the utmost importance. The best results are obtained with material taken from the body within 1 hour after death. After 4 to 6 hours the results are only fair and after 24 hours the loss in normal neuroglia fibrils is often marked. All fibrils do not, however, disappear with equal rapidity after death. The coarse fibrils of neuroglia scars, the heavy fibers of the white matter of the cord and those arising from the buried ends of the ependymal cells apparently resist disintegration longer than the finer supportive framework of the cortex and white matter of the cerebrum. Furthermore this disintegration is not entirely a postmortem change. In cases of

death following high febrile periods the neuroglia stains may fail practically entirely even on fresh material. Alzheimer has demonstrated the disintegration of the fibers in such cases and this destruction is probably to be correlated with the "soft brains" of acute infectious disease. Here also the destruction apparently is more striking in the finer normal fibers than in the coarser network of neuroglial replacements.

For best results by either the Mallory or the Weigert method, it is important to fix thin slices of fresh tissue. The phosphotungstic acid hematoxylin stain has the advantage that it is much simpler and that after fixation the material is permanent and sections can be prepared as needed. With the Weigert method practically all the steps must be carried out without delay in order to get the best results. Even after staining there is greater danger of fading in the Weigert stain. This is particularly true if the aniline oil has not been thoroughly removed. Material fixed in Zenker's fluid and prepared for the phosphotungstic acid hematoxylin method is also available for a variety of general stains and for study of the mesodermal elements in the nervous tissues. Material fixed in Weigert's neuroglia mordant is available for the demonstration of the neuroglia cells by several methods. Where striking contrast is desired, as for microphotography and demonstrations to students, the Weigert method is recommended. However, the comparative simplicity, the permanence of the stain, and the greater delicacy of the pictures without sacrifice of selectivity, make Mallory's phosphotungstic acid hematoxylin the method of choice for practically all purposes. Both methods stain fibrin intensely and also the fibroglia and myoglia fibrils, if the tissue is fresh when fixed.

Most neuroglia methods are more successful in the white matter than in the gray. In the white matter of both cord and brain the clear background furnished by practically colorless medullary sheaths serves to bring out the neuroglia cells and fibers with great distinctness. In the cortex and spinal gray matter, however, the background takes an indefinite cloudy or hazy stain which shows practically no detailed structures of its own and obscures the clear view of the contained neuroglia fibrils. In phosphotungstic acid hematoxylin preparations this background is a pale tan color, not unlike that which the bodies of neuroglia cells take. One of Cajal's methods (given later) stains the neuroglia fibrils with startling

clearness and at the same time stains in the cortex a tenuous interlacing meshwork which is apparently made up of extensions of the neuroglia cell cytoplasm, and it seems probable that diffuse staining of this material is the reason for the lack of clearness by other methods.

Mallory's Phosphotungstic Acid Hematoxylin Stain.—This has already been given under the heading of hematoxylin stains (p. 76). It is very useful also, however, for neuroglia fibrils. It has two disadvantages. It has been used ordinarily only after fixation in Zenker's fluid and it tends to stain the myelin sheaths more or less deeply.

For formalin-fixed material the following modification has been found useful.

Fixation.—Fix in 10 per cent formalin. Mordant thin sections of tissue in a saturated solution of lead chloride, as directed for axis cylinders (p. 226). Embed in celloidin or paraffin.

Method of Staining.—1. Place sections in 5 per cent aqueous solution of oxalic acid for 30 to 60 minutes.

2. Rinse in tap water.

3. Stain in phosphotungstic acid hematoxylin (p. 76) for 3 to 24 hours.

4. Rinse in water.

5. Dehydrate in 95 per cent alcohol, followed by absolute alcohol for paraffin sections.

6. Clear in xylol for paraffin, terpineol or oil of origanum for celloidin sections, and mount in balsam.

Results.—Neuroglia fibrils clear blue; collagen, axis cylinders, cell cytoplasm and processes reddish. Coarse elastic fibrils bluish. Myelin blue to bluish gray.

Weigert's Differential Stain for Neuroglia Fibrils (1895). —*Fixation.*—1. Fix thin, fresh pieces of tissue, not over 0.5 cm. thick, in 10 per cent formalin for 4 days at least. Change the fluid after the first day.

2. Mordant in Weigert's neuroglia mordant (p. 232) for 4 to 5 days at 37° C. or for 8 days at room temperature. Fixation and mordanting may be combined by placing the tissue at once in a mixture of 1 part of formalin to 9 parts of the mordant for the first 24 hours and then transferring to the mordant without formalin for 8 days.

3. Wash in water, dehydrate in graded alcohols, embed in celloidin or paraffin.

Staining Solution.—

Methyl Violet Solution

Methyl violet, saturated solution in 80 per cent alcohol (15.21 per cent)	100 cc.
Oxalic acid, 5 per cent aqueous solution	5 cc.

Make up this solution by saturating the methyl violet with heat and decanting when cold, then adding the oxalic acid.

Reducing Solution

Chromogen	5 gm.
Formic acid, sp. gr. 1,20	5 cc.
Water, distilled	100 cc.

To each 90 cc. of this add 10 cc. of a 10 per cent solution of sodium sulfite just before using, and filter carefully. The sections bleach rapidly but should be left in the solution as directed.

Method of Staining.—1. Place sections, cut not over 20 μ thick, for 10 minutes in 0.3 per cent aqueous solution of potassium permanganate.

2. Rinse twice in distilled water.

3. Put into the reducing fluid for 2 to 4 hours.

4. Rinse twice in distilled water.

5. Stain for a few minutes (staining is practically instantaneous) in the methyl violet mixture. Staining is best accomplished on the slide.

6. Drain off stain and pour over the section a saturated solution of iodine in a 5 per cent aqueous solution of potassium iodide for 30 seconds. Saturation with iodine is important.

7. Dry section with filter paper and differentiate thoroughly in a mixture of equal parts of xylol and aniline.

8. Rinse in xylol to remove the aniline completely and mount in balsam.

After Step 2 the sections may be rinsed in distilled water and left overnight in a filtered 5 per cent aqueous solution of chromogen, then rinsed and carried through as above. This affords the so-called contrast which gives the neuroglia fibers a somewhat darker color and stains the background pale yellow. Connective tissue stains blue after this step, however.

After staining, sections should be left in strong light for several days as this aids in fixing the stain.

Results.—Neuroglia fibers blue; nuclei blue; cytoplasm and connective tissue unstained.

Bailey's Stain for Neuroglia Fibrils (1923).—The success of the stain depends on the freshness of the material. The best results are obtained after fixation in Zenker's fluid, but formalin-fixed tissue may be used after mordanting in the block or on the slide in Zenker's fluid, or preferably in Bouin's fluid overnight and then passing through the bichromate solution (Step 2) in the usual way.

Fixation.—Fix tissues in Zenker's fluid. Embed in paraffin and cut sections 5 μ thick.

Staining Solution.—To a saturated aqueous solution of ethyl violet diluted with 3 volumes of distilled water add a saturated aqueous solution of orange G (about 10.86 per cent) drop by drop with a pipette, testing from time to time for the end-point. This is done by letting a drop fall on a filter paper. The ring which forms around the precipitate will be blue until the end-point is reached, when it will become orange. Care should be exercised to stop just short of this moment. The precipitated dye is filtered and the precipitate washed once with distilled water. After it has dried add enough absolute alcohol to dissolve it and keep as the stock solution.

Method of Staining.—1. Remove paraffin and pass sections through alcohol and iodine to water in the usual way.

2. Place for 3 days in a 3 per cent aqueous potassium bichromate solution.

3. Rinse and place for 12 hours or longer in a solution of neutral ethyl violet-orange G made by diluting 1 portion of the stock solution with 3 parts of 20 per cent alcohol.

4. Blot and agitate quickly in anhydrous acetone.

5. Place in toluol for a few seconds; then wipe most of it off the slide with a towel.

6. Flood slide with pure oil of cloves.

7. Differentiate in oil of cloves 3 parts, 95 per cent alcohol 1 part, agitating the slide and watching with the microscope until the small blood vessels show up as orange rings.

8. Blot and rinse in pure oil of cloves.

9. Blot and agitate in toluol; then xylol half a dozen changes, and mount in balsam.

Results.—Neuroglia fibrils appear dark violet on an orange background. This method, like other neuroglia procedures, stains also fibroglia and myoglia fibrils.

The chief methods for the neuroglia fibers, those of Mallory and Weigert, stain the fibers selectively and leave neuroglia cell cytoplasm practically unstained. In early gliosis where marked degeneration of nerve tissue is taking place and the astrocytes are proliferating rapidly to fill the defect but are not as yet actively producing fibers, the cytoplasm increases very markedly from the normal small angular cell body to a large ovoid or irregular mass ("cellular gliosis"). Often these large cells are crowded irregularly into the interstices between white fibers and form relatively enormous masses with multiple nuclei and no clear evidence of division of their bodies. These are the "ameboid" neuroglia cells of Alzheimer. They are short-lived structures occurring early in the course of degenerative lesions. Some of them probably go on to the production of fibers and thus take part in the scar formation resulting in the neuroglial replacement which is the common end result of such lesions. Many, however, undergo degeneration by fragmentation of their cytoplasm, resulting in the formation of a mosaic of small angular masses staining with variable intensity, the "Füllkörperchen" of Alzheimer. The formation of "ameboid" astrocytes is apparently an acute reaction and seems particularly profuse in rapid degenerations of considerable masses of fibers and sheaths as, for example, in subacute posterolateral sclerosis and much less frequently in the slowly progressive processes such as tabes. In chronic meningitis (*e. g.*, simple syphilitic infiltrative meningitis) there is often a distinct zone formed between the marginal neuroglia and the outermost fibers of the cord made up of closely packed "ameboid" cells. This appears at times as a protective walling off of the cord tissues proper as there is frequently no demonstrable loss of fibers underlying this mantle.

The general stains such as the phloxine-methylene blue will frequently give a fairly good clue to the presence of the pathological neuroglia cells in numbers and good pictures are sometimes obtained in alum hematoxylin-van Gieson and in toluidine blue preparations. Three special methods are given here, however, which are recommended for more accurate studies. All of these methods are credited to Alzheimer.

Alzheimer's Modification of Mallory's Phosphomolybdic Acid Hematoxylin Stain for Neuroglia Changes (1909–10).—*Fixation.*—1. Fix in Weigert's neuroglia mordant (p. 232) for 2 weeks, or in neuroglia mordant to which 10 per cent of formalin has been added for 24 hours, followed by the neuroglia mordant alone for 2 weeks. The former method is preferable.

2. Wash in running water for 8 to 12 hours to remove the excess copper which otherwise will rapidly injure the knife edge. Prolonged washing seems to have no deleterious effect on the stain.

3. Cut frozen sections not over 10 to 15 μ thick.

Method of Staining.—1. Wash sections 2 minutes in distilled water to which a little glacial acetic acid has been added, about 1 drop to 10 cc.

2. Carry over directly for 2 minutes to a very weak solution of Mallory's phosphomolybdic acid hematoxylin prepared by adding the stain (p. 156) drop by drop to a dish of distilled water until the mixture has just lost its transparency.

3. Rinse quickly in distilled water.

4. Dehydrate rapidly in graded alcohols.

5. Clear in xylol and mount in balsam.

Results.—The sections should be reddish blue. Blue sections show a less selective staining. The neuroglia cells and nerve cell bodies are stained a pale bluish red, the axis cylinders in the white matter are darker. Pathological astrocytes vary considerably, even adjacent cells, in the intensity with which they are colored and often show deeply stained granular inclusions. Neuroglia fibers are darker than the cytoplasm and well differentiated.

Alzheimer's Modification of Mann's Eosin-Methyl Blue Stain for Neuroglia Changes (1909–10).—*Fixation.*—Fix thin pieces of tissue for 2 weeks in Weigert's neuroglia mordant (p. 232) to which 10 per cent of formalin has been added. Wash 8 to 12 hours in running water. Cut frozen sections 10 μ thick.

Staining Solution.—

Mann's Eosin-Methyl Blue Solution

Methyl blue, 1 per cent aqueous solution	35 cc.
Eosin, 1 per cent aqueous solution	35 cc.
Water, distilled	100 cc.

Method of Staining.—1. Mordant sections 2 to 12 hours in a saturated aqueous solution of phosphomolybdic acid (phosphomolybdic acid is readily soluble in water).

2. Wash rapidly in 2 changes of distilled water.

3. Stain in Mann's solution 1 to 5 hours.

4. Wash rapidly in distilled water until color clouds are no longer given off.

5. Place in 95 per cent alcohol until the gray matter becomes a light blue. The white matter should have a bright red or pink color.

6. Pass through absolute alcohol quickly.

7. Clear in xylol and mount in balsam.

Results.—This method gives beautiful preparations of the white matter, and they keep well. Normal medullary sheaths are colorless to light pink, depending on the degree of decolorization in the alcohol. Degenerating myelin sheaths are deep pink to red. Normal axis cylinders are deep blue to purple. Degenerating axis cylinders are glistening red. Neuroglia cytoplasm is pale blue and the fibers dark blue. "Ameboid" astrocytes vary from pale to dark blue, as do Alzheimer's "Füllkörperchen." Collagen is dark blue. In the gray matter the preparations are less acceptable. The nerve cells stain dark blue and show relatively little detail and the background also lacks clear-cut differentiation. The method is particularly useful in demonstrating the abnormal astrocytes in the white matter and bids fair to become of service as a method for the early recognition of degenerating axis cylinders. The change from blue to red staining takes place in experimental material as early as 48 hours after a lesion, while the other method by which this degeneration is usually demonstrated (Marchi) depends on slow secondary changes in the medullary sheath, which do not begin to appear for about 7 days.

Alzheimer's Light Green-Acid Fuchsin Method for Neuroglia Changes (1909–10).—*Fixation.*—Fix in 10 per cent formalin 24 hours, not over 4 days at most. Wash 24 hours in running water. Transfer very thin pieces to Flemming's solution (p. 44) containing only 1 to 2 drops of acetic acid for 8 days in the dark. The solution should be in considerable volume in proportion to the tissue and should be changed once or twice during the period if it blackens. Embed in paraffin and cut very thin sections, 2 to 4 μ.

Method of Staining.—1. Stain sections in a saturated aqueous

solution of acid fuchsin (about 20 per cent) for 1 hour at about 60° C.

2. Let cool and wash in tap water.

3. Immerse in the following solution, varying the time with the tissue from a single immersion to 2 minutes:

Picric acid, saturated alcoholic solution (about 8.9 per cent)	30 cc.
Water, distilled	60 cc.

4. Wash in 2 changes of tap water.

5. Stain for 30 minutes to 1 hour in a 10 per cent aqueous solution of light green.

6. Wash in 2 changes of tap water.

7. Differentiate to a violet color in 95 per cent alcohol.

8. Pass through absolute alcohol quickly.

9. Clear in xylol and mount in balsam.

The procedure is somewhat uncertain and must be varied with the tissue. The material must be fresh and pieces must be small. The color of the finished specimen to the naked eye should be neither red nor green but a delicate lilac. It often proves necessary to run several experimental sections in order to determine the optimum time in picric acid and light green, but good preparations will repay this labor.

Results.—The method gives extremely delicate pictures. "Ameboid" astrocytes are varying shades of green and often contain fuchsinophile granules or brown stained lipoid inclusions. The lipoid contents of perivascular phagocytes are brown to black. Neuroglia fibers and red blood corpuscles are red, medullary sheaths unstained, connective tissue deep green, nerve cells pale green with red stippling, and nerve cell nuclei are darker green with bright red nucleoli.

Alzheimer's Stain for Granules in Neuroglia Cells (1909–10).—Alzheimer has given several methods for the demonstration of various granules produced by degenerations of the neuroglia and nerve cells. Among the most interesting of this group are the fuchsinophile granules which are brought out by the light green-acid fuchsin stain. Alzheimer considers these as precursors of the fatty degeneration products. These may be more strikingly stained by the following procedure.

Fixation.—Fix in 10 per cent formalin for 24 hours. Wash in running water. Transfer thin pieces to Flemming's solution (p. 44) in the dark for 8 days, changing the solution if it blackens. Embed in paraffin and cut sections 2 to 4 μ thick.

Staining Solution.—

Hematoxylin, 10 per cent alcoholic solution, ripened	10 cc.
Water, distilled	87 cc.
Lithium carbonate, saturated aqueous solution (about 1.33 per cent)	3 cc.

Method of Staining.—1. Mordant sections for 1 hour at 37° C. in a saturated aqueous solution of copper acetate (about 12.5 per cent).

2. Wash twice in water.
3. Stain in the hematoxylin solution for 30 minutes.
4. Rinse in water.
5. Dehydrate in 95 per cent followed by absolute alcohol.
6. Clear in xylol and mount in balsam.

Results.—The granules are stained blue-black on a pale background in which sufficient detail shows for orientation.

Two fundamental metallic staining methods originating with Cajal and del Río-Hortega have been of the greatest value in the study of the nervous system. Because of various factors they are not always successful. This has led to innumerable modifications. The following methods recommended by Dr. Wilder Penfield seem sufficient for inclusion in this type of book and they have been found reliable even in the hands of the inexpert.

It is exceedingly important for the success of the metallic stains that all chemicals used be of the highest purity and that the glassware be scrupulously clean. See page 158 for a discussion of the precautions to be taken.

The chemical formulas involved in the metallic staining reactions are not known.

Cajal's Gold Chloride Sublimate Method for Astrocytes (1923).—The procedure given here is the one recommended by Penfield (McClung, 1937).

Fixation.—1. Fix thin blocks or slices of tissue in formalin-ammonium bromide solution for 1 day in the incubator at 37° C.

Formalin, Merck's blue label	15 cc.
Ammonium bromide	2 gm.
Water, distilled	85 cc.

Cut frozen sections, 15 μ thick, and place them in 1 per cent formalin in distilled water.

Or 2. Fix the whole brain in 10 per cent formalin for 1 week at room temperature. If possible formalin should be injected into the brain at the earliest possible moment after death. In experimental animals the injection is made under ether anesthesia, using a pressure of about 2½ meters of water, which eliminates the necessity of washing out the vessels with any other solution. When the brain has been thus injected frozen sections are cut within 24 hours. The frozen sections are washed in distilled water and bromurated by Globus' method in the following way: Place sections in a covered dish containing distilled water to which 1 drop of strong ammonia water for every cubic centimeter of water has been added. Leave in this overnight. Wash sections rapidly, then put them in a 10 per cent aqueous hydrobromic acid solution for 1 hour at 37° C.

Staining Solution.—

Cajal's Gold Chloride Sublimate Solution

Mercuric bichloride crystals	0.5 gm.
Gold chloride (Merck's brown), 1 per cent aqueous solution	6 cc.
Water, distilled	35 cc.

Prepare the solution fresh each time, using scrupulously clean glassware. Pulverize the mercuric bichloride crystals and add them to the distilled water. Heat gently to dissolve the crystals; do not overheat. When dissolved add the gold chloride to the hot solution and filter. Cajal warned against using powdered mercuric bichloride. The gold chloride solution keeps well for a long time in a brown bottle.

Hortega's Carbol-Xylol-Creosote Mixture

Creosote	10 cc.
Phenol crystals, melted	10 cc.
Xylol	80 cc.

Method of Staining.—1. Wash sections quickly in 2 changes of distilled water.

2. Place not more than 6 sections for 4 to 6 hours in a flat porcelain dish with a porcelain cover containing about 35 cc. of a freshly prepared gold chloride sublimate solution with double the amount of mercuric bichloride, given above, *i. e.*, mercuric bichloride 1 gm., 1 per cent gold chloride 6 cc., and distilled water 35 cc.

The porcelain dish should be used only for the gold solution and the glassware used in the preparation of the solution should be kept for that purpose only.

The sections are flattened out on the bottom of the dish and kept in the dark. When the purple color begins to appear one section is examined wet. If astrocytes can be seen, the section is mounted. The optimum color is reddish purple. If a scum begins to form in the solution sections should be removed; a little reddish deposit is not detrimental.

The gold solution is kept at room temperature, a temperature of 18° to 20° C. being best for the human cerebrum, and 22° to 26° C. for the human cerebellum and medulla. Tissues from small animals require staining for 2 to 3 hours at 24° to 26° C., birds, reptiles and fish at 25° to 30° C. During the heat of summer the results are apt to be unsatisfactory.

3. Wash in distilled water 5 to 10 minutes.

4. Fix the sections 5 to 10 minutes in a 5 per cent aqueous sodium thiosulfate solution.

5. Wash well in several changes of water. Extra sections may be preserved in a 1 per cent aqueous solution of formalin for long periods.

6. Float sections from water onto a slide and dehydrate in 95 per cent alcohol.

7. Clear in Hortega's carbol-xylol-creosote mixture. Blot sections as soon as they are clear.

8. Mount in balsam.

Results.—Astrocytes with their processes black; background almost unstained or light brownish purple; nerve cells pale red; nerve fibers unstained.

Del Río-Hortega's Silver Carbonate Method for Astrocytes (1917).—*Fixation.*—Cut the tissue into slices less than 1 cm. thick and fix them in Cajal's formalin-ammonium bromide (p. 249). The duration of the fixation varies; it is difficult to give a definite time as the optimum duration is not constant. The fixation time may

be shortened by putting the tissue in an incubator at 37° C. At room temperature 20 to 40 days give best results for the protoplasmic astrocytes of the gray matter, a month or more for the fibrous astrocytes. At 37° C. good results may be obtained after fixation for 2 to 3 weeks.

If tissues have been fixed in 10 per cent formalin satisfactory silver stains may sometimes be obtained if the formalin-fixed tissue is bromurated by Globus' method (p. 217). Sometimes it is helpful to place sections of formalin-fixed tissue in Cajal's reinforcer solution for 4 hours at 37° C. before staining.

Cajal's Reinforcer

Formalin, neutral	30 cc.
Ammonium bromide	3 gm.
Water, distilled	70 cc.

Cut frozen sections 15 to 25 μ thick and place them in distilled water to which a few drops of ammonia water have been added.

Staining Solution.—

Hortega's Silver Carbonate Solution for Astrocytes

Silver nitrate, 10 per cent aqueous solution	5 cc.
Lithium carbonate, saturated aqueous solution (about 1.33 per cent)	20 cc.
Ammonia water, sufficient amount to dissolve the precipitate	
Water, distilled	75 cc.

When the lithium carbonate is added to the silver nitrate a precipitate of silver carbonate is formed. The ammonia water is then added drop by drop, stirring constantly, until the precipitate disappears and ammoniacal silver goes into solution. Do not add more ammonia water than is necessary to dissolve the precipitate almost completely. The small amount of black, dust-like precipitate which remains may be filtered off. The solution keeps for a long time.

In his original publication del Río-Hortega used 10 cc. of a 10 per cent silver nitrate solution to which he added an equal or larger amount of saturated lithium carbonate solution. He decanted the liquid, washed the precipitate with 50 cc. distilled water, decanted again and then added 15 to 20 cc. distilled water followed

by ammonia water sufficient to dissolve the precipitate. Distilled water was then added to make the volume up to 50 cc.

Gold Chloride Solution for Toning

Gold chloride, yellow	1 gm.
Water, distilled	500 cc.

It is not necessary to use the more expensive brown gold for toning; the yellow gold chloride seems to serve the purpose better.

Method of Staining.—1. Wash sections in 4 changes of distilled water to remove all formalin.

2. Place 6 to 12 sections in the staining solution given above in a small glass dish of about 10 cc. capacity. A few drops of pyridine are added to prevent the formation of a scum on the surface. Cover the dish with a watchglass, leaving a bubble of air beneath it and heat gently to 45° to 50° C., shaking occasionally. Stain sections 3 to 5 minutes until they have turned dark amber in color and the solution is grayish. If the sections have not been washed thoroughly the solution may become brownish.

If the fixation has been too short or the first result is unsuccessful, the sections may be heated in a second dish of the silver carbonate solution to which 12 drops of 5 per cent alcohol have been added. If desired the alcohol may be added to the first silver carbonate solution. When amber in color the sections are removed.

It is best to carry one section through to reduction and examine it in water with the microscope to see whether the impregnation is complete or whether a second heating in the silver carbonate solution is necessary.

3. Wash sections rapidly in distilled water; if they remain in water too long the staining is pale.

4. Reduce in 1 per cent formalin for 30 seconds.

5. Wash thoroughly in distilled water.

6. Tone in 1:500 aqueous gold chloride. Leave in the toning solution a few minutes until the sections become gray, then heat to 45° to 50° C. until dark purple.

7. Fix in 5 per cent aqueous sodium thiosulfate until flexible, about 30 seconds.

8. Wash in distilled water.

9. Float sections on slide and dehydrate in 95 per cent alcohol.

10. Clear in carbol-xylol-creosote (p. 249) and mount in balsam.

Results.—Background unstained; astrocytes clearly outlined; nucleus and pigment granules are stained and the fibers of the fibrous astrocytes can be seen as they pass through the cytoplasm of the cell; gliosomes are rarely stained.

When successful the results are brilliant, the astrocytes are stained completely and can easily be photographed. However, the method is variable and less reliable than Cajal's gold chloride sublimate method. If the nervous tissue has been fixed in formalin for a long time Hortega's method is more reliable than Cajal's.

Pathological astrocytes in cases of hypertrophic gliosis stain very well with silver carbonate.

If the cytoplasm of the astrocyte is to be studied the toning should be omitted and the sections mounted after reduction and washing in water. Toning reinforces the neuroglia fibrils and gives better preparations for routine work. If formalin is used for fixation without ammonium bromide the neurofibrils stain too deeply.

(*b*) Stains for Oligodendroglia

Del Río-Hortega's Silver Carbonate Method for Oligodendroglia (1921).—*Fixation.*—Fix thin pieces of tissue in formalin-ammonium bromide (p. 249) 12 to 48 hours. Warm tissue in fresh formalin-ammonium bromide for 10 minutes at 45° C. Cut frozen sections 15 to 20 μ thick.

Staining Solution.—

Del Río-Hortega's Strong Ammoniacal Silver Carbonate Solution

Silver nitrate, 10 per cent aqueous solution	5 cc.
Sodium carbonate, 5 per cent aqueous solution	20 cc.
Ammonia water, sufficient amount to dissolve the precipitate	
Water, distilled	up to 45 cc.

Add the ammonia water drop by drop until the precipitate is just dissolved, shaking vigorously. Filter and place in a dark bottle. The solution keeps well for long periods of time.

Method of Staining.—1. Wash sections in 2 changes of distilled water; the first water should contain 10 drops of ammonia water.

2. Place sections in the strong silver carbonate solution for 1 to 5 minutes. Determine by trial.

3. Agitate gently for 15 seconds.

4. Reduce in 1 per cent formalin; do not agitate.

5. Wash well in water.

6. Tone in a 1:500 gold chloride solution, at first at room temperature until sections are gray, then heating until sections are dark purple.

7. Fix in 5 per cent sodium thiosulfate solution for 30 seconds.

8. Wash in water.

9. Float sections on slide and dehydrate with 95 per cent alcohol.

10. Blot and clear in carbol-xylol-creosote (p. 249) and mount in balsam.

Results.—Oligodendroglia stain selectively; astrocytes may stain faintly; the microglia may stain well.

Penfield's Combined Oligodendroglia and Microglia Method (1928).—*Fixation.*—Fix for an indefinite period in 10 per cent formalin or in formalin-ammonium bromide (p. 249). About 1 week in formalin-ammonium bromide gives good results. Cut frozen sections 20 μ thick and place them in 1 per cent formalin in distilled water.

Staining Solution.—

Del Río-Hortega's Weak Ammoniacal Silver Carbonate Solution

Silver nitrate, 10 per cent solution	5 cc.
Sodium carbonate, 5 per cent solution	20 cc.
Ammonia water, sufficient amount to dissolve the precipitate	
Distilled water	up to 75 cc.

The ammonia water is added drop by drop shaking vigorously until the precipitate is just dissolved. Filter and keep in a dark bottle.

Method of Staining.—1. Place sections in a dish of distilled water to which 10 to 15 drops of strong ammonia water have been added; cover and leave overnight to remove the formalin.

2. Transfer sections directly to Globus' 5 per cent aqueous solution of hydrobromic acid in the incubator at 37° C. for 1 hour.

3. Wash in 3 changes of distilled water.

4. Mordant sections in 5 per cent aqueous solution of sodium carbonate for 1 hour. They may remain in the mordant 5 to 6 hours without ill effects.

5. Impregnate sections with or without washing in Hortega's weak silver carbonate solution. Leave in stain 3 to 5 minutes, or until they turn a uniform gray color when transferred to the reducer. Control by taking out a section at intervals of 1 to 2 minutes and examining with the microscope. At times good results are obtained by leaving the sections in the silver solutions until they turn a light brown.

6. Reduce in 1 per cent aqueous formalin and agitate.

7. Wash in distilled water.

8. Tone in yellow gold chloride (1:500) at room temperature until sections are a uniform bluish gray.

9. Fix in 5 per cent sodium thiosulfate 30 seconds.

10. Wash thoroughly in distilled water.

11. Float sections on slide and flatten out with needle.

12. Wash with 2 to 4 changes of 95 per cent alcohol from drop bottle.

13. Clear in carbol-xylol-creosote (p. 249), drain, blot and mount in balsam.

Results.—By this method both microglia and oligodendroglia may be stained with fairly constant results. The morphological differences of the two types of cells make it quite easy to distinguish them. This differentiation is even easier when either type of cell has undergone some pathological change. There may be at times faint staining of astrocytes, particularly if the sections are left too long in silver.

d. Microglia Stains

Most of the early intensive work on the nervous system was done with silver stains and a certain type of cell found in addition to the astrocytes and the oligodendroglia was regarded as a third form of neuroglia cell and was named microglia. This has led to much confusion because more recent work has proved that these cells are of mesodermal origin and that they occur in other tissues and organs of the body. They are well stained by Penfield's second modification of del Río-Hortega's silver carbonate method for oligodendroglia—Penfield's combined oligodendroglia and microglia method (p. 254).

Del Río-Hortega's Silver Carbonate Method for Microglia (1921).—*Fixation.*—Fix pieces of tissue, 3 mm. thick, in formalin-ammonium bromide (p. 249) 2 to 3 days at room temperature. Re-

move to fresh formalin-ammonium bromide in a covered glass dish and heat to 50° C. for 10 minutes. Cut frozen sections 20 to 25 μ thick and place them in distilled water.

Method of Staining.—1. Wash sections in 3 changes of distilled water, adding 4 to 5 drops of ammonia water to the second dish to remove the formalin.

2. Place 4 to 5 sections in Hortega's weak silver carbonate solution (p. 254) for 20 seconds to 2 minutes.

3. Reduce in 1 per cent formalin. Remove one section after 20 seconds from the silver carbonate solution. Agitate section in formalin by blowing on the surface of the solution. After 45 seconds remove a second section and after 2 minutes a third. If sections are brownish they have been in silver too long and astrocytes are stained as well as microglia. Control with microscope. Microglia cells are small, spider-like in shape; if neuroglia cells stain, they are larger and tan in color. Reduce for 1 minute.

4. Wash in distilled water.

5. Tone in 1:500 gold chloride until sections are gray or slightly purple, 10 to 15 minutes at room temperature.

6. Fix in 5 per cent sodium thiosulfate 1 minute.

7. Wash in water.

8. If desired the sections may be counterstained with van Gieson's picro-acid fuchsin (p. 92).

9. Float sections on slide, dry with filter paper.

10. Clear in carbol-xylol-creosote (p. 249).

11. Mount in balsam.

Results.—If successful the microglia are deeply impregnated; the neuroglia cells and fibers are slightly stained.

e. Stains for Connective Tissue and Blood Vessels of the Nervous System

The stroma of the nervous system consists of blood vessels and connective tissue as in other organs. Under normal conditions it is only moderate in amount but as the result of inflammatory conditions of various sorts it may be greatly increased either focally or diffusely.

The stroma is well brought out by the phloxine-methylene blue stain after Zenker fixation as are also the exudative cells present in inflammatory processes of all kinds. Special methods are van Gieson's picro-fuchsin and Mallory's aniline blue stain for collagen

which color the collagen more or less intensely. Elastic tissue stains are rarely important.

For the demonstration of the plasma cell exudate in paresis two special methods are valuable. The first is the toluidine blue method for staining the Nissl bodies. The other is the Unna-Pappenheim pyronine-methyl green stain. Both of these are more successful after fixation in 95 per cent alcohol. The pyronine-methyl green stain is less dependable than the toluidine blue but gives a contrast differentiation as the cytoplasm of the plasma cell is stained red and its nucleus green.

Masson's trichrome method (p. 156) gives an excellent picture of the connective tissue of the nervous system; it brings out fibrin and blood vessel walls especially well. It may be used on material fixed in any of the usual fixing fluids, even on sections chromatized for Weigert's method, so that alternating sections of tissue stained by Masson's and Weigert's methods can be made.

For the study of the brain capillaries an excellent method is that devised by Lepehne (1919) and applied to the cerebral vascular pattern by Pickworth (1934–35). The method is an application of the peroxidase reaction to thick frozen sections of brain tissue. Pickworth's original method is very difficult and complicated, and therefore a simplified method devised by Dr. A. C. P. Campbell and Dr. L. Alexander is given.

Lepehne-Pickworth Method for the Study of Brain Capillaries.—*Fixation.*—Fix in 10 per cent formalin for 1 to 3 weeks. Cut frozen sections 200 to 300 μ thick.

Staining Solutions.—

Solution A.—Dissolve 0.1 gm. of benzidine in 0.5 cc. of glacial acetic acid. Add 20 cc. of distilled water. Then dissolve 0.1 gm. of sodium nitroprusside in 10 cc. of distilled water and add it to the benzidine solution. Add water up to 100 cc. If a precipitate forms, filter. This solution must be made up fresh for use as it does not keep.

Solution B.—Add 2 to 3 drops of a 30 per cent hydrogen peroxide solution to 100 cc. of distilled water.

Method of Staining.—1. Wash sections in distilled water for ½ hour.

2. Put sections in Solution A for ½ hour at 37° C., shaking frequently.

3. Wash in distilled water for 10 seconds.
4. Place in Solution B for ½ hour at 37° C., shaking frequently.
5. Wash in distilled water.
6. Dehydrate in 70 and 95 per cent alcohol, followed by absolute.
7. Clear in xylol and mount in balsam.

Results.—The blood vessels stand out black against a light, almost colorless background.

(2) EYE

The pathologist is concerned chiefly with the lesions of the eye, not with its normal histology. Therefore, the routine fixatives and staining methods recommended are the most important. Ideal sections through the entire bulb count for much less than perfect fixation of the part where the lesion is present.

Fixation.—The simplest method is to cut into the bulb transversely in the middle with a sharp razor blade or scalpel to a slight depth only, and then with scissors and forceps to cut the bulb into halves. This can be done without disturbing the retina. The two pieces of tissue can then be fixed in Zenker's or any other fluid desired. Practically the same procedure is followed in removing the posterior half of an eye at autopsy. For preservation of the entire bulb Verhoeff (1926) recommends fixation for 48 hours or longer in the following fluid:

Formalin	10 cc.
Alcohol, 95 per cent	48 cc.
Picric acid	1 gm.
Water, distilled	36 cc.

Transfer directly to 70 per cent alcohol. Embed in celloidin after cutting in slices.

Staining.—The phloxine-methylene blue method discloses well the nature of lesions, whether inflammatory or a newgrowth, while phosphotungstic acid hematoxylin is of value for bringing out sharply the neuroglia fibrils in the retina and optic nerve, especially when sclerosis is present. It also stains fibroglia fibrils and fibrin in any acute inflammatory reaction.

(3) EAR

Lesions of the internal ear of interest to the pathologist are chiefly inflammatory in nature. Cultures and coverslip preparations may disclose the infectious agent. Tissue must be fixed, de-

calcified and stained in the same manner as lesions of the bone from other parts of the body. The petrous bone enclosing the middle ear is exceedingly dense and requires thorough decalcification. On this account it is often advisable to cut it into thin parallel slices with a fine jeweler's saw before or after fixation.

11. Skin and Appendages

Under this heading are included the epidermis and its supporting connective tissue, the corium, and in addition various epithelial appendages, namely the coil, mammary and sebaceous glands, the hairs and the nails.

Fixation.—The routine fixatives, Zenker's fluid, 10 per cent formalin and alcohol, are sufficient for most purposes.

Embedding.—It is often difficult to cut good paraffin sections of the skin because dehydration in alcohol is apt to harden the tissue. Therefore, the length of time in alcohol should be cut to a minimum. Celloidin embedding is on this account often advisable. For paraffin embedding oil of cedarwood should be used as a clearing reagent.

Staining.—Phloxine-methylene blue and alum hematoxylin followed by phloxine or van Gieson's stain are used for general purposes and bring out clearly a variety of structures. Phosphotungstic acid hematoxylin will stain sharply the epithelial prickles and fibrils in the epidermis, and the fibrils in the outer layer of epithelium lining the coil and mammary glands. It also stains the myoglia fibrils in the smooth muscle cells and the fibroglia fibrils in the stroma. Collagen is stained by the aniline blue methods, and elastic fibers by the special methods devised for them. Other stains and reactions often of value are those for fat in the sebaceous glands, for pigments, especially melanin, in the epidermis and hairs, and for certain retrograde products in the epidermis (eleidin, keratohyalin and cornification).

The structures and retrograde products that require special demonstration in the different parts of the skin and in the tumors arising from them are the following:

Epidermis.—Nuclei, cytoplasm, prickles and epithelial fibrils, eleidin, keratohyalin and cornification. The epidermis is thickest over the palms of the hands and the soles of the feet.

Keratohyalin is stained fairly deeply by alum hematoxylin. A more intense color can be obtained by overstaining and then de-

colorizing for a few seconds in a very dilute solution, 0.05 per cent, of potassium permanganate.

Eleidin must be fixed in formalin or in Orth's or Bouin's fluids. For staining it Unna recommends the picro-nigrosin method as follows:

Method for Staining Eleidin (Buzzi, 1889).—*Fixation.*—Fix in 10 per cent formalin. Cut frozen sections.

Method of Staining.—1. Stain sections with a saturated aqueous solution of picric acid (about 1.22 per cent) for 5 minutes.

2. Rinse in distilled water.

3. Stain in a 1 per cent aqueous solution of nigrosin for 1 minute.

4. Rinse in water followed by 95 per cent alcohol.

5. Clear in terpineol or oil of origanum and mount in balsam.

Results.—Eleidin blue-black; horny material bright yellow.

Coil or Sweat Glands.—Epithelial fibrils in the outer layer of the lining epithelial cells.

Mammary Glands.—Structurally the same as the coil glands but much more developed. Fat stains are sometimes of value.

Sebaceous Glands.—Fat is usually present in the cells.

Hairs.—Epithelial cells of the matrix produce great numbers of epithelial fibrils to which they owe their strength, also the pigment melanin from which their color is derived. The cells of the carcinomas arising from them are characterized by the production of numerous epithelial fibrils.

Nails.—Composed chiefly of the greatly thickened stratum lucidum with its excessive production of eleidin.

Corium.—Vascularized connective tissue supporting and nourishing the various epithelial structures. Contains numerous elastic fibrils, clumps of smooth muscle cells and fat cells and frequently pigmented cells (nerve fibers, tactile corpuscles and so on). Very rarely it produces amyloid.

In the skin there exists one pigment, melanin. For the demonstration of its origin and manufacture Bloch's dopa reaction is of importance. This reaction is an oxidation of 3,4-dioxyphenylalanine within pigment-forming cells by an oxidase.

Laidlaw's Modification of Bloch's Dopa Reaction (1932).—*Fixation.*—Use fresh tissue or tissue fixed only 2 to 3 hours in 5 per cent formalin. Cut frozen sections. Rinse in distilled water

for 5 seconds; do not allow the sections to remain in water more than a few seconds as it weakens the reaction.

Staining Solution.—

Stock Dopa Solution.—Dissolve 0.3 gm. 3,4-dioxyphenylalanine (levorotatory) in 300 cc. distilled water. Keep it in a tightly stoppered bottle in a refrigerator where it will remain good for many weeks. The solution is usable as long as it remains colorless or becomes but slightly reddish.

Buffer Solutions

A

Disodium acid phosphate ($Na_2HPO_4.2H_2O$)	11 gm.
Water, distilled	1000 cc.

B

Monopotassium acid phosphate (KH_2PO_4)	9 gm.
Water, distilled	1000 cc.

Keep tightly stoppered in the refrigerator. For use add 2 cc. of Solution B and 6 cc. of Solution A to 25 cc. of the stock dopa solution. At a given temperature, the speed of the reaction depends upon the *p*H. At 7.4 the reaction will be complete at 37.5° C. in 4 or 5 hours. The reaction may be hastened by using 1 cc. of Solution B, giving a *p*H of 7.7; or Solution B may be entirely omitted, giving a *p*H of 8.2. Such solutions react in about 60 minutes at 37.5° C. These hurried reactions tend to be overstained, and the slow ones give more delicate pictures. A trace of alkali hastens the reaction; a trace of acid inhibits it. All glassware, therefore, must be clean.

Method of Staining.—1. The sections in the buffered dopa solution are placed in the incubator at 37.5° C. for about 30 minutes.

2. Change the solution, adding fresh which has been kept cold in the refrigerator.

3. Inspect the reaction every 30 minutes with the microscope. In 2 or 3 hours the solution turns reddish, in 3 or 4 hours sepia brown. The appearance of the sepia tint means the completion of the reaction.

4. Wash in water.

5. Dehydrate in 95 per cent alcohol.

6. Clear in oil of origanum and mount in balsam, or

7. If a counterstain is desired, bring the sections back to water and stain. For this, Laidlaw recommends 0.5 per cent aqueous solution of cresyl violet, well differentiated with alcohol. We have found a 1 per cent aqueous solution of safranine also satisfactory.

Results.—The cytoplasm of the dopa-positive cells (melanoblasts and leukocytes) is gray or black. Melanin retains its natural yellow-brown color and collagen is colorless or light gray.

For stains for bacteria and fungi in hairs and scales of the epidermis see Chapters XIV and XV.

12. Endocrine System

Under this heading are grouped a number of organs, some of which have to be considered elsewhere also. The usual fixatives and staining methods recommended for obtaining a good insight into the pathological changes present are as necessary here as with other organs and tissues of the body. In addition, certain special methods are valuable for some of the tissues and they will be discussed briefly.

(1) THYROID GLAND

The colloid secretion is brittle after fixation in Zenker's fluid and embedding in paraffin so that it cracks in parallel lines. It is much better preserved by fixation in formalin or Orth's fluid and embedding in celloidin. It is acidophilic but so far no specific stain for it has been discovered. Fine acidophilic granules and occasionally coarser ones are present in the epithelial cells of the follicles and are believed to precede the formation of colloid. Special staining methods are needed for iron pigments frequently present following hemorrhage. Calcification is common and amyloid occurs rarely but may be extensive.

(2) PARATHYROID GLANDS

These are epithelial organs, usually four in number, of entodermal origin. They are composed of masses and strands of polygonal cells of four major types: (1) normal, chief or principal cell with deeply staining nucleus and faintly acidophilic cytoplasm—the only cell present until puberty; (2) water-clear cell; (3) pale oxyphil cell with finely granular cytoplasm; and (4) dark oxyphil cell. Fat cells are often present in the stroma.

Fixation.—Zenker's, Helly's and Bouin's fluids and 10 per cent formalin or alcohol are all recommended.

Staining.—The usual routine stains are advised. The oxyphil granules are brought out more or less sharply by phloxine, eosin and acid fuchsin. Special fixatives recommended for the granules are Regaud's, Champy's and Altmann's. One special stain is of much importance pathologically, namely Best's carmine stain for glycogen after alcohol fixation. Glycogen is usually present, often in large amounts.

(3) THYMUS

The two points of greatest interest are Hassall's corpuscles, because they are peculiar to the organ, and the marked phagocytosis of the lymphoid cells often present in infectious diseases, especially in diphtheria. The phagocytes are brought out particularly sharply by phosphotungstic acid hematoxylin after fixation in Zenker's fluid.

(4) PINEAL BODY

The pineal body arises by differentiation of cells from the ependyma. It contains numerous neuroglia fibrils; also, even in the first decade, concretions composed of calcium and magnesium phosphate and carbonate.

Fixation.—The routine fixatives serve all purposes. Zenker's fluid is especially important on account of the neuroglia fibrils present. Decalcification is often necessary because of the concretions present, but Zenker's fluid usually decalcifies the small ones.

Staining.—Of the routine stains phosphotungstic acid hematoxylin is especially valuable because it colors the neuroglia fibrils an intense blue, provided the tissue was fresh when put into Zenker's fluid. Collagen and elastic fibrils are stained by the usual methods. For special investigation of the histology of the pineal body, del Río-Hortega recommends two methods: the gold-sublimate method of Cajal for astrocytes, and the following modification of his own silver carbonate method for neuroglia cells and fibrils.

Del Río-Hortega's Modification of the Silver Carbonate Method for the Pineal Body (1923).—*Fixation.*—Formalin, 10 per cent, at least for 2 days. Cut frozen sections.

Staining Solution.—To 5 cc. of a 10 per cent aqueous silver nitrate solution add 20 cc. of a 5 per cent aqueous solution of sodium carbonate. Add carefully ammonia water drop by drop until the slightly yellow precipitate is just dissolved. Add 15 cc. of distilled water.

Method of Staining.—1. Wash sections carefully with distilled water several times.

2. Place in a 2 per cent aqueous silver nitrate solution, to 10 cc. of which 3 drops of pyridine have been added. Heat to 50° C. for 5 to 10 minutes, then let stand for several hours in the oven at 37° C., or leave the sections in the solution at room temperature for 24 hours. The sections should be dark brown in color.

3. Wash in distilled water, to 10 cc. of which 2 drops of pyridine have been added.

4. Impregnate in the silver carbonate solution given above, to 10 cc. of which 3 drops of pyridine have been added, heating the sections to 50° C. until they are sepia brown.

5. Wash in distilled water.

6. Reduce in 10 per cent formalin.

7. Tone in a 0.2 per cent gold chloride solution, heating slightly until the sections are violet in color.

8. Fix in 5 per cent sodium thiosulfate for 30 seconds.

9. Wash in water thoroughly.

10. Mount on slide, dry with filter paper and dehydrate with 95 per cent alcohol.

11. Clear in carbol-xylol-creosote (p. 249).

12. Drain and mount in balsam.

Results.—Neuroglia cells with their fibers are impregnated, as are also the parenchyma cells and connective tissue.

(5) PITUITARY GLAND

The organ consists of three parts—an anterior and a posterior lobe with a thin pars intermedia between. The anterior lobe is the largest, is of ectodermal origin, and contains cells of three different types—chromophobic, basophilic and acidophilic. The posterior lobe consists mostly of neuroglia tissue. The pars intermedia is composed of groups of epithelial cells and of follicles containing a colloid secretion.

Fixation.—Zenker's fluid, formalin and alcohol will serve every purpose, but others may be used if desired.

Staining.—For general purposes the routine stains of phloxine-methylene blue, Mallory's aniline blue, and phosphotungstic acid hematoxylin are recommended after Zenker fixation.

The following points will be found helpful. Methylene blue

stains the nuclei and in addition the hemofuscin granules so often present in the posterior lobe. Phloxine stains the acidophilic granules in the anterior lobe a deep red. After fixation in neutral formalin, alum hematoxylin stains the basophilic granules a deep blue and the acidophilic granules may be brought out at the same time by counterstaining with phloxine. Another excellent color contrast of the granules is obtained by using Mallory's aniline blue stain after formalin fixation: the acidophilic granules stain deep red and the basophilic blue. Phosphotungstic acid hematoxylin stains the basophilic granules intense blue after Zenker fixation.

Pigments often present, most abundantly in all cases of hemochromatosis, are best demonstrated by means of the ferrocyanide stain for hemosiderin, preferably after alcohol fixation, followed by Mallory's hemofuscin stain. The hemofuscin is colored bright red and the iron blue when these pigments are present in the posterior lobe. The neuroglia fibrils of the posterior lobe are stained blue by phosphotungstic acid hematoxylin after Zenker fixation, but only if the tissue is obtained very fresh, within 1 hour postmortem if possible.

MacCallum's Modification of Cowdry's Copper Hematoxylin Stain for the Pituitary Gland (1935).—According to MacCallum, Cowdry's copper hematoxylin stain for mitochondria, slightly modified and used on tissue fixed in Helly's fluid, is a more or less specific method of differentiating the cells of the pars intermedia from the basophilic cells of the anterior lobe of the pituitary gland.

Fixation.—Fix in Helly's fluid and embed in paraffin.

Method of Staining.—1. Remove the paraffin, treat with iodine and sodium thiosulfate and wash in water.

2. Place sections in a saturated aqueous solution of normal copper acetate (about 12.5 per cent) for 5 minutes.

3. Wash in several changes of distilled water.

4. Stain for 1 minute in a weak hematoxylin solution made by adding 2 cc. of a well ripened 10 per cent hematoxylin solution in absolute alcohol to 50 cc. of distilled water.

5. Wash in distilled water.

6. Immerse in a 3 per cent aqueous solution of potassium bichromate for 1 minute.

7. Wash in distilled water.

8. Differentiate, controlling with the microscope, in Weigert's borax-ferricyanide mixture until the color is deep grayish yellow.

Borax	2	gm.
Potassium ferricyanide	2.5	gm.
Water, distilled	100	cc.

9. Wash several hours in tap water.
10. Dehydrate in 95 per cent followed by absolute alcohol.
11. Clear in xylol and mount in balsam.

Results.—Basophilic granules in the cells of the anterior lobe of the pituitary gland black; granules in the cells of the pars intermedia unstained.

(6) ADRENAL GLANDS

The cortex and the medulla of the adrenal glands originate separately; the cortical cells from the celomic epithelium (mesoderm), the medulla from the sympathetic nervous system (ectoderm).

The cortical epithelial cells form three layers. The cells of the inmost, zona reticularis, contain brownish pigment; those of the middle, zona fasciculata, often have fat droplets, the majority of which are doubly refractive; those of the outer, zona glomerulosa, show nothing characteristic.

The medulla is composed of polymorphous cells with delicate cytoplasm containing granules and droplets which can be fixed and colored brown by chrome salts. These cells belong to the chromaffin system. The medulla also contains typical ganglion cells of the sympathetic nervous system.

Fixation.—For general purposes the routine fixatives are advisable. Thin, transverse slices should be cut through the middle of the organ so as to include the medulla, and the tissue should be handled very gently because of its fragility, especially if at all postmortem. The browning of the chromaffin cells is due to reduction of potassium bichromate or of chromic acid to chrome dioxide.

Staining.—The cells of the cortex are well demonstrated by the routine stains. Fat, of course, must be colored by scarlet red or by osmic acid. The cells that require special treatment in order to demonstrate them are the chromaffin cells of the medulla. Not all fixatives containing chrome salts are suitable, as for example Flemming's and Zenker's. The two solutions recommended are

Champy's and Orth's. The browning is best seen in frozen sections mounted, unstained or colored with carmalum, in glycerin. They may also be blackened by the silver method devised by T. and A. Ogata (p. 268). The lead chloride hematoxylin stain (p. 226) shows that the granules in the chromaffin cells must take up lead because they are colored blue by the hematoxylin solution. This affords a simple way for obtaining a permanent stain.

(7) CHROMAFFIN SYSTEM

Under this heading are included the medulla of the adrenal glands, the paraganglia and the carotid glands. They are characterized by containing cells that possess the property of reducing chrome salts.

Fixation.—The tissue must be as fresh as possible. The action of alcohol, corrosive sublimate and acetic acid is unfavorable; therefore, fixatives containing them should be avoided. Champy's and Orth's fluids are excellent. Müller's fluid is also recommended.

Staining.—Three different types of staining may be employed: (1) a simple nuclear stain such as carmalum, which allows the cytoplasm of the chromaffin cells (fixed in a suitable chrome salt solution) to show its brown coloring; (2) a toluidine blue stain, which turns the chromaffin cells green; and (3) a silver stain, which blackens the chromaffin substance.

Wiesel's Method for Chromaffin Cells (1902).—*Fixation.*—Fix for 1 to 4 days in the following solution:

Potassium bichromate, 5 per cent	10 cc.
Formalin, 10 per cent	20 cc.
Water, distilled	20 cc.

Transfer to 5 per cent potassium bichromate for another 1 to 2 days. Wash in running water 24 hours and embed in paraffin.

Method of Staining.—1. Stain sections for 20 minutes in a 1 per cent aqueous toluidine blue solution.

2. Wash in tap water 5 minutes.

3. Stain for 20 minutes in a 1 per cent aqueous solution of safranine.

4. Differentiate in 95 per cent alcohol until the sections appear blue again.

5. Clear in carbol-xylol, followed by xylol and mount in balsam.

Results.—Nuclei red; cytoplasm of the chromaffin cells green; cytoplasm of all other cells blue.

Schmorl's Method for Chromaffin Cells (1914).—*Fixation.*—Fix in 10 per cent formalin or Orth's fluid. Cut frozen or paraffin sections.

Method of Staining.—1. Stain sections for 24 hours in a dilute Giemsa solution (10 drops of stain [p. 183] to 10 cc. distilled water).

2. Rinse in distilled water.

3. Differentiate quickly in a 0.25 per cent aqueous solution of acetic acid.

4. Dehydrate rapidly in 95 per cent and absolute alcohol.

5. Clear in xylol and mount in balsam or preferably in oil of cedarwood.

Results.—Nuclei deep blue; cytoplasm of chromaffin cells green.

Silver Method of T. and A. Ogata for Chromaffin Cells (1922–23).—*Method of Staining.*—1. Place thin slices of fresh unfixed tissue for 2 hours in a 1 per cent solution of ammonia water in the dark.

2. Place tissue in Bielschowsky's ammoniacal silver solution (p. 222) diluted to 5 per cent for 3 to 5 hours in the dark.

3. Place in 1 per cent solution of ammonia water renewed several times for 30 minutes in the dark.

4. Fix in a 3 per cent aqueous solution of sodium thiosulfate for 1 hour in the dark.

5. Wash in water for 1 hour.

6. Fix in 10 per cent formalin.

7. Cut frozen sections.

8. Place sections on slide, treat with 95 per cent alcohol, blot and clear with xylol. Repeat the blotting process at least twice and then mount in balsam.

Results.—In the chromaffin cells numerous black granules are present.

(8) GLOMUS COCCYGEUM

For the glomus coccygeum the aniline blue stain after fixation in Zenker's fluid is useful for showing the reticulum surrounding the individual cells.

CHAPTER XIV

BACTERIAL STAINS

Bacteria are demonstrated in sections of tissues almost entirely by means of aniline dyes, of which three thus far have proved to be particularly valuable, namely, methylene blue, basic fuchsin, and crystal violet. One or more solutions of each of these dyes have become famous because of their efficacy in staining and their good keeping qualities. They will be referred to later.

All bacteria yet known will stain when placed in appropriate staining solutions. Some, however, are stained quickly, while others are stained with difficulty; some give up the stain readily to decolorizers, while others retain it tenaciously. In consequence of their reactions to certain dyes and to certain decolorizers, bacteria, from the point of view of staining, may be divided into three groups: (1) bacteria that stain by Gram's method (Gram-positive); (2) bacteria that do not stain by Gram's method (Gram-negative); and (3) bacteria that stain by the acid-fast or tubercle bacillus method.

The most important pathogenic bacteria, listed according to their reaction to Gram's stain, are the following:

Gram-Positive Bacteria

Streptococci
Pneumococci (*Diplococcus pneumoniae*)
Staphylococci
Micrococci
Bacillus subtilis
Bacillus anthracis
Bacillus tetani (*Clostridium tetani*)
Bacillus welchii (*Clostridium welchii*)
Bacillus botulinus (*Clostridium botulinum*)
Bacillus diphtheriae (*Corynebacterium diphtheriae*)
Bacillus tuberculosis (*Mycobacterium tuberculosis*)
and other acid-fast bacteria
Many actinomycetes

Gram-Negative Bacteria

Meningococcus (*Neisseria intracellularis*)
Micrococcus catarrhalis (*Neisseria catarrhalis*)
Gonococcus (*Neisseria gonorrhoeae*)
Bacterium coli (*Escherichia coli*)
Bacterium aerogenes (*Aerobacter aerogenes*)
Proteus
Bacillus typhosus (*Eberthella typhosa*)
Paratyphoid bacilli (*Salmonella paratyphi*)
Dysentery bacilli (*Shigella dysenteriae*)
Friedländer's bacillus (*Klebsiella pneumoniae*)
Bacillus pyocyaneus (*Pseudomonas aeruginosa*)
Bacillus influenzae (*Hemophilus influenzae*)
Koch-Weeks bacillus (*Hemophilus conjunctivitidis*)
Morax-Axenfeld bacillus (*Hemophilus lacunatus*)
Ducrey's bacillus (*Hemophilus ducreyi*)
Bacillus mallei (*Actinobacillus mallei*)
Bacillus pestis (*Pasteurella pestis*)
Brucella group (infectious abortion and undulant fever)
Bacterium tularense (*Pasteurella tularensis*)
Vibrio cholerae (*Vibrio comma*)

It has been customary in the past to fix all tissues in which bacteria were to be demonstrated in alcohol. In recent years formalin has been used for the same purpose. Fixation in Zenker's fluid is superior to either because of its perfect preservation, not only of all bacteria but also of the tissues, so that by means of proper staining both the pathogenic organism and the lesion it produces can be perfectly and faithfully demonstrated.

Sections to be stained for bacteria may be divided into two classes—paraffin and celloidin. Paraffin sections should, as a rule, be attached to the slide by means of Mayer's albumin-glycerin mixture. Celloidin embedding is to some extent a drawback to stains for certain organisms because the celloidin tends to hold the color so that the bacteria are not so distinct as they otherwise would be. Still, it is sometimes necessary to stain celloidin sections for bacteria. It will usually be found advisable to attach the sections to the slide by means of ether vapor. They will then keep perfectly flat in any

staining solution, and may be heated without danger of wrinkling or shrinking.

1. GENERAL METHODS

Of the general stains, the most useful for demonstrating bacteria in sections are phloxine-methylene blue and Giemsa's.

The Phloxine-Methylene Blue Stain.—Directions for this stain are given on page 86. It is used after fixation in Zenker's fluid and is especially useful, not only as a stain for bacteria, but also for the faithful demonstration of the histological changes present in the tissues.

Wolbach's Modification of Giemsa's Stain for Bacteria in Paraffin Sections.—For sections this modification gives better results than the original Giemsa stain. Directions for this method are given under bone marrow (p. 195).

Methyl Green-Pyronine Stain (The Unna-Pappenheim Method, as Modified by Saathof for Bacteria [1905]).—*Fixation.*—Absolute alcohol or Carnoy's fluid. Embed in paraffin or celloidin.

Staining Solution.—

Methyl green	0.15	gm.
Pyronine	0.5	gm.
Alcohol, 95 per cent	5	cc.
Glycerin	20	cc.
Carbolic acid water, 2 per cent	75	cc.

Method of Staining.—1. Stain sections in the methyl green-pyronine stain for 2 to 4 minutes.

2. Wash in distilled water.

3. Dehydrate quickly in absolute alcohol. If acetone is used instead of absolute alcohol for dehydration there is less danger of decolorizing the cytoplasm of the cells.

4. Clear in xylol and mount in balsam.

Celloidin sections are cleared in terpineol or in oil of origanum and mounted in balsam.

Results.—Chromatin green; bacteria bright red; all other structures varying shades of red.

2. SPECIFIC METHODS

Gram's Stain for Bacteria in Smears (1884).—*Fixation.*—Dry thin, evenly spread smears in the air and fix by passing through a flame.

Staining Solutions.—

Stirling's Crystal Violet Solution (p. 90)

Iodine Solution

Iodine	1 gm.
Potassium iodide	2 gm.
Water, distilled	100 cc.

Method of Staining.—1. Apply the crystal violet solution to the smear for 20 seconds.

2. Wash thoroughly in tap water.

3. Treat with the iodine solution for 20 seconds.

4. Wash in tap water.

5. Decolorize with 95 per cent alcohol until no more color comes off.

6. Wash in tap water.

7. Counterstain with 0.1 per cent aqueous solution of basic fuchsin, or with a 1 per cent aqueous solution of safranine or pyronine for 20 seconds.

8. Wash in tap water and dry.

Results.—Gram-positive bacteria deep violet; Gram-negative bacteria take the color of the counterstain.

The Gram-Weigert Method of Staining Bacteria in Sections (1887).—*Fixation.*—Preferably Zenker's fluid. Embed in paraffin or celloidin.

Method of Staining.—1. Stain sections lightly in alum hematoxylin (p. 70).

2. Wash in several changes of tap water.

3. Place in a 2.5 per cent aqueous solution of phloxine or eosin for 5 to 15 minutes in the paraffin oven.

4. Wash in water.

5. Stain in aniline crystal violet (Ehrlich's or Stirling's formula with crystal instead of gentian violet) for ½ to 1 hour.

6. Wash in water.

7. Treat with Gram's iodine solution (p. 82) 1 to 2 minutes.

8. Wash in water.

9. Blot with filter paper and decolorize and clear in several changes of aniline. Aniline and xylol in equal parts may be used instead of aniline for decolorizing organisms that stain delicately.

10. Rinse in xylol and mount in balsam.

Celloidin sections are best stained by attaching to the slide with ether vapor before Step 1, or by blotting on the slide after staining with phloxine (Step 3).

It is advisable to shorten the staining time for celloidin sections. Remove aniline by rinsing in xylol or in oil of origanum or terpineol before mounting in balsam.

Results.—Gram-positive organisms deep violet; nuclei blue to violet; connective tissue red; Gram-negative bacteria unstained.

Glynn's Modification of the Gram Stain for Paraffin Sections (1935).—*Fixation.*—Zenker's fluid (preferably without acetic acid), Helly's or Bouin's solutions, or 10 per cent formalin. Embed in paraffin.

Staining Solution.—

Carbol Crystal Violet

Crystal violet	1 gm.
Phenol crystals	1 gm.

Triturate in a mortar and add:

Alcohol, absolute	10 cc.

Dilute this stock solution 10 times with distilled water; allow to stand for 48 hours and filter before using.

Method of Staining.—1. Treat paraffin sections in the usual way up to water.

2. Stain in the carbol crystal violet solution for 2 minutes.
3. Drain but do not wash.
4. Apply Gram's iodine solution (p. 82) for 1 minute.
5. Place in acetone until no more color is removed, 10 to 15 seconds.
6. Wash in water.
7. Apply 0.05 per cent basic fuchsin in N/500 hydrochloric acid (*p*H 2–3) for 3 minutes.
8. Drain but do not wash.
9. Apply a saturated aqueous solution of trinitrophenol (about 1 per cent) for 30 seconds to 1 minute.
10. Wash in water.
11. Differentiate and dehydrate in acetone for 10 to 15 seconds.
12. Clear in xylol and mount in balsam.

Results.—Gram-positive bacteria deep violet; Gram-negative

bacteria deep red; nuclei lighter red; cytoplasm faint yellow. Nuclei of nerve cells show differentiation of nucleoli and Nissl bodies; axones unstained; myelin sheaths violet. Edema fluid, fibrin and fibers of connective tissue pale pink. Erythrocytes yellow. Diphtheria bacilli show barred and granular forms. Human tubercle bacilli are less deeply stained than other Gram-positive organisms and show distinct beading. The clubs of actinomycetes are red.

MacCallum's Modification of Goodpasture's Method for Gram-Positive and Gram-Negative Bacteria (1919).—*Fixation.*—Zenker's or Helly's solution. Embed in paraffin and cut thin sections.

Staining Solutions.—

Goodpasture's Stain

Basic fuchsin	0.59	gm.
Aniline	1	cc.
Phenol crystals	1	gm.
Alcohol, 30 per cent	100	cc.

Stirling's Crystal Violet Stain (p. 90)

Method of Staining.—1. Stain sections for 10 to 30 minutes in Goodpasture's stain.

2. Wash in water.

3. Differentiate in strong formalin for a few seconds, until the bright red color changes to a clear rose.

4. Wash in water.

5. Counterstain in a saturated aqueous picric acid solution (about 1.22 per cent) 3 to 5 minutes or less, until section assumes a purplish yellow color.

6. Wash in water.

7. Differentiate in 95 per cent alcohol. This causes the red color to reappear; some of it is washed out together with some of the yellow color.

8. Wash in water.

9. Stain for 5 minutes in Stirling's crystal violet solution.

10. Wash in water.

11. Immerse in Gram's iodine solution (p. 82) for 1 minute.

12. Blot dry without washing in water.

13. Treat with equal parts of aniline and xylol until no more color comes away.

14. Rinse with 2 changes of xylol and mount in balsam.

Results.—Gram-positive organisms blue; Gram-negative organisms red; all other tissue elements various shades of red to purple.

W. H. Smith's Method for Staining Bacterial Capsules in Sections.—*Fixation.*—Zenker's fluid. Embed in paraffin.

Staining Solution.—Shake 1 part of aniline green in 200 parts of a 3 to 6 per cent aqueous solution of yellowish water-soluble eosin. Allow the solution to stand for 1 to 2 hours before filtering to remove the precipitate.

Method of Staining.—1. Cover sections with Ehrlich's or Stirling's aniline crystal violet solution (pp. 89, 90) for a few seconds and warm by drawing the slide through a flame 2 or 3 times.

2. Wash in Gram's iodine solution (p. 82).

3. Wash with formalin.

4. Decolorize with 95 per cent alcohol.

5. Wash quickly with Gram's iodine solution.

6. Cover with the aniline green-eosin mixture, warming in a flame for a few seconds.

7. Wash in distilled water.

8. Dehydrate with 95 per cent followed by absolute alcohol.

9. Clear in xylol and mount in balsam.

To obtain the best results the duration of the application of the various reagents must be varied with each preparation, and in some instances, where a deep stain is desired, the stronger solution of iodine (p. 82) may give better results.

Decolorization by alcohol may have to be supplemented by rinsing with ether or with aniline-xylol, as the Gram stain may be so intense as to mask the red staining capsules.

Results.—Bacterial capsules red; Gram-positive bacteria blue.

Stain for Acid-Fast Bacilli in Smears.—*Fixation.*—Dry smears in the air and fix by passing through a flame.

Method of Staining.—1. Apply Ziehl-Neelsen's (p. 88) or Verhoeff's (p. 88) carbol fuchsin stain for 3 to 5 minutes, steaming gently.

2. Wash in tap water.

3. Decolorize in acid alcohol (70 per cent alcohol containing 1 per cent hydrochloric acid) for 10 seconds.

4. Finish decolorizing in 95 per cent alcohol until no more color comes off, usually about 20 seconds longer.

5. Wash in water.

6. Counterstain with a saturated aqueous solution of methylene blue or with Loeffler's methylene blue (p. 84) for 10 seconds.

7. Wash in tap water.

8. Dry and examine.

Results.—Acid-fast bacteria red; non-acid-fast bacteria blue.

Stain for Tubercle Bacilli in Sections.—*Fixation.*—Alcohol, 10 per cent formalin, corrosive sublimate, Zenker's or Flemming's solution. Embed in paraffin or celloidin.

Staining Solutions.—Verhoeff's carbol fuchsin (p. 88), or Ziehl-Neelsen's carbol fuchsin (p. 88).

Method of Staining.—1. Stain sections lightly in alum hematoxylin (p. 70).

2. If overstained, sharpen nuclear detail with acid alcohol (1 per cent hydrochloric acid in 70 per cent alcohol) and wash thoroughly in water.

3. Stain either with Verhoeff's carbol fuchsin or with Ziehl-Neelsen's carbol fuchsin solution by steaming the sections for 5 minutes, or by leaving them in the paraffin oven for 1 hour or at room temperature overnight.

4. Decolorize in the acid alcohol for 20 seconds.

5. Wash thoroughly, to remove the acid, in water to which 2 to 3 drops of ammonia water have been added.

6. Differentiate in 95 per cent and dehydrate in absolute alcohol.

7. Clear in xylol and mount in balsam.

If celloidin sections are used differentiate in 95 per cent alcohol, clear in terpineol or in oil of origanum, blot on the slide and mount in balsam.

Results.—Tubercle bacilli brilliant red; nuclei blue.

Flexner's Method for Staining Leprosy Bacilli.—The leprosy bacillus is more difficult to stain than the tubercle bacillus because it does not retain the carbol fuchsin dye so well.

Fixation.—Alcohol, 10 per cent formalin, Zenker's fluid. Paraffin sections are preferable.

Method of Staining.—1. Stain in alum hematoxylin (p. 70) to get a sharp nuclear stain.

2. Wash in water.

3. Stain in one of the carbol fuchsin solutions mentioned above for 2 to 5 minutes by steaming, or for 30 to 60 minutes at room temperature.

4. Wash in water.

5. Treat with Gram's iodine solution (p. 82) for ½ to 1 minute.

6. Wash in water.

7. Blot section, and decolorize in aniline.

8. Rinse in xylol and mount in balsam.

Results.—Leprosy bacilli red; nuclei blue.

CHAPTER XV

MISCELLANEOUS INFECTIOUS AGENTS

1. ACTINOMYCETES

ACTINOMYCETES are regarded as occupying a position intermediate between bacteria and molds. These organisms grow as branching threads or mycelia which are Gram-positive. In animal tissues these mycelia are often surrounded by dense hyaline sheaths, the so-called "clubs."

(1) ACTINOMYCOSIS

Caused by *Actinomyces hominis*. In specimens of pus from subacute and chronic lesions the organisms appear as gray or yellowish granules, known as "sulfur granules." The organism is best demonstrated by examining unstained the granules, gently crushed beneath a coverslip. They are seen to be made up of a central, tangled mass of filaments from which radiate delicate threads that may terminate in typical club-shaped bodies. A Gram stain on a crushed granule shows isolated and matted Gram-positive filaments, many of which are branched.

In staining the actinomyces it is important to stain not only the filaments and other forms of the organism, but also the hyaline sheaths that surround the ends of the filaments. Phloxine followed by methylene blue sometimes gives excellent results. Satisfactory preparations may also be obtained by staining with alum hematoxylin followed by a strong solution of phloxine or eosin, then placing the sections for 5 to 30 seconds in acid alcohol and washing thoroughly in water before dehydrating in alcohol.

It is believed that the two following methods will give better results than can be obtained by any of the other methods published for this purpose. The first is, perhaps, the better and surer, although the clubs are sometimes stained more intensely by the second method. Both methods can be used on either paraffin or celloidin sections. Method 1 is described below for paraffin, Method 2 for celloidin sections.

Mallory's Stains for the Actinomyces (1895).—**Method 1.**—*Fixation.*—Alcohol or 10 per cent formalin is preferable to Zenker's fluid as a fixative for the study of this microorganism, but not for study of the lesions it produces.

Method of Staining.—1. Stain paraffin sections in alum hematoxylin (p. 70) for 3 to 5 minutes.

2. Wash in water.

3. Stain in a 2.5 per cent aqueous solution of phloxine, or a 5 per cent aqueous solution of eosin, for 15 minutes in the paraffin oven.

4. Wash in water.

5. Stain in Stirling's or Ehrlich's aniline crystal violet solution (pp. 90, 89) for 5 to 15 minutes.

6. Wash in water.

7. Treat with Gram's iodine solution (p. 82) for 1 minute.

8. Wash in water and blot with filter paper.

9. Differentiate in several changes of aniline until no more color comes off.

10. Rinse in several changes of xylol and mount in balsam.

Results.—Branched organisms blue; hyaline sheaths ("clubs") pink to red.

Method 2.—*Fixation.*—The same as in Method 1.

Method of Staining.—1. Stain celloidin sections lightly in alum hematoxylin (p. 70) 3 to 5 minutes.

2. Wash in water.

3. Place sections in 95 per cent alcohol and attach to the slide with ether vapor.

4. Stain in Stirling's or Ehrlich's aniline crystal violet solution (pp. 90, 89) for 5 to 10 minutes.

5. Wash in water and blot with filter paper.

6. Place in aniline saturated with basic fuchsin for 1 to 3 minutes.

7. Wash out the fuchsin with pure aniline until the clubs are sharply differentiated, watching the process under the low power of the microscope.

8. Rinse in several changes of xylol and mount in balsam.

Results.—Branched organisms blue; hyaline sheaths ("clubs") pink to red. In sections containing young colonies it is possible by these methods to demonstrate the ends of the threads stained blue, surrounded by the hyaline cell membrane stained pink.

(2) Mycetoma (Madura Foot)

The maduromycoses are characterized by the presence in pus or in the tissues of granules of various colors—white, black and red. The pale or white granules are usually due to infection with *Actinomyces madurae,* although certain other varieties have been described in connection with these granules. The black granules are due to a variety of organisms, the best known one being *Madurella mycetomi.* The red granules are associated with infection with *Actinomyces pelletieri.*

The methods for examining these organisms in pus and tissues are the same as those employed for *Actinomyces hominis.*

2. YEASTS AND MOLDS (ASCOMYCETES AND HYPHOMYCETES)

These are unicellular or multicellular organisms usually larger than bacteria. They multiply by cell fission, budding, and by the formation of asexual and sexual spores. The exact classification of the various members of these groups is still unsettled in many instances. The terminology employed below, while in some cases botanically incorrect, is that generally used in medical mycology. In general, the organisms are all Gram-positive and hence are well brought out by the Gram-Weigert stain. Stained with phloxine-methylene blue or Giemsa's stain the organisms appear blue.

The important diseases and the causative organisms are given below:

(1) Blastomycosis

An infection of the skin or internal organs due to *Blastomyces hominis.* In sections the organisms appear as round and budding forms surrounded by a dense capsule. This capsule stains red with the phloxine-methylene blue stain. The capsules are especially well brought out by Mallory's aniline blue collagen stain, which stains them an intense blue while the microorganisms themselves stain yellow.

(2) Torulosis

The infecting agent is known as *Torula histolytica.* This organism produces lesions in the lungs and central nervous system. In sections it appears as a round or ovoid, often budding form, surrounded by a heavy capsule which stains similar to that of the blastomyces.

(3) Moniliasis

The main types of this disease are the following:

Thrush.—Caused by *Monilia albicans.* Thrush is a disease of the oral cavity and pharynx, characterized by the formation of a

membrane. In sections the organisms appear as simple and branched mycelia.

Cutaneous Moniliasis.—As its name implies, an infection of the skin due to *Monilia albicans*.

Pulmonary Moniliasis.—Moniliae, while probably secondary invaders, in the majority of instances, nevertheless apparently play a rôle in producing some diseases of the lung.

(4) Dermatomycoses

These are diseases of the skin due to members of the hyphomycetes group. The diagnosis of these diseases is usually made by placing scrapings from the lesion on a slide in a drop of 20 per cent sodium or potassium hydroxide, covering with a coverslip and examining unstained. The hydroxide is used to dissolve or clear the tissue elements, thus rendering the microorganisms more easily visible. These appear as branched, segmented mycelia or spores, or as both. The differential diagnosis is based on the type of mycelia, the number and size of the spores, and also on the location of the spores with respect to the hairs. The commonest diseases belonging to this group are the following:

Favus.—Commonly a disease of the scalp but may involve the skin and nails. The organism causing this disease is known as *Achorion schoenleinii*.

Ringworm or Tinea.—A disease usually of the scalp but may occur elsewhere. The causative organisms are Microsporon and Trichophyton. A considerable number of species of each are recognized and cultural study is necessary for their final classification.

Eczema Marginatum.—A skin disease of the groin due to *Epidermophyton inguinale*.

Interdigital Ringworm (Athlete's Foot).—A dermatomycosis of the hands and feet due apparently to various fungi. Among them may be mentioned Trichophyton, Epidermophyton and Monilia.

3. RICKETTSIAE

The Rickettsiae are minute bacillary organisms varying in size from filaments approaching bacilli in dimensions to coccoid forms which are just visible. The exact nature of this group of organisms is as yet undetermined. In tissues they always occur in an intracellular position. They are Gram-negative and stain poorly or not

at all with the usual bacterial stain. Wolbach's modification of Giemsa's stain (p. 195) and the stain given below, used on tissues fixed in Regaud's fluid, are to be recommended. So stained, the Rickettsiae are red to reddish purple. With this same stain on Zenker-fixed tissue, they appear blue.

Stain for Rickettsiae and Rickettsia-like Organisms in Tissues.—This method is recommended by Dr. S. B. Wolbach and Dr. Henry Pinkerton.

The combination of fixation in Regaud's fluid and staining by the Giemsa method is invaluable for the demonstration in tissues, not only of Rickettsiae but also of *Bartonella bacilliformis* and *Bacterium tularense*. The peculiar virtue of the method lies in the sharpness with which it differentiates minute Gram-negative intracellular organisms from the cytoplasm in which they lie.

Fixation.—Fix thin pieces of tissue in Regaud's fluid, made up as follows, for 24 to 48 hours, renewing the fixative every 12 hours.

Potassium dichromate	25 gm.
Sodium sulfate	10 gm.
Water, distilled	1000 cc.

Before using add 20 cc. of strong (40 per cent) formalin to each 100 cc. of the above solution.

After washing tissues for 24 hours in running water, pass through graded percentages of alcohol, clear in oil of cedarwood and embed in paraffin. Cut sections as thin as possible.

This method has been successfully used for Rickettsiae in tissues previously fixed for several months in formalin and thoroughly washed before refixing in Regaud's fluid.

Staining Solution.—Dissolve 3 gm. of azure II eosin and 1 gm. of azure II as completely as possible in 250 cc. of acetone-free absolute methyl alcohol at 60° C. Add 250 cc. of glycerin (C.P.) heated to 60° C., and allow the solution to stand overnight in a desiccator in a loosely stoppered bottle. Transfer to small bottles and keep tightly sealed when not in use.

Method of Staining.—1. After passing sections through xylol and descending percentages of alcohol in the usual way, place in distilled water for 5 minutes. All distilled water should be made neutral in reaction. Freshly boiled distilled water usually requires no further treatment.

2. Place in the following dilution of Giemsa's stain for 1 hour.

Giemsa's stain	2.5	cc.
Alcohol, absolute methyl, acetone-free	2.5	cc.
Sodium carbonate, 0.5 per cent aqueous solution	5	drops
Water, distilled	100	cc.

Pour off the solution and replace with a fresh solution identical with the first, but diluted with an equal volume of distilled water. Leave overnight in this dilute solution.

3. Rinse quickly in distilled water and place in a second change of distilled water until the sections take on a pink color, usually 10 to 15 minutes.

4. Differentiate in 95 per cent alcohol to which a few drops of colophony have been added.

5. Dehydrate in absolute alcohol.

6. Clear in xylol and mount in neutral oil of cedarwood.

Results.—In a well differentiated section the Rickettsiae or other organisms should be stained an intense reddish purple, unlike any other color in the section, so that cells containing them can be located under low power. Nuclei stain blue or dark purple, cytoplasm pale to deep blue, and collagen and muscle fibers light pink. Erythrocytes are at a transitional stage, varying from pale slaty blue to pale olive yellow or pink.

Well stained sections generally have a fairly deep bluish purple appearance to the naked eye. Organisms may be well stained at times even when there is no color other than blue in the sections, and such sections will often differentiate well with prolonged exposure to sunlight.

Perfect results can be expected only after some experience with the method, and sections must be treated individually since certain tissues, such as skin, differentiate quickly and well, while others, such as brain, are extremely difficult to differentiate properly.

The diseases due to the Rickettsiae are all insect-borne and probably all require an intermediate host (a rodent). Two diseases belonging to this group are the following:

(1) **Typhus Fever.**—Caused by *Rickettsia prowazeki.* In sections the organisms are found in vascular lesions especially in the skin but also in the skeletal muscles, brain, kidneys and testes.

In such lesions they occur in the endothelial cells of the blood vessels and also in perivascular mononuclear phagocytes.

(2) **Rocky Mountain Spotted Fever.**—Due to *Dermacentroxenus rickettsi.* In sections the Rickettsiae are present in the endothelial cells and smooth muscle cells of the blood vessels, of the skin, skeletal muscles, testes and scrotum. They also occur in mononuclear cells in the adventitia of the blood vessels.

4. FILTRABLE VIRUSES

A number of diseases are caused by infectious agents that are characterized by being not visible by any of the ordinary methods and by being able to pass through bacteria-retaining filters. Certain of these viruses cause the formation in the cytoplasm or nuclei of the cells of the infected tissue characteristic bodies known as "inclusion bodies." The true nature of these bodies is not known. Only those virus diseases that are important for man and that are characterized also by the formation of inclusion bodies will be mentioned below.

The phloxine-methylene blue stain or Wolbach's modification of Giemsa's stain on tissues fixed in Zenker's fluid or in the same fluid without acetic acid will be found satisfactory for the demonstration of inclusion bodies.

(1) **Rabies.**—A disease transmitted to man usually from dogs or wolves. The inclusion bodies diagnostic of this disease are known as Negri bodies. They are best demonstrated in the brain in the hippocampus major and in the Purkinje cells of the cerebellum. These bodies are round or oval, but may be irregular, pear shaped, or triangular in form. They vary in diameter up to 23 μ. They contain small vacuoles, in some of which are granules of varying size and number; generally there is a central larger structure surrounded by smaller ones. In preparations stained by phloxine-methylene blue the bodies generally are colored deeply with phloxine, with the exception of the granules, some of which take the methylene blue.

The bodies may be sought for in smear preparations or in sections. Pieces of gray brain substance should be taken for examination from the cortex in the region of the fissure of Rolando, from the hippocampus, and from the vermis of the cerebellum.

For demonstrating the Negri bodies in smear preparations pro-

ceed as follows: A small bit of the gray matter of the brain chosen for examination is cut out and placed on a clean slide. The cut in the brain should be made at right angles to the surface and a thin slice taken, avoiding the white matter as much as possible. A coverslip is now pressed down upon the thin piece of tissue, spreading it out in a moderately thin layer, then the coverslip is drawn slowly and evenly along the slide.

Williams' Modification of Van Gieson's Stain for Negri Bodies in Smears (1908).—*Fixation.*—Smears are partially air-dried and fixed for 10 seconds in neutral methyl alcohol to which 0.1 per cent picric acid has been added. Remove excess of fixing solution with filter paper.

Staining Solution.—

Basic fuchsin, saturated alcoholic solution (about 5.93 per cent)	0.5 cc.
Methylene blue, saturated alcoholic solution (about 3.55 per cent)	10 cc.
Water, distilled	30 cc.

This staining mixture keeps for a long time in the ice-box.

Method of Staining.—1. Cover smear with the above stain and heat to steaming.

2. Wash in tap water.

3. Blot with filter paper.

Results.—Negri bodies magenta with blue granules; nerve cells blue; red blood cells yellow or salmon pink.

Bond's Modification of Mann's Eosin-Methyl Blue Stain for Negri Bodies in Smears.—*Fixation.*—Smears are partially dried in the air and fixed in methyl alcohol for 5 to 6 minutes.

Staining Solution.—

Eosin, 1 per cent aqueous solution	1 cc.
Methyl blue, 1 per cent aqueous solution	0.7–1 cc.
Water, distilled	6 cc.

Prepare the mixture fresh each time. The stock solutions keep well.

Method of Staining.—1. Wash for 30 seconds in running tap water.

2. Stain for 4 to 5 minutes with the eosin-methyl blue stain.
3. Wash in running tap water for 30 seconds.
4. Blot with filter paper.
5. Dehydrate in absolute alcohol.
6. Clear in a mixture of 1 part xylol and 2 parts aniline.
7. Rinse in xylol and mount in balsam.

Results.—Negri bodies stain red; the differential staining between red blood corpuscles and Negri bodies is not always clear in this method.

For the demonstration of Negri bodies in sections it is preferable to fix brain tissue from the hippocampus in Zenker's fluid, then to embed in paraffin and cut sections. The sections are best stained with the phloxine-methylene blue stain (p. 86) which colors the cytoplasm of the Negri bodies red and the granules blue; or with Wolbach's modification of Giemsa's stain (p. 195). Negri bodies by the latter stain are brought out as lilac to red bodies in the blue cytoplasm of the nerve cells. The following rapid method devised by Schleifstein permits early identification of the Negri bodies.

Schleifstein's Rapid Method for Demonstrating Negri Bodies in Sections (1937).—This method has the advantage that the entire procedure, from the removal of the brain to the completion of the sections, requires only about 8 hours.

Fixation.—Fix blocks of tissue, not more than 3 mm. thick, from the hippocampus major and the cerebellum in Zenker's fluid for 4 hours at 37° C.

Embedding.—1. Wash for 30 minutes in running tap water or, if desired, overnight.

2. Place the blocks in a glass stoppered bottle which contains 80 cc. of dioxane, a few flakes of iodine, and anhydrous calcium chloride to a depth of 1 cm. The blocks of tissue should be supported in the dioxane well above the layer of calcium chloride. (A non-corrosive wire tripod with a fine mesh screen has been used.) Leave for 1 hour at 37° C.

3. Transfer to a mixture of equal parts dioxane and paraffin for 1 hour at 56° C. Care should be taken to have the blocks rest a few centimeters above the bottom of the bottle, since dioxane tends to settle.

4. Place in a paraffin bath at 56° C. for 1 hour.

5. Embed in paraffin.

Staining Solutions.—

Solution A

Rosaniline	1.8	gm.
Methylene blue	1	gm.
Glycerin	100	cc.
Alcohol, methyl	100	cc.

Shake for several minutes. The mixture keeps indefinitely.

Solution B

Potassium hydroxide, 1:40,000 aqueous solution

For staining add 1 drop of Solution A to 2 cc. of Solution B. This mixture should be freshly prepared for use each time.

Method of Staining.—1. Cut sections 4 μ thick and attach to slides with Mayer's albumin-glycerin.

2. Heat slides carefully, section up, over a low Bunsen flame until the paraffin begins to melt. By that time, the water will have evaporated and the slides can be put into xylol.

3. Remove paraffin in the usual manner and place slides in distilled water.

4. Place slides on an electric plate; flood with freshly prepared stain and gently steam for 5 minutes.

5. Cool and wash quickly in tap water.

6. Decolorize and differentiate each slide separately by gently agitating in a jar of 90 per cent alcohol until the section assumes a faint violet color. This is a particularly important step.

7. Pass sections rapidly through 95 per cent and absolute alcohol.

8. Clear in xylol and mount in balsam.

Results.—Negri bodies stain a deep magenta red color; the granular inclusions are dark blue. Nucleoli bluish black; cytoplasm bluish violet; red blood cells copper color.

(2) **Vaccinia.**—The inclusion bodies are known as Guarnieri's bodies. They occur only in the cytoplasm. Following the phloxine-methylene blue or the Giemsa stain they appear as dense, red to pink to lilac bodies, round to oval in shape.

(3) **Variola.**—The inclusion bodies are likewise known as Guarnieri's bodies. Morphologically they resemble those of vaccinia but are found in the nuclei as well as in the cytoplasm.

(4) **Herpes.**—The inclusion bodies of the different types of

herpes are similar in appearance. They are exclusively intranuclear and are acidophilic. Following the phloxine-methylene blue or the Giemsa stain, they appear as homogeneous or finely granular pink to red staining masses; the nucleolus and chromatin marginate on the nuclear membrane.

(5) **Varicella.**—The inclusion bodies are similar to those of herpes.

(6) **Molluscum Contagiosum.**—The inclusion bodies in their fully developed state are large, often practically filling the cell and compressing the nucleus. They are cytoplasmic in situation and are acidophilic, staining reddish to purplish with the phloxine-methylene blue or with Giemsa's stain.

(7) **Yellow Fever.**—The inclusion bodies are intranuclear and occur exclusively in liver cells. Following the phloxine-methylene blue or Giemsa's stain, they are colored pink to red. They are made up of spherical clumps of fine particles. The nucleolus preserves its central position in contrast with its marginal position in herpes.

5. SPIROCHAETALES

These organisms are slender, flexible spiral filaments. In addition to the spirals they often have a variable number of waves. They are actively motile but possess no flagella. The exact nature of this group has not yet been definitely settled, that is, whether they are bacteria or protozoa. This uncertainty has led to a confusion in nomenclature. I shall follow that given in Bergey's Manual of Determinative Bacteriology (1934) and shall deal only with the important parasitic forms.

These organisms can be studied in unfixed specimens of blood, body fluids or exudates by means of dark-field illumination. The diagnosis of the type depends on the size, number, constancy and angles of the spirals. For staining them in fixed smears and sections a variety of special methods have been devised, since in general they do not stain by the usual bacteriological methods.

(1) Syphilis

Caused by the *Treponema pallidum*. The organisms are 6 to 14 μ long, 0.25 to 0.5 μ in diameter and have 3 to 20 sharp deep spirals which are constant.

The organisms are to be sought for in different locations, ac-

cording to the type or stage of the disease. In the primary stage they are best demonstrated by dark-field illumination in material from the primary lesion (chancre). In the secondary stage they may be found by similar means in the skin lesions or lymph nodes. In the tertiary stage they are to be sought for in sections of the lesions characteristic of this stage, *e. g.*, gummas in any organ, lesions of aortitis and those of general paresis (brain). It may be stated that the demonstration of treponemas in the tertiary stage is often unsuccessful even in active lesions. In congenital syphilis the liver, pancreas, adrenal and heart should be examined either by dark-field illumination of fluid expressed from the organs or in fixed material by special stains. Even dead forms may show up distinctly, as for example in fluid squeezed from the liver at autopsy of a case of congenital syphilis. The treponemas are often extremely numerous in this form of syphilis.

The organisms may be demonstrated in fixed smears and sections by various staining methods. For this purpose lesions are cleaned of any adherent exudate. Smear preparations are then made from the tissue exudate obtained by pressure or scraping. An excess of blood should be avoided. The preparations are dried in the air and may be stained by the following methods:

Method for Demonstrating Treponemata Pallida in Smears with Wright's Blood Stain.—The material should be thinly spread on a coverslip, not on a slide. The coverslip is held level with forceps during the staining.

Staining Solution.—In a test tube mix 10 cc. of distilled water, 1 cc. of Wright's stain (p. 181), and 1 cc. of a 0.1 per cent aqueous solution of potassium carbonate. Heat to boiling.

Method of Staining.—1. Cover smear preparation with the hot staining solution for 3 to 4 minutes.

2. When the fluid in the preparation has become a violet color and a thin, yellow metallic scum has formed on the surface, pour the staining solution off and again cover the preparation with the hot mixture after again heating in the test tube.

3. Repeat once more.

4. Wash in water.

5. Dry and mount in balsam.

Results.—Treponemas intensely violet.

Giemsa's Method for Demonstrating Treponemata Pallida in Smears (1905).—Giemsa's stain is very sensitive to changes in the hydrogen ion concentration. Therefore the glassware used must be clean and any traces of acid should be avoided.

Fixation.—Very thin smears are dried in the air and fixed for at least 15 minutes in absolute alcohol.

Staining Solution.—

Giemsa's stock solution (p. 183)	10 drops
Water, distilled	10 cc.
Potassium carbonate, 0.1 per cent aqueous solution	5–10 drops

Method of Staining.—1. Pour on freshly prepared dilute Giemsa solution and stain for 10 to 30 minutes.

2. Rinse quickly in a stream of water.

3. Blot with filter paper and mount in balsam.

Results.—Treponemas dark red.

Giemsa's Rapid Method for Treponemata Pallida in Smears (1909).—*Fixation.*—Fix in absolute alcohol 15 minutes or draw the smear 3 times through a flame.

Method of Staining.—1. Pour on freshly prepared dilute Giemsa solution (10 drops of stock Giemsa to 10 cc. distilled water) and steam gently, then leave for 15 seconds.

2. Pour off staining solution and immediately pour on fresh dilute Giemsa solution, warm and then allow to cool for 15 seconds.

3. Repeat this 4 times, leaving stain on 1 minute the last time.

4. Rinse quickly in a stream of water.

5. Blot with filter paper and mount in balsam.

Results.—Organisms intensely dark red.

Benian's Method for the Demonstration of Treponemas in Smears.—*Procedure.*—Thoroughly mix on a coverslip 1 or 2 platinum loops full of 2 per cent aqueous solution of Congo red with a small amount of serum or exudate from the lesion. Spread the viscid mixture evenly and rather thinly. Dry in the air. Wash with 1 per cent aqueous solution of hydrochloric acid. Drain off at once and dry in the air. Do not wash in water or blot.

Results.—The treponemas appear white and unstained on a blue background, which should be homogeneous. Too large a proportion of exudate results in a granular background.

The Fontana-Tribondeau Method for Demonstrating Treponemas in Smears (1925–26).—*Fixation.*—Thin smears of serum are air-dried.

Staining Solution.—

Ruge's Solution

Acetic acid, glacial	1 cc.
Formalin	2 cc.
Water, distilled	100 cc.

Mordant

Carbolic acid, liquid	1 cc.
Tannic acid	5 gm.
Water, distilled	100 cc.

Fontana's Ammoniacal Silver Nitrate Solution.—To a 1 per cent silver nitrate solution add ammonia water, diluted 1:20, drop by drop until a coffee-colored clouding of the solution occurs. Use at least 50 to 100 cc. of the silver nitrate solution, because otherwise the optimum is easily passed.

Method of Staining.—1. Pour a few drops of Ruge's solution on the smear for 1 minute, changing the fluid several times.

2. Rinse in running water.

3. Mordant with a few drops of the tannic acid solution, heating for 20 seconds over a small flame to steaming.

4. Rinse in distilled water.

5. Stain in Fontana's ammoniacal silver nitrate solution for 30 seconds, heating slightly.

6. For preservation, wash in water, dry in the air and mount in balsam.

Results.—Treponemas and other spirochetes and almost all microorganisms brown to deep black.

Burri's India Ink Method for Demonstrating Treponemas (1909).—*India Ink Suspension.*—A solution of India ink in distilled water, 1:10 or better 1:4, is sterilized in test tubes in the autoclave for 15 minutes.

Procedure.—Approximately equal parts of the fluid from the lesion and of the India ink solution are quickly mixed together on a slide with the aid of a platinum loop, spread thinly, and allowed to dry. When dry examine the slide with an oil immersion lens.

Results.—The preparation should have a brown color. The

treponemas and bacteria appear as unstained, brilliant white bodies in a brown to black background. Particles of India ink surround the organisms like a capsule.

The Warthin-Starry Silver-Agar Method for Staining Treponemas in Smears (1922).—*Fixation.*—Prepare smear on coverslip and dry thoroughly in the air. Then fix in absolute alcohol 3 to 5 minutes.

Staining Solution.—

Silver nitrate, 2 per cent aqueous solution	3 cc.
Gelatin, warm 10 per cent aqueous solution	5 cc.
Glycerin, warm	5 cc.
Agar suspension, warm 1.5 per cent solution	5 cc.
Hydroquinone, 5 per cent aqueous solution	2 cc.

Mix silver nitrate, gelatin and glycerin thoroughly, then stir in the agar suspension last. The hydroquinone is added just before use. Make the agar suspension by breaking up fine 1.5 gm. of agar and place in 20 to 30 cc. of distilled water. Allow the agar to soak for a few minutes until it is saturated with water. The excess is poured off and the agar washed with several changes of distilled water. Then 100 cc. of distilled water are added and with constant stirring brought to the boiling point. When the agar is in a fine suspension pour into a clean bottle. As the agar thickens shake occasionally and, when it begins to set, break it up by violent shaking. Keep on top of a paraffin oven.

Method of Staining.—1. Wash in 2 changes of distilled water. Hydrogen peroxide may be used to clear the background, and in that case the smear is placed in concentrated hydrogen peroxide for 5 to 20 minutes and then washed thoroughly in distilled water.

2. Rinse the smear in a 2 per cent solution of silver nitrate, which should not be over 6 to 7 days old. Cover the smear side with another clean coverslip, which has also been rinsed in the silver nitrate solution. Place the two adherent coverslips carefully in a wide-mouthed bottle of 2 per cent silver nitrate (smeared coverslip next to the wall) and put in the incubator for 1 to 2 hours; then remove the coverslips from the silver nitrate solution and separate them.

3. Put the coverslip smear side up in the solution given above for 30 seconds to 2 minutes, until the smear is light brown.

4. After the solution is reduced remove and rinse in 5 per cent sodium thiosulfate for a few seconds.

5. Rinse in distilled water.

6. Dehydrate in absolute alcohol.

7. Clear in xylol and mount in balsam.

Results.—Treponemas black against a light background. The stained smears are not always permanent.

Levaditi's Method for Staining Treponemata Pallida in Sections (1906).—*Fixation.*—Pieces of tissue about 1 mm. thick are placed in 10 per cent formalin for 24 hours.

Method of Staining.—1. Rinse tissue in water.

2. Place in 95 per cent alcohol for 24 hours.

3. Place in distilled water until the tissue sinks to the bottom of the container.

4. Place in a freshly prepared 1.5 to 3 per cent aqueous solution of silver nitrate and keep at 37° C. in the dark for 3 to 5 days, changing the solution 3 times. The stronger solution of silver nitrate is preferable for tissues removed during life.

5. Wash in distilled water.

6. Place in the following solution for 24 to 72 hours at room temperature in the dark:

Pyrogallic acid	2–4 gm.
Formalin	5 cc.
Water, distilled	100 cc.

7. Wash in distilled water.

8. Dehydrate in 80 and 95 per cent followed by absolute alcohol.

9. Clear in oil of cedarwood and embed in paraffin in the usual manner.

10. Cut sections 5 μ thick and mount after removing the paraffin.

Results.—The treponemas are stained intensely black by the precipitation of metallic silver on them and the tissue appears yellow to brown. The sections may be counterstained with Giemsa's blood stain, but this is of doubtful advantage.

Heitzman's Modification of the Warthin-Starry and Nieto's Methods for Staining Treponemata Pallida.—*Fixation.*—Fix in 10 per cent formalin. Cut frozen sections at 15 μ or less. Deparaffinized sections can be used, but are not recommended.

Method of Staining.—1. Place sections directly in pyridine for 10 minutes.

2. Wash in 3 changes of distilled water.

3. Place in a 1 per cent aqueous solution of uranium nitrate for 15 minutes at 37° C.

4. Wash quickly in 2 changes of distilled water.

5. Place in a 0.25 per cent aqueous solution of silver nitrate for 15 to 30 minutes in the paraffin oven at 56° C.

6. Place sections in the following developing solution, freshly prepared, until they are dark brown (2–3 minutes).

Pipette rapidly into a 50 cc. beaker and mix each solution by whirling. Place sections in fluid immediately after adding hydroquinone.

Gelatin, 5 per cent aqueous solution at 56° C.	15	cc.
Silver nitrate, 2 per cent aqueous solution	3	cc.
Hydroquinone, 1 per cent aqueous solution	0.5	cc.

7. Remove sections and wash thoroughly in warm distilled water.

8. Dehydrate on a slide, using a pipette, and gradually increase the strength of alcohol to absolute.

9. Clear in benzol and mount in balsam.

Results.—Sections should be examined under an intense yellow light. Treponemas black; nuclei dark brown; other tissue elements yellowish brown.

It is important not to leave sections in the developing solution too long as a heavy precipitation will take place, obscuring all detail.

(2) Yaws

Frambesia tropica, or yaws, is caused by *Treponema pertenue*. This organism is 18 to 20 μ in length and has 6 to 20 uniform spirals.

Yaws is primarily a disease of the skin although visceral lesions, in the aorta and bone, do occur also. In addition the organisms may be found in the spleen, lymph nodes and bone marrow. These treponemas can be demonstrated by the same means as those employed for the *Treponema pallidum*.

(3) Relapsing Fever

Caused by *Borrelia recurrentis*. These organisms are 8 to 16 μ long and 0.35 to 0.5 μ in diameter; the spirals are large, wavy and inconstant.

In this disease the organisms are best demonstrated in the blood, either by dark-field illumination or in fixed smears stained by Wright's or Giemsa's stain as given for the *Treponema pallidum*. Specimens of blood should be taken during the febrile paroxysms.

(4) Vincent's Angina and Fusospirochetal Diseases

The organism, *Borrelia vincentii*, occurs in association with fusiform bacilli, especially *Fusiformis dentium* (Vincent's fusiform bacillus). The organism is 12 to 25 μ in length and has shallow irregular spirals.

The organisms are found in diseases of the oral cavity, tonsils and pharynx (Vincent's angina) and have been assigned an important rôle in the production of certain diseases of the lung and elsewhere. They may be looked for in unfixed preparations by dark-field illumination or may be stained in smears and sections by the methods given for the *Treponema pallidum*. However, since *Borrelia vincentii* stains more easily than others of the spirochaetales, staining heat-fixed smears with Stirling's aniline crystal violet (p. 90) for 10 seconds is sufficient to bring them out distinctly.

(5) Weil's Disease

Infectious jaundice or Weil's disease is caused by *Leptospira icterohaemorrhagiae*. The organisms are 6 to 9 μ long and 0.25 to 0.3 μ in diameter. The spirals are rigid and regular.

The leptospirae occur in the blood and urine but usually are difficult to demonstrate by microscopic means. In tissues they are well brought out by Levaditi's stain for the *Treponema pallidum* and are to be sought for especially in sections of the liver and kidney.

6. PROTOZOA

(1) Amebic Dysentery

Several species of amebas occur in the intestine but only one species, *Endamoeba histolytica*, is pathogenic and is the cause of amebic dysentery. The others are harmless parasites.

In cases of dysentery suspected of being due to amebas the stools are best examined as soon as passed, although the amebas will sometimes remain active in stools even after 24 hours. A warm stage during the examination is an advantage, but not a necessity. A drop of the fluid material, preferably that containing mucus or

blood, is placed on a slide and lightly covered with a coverslip. If the slide is cold and the organisms do not move, warm the slide gently and the movements of the amebas will often begin. Pus from abscesses due to amebas is examined in the same way. The diagnosis is made by finding the characteristic large cell, with single round nucleus and glassy refringent peripheral cytoplasm, which projects itself actively in the form of pseudopods. The cytoplasm often contains vacuoles and red blood corpuscles. The presence of the latter is an important diagnostic feature for the pathogenic amebas; they are not found in non-pathogenic forms.

Amebas and their nuclei may be made more clearly visible in wet smear preparations by emulsifying the fecal material or pus in Gram's iodine solution (p. 82), which stains the glycogen present, or by mixing the feces with a drop of 1 or 2 per cent formalin, then adding a drop of 2 per cent acetic acid and tinging with 1 drop of 1 per cent neutral red solution.

Hematoxylin Stains for Amebas.—Permanent, stained smear preparations may be made as follows:

Fixation.—Make a thin smear and fix, while moist, for 15 minutes in a mixture of 95 per cent alcohol 1 part, and a saturated aqueous solution of mercuric bichloride (about 6.9 per cent) 2 parts.

Method of Staining.—1. Wash in water for a few seconds and cover with a 1 per cent alcoholic solution of iodine for 3 minutes.

2. Wash with 95 per cent alcohol until the iodine stain disappears.

3. Wash in water and stain with Mallory's iron hematoxylin (p. 75), or with phosphotungstic acid hematoxylin (p. 76) for 30 minutes.

4. Wash in water.

5. Dehydrate with 95 per cent followed by absolute alcohol.

6. Clear in xylol and mount in balsam.

Results.—Nuclei and ectosarc deep blue; cytoplasm bluish.

In sections of fixed tissues the nuclei of the amebas do not stain particularly well with the ordinary nuclear stains, such as alum hematoxylin and methylene blue, although the phosphotungstic acid hematoxylin stain brings them out with great sharpness. The following method of staining has been found to give very satisfactory results and to render the recognition of the organisms easy. This method may be applied also to bits of mucus in the stools.

Mallory's Differential Stain for Amebas in Sections (1897). —*Fixation.*—Fix tissue in 95 per cent or absolute alcohol. Embed in paraffin or celloidin.

Method of Staining.—1. Stain sections in a saturated aqueous solution of thionin (about 0.25 per cent) for 3 to 5 minutes.

2. Differentiate in a 2 per cent aqueous solution of oxalic acid for ½ to 1 minute.

3. Wash in water.

4. Dehydrate in 95 per cent and absolute alcohol.

5. Clear in xylol and mount in balsam.

Clear celloidin sections in terpineol or in oil of origanum after 95 per cent alcohol.

Results.—Nuclei of the amebas and granules of mast cells are brownish red; nuclei of mast cells and of all other cells are blue.

The results obtained with feces examined in the same way or after embedding in celloidin were much less satisfactory, for the reason that various substances in the feces precipitate the thionin in the form of reddish crystals and give rise to deceptive pictures. A similar differential stain can be obtained by Unna's method for staining the granules of mast cells (p. 175).

(2) Balantidial Dysentery

Balantidium coli is a large protozoal parasite that infects man but rarely and causes balantidial dysentery. Phosphotungstic acid hematoxylin stains deep blue the bean shaped macronucleus, the spherical micronucleus, and also the cilia surrounding the body but best developed in the funnel shaped opening at one end.

(3) Malaria

Three varieties of the *Plasmodium malariae* have been described, namely the tertian, quartan and aestivo-autumnal. They develop within or upon the red corpuscles of the blood and cause the destruction of the corpuscles affected.

The three varieties of parasites differ from one another in a number of ways. The chief differences are the length of the cycle of development; the size of the full-grown organisms; the difference in the refractivity of the organisms; the quantity, size and color of the pigment granules; the degree of ameboid movement; and the number and shape of the segments into which the full-grown organ-

isms divide. In the earliest stage the varieties cannot be distinguished from each other.

The organisms of malaria can be detected in fresh specimens of blood or in specimens of blood that have been fixed and stained. In doubtful cases the parasites are more surely and easily found in coverslip preparations of the blood fixed and stained by special methods. The method employed in making coverslip preparations of the blood has been described (p. 180).

Wright's Stain for Malarial Parasites (1902).—This stain is identical with his blood stain and is applied in the same way (p. 181). It gives the so-called Romanowsky stain to the parasites.

Results.—With this method the body of a malarial parasite stains blue, while the color of the chromatin varies from a lilac color through varying shades of red. In the young forms of the tertian and aestivo-autumnal parasites the chromatin appears as a spherical, dark red body, while in the older forms of the tertian parasite it has a more lilac or purplish red color, and may appear in the form of a reticulum. In the intermediate forms the color of the chromatin may present variations between these extremes.

Blood platelets, apparently situated within red blood corpuscles, may be mistaken by the inexperienced for young malarial parasites. This ought never to occur if one bears in mind the fact that the young parasite of all three varieties should present by this method a dark red spherical nucleus and a homogeneous blue cytoplasm which is usually in the form of a definite ring.

Ross' Method for Staining Malarial Parasites.—This method permits a relatively large amount of blood to be examined.

Fixation.—A large drop of blood is spread on a slide over an area the size of a coverslip. Dry and fix by warming over a flame.

Method of Staining.—1. After treating with a solution of acetic acid and washing in water, cover smear with a 10 per cent aqueous solution of eosin for 15 minutes.

2. Wash in water.

3. Cover with a dilute solution of alkaline methylene blue for a few seconds.

4. Wash again in water.

5. Dry and mount.

Results.—The blue stained parasites stand out well since the red blood corpuscles are eliminated by this procedure.

Examination of Fresh Blood Specimens for Malarial Parasites.—In examining a fresh specimen of the blood for malarial organisms a slide is substituted for one of the coverslips, and the coverslip which has the drop of blood on its surface is dropped lightly upon the slide and allowed to remain there. When taking the specimen of blood the first 4 or 5 drops should be quickly wiped away from the ear until a very small drop is obtained. Great care must be exercised to touch only the tip of the drop with the coverslip so as to avoid smearing the blood. If the blood is smeared on the coverslip the edges of the blood drop will dry before the coverslip can be transferred to the slide and the blood will not spread. It is necessary that the blood should spread in a thin layer in order to study satisfactorily the individual red blood cells. If one desires to study the preparation for several hours the edges of the coverslip can be sealed by melted paraffin or vaseline to exclude the air. The examination should be made with an oil immersion lens. It should be remembered that the action of cold inhibits the ameboid movements of the parasites; it may be necessary, therefore, at times to warm the slide before examining the specimen. Evaporation not infrequently occurs, caused by the air penetrating beneath the coverslip. This produces changes in many of the corpuscles which may be mistaken for hyaline bodies; the central depression becomes paler and less refractive than the periphery of the corpuscles; later a number of corpuscles contain small glistening points, and still later the corpuscles become crenated.

Giemsa's Stain for Malarial Parasites in Smears.—This method gives the Romanowsky staining also. It is carried out exactly as described for blood smears (p. 183) fixed in methyl alcohol.

Results.—The malarial parasite, consisting of a nucleus which stains red and cytoplasm which stains blue, forms a rounded body inside of the pale red staining erythrocyte. The parasite is seen typically in the signet-ring form, with a blue ring with red nucleus at one side, and dark brown pigment granules in the blue cytoplasm.

Giemsa's Method for Staining Protozoa and Bacteria in Sections.—*Fixation.*—1. Fix pieces of tissue, not more than 2 mm. thick, in sublimate alcohol, consisting of 2 parts of a saturated aqueous solution of mercuric bichloride (about 6.9 per cent), and 1 part of absolute alcohol. The duration of fixation should be at least 48 hours, renewing the fixing fluid after 24 hours.

Or, fix in Maximow's Zenker-formol, consisting of Zenker's fluid, to 100 cc. of which 10 cc. of formalin are added just before use. Fix for 6 to 24 hours at room temperature.

2. Wash in water for 24 hours if fixed in Maximow's solution.

3. Dehydrate in graded alcohols and clear in xylol.

4. Embed in paraffin.

5. Cut sections 2 to 4 μ thick. Tissues must not be handled with metal instruments until after they have been cleared in xylol.

Method of Staining.—1. Remove paraffin with xylol and pass sections through graded alcohols to water.

2. Place sections for 10 minutes in an iodine solution consisting of 2 gm. potassium iodide, 100 cc. distilled water, and 3 cc. Gram's solution (p. 82). Instead of this mixture it is possible to use Gram's iodine solution, 1 to 3 cc. of which are mixed with 100 cc. of water or of 70 per cent alcohol, or a weak alcoholic solution of iodine. The use of the latter is indicated when a more intense blue staining of the cytoplasm is desired, and demands a longer time, 20 to 30 minutes.

3. Wash in 95 per cent alcohol until the yellow color is removed.

4. Wash quickly in distilled water.

5. Place sections for 10 minutes in 0.5 per cent aqueous solution of sodium thiosulfate.

6. Wash in tap water for 5 minutes.

7. Rinse in distilled water.

8. Stain in a freshly diluted solution of Giemsa's stain for 2 to 12 hours or longer. The dilution is made by adding 1 drop of Giemsa's stain (p. 183) to 1 cc. of distilled water; or for longer staining add 1 drop of Giemsa's stain to 2 cc. of distilled water. After the first 30 minutes the staining mixture is replaced by a fresh solution.

9. Wash in distilled water.

10. Dehydrate successively in the following mixtures:

Acetone 95 cc., xylol 5 cc.
Acetone 70 cc., xylol 30 cc.
Acetone 30 cc., xylol 70 cc.

The duration in these mixtures depends on the degree of differentiation required.

11. Clear in xylol and mount in balsam.

The distilled water used for diluting the staining fluid must be absolutely free from acid. The slightest trace of organic or mineral acids, or even the presence of a considerable amount of carbonic acid, spoils the staining. Instead of distilled water a buffered solution made up as given in the section on blood (p. 184) may be used.

Results.—Nuclei of protozoa brilliant red; blood platelets blue. Nuclei of cells red-violet; neutrophil granules red-violet; basophil granules blue to blue-violet; cytoplasm of lymphocytes blue; azure granules and erythrocytes pale red.

After considerable experience with Giemsa's methods, S. B. Wolbach suggested an excellent modification for Zenker-fixed, paraffin embedded material (p. 195).

7. PARASITIC WORMS

The important means of diagnosis of infection with worms and of the kind of worm concerned are the finding and identification of the adult parasite, its larvae or ova in the feces, body fluids or tissues.

(1) Methods of Examination of Feces

Direct Smear Method for Ova and Larvae.—A small bit of feces on the end of a match is thoroughly mixed with a small drop of tap water on a slide. Place a coverslip gently on the smear, which should not be too dense, so that the material spreads out. This method is of value only for heavy infestations.

Sugar Solution Method for Concentrating Ova.—To a small amount of feces add a sugar solution (saccharose, in the form of common granulated sugar, 500 gm., water 360 cc., phenol 1 per cent) in a centrifuge tube, almost filling the tube. Cover the top of the tube and gently mix the contents. Centrifuge at a speed of 1000 revolutions for 5 to 6 minutes. The heavy sugar solution causes the eggs to be concentrated at the surface and they can be removed by a large wire loop and placed on a slide, covered with a coverslip and examined with the microscope.

Hypertonic Salt Solution Method for Concentrating Ova.—Mix a small amount of feces with a concentrated solution of sodium chloride in a proportion not exceeding 1 part of feces to 20 parts of salt solution. Centrifuge as for the sugar solution method and examine with the microscope. It may be necessary to push coarse

floating particles to the bottom of the tube by means of a disk of No. 0 steel wool before centrifuging.

Examination for Adult Worms and Segments.—Emulsify the feces with a small amount of tap water and pass through a wire screen of medium mesh. Adult worms and segments can be seen on the screen. Washed with physiological salt solution and placed on a slide they may be examined with a low power dissecting microscope.

(2) Identification of Parasites

a. Platyhelminthes or Flat Worms

(*a*) **Cestodes or Tapeworms.**—It is not always easy to recognize the variety of tapeworm by a single segment passed with the feces because the uterus, which furnishes the most characteristic points of difference, is not developed in the young segments and is atrophied in the old. When the whole worm is obtained the problem is much simpler. The uterus is identified by squeezing a segment between two slides and holding it up to the light. The heads are examined under the microscope in water, salt solution or glycerin.

Taenia Solium.—The head has four suckers and a circle of hooklets; the uterus is noticeably but little branched; and the genital tract opens laterally. The eggs develop into the *Cysticercus cellulosae*, a larval form which occurs in the pig and which is not infrequently found in man. The scolex is obtained for examination by tearing open the cyst and examining the inner wall. The suckers and hooklets are best studied after mounting fresh and pressing under a coverslip.

Taenia Saginata.—The head has four strong suckers, but no hooklets; the uterus is very much branched; the segments show marked muscular development; and the genital tract opens laterally. The eggs develop into the *Cysticercus bovis*, a larval form occurring in cattle but not in man.

Taenia Echinococcus.—This occurs in dogs; in man it produces hydatid cysts of the liver and other organs. The echinococcus cysts which occur in man are recognized by the very characteristic laminated structure of the cyst wall. The heads of the scolices have four suckers and a double circle of hooklets.

Diphyllobothrium Latum.—The opening of the genital tract lies in the median line. The head is flattened, and has two small suckers situated at the sides.

(*b*) **Trematodes or Flukes.**—Of the blood flukes the three that occur in man are *Schistosomum haematobium*, *Schistosomum mansoni* and *Schistosomum japonicum*. The male and female parasites of these human blood flukes occur in the venous system.

Schistosomum Haematobium.—This tropical parasite is found especially in the veins of the bladder. The ova escape from the blood vessels into the bladder and cause violent inflammation. The process may extend to the kidneys. The eggs which have a terminal spine are usually passed with the urine and frequently with the feces.

Schistosomum Mansoni.—The habitat of this species is similar to that of *S. japonicum*. The eggs have a distinct lateral spine and are passed with the feces, only rarely with the urine. The ova infect the rectum, causing a type of dysentery and may involve even the appendix.

Schistosomum Japonicum.—This species is present mostly in the veins of the large intestine, the hemorrhoidal plexus and the portal system. The eggs, which are smaller than those of the other species and have an abbreviated lateral spine, are passed only in the feces. The liver and spleen become enlarged in cases of schistosomiasis japonicum, the walls of the portal veins are thickened, and ulcers are present in the intestine.

b. NEMATHELMINTHES OR ROUND WORMS

(*a*) **Filaria Bancrofti.**—This is the most important of the filarial worms as it is the only one known to be pathogenic, causing the common manifestations of filariasis such as elephantiasis, chyluria and so on. It is usually seen as a thread-like writhing worm which lives in the lymphatic system.

Its embryos are usually found in the peripheral blood stream at night and therefore in suspected cases a drop of fresh blood or chylous or bloody urine is examined on a slide with the low power of the microscope. The embryos are readily detected when present because of their very active movements. Permanent specimens can be made by fixing ordinary coverslip preparations of the blood or chylous fluid by heat or by use of a saturated solution of corrosive sublimate, and staining for a few seconds with Loeffler's methylene blue or with a 2 per cent aqueous solution of thionin.

(*b*) **Ankylostoma Duodenale and Necator Americanus.**—These are the two species of hookworms that infect man. Their eggs occur in the feces and can be most easily found by emulsifying

the feces in equal parts of glycerin and saturated salt solution on a slide. The eggs rise to the surface and can be identified. The adult worms may at times be found in the feces after the administration of an anthelmintic.

(*c*) **Trichinella Spiralis.**—This parasite is the cause of trichinosis. No eggs are found in the feces, but encysted trichinae are obtained from muscle tissue. A rapid method of diagnosis is to squeeze small bits of muscle tissue between two slides and to examine with the lower power of the microscope. Pieces of muscle nearest the insertion of the tendon are chosen from the diaphragm or from the muscles of the jaws. Encapsulated and calcified trichinellae may be cleared by means of acids.

Infected tissues may be fixed in 10 per cent formalin or Zenker's fluid and stained with alum hematoxylin and phloxine. The trichinellae are studied in longitudinal sections of the muscle fibers.

During the wandering stage of the trichinella embryos they may be demonstrated in the circulating blood. With a syringe containing 3 per cent acetic acid, take some blood from a vein in the arm, centrifuge, and examine the sediment for larvae. Always examine the blood for eosinophilia.

PART III. AUTOPSY METHODS

CHAPTER XVI

POSTMORTEM TECHNIQUE

1. Virchow's Postmortem Technique

(1) INTRODUCTION

One of the commonest mistakes made in performing postmortem examinations is that, in studying the details of the pathological changes found in the individual organs, structures and relationships which are of importance in explaining the disease process as a whole are destroyed. Regardless of the method used, this danger can be avoided to a large extent if the prosector intelligently plans his method of procedure after a careful study of the clinical history of the case followed by a painstaking external examination of the body and of the organs while still *in situ*. Every autopsy presents an individual problem and no standard technique will be found entirely satisfactory for every case.

Almost every laboratory has its own individual autopsy technique, but most of these are only minor variations of one of four major types. The four differ chiefly in the methods used in the removal of the organs and in the order in which they are opened.

The first and probably the oldest of these is the procedure originally taught by Virchow. In this the organs are removed one by one and dissected and examined as removed.

In the second, groups of organs that are anatomically and functionally related are removed together and dissected without disturbing their anatomical relationships. The contents of the thorax compose one group; the stomach, duodenum, liver, biliary apparatus, pancreas and spleen, another; the genito-urinary organs with the adrenals, a third; the intestines with mesentery, a fourth; and the central nervous system, a fifth. This method was devised by Ghon and not only keeps functional units together but also preserves lymph nodes with the organs which they drain.

In the third method the organs of the chest and abdomen with the diaphragm are all removed in one block. These are then dissected in a routine manner beginning posteriorly and going anteriorly.

The fourth is that used by Rokitansky and published by Chiari (1907). Its fundamental principle is to examine and open every organ *in situ* before removing it. This method, at least in its entirety, is not used to any extent in this country.

In this book the Virchow (1893) technique will be given in detail accompanied by a general discussion of the problems associated with postmortem examinations and then a briefer summary of the other techniques will be presented. A knowledge of all four forms of procedure is useful. It broadens one's point of view, and tends to keep one's mind open to possibilities. A list of the best publications on postmortem technique may be found in the bibliography at the end of the book.

The problem offered by an autopsy is often solved in part or wholly by the macroscopic postmortem examination. More frequently, however, the complete and final solution is reached only after careful bacteriological and histological study. The postmortem examination may, therefore, be looked upon as the beginning of the solution of the problem. Its particular function is to demonstrate in the individual case all congenital or acquired abnormalities, all macroscopic lesions, and to explain all gross mechanical questions. It furnishes the material for bacteriological and histological study. Perfectly to accomplish its purpose a postmortem examination must be made in a careful, systematic and thorough manner.

While a general routine method of procedure is advisable, it will often be found advantageous, or even necessary, to depart from it. According to Orth (1900), the chief requisite of every exact postmortem examination is this, that no part shall be displaced from its position until its relations to the surrounding parts are established, and that no part shall be taken out by whose removal the further examination of other parts is affected.

In the Virchow technique the order and method of procedure in making a postmortem examination, including the various incisions, may be said to have been planned for the routine examinations of normal or diffusely diseased organs. As soon as a noticeable focal

lesion is present the order of procedure and the customary method of removal and of incision must be so altered as best to display the lesion.

a. Autopsy Permissions

No autopsy should be performed without a written permission signed by the person who has the right of custody of the body. The laws concerning this differ somewhat from state to state, but usually the surviving relative nearest of kinship is responsible. In cases in which any medicolegal problems are apt to arise, no permission should be asked for and no autopsy done until the medical examiner in charge of the district in which the patient has died has been consulted. A more thorough discussion of this problem may be obtained by reading an article by O. T. Schultz (1930) entitled "The Law of the Dead Human Body."

Frequently, permission for only a partial autopsy is obtained. The autopsy is restricted to the examination of the head, the contents of the thorax, the abdomen or single organs. Sometimes the size or the location of the incision is restricted. Permissions should be examined carefully to see whether or not such restrictions exist. From the standpoint of the pathologist such cases are frequently very unsatisfactory, as the partial examination is insufficient to explain many features of the disease process as a whole.

Much misunderstanding between the clinician, the relatives and the pathologist can be avoided if restrictions are carefully worded. The usual understanding is that, in cases where only the size and location of the incision are limited, it is legitimate for the pathologist to remove and examine all organs that he can reach through this incision. If, on the other hand, the restrictions read that only certain organs can be examined he should limit his examination to those organs.

Even in cases where no restrictions are mentioned it is usually better to limit the examination to the organs of the thorax and abdomen, the brain and spinal cord. If further examination is indicated it should be done only after due consideration, always keeping the disfigurement of the body down to a minimum.

b. Instruments

The following instruments will be found extremely useful in the autopsy room, although not all of them are necessary.

Autopsy Table.—The table (Fig. 6) should be large, in order to accommodate on it the instruments and several dishes in addition to the body. It should have a slightly raised edge, and drainage toward an opening in the center of the table should be provided for the escape of fluids. The autopsy tables found in different laboratories vary greatly. Many are made of stainless steel or similar stain and corrosive resistant preparations, some of stone or porcelain. Wooden tables lined with zinc or copper are probably cheapest and are very practical. The height of the table is important. Thirty-two to 33

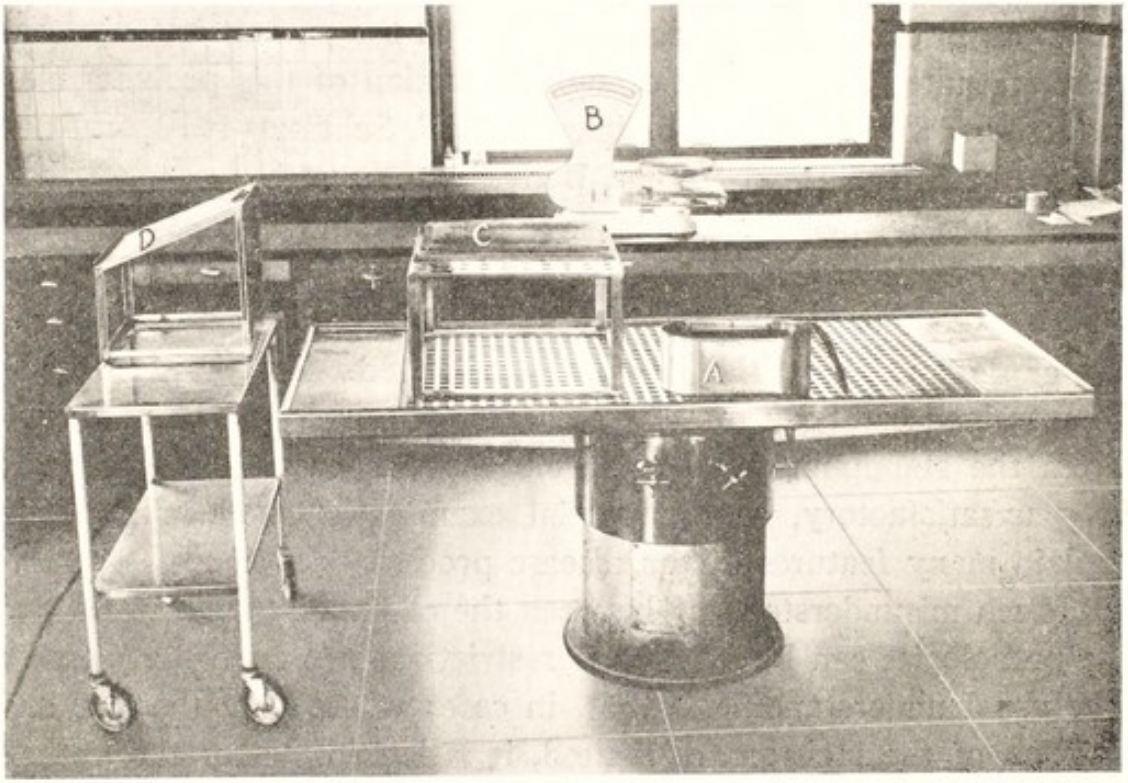

Fig. 6.—Autopsy table in use at the Boston City Hospital. A, Sponge basin; B, scales; C, small dissecting table; D, elevated, movable writing desk.

inches (81–84 cm.) is a very satisfactory height which both short and tall prosectors can use with comfort. Running water should be present at the table. This can be brought by overhead pipes but, when practical, is best supplied by connections running up through the center of the table. Both cold and hot water should be available, joining in a mixing faucet to which a rubber hose can be attached. A 6 to 8 inch (15–20 cm.) piece of metal pipe can be attached to the free end of the hose and should be of sufficient weight to hold the hose in position when it is put in the sponge basin.

Sponge Basin.—A rectangular or oval copper or zinc basin with a flat bottom measuring about 9 inches (23 cm.) in height, 15 inches (38 cm.) in length and 8 inches (20 cm.) in width with a row of perforations on one side about three quarters of an inch (2 cm.) below the upper edge so as to allow the overflow to escape.

Dissecting Table.—A small table about 12 inches (30 cm.) in height with a top measuring about 20 by 24 inches (51 by 61 cm.) is a great convenience for dissection of organs. It should be supported by legs so that it can be put over the feet of the cadaver. It should also have a half inch (1.3 cm.) rim to prevent dripping of fluid. A removable cork top for this table is practical and saves the edge of the section knives.

Scales.—It is impossible to obtain a single scale sufficiently delicate for small structures such as the pituitary and parathyroid glands and large enough for the liver, large tumor masses or the whole body. The ideal combination is (1) a platform scale of a capacity to weigh whole bodies, *i. e.*, 10 to 100 kg.; (2) a balance accurate between 100 gm. and 10 kg.; (3) a balance with weights accurate between 0.2 gm. and 100 gm. A direct reading balance is of great convenience for the medium sized scale. All scales should be graduated in the metric system.

Cutting Instruments.—The best autopsy knife is a stout, broad-bladed knife with bellied edge and heavy handle. The blade should measure about 12 cm. in length and 3 cm. in width; the handle should be 12 cm. in length. Many operators prefer a somewhat smaller knife than this.

Amputating knives of different sizes are useful for long, deep cuts into organs and tumors.

A myelotome consists of a short, thin, narrow knife blade, 1.4 cm. long and 4 mm. wide, set obliquely on a slender steel stalk ending in a wooden handle. It is used only for cutting the cord squarely across while removing the brain.

Cartilage knives and scalpels of different sizes are useful for a variety of purposes. The modern surgical knife with changeable blade is very convenient.

Scissors, both straight and curved, should be of various sizes. A medium sized and a fine pair should each have one probe-pointed blade.

An enterotome is a long, straight pair of scissors, of which one

blade is longer than the other and blunt at the extremity. A hook at the end is not advisable. The instrument is used in opening the intestines and the heart.

A saw with movable back and rounded end will be found the most generally useful for opening the skull and the spinal canal. An ordinary meat saw is preferred by some, but cannot be used on the vertebrae.

Luer's double rachiotome, or adjustable double saw, is very useful in removing the cord, and is the safest instrument to put into the hands of beginners.

A costotome, heavy bone shears, is employed for cutting the ribs.

A powerful bone cutter, with short blades 5 cm. long, set at an angle of about 45° to the handles, which are 36 cm. in length, is employed for dividing the arches of the cervical vertebrae and for other purposes for which ordinary bone cutters cannot be used.

An electric band saw for splitting bones that have been removed is very useful. It is a dangerous instrument in the hands of a beginner and should be used with the greatest care. It is safer to saw fixed rather than fresh material.

Other Instruments.—Forceps should be of several sizes, large and small, mouse-toothed and smooth.

A chisel with a 2 cm. cutting edge is used for exposing the marrow of the long bones, removing portions of the base of the skull and so on.

A hatchet-chisel of steel is helpful in removing the calvarium and the spinous processes of the vertebrae after sawing the skull and the vertebral column.

Soft iron hammer with wooden handle.

Steel hammer with wedge end and blunt hook on the handle.

Holder for the head during the sawing of the skull.

Probes of flexible metal as well as fine glass probes for small blood vessels or ducts.

Catheters.

Pans for holding water, organs, and so on.

Sponges.

Glass graduates for measuring fluids.

Although not necessary some sort of suction apparatus for the removal of body fluids is very useful. An electric suction pump, leading through first one bottle to act as a trap, and then to an-

other larger bottle (1–2 liters' capacity) with graduations marked in its side, and finally by a rubber tube to the body itself, is very satisfactory. The whole apparatus is best mounted on a small movable table.

A small cup or dish for removing fluid from cavities.

A searing apparatus for cultures. In order to prevent contamination of the cultures taken from organs, the external surface should be seared with some hot object and the culture taken by puncturing through this seared area into the organ itself. The simplest apparatus for this, when illuminating gas is available, is a Bunsen burner, an old autopsy knife or a soldering iron, and a ring stand of such height that the object to be heated can be rested on it and be exposed to the flame of the burner. An electric soldering iron is also a very good instrument for this purpose.

Autopsy needles, long and slightly curved.

Autopsy twine for sewing up the body.

c. General Rules

The autopsy room (Fig. 7) should be well lighted, otherwise the finer changes in the tissues cannot be recognized. As it is necessary to use artificial light in many instances, a good source of artificial illumination should be available. Three overhead lights distributed 6 to 10 feet above the body and focused on it are very satisfactory. An adjustable and movable floor lamp is convenient in order to be able to throw light into the various cavities.

Before beginning an autopsy the necessary instruments should be arranged on a short board or dissecting table on the autopsy table in the order in which they are most likely to be used.

The operator stands on the right side of the body. This position he rarely leaves except for some definite purpose; for example, in opening the skull he stands at the head.

Order and cleanliness are the first points to be insisted upon at every autopsy. Clean water should always be at hand for washing the instruments and for keeping the hands free from blood and pus. The cut surface of an organ should not be washed with water except to remove blood; gently scrape the surface with the knife held obliquely.

In cutting, the knife should be drawn, not pressed or shoved into the tissues. According to Virchow, a broad, clean cut into an or-

gan, even if incorrectly made, is much better than several short cuts which leave a ragged surface.

The autopsy knife should be grasped in the hand as if to cut bread. In using this knife the main movement should be from the shoulder, not from the wrist as in dissecting. It goes without saying that the sharper the knife the better.

In cutting the brain and cord, especially if their consistence is soft, moisten the knife to prevent the tissue from sticking and tearing.

Fig. 7.—View of the autopsy room at the Boston City Hospital.

Before beginning an autopsy it is important to know the main points in the clinical history of the case, as they may greatly lighten the work of investigation by calling attention to those organs that require special examination.

The record of an autopsy should preferably be dictated by the operator as he proceeds with the examination of the body, and should be as nearly as possible an objective description of the appearances found. Only the anatomical diagnoses should express the opinion of the operator. If it is not convenient to dictate the

autopsy during its performance, a description of the lesions certainly ought to be made with the organs in sight, and not from memory after the lapse of hours or even days, when many of the details may be forgotten. Later, the results of the bacteriological and histological examinations should be added to the autopsy report, to make the record complete.

Rubber gloves similar to those used by surgeons should be worn whenever a postmortem examination is made. For an ordinary unrestricted examination the usual short gloves are sufficient, but when in a restricted case the arm must reach into distant cavities through a small incision, long obstetrical gloves should be worn. Many pathologists prefer to wear thin cotton gloves over their rubber gloves in order to be able to grasp slippery tissues more firmly. If a glove is torn or cut it should be removed, the hands thoroughly washed and a new glove put on before continuing the autopsy.

After an autopsy the operator should scrub his hands thoroughly with soap and a brush, just as a surgeon does before an operation, and then use, if he so desires, an antiseptic solution, such as corrosive sublimate (1:2000) or 70 per cent alcohol. For removing odors from the hands, turpentine will often be found serviceable, or a ¼ per cent aqueous solution of potassium permanganate followed by 5 per cent aqueous oxalic acid. Autopsy infections are easier to prevent than to cure. They are also potentially dangerous. A cut received during an autopsy should immediately be washed thoroughly and an antiseptic, such as iodine, applied. If the cut is small cover it with a finger cot and glove; if it is extensive it is much safer to let someone else finish the autopsy. A bottle with a 20 per cent argyrol solution should be kept available in every laboratory, together with a medicine dropper. If infected material splashes in the eyes a drop of this should be instilled in the conjunctival sac.

To prevent infections of slight wounds, such as scratches, or such as occur in hair follicles, the best treatment within the first 24 hours is to bore into them with a sharp pointed orange-wood stick dipped in strong carbolic acid, followed by washing with 95 per cent alcohol. The procedure is practically painless, and the infection is stopped in the very beginning. Where the infection has spread, surgical treatment must be resorted to.

d. Suggestions to Beginners

In a case of generalized miliary tuberculosis the older focus from which the organisms have spread must always be sought for. Look especially for tuberculous thrombi in the pulmonary veins and for tuberculous involvement of the thoracic duct as a frequent source of the general infection.

In a case of embolism search for the thrombus, bearing in mind, however, that an entire thrombus may become free and form an embolus. An arterial embolus may be due to a venous thrombus, in which case it must have passed through an open foramen ovale, except in cases of thrombosis of the pulmonary veins.

In acute peritonitis always seek for a source of infection (appendix, female genitals, gastro-intestinal tract). It cannot always be found.

In hemorrhage from the stomach associated with cirrhosis of the liver look for rupture of dilated esophageal veins.

In cases of more or less sudden death, especially if preceded by signs of asphyxia, always examine the pulmonary artery *in situ* for possible emboli. In cases of instantaneous death examine the coronary arteries.

The following general rules should be kept in mind by the inexperienced:

1. Never start an autopsy in a hospital or elsewhere until you have seen the written permission.

2. The autopsies are for both the clinicians and the pathologists. It is not fair to keep clinicians waiting while a slow technique is followed out. Find out what they are interested in and show them this as soon as possible. This usually can be done without spoiling relationships for the pathologists.

3. It is often difficult to obtain autopsies because of the objections of the undertakers. Therefore, every attempt should be made to coöperate with them and the body should not be unnecessarily disfigured. The most important thing from the standpoint of the undertaker is that the circulation to the head and extremities is not damaged. In these regions any vessels cut should be tied in such a way as to permit embalming.

4. If you are in doubt about any procedure, do not hesitate to call for advice.

5. Autopsies can be done neatly if care is taken. Sloppiness

makes a bad impression on onlookers and visitors. Therefore keep the body and yourself clean. If accidents occur, change your apron.

6. When signs of inflammation are found, make both a culture and a smear from the involved area. The culture may be badly contaminated but the predominant organism can often be determined from the smear.

7. Once the autopsy is completed and the body has been removed, you can never go back and do the things you have forgotten to do. Therefore, try to think of everything important while you are doing the autopsy.

8. On interesting or unusual cases, gross material should be saved for conferences and teaching. A specimen can be completely spoiled for this purpose by one careless cut. In such cases, alterations in the usual technique may be necessary. Do not spoil a good gross specimen for the sake of histology. Usually a suitable compromise can be reached. In such cases ask for advice.

e. Private Autopsies

Private autopsies must often be made under many difficulties and, when out of town, not infrequently in a short space of time. It is always important to warn the attending physician not to allow the undertaker to inject the body before the autopsy, because the color and consistence of the organs are so changed by most injecting fluids that it is difficult to recognize the pathological processes. If there is danger of postmortem changes, have the body packed in ice.

A bag equipped for postmortem examinations will be found very convenient for carrying instruments to private autopsies. The instrument case should contain 1 or 2 autopsy knives, 2 scalpels, a pair of forceps, 1 or 2 pairs of scissors, an enterotome, a steel hammer with wedge-end and blunt hook on the handle, a small chisel, a saw with detachable handle and back, a celluloid ruler, an autopsy needle and a probe; free within the bag should be carried a spool of strong twine, a costotome, a long slender knife for use in removing the brain, a hammer with soft iron head and a sponge. In rare cases additional instruments may be required. A spring scale reading up to 2 kg. is convenient but somewhat bulky. It is also well to carry along several plates and tubes of culture media and sterile swabs for bacteriological examination. An alcohol lamp

with an old autopsy knife for searing, or better, a small electric soldering iron, should be taken. Bottles containing fixative for tissue must be included. For fixation take 2 one-half pint jars, one containing Zenker's fluid, the other 10 per cent formalin.

At the house can always be obtained a slop-pail, a wash-bowl, a pitcher of water, several newspapers and an old sheet. The body is usually on an undertaker's frame, but it may be on the bed. The examination of the chest and abdomen can be made in either of these positions. On the thighs of the body place one or two folded newspapers, and on these the necessary instruments. On the legs place the bowl containing a dampened sponge. If the undertaker has not put a rubber sheet on the floor beneath the body and on the side where the operator is to stand, newspapers should be spread to protect the floor. Place the slop-pail on the rubber sheet within convenient reach. Having thus made all arrangements, even to the threading of his needle, the operator is ready to begin.

If the cord and brain have to be examined as well as the body, it is best to do the cord first, so as to avoid the leakage that might otherwise occur from the trunk cavities if they have been opened first. To support the head while opening it use a stick of wood, a brick, or, in case of necessity, the instrument box wrapped in a newspaper.

At a private autopsy cleanliness is extremely important. If there is no undertaker or nurse present, the operator himself must see that everything is cleaned and put in order before leaving, that all the blood stains are removed from the dishes, and that all papers and soiled clothes are burned or rolled up and left in a neat bundle for the undertaker to dispose of.

(2) EXTERNAL EXAMINATION OF THE BODY

External examination is often of great importance, especially in medicolegal autopsies, and should never be neglected, as it may throw light on lesions found within the body. It should be systematic and careful, and is best taken up in the following order:

a. Inspection of the Body as a Whole

Sex.

Age.

The body length should be measured on the table beside the body from the vertex of the head to the heel of the foot.

The development of the skeleton has reference to the bony framework, which may be powerful, slender or deformed.

The general nutrition is shown by the amount of subcutaneous fat tissue and muscular development. The former is judged by pinching up folds of skin.

The general condition of the skin is examined for amount of elasticity, bronzing, jaundice, edema and decubitus.

Postmortem discolorations may be divided into three varieties:

1. Hypostasis of blood, or the settling of blood into the lowest lying blood vessels; this form of discoloration disappears on pressure.

2. Diffusion of blood-coloring matter out of the vessels into the surrounding tissues (due to blood pigment being set free by postmortem decomposition); this does not disappear on pressure.

3. Greenish discoloration, usually seen earliest over the abdomen, is due to iron sulfide formed through decomposition of the tissues. This discoloration is important, as it may modify the interpretation of appearances observed in the internal organs.

Postmortem rigidity is observed as to degree and extent. It begins in the maxillary muscles and spreads gradually from above downward, disappearing later in the same order. It is most marked, and lasts longest, in muscular individuals who have been ill but a short time. Cholera furnishes the most marked cases. The rigor disappears quickest in cachectic diseases. When once it has been forcibly overcome, it does not recur. The time at which it begins after death varies greatly, from 10 minutes to 7 hours.

b. Special Inspection of the Different Parts of the Body

The examination should begin with the head. Any lesion or abnormality found should be carefully noted. Particular attention should be paid to the condition of the pupils and to the color of the sclerae. Then observe in order the neck, the thorax (size and shape), the abdomen (distended or retracted), the genitals and the extremities.

(3) INTERNAL EXAMINATION OF THE BODY

The opening of the body cavities is described first, because the brain is less frequently the seat of disease, and because in this country it is often impossible to obtain permission to open the head. Moreover, the lesions in the body often throw much light on those

to be expected in the brain. The advantage of examining the brain first, particularly in those cases in which the important lesions are cerebral, is said to be that the amount of blood in the cerebral vessels can be more accurately determined. After the heart has been removed some of the blood in the brain may escape through the severed vessels below.

In routine examinations, however, the body is usually examined first, then the brain, and finally the cord. It is not a bad practice to remove the calvarium, to examine the meninges over the upper surface of the cerebrum, and then to make the examination of the body before removing the brain. In this way any change in the blood supply of the cerebral vessels will be observed.

a. Opening of the Abdominal and Thoracic Cavities and Inspection of the Organs in Situ

(*a*) *Opening of the Abdominal Cavity.*—In the examination of the body the peritoneal cavity is opened first, the two pleural cavities next, and the pericardial cavity last. The cavities and their contents are inspected in the order and at the time that each is opened, but the organs are removed from the cavities for further examination in the reverse order, beginning with the heart.

A Y-shaped incision is probably the most satisfactory and practical type of primary incision to make in all unrestricted autopsies, both male and female. The incision is started at the left acromion process, passes just medial to the breast and is continued to a point in the midline over the lower end of the sternum. At this point it is met by a similar incision from the right acromion process and is then continued down the midline of the abdomen passing just to the left of the umbilicus and extending to the symphysis pubis. In cutting, the handle of the knife is depressed so as to use the belly of the blade rather than the point. Over the sternum the cut should extend down to the bone; over the abdomen, however, only into the muscles, or in fat people through the muscles into the fat tissue. To open the abdominal cavity, nick carefully through the peritoneum in the lower third of the abdomen, introduce the first and second fingers of the left hand and, while making strong upward and outward traction on the right abdominal flap, extend the incision down to the pubis and up to the xiphoid. Some operators prefer to separate the fingers and to cut between them.

The abdominal flaps are rendered much less tense by cutting the pyramidales and recti muscles transversely from below just above the pubis. Care must be taken not to injure the overlying skin. The abdominal cavity can now be examined, but more space will be obtained if the skin and the underlying muscles are first stripped back from the thorax to about 5 cm. lateral to the costochondral line.

The operation is most easily and neatly done by lifting the skin directly away from the chest wall or turning it forcibly out with the left hand, and then cutting the tense tissue close to the cartilages and ribs with long sweeps of the knife held almost flat. The operation begins over the lower border of the ribs and extends upward. In dissecting off the skin and muscles from the left side, the right hand works underneath the left.

Next, the "V" flap is also dissected up as far as the clavicles or up into the neck if examination of the neck organs is to be made. Great care should be taken not to nick through the skin. The mammae can easily be incised from the under side of the flap, and if necessary the axillary lymph nodes can be reached by dissecting the skin farther out, especially over the clavicle. Before beginning the inspection of the peritoneal cavity it is important to examine first the surface of the incision into the abdomen, noting the thickness and color of the fat tissue and the condition of the muscles.

(*b*) *Inspection of the Abdominal Cavity.*—The character of any fluid present should be determined and its amount measured or estimated. The simplest way to remove it is to dip it up with a small cup or dish and pour it into a glass graduate for inspection and measurement. If the presence of gas within the peritoneal cavity is suspected, form a small pocket in the primary incision (undercutting the skin a little if necessary) and fill it with water. Puncture through the water into the peritoneal cavity and the gas, if present, will escape in bubbles.

The various abdominal organs and their relations to each other should be investigated *in situ* by sight and by touch. As a rule, examine first the gastro-intestinal tract, including the appendix and the mesenteric lymph nodes. Ulcerations of the intestine can often readily be made out through the walls. The examination of the spleen, liver, kidneys and pelvic organs follows. The pancreas is

easily reached by tearing through the omentum between the stomach and the colon, so as to open the lesser peritoneal cavity.

After the inspection of the abdominal organs the position of the diaphragm is ascertained on both sides in the costochondral line by reaching with the right hand, passed palm upward, underneath the ribs, and the left hand outside at the corresponding height to mark the position of the ribs or intercostal spaces. On the right side the hand is passed up on the outside of the falciform ligament. Normally, the diaphragm stands at the fifth rib on the left side, and at the fourth rib or fourth interspace on the right.

(*c*) *Opening of the Thorax.*—To open the thorax, cut through the costal cartilages at a distance of 5 mm. from their junction with the ribs, beginning at the second cartilage and holding the scalpel nearly horizontal, so that as one cartilage is cut through the handle of the scalpel will strike the next below and prevent the blade from penetrating too far and injuring the lung. In young people the cartilage can be cut easily by one long stroke on each side, but care must be taken not to go too deep. If the intercostal muscles are not divided by the same operation, the sternum can be depressed by the left hand and the muscles severed by one pass of the knife on each side. The lower end of the sternum can now be elevated and freed from below upward from the diaphragm and pericardium until the first rib is reached. The first rib is cut about 1 cm. farther out than the others, and from below upward toward the clavicle, with the handle of the knife directed upward and a little outward. The sternum is then still further freed from the anterior mediastinal connective tissue until its upper end is reached. The sternoclavicular joint on the left side can now be easily opened from below by entering a scalpel just above the cartilage of the first rib, and following the irregular line of the joint around the end of the clavicle, while at the same time drawing the sternum over to the right side of the body. The right sternoclavicular articulation is opened by continuing the incision of the scalpel over the upper end of the sternum into the joint. The advantage of this method is that there is much less danger of wounding the large vessels at the base of the neck, and thus of mingling blood with any exudate which may happen to be present in the pleural cavities. If preferred, however, the articulations can be opened and the cartilages of the first ribs cut from above before freeing the sternum from the diaphragm.

In this case enter a short, sharp, narrow-bladed scalpel held vertically and loosely into the left joint on its upper side, starting the incision just outside of the attachment of the sternal end of the sternomastoid muscle, and cutting around the end of the clavicle by a series of short up-and-down strokes, allowing the blade to follow the irregular line of the joint. After cutting through the joint continue the incision outward and cut through the cartilage of the first rib.

If the cartilages are calcified, use the costotome and cut through the ribs, as more room can be gained in this way and the ribs are more easily cut than the calcified cartilages. The sternoclavicular joint can also be disarticulated by cutting with the costotome. If the autopsy restrictions allow only an abdominal incision, the organs of the thorax can be obtained by removing them from below. The diaphragm is first freed by cutting its attachments to the ribs. Then by blunt dissection with the fingers of the right hand the aorta and mediastinal contents are freed from the vertebral column. When this is complete, the arch of the aorta and the great vessels are located by palpation and the contents of the thorax put under traction by pulling with the left hand while the great vessels are severed just at their origin from the aortic arch. Now the whole contents of the thoracic cavity can be pulled down into the abdomen. The sternum should be inspected at the time of its removal. It is perhaps best to examine next, especially in children, the epiphyses of the ribs at the costochondral line for any evidence of thickening.

(*d*) *Inspection of the Thoracic Contents.*—In the pleural cavities, as in the peritoneal cavity, the character and amount of any abnormal contents must be determined. If, from the clinical history or for any other reason, the presence of air in a pleural cavity is suspected, a pocket should be formed over the ribs by aid of the skin-flap and filled with water. The pleural cavity is then pierced with a scalpel through the bottom of the pocket. Air, if present, will bubble up through the water.

Slight adhesions are best torn through or cut. If the lungs are firmly attached, it is best to strip off the costal layer of the pleura with the lung. This is most easily done by freeing the anterior edge of the costal pleura with the handle of the scalpel, and working in first a finger and then the whole hand until the pleura is entirely free. In passing the hand into the pleural cavities protect the back

of it, especially if the ribs have been cut through, by folding the skin-flap in over the edge of the ribs.

If desired, the lungs can be drawn forward, examined over their whole extent, even incised, and then replaced until the heart has been removed. In the connective tissue of the anterior mediastinum there is almost always a certain amount of emphysema due to the removal of the sternum. Emphysema due to laceration of the lung tissue is more marked in the upper half of the mediastinum, and usually extends up into the neck. The thymus gland attains its full development at the end of the second year, after which time it usually slowly atrophies.

b. Removal and Opening of the Thoracic Organs

(*a*) *Opening of the Pericardium.*—To open the pericardium, seize the sac near the middle with fingers or forceps, snip through the wall with a knife or scissors, and with either instrument cut upward to where the pericardium is reflected over the large vessels, downward to the lower right border, and lastly to the apex. By gently raising the apex of the heart the amount of fluid in the pericardial cavity can be seen. The normal amount ranges between 5 and 50 cc. Pericardial adhesions should be broken through with the fingers. If this is impossible, the heart must be incised through the pericardium.

(*b*) *External Inspection of the Heart.*—Determine first the position, size and shape of the heart, and the degree of distention of the different parts. The right ventricle and both auricles are usually distended with blood, which may be fluid, as in death from suffocation, or more or less coagulated. The left ventricle is contracted and empty unless the individual has died from paralysis of this part of the heart, in which case it will be found distended with blood (condition of greatest diastole).

(*c*) *Removal and Opening of the Heart.*—To remove the heart, grasp it gently near the apex with the left hand, supporting it further, if necessary, by one or two fingers placed above the coronary sulcus, and lift the whole heart vertically upward. Then cut its vessels from below upward with the knife held transversely and obliquely. Sever in turn the inferior vena cava, the pulmonary veins on both sides, the superior vena cava, the pulmonary artery

and the aorta. Go deep enough to remove the heart entirely, but avoid injury to the underlying esophagus.

For making the incisions (Fig. 8) to open the heart, either a long, slender-bladed knife or long, straight scissors may be used. The heart should be placed on the dissecting table with its anterior surface up. The right auricle is opened by cutting from the orifice of the inferior vena cava into that of the superior, and from the latter into the auricular appendage. Before proceeding, examine the tricuspid valve and estimate whether its orifice is dilated or narrowed by determining how many fingertips it will admit. The

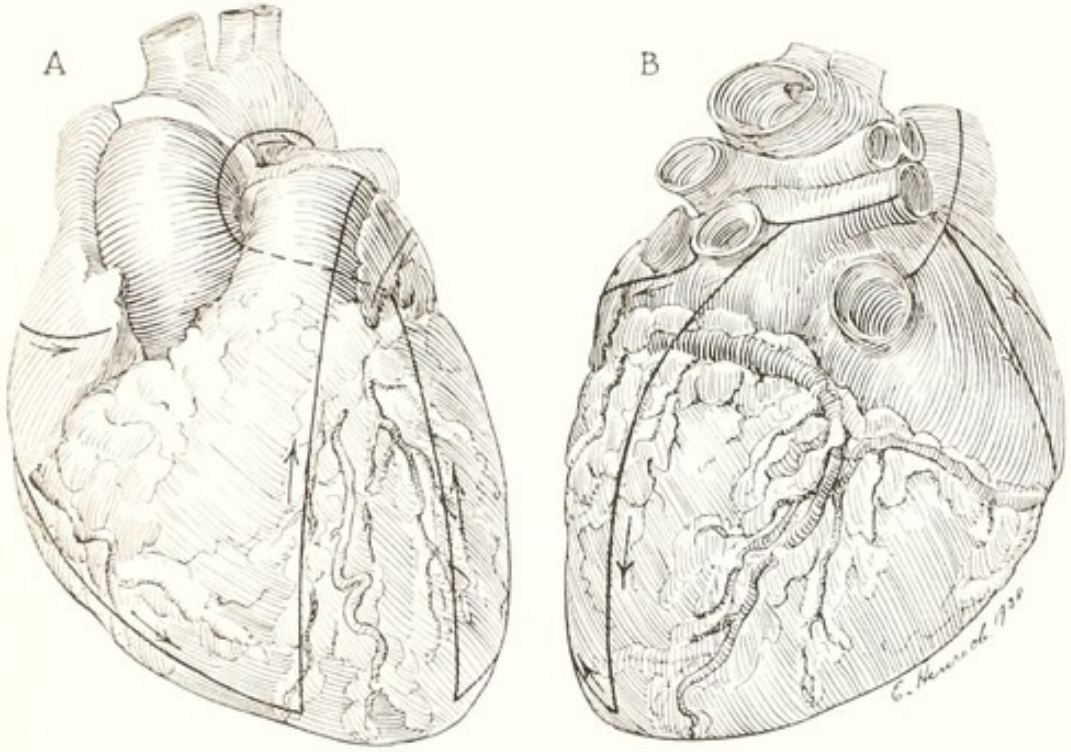

Fig. 8.—Method of opening the heart showing lines of incisions followed. A, Anterior view; B, posterior view.

first incision to open the right ventricle is made through the tricuspid valve and the wall of the ventricle along the under surface of the right border of the heart. It should be carried to the end of the ventricle, which does not reach quite to the apex of the heart. The second incision begins about the middle of the first, just above the insertion of the anterior papillary muscle, which should not be cut, and is carried through the pulmonary valve well over on the left side along the left border of a narrow, projecting ridge of fat tissue usually present, so as to pass between the left anterior and the posterior segments of the valve.

The left auricle is opened in a manner similar to the right by incisions joining the four orifices of the pulmonary veins and extending into the auricular appendage. The lumen of the mitral valve is measured with fingertips in the same manner as indicated for the tricuspid valve.

The first incision into the left ventricle is made through the mitral valve along the left border of the heart (*i. e.*, the middle of the external wall of the left ventricle), between the two bundles of papillary muscles, to the apex of the heart. The second incision begins at the termination of the first at the apex, and is carried up close to the interventricular septum, parallel to the anterior descending branch of the left coronary artery and about 1 cm. from it. The upper portion of the incision should pass midway between the pulmonary valve and the left auricular appendage. Ordinarily, one of the aortic cusps is divided, but this may be avoided, if desired, by dissecting away to some extent the pulmonary artery from the aorta and carrying the incision well over to the right between the right posterior and anterior valve segments. As each auricle is opened the blood and clots it contains should be carefully inspected from above. In certain cases, as for instance extreme stenosis, it may be preferable not to cut through the valve, but to begin the incision in the ventricular wall below the valve. The ventricular cavities should in like manner be freed from clots and the valves closely inspected. The coronary arteries should usually be opened by means of small, narrow-bladed, probe-pointed scissors as far as they can be followed. The examination of the descending branch of the left coronary artery is especially important. The right coronary artery is best opened by placing the tip of the left forefinger in the aorta over the orifice of the artery, and cutting from without in toward the fingertip until the vessel is reached, when it can easily be slit up. In this way injury to the aorta is avoided. Sometimes it is advisable to examine the coronary vessels by means of multiple transverse sections instead of by slitting them longitudinally; the danger of disturbing emboli or thrombi within the lumen is less.

In cases of more or less sudden death with symptoms of asphyxia, the pulmonary artery should always be opened *in situ*, before removal of the heart, in order to examine it for possible emboli, because they often lodge just at the point where the vessels are severed in removing the heart and lungs and easily may slip out

unobserved. The simplest operation is to thrust a sharp pointed scalpel through the artery just above the valve on the left side in the line of incision already described, and to cut upward until the branches to the right and left lungs are reached. If desired, this incision may be extended down through the pulmonary valve and the ventricular wall along the line given for the second incision in the right ventricle.

The water test for the competence of the valves of the heart is not very reliable, especially for the auriculoventricular valves, and is not so much used as formerly. Inspection and measurement of the valve after the heart has been opened will usually enable one to judge fairly accurately concerning the degree of competence. Before applying the test to the aortic valve the first incision into the left ventricle must be made and the cavity freed from clots, so that no obstruction will exist below the valve. Then the heart is held so that the aortic valve is perfectly horizontal, and water is poured in from above to float the cusps out. If competent, they should keep the water from flowing through. If, however, in holding the heart the normal relations of the valve and the surrounding parts are not maintained, the valve may leak. A second source of error is that the water may escape through the coronary arteries, branches of which have been cut in opening the ventricle. In testing the mitral valve the left auricle is first opened and the clots removed, so as to expose the upper surface of the valve. Then the nozzle of a syringe is introduced through the aortic valve and water forced in so as to float the mitral curtains up. The test, however, is very unreliable, because the parts cannot be placed under natural conditions.

The pulmonary and tricuspid valves can, of course, be tested by methods similar to those already described.

Increase or diminution in the size of the heart is best determined by weighing the organ after the removal of the clots. In certain cases, however, and in special investigations, measurements of different parts of the heart are desirable. Roughly, the heart is the size of the individual's fist.

Opening and examination of the heart after its removal are satisfactory only when the organ is normal or contains lesions within itself which are not in continuity with any of the vessels entering into it. In aneurysm of the ascending aorta, in thrombosis of a

vena cava, and in a number of different lesions connected with the heart or with the vessels given off from it, it is important to examine these vessels and to open them while they are still in continuity with the heart. For this purpose the Ghon or the "en masse" technique is better. These will be described later.

More space for the examination *in situ* of the vessels at the base of the neck can be obtained by freeing the clavicles from all attachments above and to the first ribs and drawing them forcibly outward; this operation will be found especially useful in following up the subclavian vessels.

(*d*) *Removal and Examination of the Lungs.*—Pleural adhesions have already been spoken of. If the base of the lung is adherent to the diaphragm, it is usually advisable to remove the latter with the lung by cutting through its insertion into the ribs. According to Orth (1900), there is less danger of wounding the abdominal organs if scissors are used for the performance of the operation. After the lung is free it is drawn forward out of the pleural cavity, and its root is grasped from above downward between the separated first and second fingers of the left hand. The lung is drawn downward toward the pubis and the primary bronchus is divided by a nearly vertical incision above and behind the left hand. Then the lung, supported by the flat surface of the knife, is lifted vertically from the thorax, avoiding injury to the esophagus and aorta.

The procedure is the same for both lungs. Occasionally the apex of a lung will be found so firmly adherent by dense scar tissue that it can be freed only by using the knife.

The primary or main incision into a lung is a long, deep cut from the apex to the base and from the convex surface to the root, slitting the primary bronchus, but not cutting it off from its branches to the upper and lower lobes. To incise the left lung, place it with its inner or medial surface and root downward on a board and with its base toward the operator. The left thumb steadies the lower lobe, the first finger reaches between the two lobes almost to the primary bronchus, and the rest of the fingers should hold the upper lobe.

The right lung is most easily incised by placing it in the same position but with the apex toward the operator; in other words, always place the anterior edge of a lung beneath the palm of the hand. Some prefer to place each lung on its lower or diaphragmatic

surface for incision. The right middle lobe is incised separately by a cut extending transversely in its greatest diameter.

The bronchi and blood vessels should be opened up for some distance with small probe-pointed scissors, as a rule from the surface of the section, cutting through the overlying lung tissue. In some cases, however, it is best to open up both the blood vessels and the bronchi from the outside of the lung before incising it. They should be opened in the following order: the vein first, then the artery, and finally the bronchus.

Secondary cuts into the lungs are made parallel to the main incision.

The peribronchial lymph nodes should be incised.

c. Examination of the Organs of the Neck

If it is desired to dissect the organs of the neck this can be accomplished successfully without making further skin incisions. A better exposure is obtained by elevating the shoulder of the subject on a wooden block and allowing the head to fall backward. Now the skin of the neck can be turned back and dissected away from the underlying structures without injury. This can be continued up to the floor of the mouth. Great care should be taken not to injure the carotid arteries, and all large branches of these blood vessels that are cut should be ligated. Dissect the skin from the larynx and muscles of the neck as far up as possible. In like manner free the muscles, esophagus and trachea from their attachments laterally and posteriorly. Then allow the head to drop well back over the end of the table and pass a long, slender-bladed knife up between the skin and the larynx, just behind the symphysis of the lower jaw, until the tip of the knife appears beneath the tip of the tongue. From this point the knife is carried with a sawing motion down first one ramus of the jaw and then the other, dividing laterally the glossal muscles as far back as the posterior pharynx. The knife is next carried up behind the esophagus, and the posterior wall of the pharynx is divided as high as possible. Pass the left hand up inside of the neck and draw down the tongue. Then cut the attachments of the soft to the hard palate, carrying the knife well out so as not to injure the tonsils. Any remaining attachments are usually easily severed by pressing the tongue first to one side and then to the other, and cutting close to the roof of the pharynx.

Next cut through the middle of the uvula and examine all of the pharynx removed. Incise the tonsils vertically. The esophagus is slit in the median line posteriorly; if it is normal, the larynx and trachea are then slit in the posterior median line also, thus splitting the esophagus in two.

Each lobe of the thyroid gland is incised in its greatest diameter.

The parathyroid glands are most commonly located on the median portion of the posterior surface of the lateral lobes of the thyroid gland close to the esophagus. They are usually four in number, a superior and an inferior being present on each side. They are flattened, ovoid, yellowish brown structures, 6 to 8 mm. in length, 3 to 4 mm. in width and 1.5 to 2 mm. in thickness. They are less transparent than lymph nodes and of a denser consistence than fat. In young people they are light gray in color, while in old individuals they become atrophic and infiltrated with fat tissue.

The parathyroids are best found by first identifying the inferior thyroid artery and dissecting this vessel until it subdivides just before entering the thyroid substance. The inferior parathyroid will usually be found just below this point of branching, the superior ones several millimeters above it. Their location, particularly that of the inferior pair, is, however, somewhat variable. They may be located in the lower pole of the thyroid, farther downward in the fat tissue of the trachea, on the anterior surface of the lowest portion of the lateral lobes of the thyroid, in the thyroid substance itself or in the thymus.

If care is taken the carotid bodies (glomus caroticum) can be located in the neck. These are bilateral structures, reddish brown, oval, each about 5 mm. long. They lie immediately at or just above the division of the common carotid to form the internal and external carotid arteries. They are situated on the medial surface of the artery.

d. Removal and Examination of the Abdominal Organs

(*a*) *The Abdominal Cavity.*—The order of removal of the abdominal organs varies with different operators, and under varying circumstances with the same operator. The gastro-intestinal tract, including the liver and pancreas, may be taken out before or after the genito-urinary tract. The spleen as an organ by itself is often the first to be taken out. The early removal of the liver is occa-

sionally advantageous for the sake of the additional space obtained for the examination of the other organs. It is well to practice the different methods of procedure, so that in a difficult case the best may be selected, because the examination of the abdominal cavity, especially in cases of extensive disease with numerous adhesions, is often one of the hardest tasks in postmortem technique. As a rule, it is best to follow the usual order as far as possible, gradually removing the more or less normal or uninvolved organs. Occasionally it may be advisable to remove the organs *en bloc*, so as to be able to approach the problem from all sides.

In all cases of acute peritonitis it is best, before removing any organ, to search for the source of the infection, paying particular attention to the vermiform appendix, to the gastro-intestinal tract and, in females, to the pelvic organs.

The order of removal of the abdominal organs now to be described is that which seems the simplest and most natural for the majority of cases. First remove the spleen; secondly, the gastro-intestinal tract, including the pancreas and the liver, which forms the upper layer; thirdly, the genito-urinary tract or middle layer, leaving the circulatory tract, the lowest layer, to be opened and inspected *in situ*. If, however, it proves necessary to open a part of the gastro-intestinal tract *in situ*, it will be neater perhaps to remove the kidneys and spleen first.

(*b*) *The Spleen.*—As a rule, the spleen can easily be drawn forward from its position behind the fundus of the stomach, beneath the diaphragm, and lifted on to the lower edge of the ribs on the left side without cutting its vessels. The organ is then incised in its greatest diameter while thus firmly fixed between the left hand and the ribs. On the other hand the vessels may be cut close to the hilus and the spleen incised after removal.

In cases of adhesion to the diaphragm the spleen must be handled carefully while the fibrous attachments are torn or cut through, for the capsule is easily ruptured. Occasionally it is advisable to cut out with the spleen the portion of diaphragm attached to it.

The important anatomical structures to be noted in the macroscopic examination are the capsule, the trabeculae, the blood vessels, the lymph nodules and the pulp.

(*c*) *The Gastro-Intestinal Tract.*—The first step is to examine

externally, more or less carefully according to the clinical symptoms, the whole tract from the stomach to the rectum, if it has not already been done at the primary inspection of the peritoneal cavity. The main points to notice are distention or contraction of the intestines, injection of the blood vessels, thickening of the wall, especially in the lower part of the ileum, adhesions, exudates, and so on. Inspect the mesentery, its length, the amount of fat and the size of the lymph nodes; incise the latter to determine color and consistence. Examine the mesenteric vessels in case any evidence of infarction of the intestine is noticed. The portal vein and its branches should be opened up *in situ* in all cases of abscess of the liver or of secondary deposits in it of malignant growths, before the gastro-intestinal tract is removed. As a rule, it is not necessary to open any part of the gastro-intestinal tract *in situ*. The operation can be performed much more neatly at the sink. The duodenum is often opened for the sake of investigating the flow of bile from the bile duct.

Free the omentum from the transverse colon by putting it under tension and dividing it with the knife close to the colon. Then begin the removal of the large intestine by drawing the sigmoid flexure forcibly forward and cutting the mesocolon close to the gut, first down to the rectum, then upward to the transverse colon. Free the latter by dividing the two folds of the lesser omentum which unite it to the stomach. The ascending colon is freed in the same manner as the descending portion. Care should be taken not to injure the appendix. If the lower part of the sigmoid flexure is now stripped upward a short distance with the fingers, so as to force the intestinal contents out of the way, the gut can be divided just above the rectum without fear of the feces escaping.

Place the freed intestine in a pan or pail, and as the small intestine is divided from its mesentery deposit it in the same receptacle. To remove the small intestine, begin at the cecum and, while lifting the ileum with the left hand strongly enough to keep the mesentery constantly tense, cut the latter close to the intestine by playing the knife easily backward and forward across it with a fiddle-bow movement. Continue the operation until the duodenum is reached. The mesentery can now be dissected from the duodenum and removed, or the mesentery, duodenum, pancreas, and stomach can be removed in continuity with the intestine by care-

fully dissecting them off the underlying structures. The operation is perhaps more easily accomplished by freeing the organs from below upward after certain preliminary incisions. First cut down through the diaphragm and free it around the esophagus. Then separate the stomach from the liver by means of the thumb and fingers of the left hand in such a way as to stretch the vessels of the hepatoduodenal ligament. These vessels (hepatic artery, common bile duct, and portal vein) are then carefully divided in the order named. As each vessel is cut the character of its contents should be observed to see if anything abnormal is present.

The mesentery, if still present, the duodenum, the pancreas and the stomach are now dissected carefully away from the underlying vessels from below upward until the esophagus is reached. This may be constricted by the fingers at any point desired, and cut across without danger of the gastric contents escaping and without the necessity of tying. In certain cases of hemorrhage from the stomach associated with cirrhosis of the liver, it is important to remove the esophagus in continuity with the stomach, because in these cases the hemorrhage usually takes place from dilated esophageal veins.

The stomach and intestines are now opened at the sink by means of the enterotome, the colon along one of its longitudinal muscular bands, the small intestine along its mesenteric attachment, because the most important lesions usually occur opposite this line in the lymph nodules and Peyer's patches. The stomach is opened along the greater curvature by many; others, however, prefer to cut along a line 3 cm. from the lesser curvature, because better museum preparations are thus obtained. In case any tumor or focal lesion is perceived from the outside, it is advisable to cut the stomach, if possible, in such a way as to leave the pathological part uninjured.

Whenever jaundice is present the duodenum must be opened *in situ* in order to examine the bile apparatus in continuity, and thus determine whether the icterus is due to obstruction of the hepatic or common bile duct, or whether it is of so-called hematogenous origin.

To open the duodenum make a transverse fold in the anterior wall and incise with the scissors. Continue the longitudinal slit thus made up as far as the pylorus and down to where the duodenum

passes beneath the mesentery. Notice the contents of the duodenum and their color both above and below the opening of the bile duct. The common bile duct usually opens in common with the pancreatic duct on the medial side of the descending portion of the duodenum, a little below the middle of the head of the pancreas, at a point marked by a small papilla which can easily be recognized by stretching the mucous membrane transversely. Press first on the common duct gently and in the direction of the papilla, watching the opening to see if any obstructing material is forced out. Pressure is then put on the gallbladder to see if its contents also will flow through the bile ducts. If necessary, the common duct and its branches are opened *in situ*. In certain cases the pancreatic duct is similarly examined.

Several cross sections of the pancreas are usually better than one in the greatest diameter, because the duct is left in a better condition for slitting up if necessary.

(*d*) *The Liver.*—The liver is usually the last organ of the gastro-intestinal tract to be removed. This is ordinarily done by lifting up the right lobe and freeing it from all attachments as far as the vertebral column. The right lobe is then lifted and placed on the edge of the ribs on the right side, while the left lobe is elevated and freed. If the diaphragm is firmly adherent, remove it with the liver. The incision to display the liver is a long deep cut passing through the right and left lobes in the greatest diameter of the organ.

In a good many cases it is very convenient to remove the liver at the beginning of the special examination of the abdominal cavity, because more room can be obtained for the investigation of the other organs. This latter fault can to some extent be obviated by cutting the diaphragm on the right side and allowing the liver to slide forward somewhat into the right thoracic cavity.

There can be no objection to the removal of the liver when jaundice is not present or when the liver is not connected by continuity with the lesion of some other organ, such as pylephlebitis in the kidney or a malignant growth extending through the portal vein or along bile ducts.

The operation is performed as follows: Pass the left hand in between the diaphragm and the right lobe and push the liver forward out of the right hypochondrium. Incise it deeply in its great-

est diameter through the left and right lobes. Next free the gall-bladder from its bed by means of the fingers, and cut it off near the hepatic duct after compressing its lower end. It can then be opened lengthwise and washed without danger of discoloring the liver or other organs. The liver is now grasped by placing the thumb on the under surface of the liver and the fingers in the incision. Elevate the organ and, while carefully watching, cut through the hepatoduodenal ligament, which includes the blood vessels and the hepatic duct. The hepatogastric ligament, the inferior vena cava, the falciform ligament, the coronary ligament, and the tissue between the inferior surface of the liver and the upper end of the kidney follow next; the adrenal is left on the kidney, and the diaphragm ought not to be injured.

Even in the ordinary way of removing the liver the organ will be found much easier to handle if the incision is made *in situ*, so as to furnish a hold for the left hand.

Other cuts into the liver are best made parallel to the primary one.

(*e*) *The Kidneys and Adrenals.*—If the adrenals are to be removed with the kidneys, it is necessary to cut first medial to and secondly above the adrenal, and then to make a curved incision along the outer convex border of the kidney from the outer end of the second cut through the peritoneum and the perinephric fat tissue. The left hand is inserted into the cut, the mass of tissue drawn forcibly forward and the vessels divided as close to the aorta as possible, so that the renal vessels may be slit up and examined in connection with renal lesions if present. The adrenal should be incised crosswise. The kidney is held firmly in the left hand between the thumb and fingers while a longitudinal incision is made from the convex border to the hilus. As a rule, it is better to shell it out of its investing fat tissue before incising it.

It will often be found convenient to make simply the curved incision given above, to shell the kidney out of its fat capsule and then to divide its vessels, leaving the adrenal behind to be incised *in situ* or removed separately. As a rule the left kidney is removed first.

In all cases in which the bladder is involved in pathological changes, in common with the kidneys, the whole urinary tract should be removed intact, so that the lesions may be examined in con-

tinuity. For this reason it is a good plan to open up the pelvis of the kidney and the ureter from the primary incision, in order to see if any lesion is present before dividing the ureter.

If it is desired to remove the kidneys before the intestines, the latter must to some extent be freed from their attachments.

The splenic flexure of the colon is first drawn forcibly forward and its attachments divided where they hide the left kidney. If the ureter is to be taken out also, it is best to free the whole of the descending colon from its mesocolon. Then the colon and the coils of small intestine are drawn over to the right side of the body, so as to leave the left kidney and adrenal exposed. They are then removed in exactly the same manner as already described.

To remove the right kidney the hepatic flexure must be freed from over it. If the ureter is to be taken out, the descending colon and the cecum are dissected from it. The right adrenal is firmly attached to the under surface of the liver and must be carefully dissected from it by turning the latter upward.

If the urinary tract is to be removed in continuity, each ureter is dissected down to the brim of the pelvis and then left with its kidney attached until the pelvic organs have been taken out.

After the kidney has been incised the capsule is stripped off, at least in part, so that the appearance of the surface of the kidney and the presence or absence of adhesions between the capsule and the renal tissue can be determined.

The points to be noted in the macroscopic examination of the kidney are size, consistence, and on section, color, relative proportions of cortex to medulla and thickness of each; finally, the details of the structure of the kidney, including the blood vessels, the glomeruli, the convoluted and straight tubules of the cortex, the collecting tubules of the pyramids, and the pelvis.

(*f*) *The Pelvic Organs.*—The pelvic organs are most easily and neatly removed by stripping the peritoneum from the pelvic wall with the fingers. Begin over the bladder and extend down the sides of the pelvis until the fingers meet beneath the rectum. Brace the backs of the hands laterally on the brim of the pelvis and lift the fingers forcibly upward; this movement will free the pelvic organs cleanly from the sacrum and leave them attached only anteriorly at the rectal and genital openings, and posteriorly by the peritoneum and the vessels at the brim of the pelvis.

Anteriorly, the attachments may then be divided with the knife at whatever point seems advisable, ordinarily close to the pubes just anterior to the prostate (or through the urethra and vagina in females) and through the lower end of the rectum. Posteriorly, cut through the tissues at the brim of the pelvis, taking care not to cut the ureters, if the kidneys are still attached to them. The rectum is opened with the enterotome along the posterior wall and the inner surface thoroughly washed off so as to avoid soiling the other organs.

To open the bladder in males, especially if the penis has been removed together with it, incise with the scissors a transverse fold in the anterior wall of the bladder, and carry the incision through the urethra and along the dorsum of the penis. To accomplish the latter act perfectly the penis must be firmly stretched by having an assistant pull at the prepuce while the bladder is held fixed by the operator.

In females it is usual to enter the scissors into the bladder through the urethra and to cut through the middle of the anterior wall of the fundus.

In males the rectum should be dissected away from the bladder so as to lay bare the seminal vesicles and the prostate, which are examined by means of several transverse incisions.

In females, if the bladder is normal, the vagina is opened by an incision through the posterior wall of the bladder and through the anterior wall of the vagina. Or the vagina may be incised laterally until the cervix is reached, and then the cut be carried up to the median line.

The uterus is incised in its anterior wall from the cervix to the fundus. From the upper part of this incision secondary incisions are carried out on each side to the orifices of the fallopian tubes.

The ovaries are incised in their greatest diameter, from the convex border to the hilus.

The testes can readily be examined without external injury to the scrotum by cutting underneath the skin over the pubes down to the scrotum on either side of the penis, and shoving the testes up through the incision. Cut carefully through the overlying tissues until the cavity of the tunica vaginalis is opened. Remove the testis by severing the cord. The incision to display a testis should be in the long diameter, beginning on the side opposite the epidid-

ymis and extending through into it. In cases of tuberculosis of the testis and epididymis it is advisable not to cut through the cord but to remove the testes and cords with the bladder, so that the whole genital tract may be examined in continuity and the associated lesions in the seminal vesicles demonstrated, if present.

The penis, or at least the larger portion of it, can be removed in connection with the bladder by continuing the primary body incision out to about the middle of the dorsum of the penis, which is then freed from the investing skin and divided just proximal to the corona. It is next dissected back to the pubic arch and freed from it partly by cutting from without, partly from within, the pelvis, until the penis can be passed underneath the arch into the pelvis. Other methods are to cut through the symphysis pubis, which can then readily be sprung apart by swinging one of the legs out in a horizontal plane, or even to saw out a small section of bone including the symphysis, so as to have more room for freeing the attachment of the penis and for removing it.

e. Examination of the Structures Remaining in the Body Cavities

There still remain several structures which should be examined.

(*a*) The inferior vena cava and its branches are opened by slitting them with scissors along their anterior walls. If it is necessary to follow the iliac vessels into the thigh it will be found easier, in sewing up, if the primary abdominal incision is continued off to the side in question, thus giving a single though curved incision. It is sometimes advisable to open the inferior vena cava and its branches before removing the pelvic organs, so that thrombi extending into the pelvic vessels may be examined before they are disturbed.

(*b*) The aorta is opened *in situ* along the anterior wall throughout its whole extent, and the iliac arteries as far as the femoral ring.

(*c*) The thoracic duct lies behind and to the right of the aorta. In the thorax it is most easily found by dissecting between the aorta and azygos vein on the right side. The cisterna chyli lies to the right and behind the aorta upon the second and third lumbar vertebrae. Although the thoracic duct can theoretically be found and examined in the course of each of the autopsy techniques, in practice it is very difficult to find it after the organs have been re-

moved from the body. Therefore, in cases in which its examination may be of great importance, such as in miliary tuberculosis or chylous hydrothorax, it is wise to alter the routine procedures to a certain extent in order to make sure of finding it. A quite satisfactory technique is the following. First remove the left lung by cutting through the hilus. Then the right lung is lifted out of the right pleural cavity and it and the heart are displaced over to the left. In this way a good exposure is obtained so that the parietal pleura can be incised along the upper lateral aspect of the bodies of the thoracic vertebrae. The duct is most easily found about 2 or 3 cm. above the diaphragm. The azygos vein is first identified and then by careful dissection the duct itself can be found lying behind the aorta between the azygos and hemiazygos veins along the anterior surface of the bodies of the vertebrae. Once it has been identified, place a ligature loosely around it. If the duct is now kept under slight tension it can be dissected up and down throughout its length.

(*d*) The celiac ganglia composing the largest of the sympathetic plexuses lie on either side of the midline in front of the crura of the diaphragm, close to the adrenal glands on the aorta above the pancreas.

(*e*) The coccygeal body is a vascular skein situated beneath the tip of the coccyx and can be best found by tracing the course of the middle sacral artery to its termination.

(*f*) The vertebral bone marrow at least should be examined routinely. This is usually best done by sawing the bodies of the lower lumbar vertebrae parallel to their anterior surface at a depth of about 1 cm. In cases where the bone marrow is of special interest, as for instance in leukemias and anemias, the bone marrow from the sternum, the ribs, the femur and the tibia should also be examined. A small wedge shaped piece is easily sawed from the sternum and a small piece of rib can be removed where it has been cut in removing the sternum. With the femur and tibia a short 5 to 7 cm. incision is made over the anterior surface of the bone and it is exposed by dissecting away the muscles. The muscles are then retracted from the bone and a small segment removed by first sawing the bone about half way through in a transverse direction in two places and then removing the intervening portion with a hammer and chisel. Care should be taken not to fracture the bone.

Before taking up the methods used for opening the head and examining the brain, three other main types of technique for the removal and the examination of the organs of the chest and abdomen will be described briefly.

2. En Bloc Technique, a Modification of Ghon's Method

(1) Removal of the Thoracic Organs

First, all adhesions between the lungs and the thoracic walls must be noted, then broken or cut. Next, dissect with the fingers between the vertebral column and the contents of the superior mediastinum. When this is complete pass the forefinger of the left hand beneath the organs and bring it in contact with the left thumb. Holding the autopsy knife in the right hand cut through the vessels, the trachea and the esophagus down to the vertebral column. The vessels should be cut as close to their origin from the arch of the aorta as possible, so that they can be easily reached and tied for the undertaker. Still keeping one's grasp with the left hand, the whole thoracic contents can be stripped from the vertebral column by pulling in a caudal direction. Usually no dissection will be necessary, but the more adherent portions can be freed by cutting with a knife. Then the aorta and esophagus are severed just as they pass through the diaphragm and the whole thoracic contents lifted out and placed in a pan.

The thoracic cavity should be sponged out and any abnormalities in the parietal pleura, the ribs and the thoracic vertebral column noted.

(2) Removal of the Abdominal Organs

The contents of the abdominal cavity are removed in three groups.

a. Jejunum, Ileum and Colon.—Start at the rectum, leaving it attached, and dissect with large scissors, cutting the colon from its mesentery. In this manner the whole colon and the first few centimeters of the ileum are freed. Next, dissect the base of the mesentery from its attachment. To do this it will be necessary to cut the superior mesenteric artery and vein. Clamp or tie the intestine at the junction of the duodenum with the jejunum and cut it. Strip as much of the fecal contents as possible from the rectum

back into the sigmoid and cut through the rectum about 3 cm. above the anus. This group of organs can now be lifted out of the abdomen.

b. Liver, Biliary System, Stomach, Duodenum, Spleen and Pancreas.—These organs are all removed as one group. Cut each leaf of the diaphragm in the midline in an anteroposterior direction. This allows the liver to be tilted backward and makes subsequent procedures easier.

Start on the left and free the spleen, the pancreas and all structures as far as the vertebral column without cutting the esophagus. Leave the aorta intact but cut the vena cava and celiac artery just below the liver. Next, proceed with a similar dissection on the right side. The area of the normal attachment of the liver to the diaphragm can be separated from the diaphragm by blunt dissection with the fingers. Take care not to remove or injure the right adrenal gland. Finally, grasp the liver with the left hand, pull it downward and sever the esophagus and the vena cava just below the diaphragm with a knife. Lift the whole group of organs from the abdomen.

c. Urogenital Apparatus, Rectum and Adrenals.—Starting on the left, grasp the kidney with the left hand and dissect it and the adrenal from the underlying muscles. Continue the dissection down to the brim of the pelvis being careful to avoid cutting the ureter. The dissection should be extended to the midline of the vertebral column behind the aorta. The same procedure is then carried out on the right side.

The next step is to free the organs of the pelvis. This is done by blunt dissection with the fingers. The bladder is first separated from the symphysis pubis and then the fingers are passed down around and posterior to the urethra, the vagina in the female, and the rectum until they meet in the midline. Next, the pelvic contents can be freed from the pelvic wall posteriorly by gradually extending the dissection to the brim of the pelvis. The organs of the pelvis are grasped with the left hand, pulled up and the only remaining attachments (urethra, vagina and rectum) cut in the floor of the pelvis. Cut the common iliac vessels just below the brim of the pelvis and dissect the organs away from the sacral promontory, thus freeing the whole group for removal from the body.

(3) EXAMINATION OF THE VERTEBRAL COLUMN AND BONE MARROW

Examine and incise each psoas muscle. With a saw cut through the bodies of at least two or three lumbar vertebrae tangentially to the anterior surface in order to examine the bone marrow and obtain material for sections.

(4) EXAMINATION OF THE THORACIC ORGANS

The examination is started from the posterior surface. Dissect the thoracic aorta free and lay it forward. Open the esophagus along the posterior surface with the enterotome, examine it and then dissect it away from the mediastinum. Thus, the lower end of the trachea, its bifurcation and the mediastinal lymph nodes are exposed. Dissect out the mediastinal lymph nodes and incise each. Open the trachea and the two main bronchi, noting the mucosa and the contents of each.

Before going further, the whole specimen should be turned over and the pulmonary artery opened. A small incision is made in its anterior wall about 1 cm. above the valve with a scalpel, and then with scissors prolong this incision to the hilus of each lung. In order to follow its branches to the right lung it is necessary to dissect the already partially freed aorta back from the pulmonary artery. Examine the artery for emboli.

Next, the heart may be separated from the lungs by cutting through each hilus with the enterotome. The heart and lungs are examined individually as described under the Virchow technique or the lungs may be left attached and opened and examined together as a unit. This latter method is the one described in the original Ghon (Halpert, 1924) technique.

To carry this out the heart is grasped by the apex and rotated so that the left border of the left ventricle faces anteriorly. With the section knife an incision is made through the left ventricular wall along this border but not high enough to injure the mitral valve. The inside of the ventricle is examined and the mitral valve palpated. If the mitral valve is not stenotic the incision is then continued up through the mitral valve into the auricle in a line leading toward the junction of the two left pulmonary veins. This is best done with the enterotome. The left auricle and ventricle and the mitral valve can now be inspected.

The right ventricle is opened in a similar fashion. The heart, held by the apex, is rotated to the right, an incision is made through the right ventricular wall along its left border and the incision is carried up into the right auricle by means of the enterotome in a line half way between superior and inferior venae cavae.

Then the pulmonary conus and pulmonary artery can be opened. The blunt end of the enterotome is passed through the pulmonary conus into the pulmonary artery and the conus and artery cut, keeping as close to the right border as possible.

The next step is to open the aortic valve and the ascending aorta. To do this the blunt end of the enterotome is inserted into the left ventricle beneath the mitral valve and passed into the aorta. The cut is then made across the pulmonary artery just above the pulmonary valve cusps and through the aortic valve.

The heart is now completely opened and the auricles, the ventricles and the valves can be examined. Although it is possible to open the coronary vessels without detaching the organ from the lungs, it is easier to do so after this has been done.

Palpate the lungs throughout and examine the visceral pleura. With a section knife cut each lobe of the lung from the midaxillary surface to the hilus. Blood vessels and bronchi can be traced into the lung substance and further sections made if indicated.

(5) EXAMINATION OF THE ABDOMINAL ORGANS

a. Liver, Biliary System, Stomach, Duodenum, Pancreas and Spleen

Place the organs on the sectioning table in the position in which they lie in the body (the liver away from the prosector, the stomach to the right). Incise the stomach half way between the greater and lesser curvature starting about 4 cm. from the pylorus. Examine its contents and then continue the incision up through the cardiac portion and the lower end of the esophagus, and down through the pylorus and duodenum along the midanterior surface. Sponge off the mucosa and examine.

Dissect out the common bile duct. It can be found most easily just before it passes under the duodenum. It should be nicked transversely, and then opened with coronary scissors up to its bifurcation to form the hepatic and cystic ducts, and down through the head of the pancreas to the ampulla of Vater. The opening of the cystic duct into the common bile duct can now be found and

the cystic duct incised as far as the neck of the gallbladder. Open the gallbladder by an incision in the middle of its free surface. Examine the contents and the mucosa.

The spleen is hemisected by one longitudinal section starting in the middle of the diaphragmatic surface and passing to the hilus.

Make a transverse incision in about the midportion of the tail of the pancreas. Identify the pancreatic duct and follow it to the duodenum. When indicated dissect out the splenic artery and vein.

Examine the external surface of the liver and make one or more transverse sections in an anterior to posterior direction.

b. Genito-urinary System

(*a*) **Excretory Organs.**—The dissection is started with the organs lying on the dissecting table in the position in which they are found in the body. Locate the inferior vena cava where it has been cut below the liver. Open it to a point just above the junction of the iliac veins. Open the renal veins as far as the kidneys. The suprarenal and spermatic or ovarian veins may also be opened if indicated. Incise each adrenal in the midline. Open the urinary bladder by entering through the urethra and cutting along the middle of the anterior wall. Sponge it out and examine it.

Turn the whole group of organs over so that the anterior surface rests on the table. Find the upper end of the aorta where it has been cut and open it along the posterior surface following down into the iliac arteries to the point where they have been severed. Open the renal arteries as far as the kidneys. Open up and clean the rectum.

Take each kidney and make a median sagittal section from the outside border to the hilus. Find the renal pelvis. Open this completely and follow it down with probe-pointed scissors into the ureter and then open the ureter into the bladder. Strip the capsule from one-half of each kidney and examine the capsular surface.

(*b*) **Male Genital Organs.**—*Seminal Vesicles.*—Place the fingers of the left hand in the urinary bladder so that they cover the trigone with the tips of the fingers at the base. Pull the fundus of the bladder down over the fingers and with a section knife cut transversely through the peritoneum over the posterior surface of the bladder. Then with forceps strip back the peritoneum and underlying fat. This should expose the seminal vesicles and the

entrance of the vasa deferentia. Make several transverse sections through these.

Prostate.—Make several partial transverse sections.

Testes.—By blunt dissection with the fingers make an opening to the inside of the inguinal canal between the pubis and the skin. Pull the testis up through this and make a longitudinal incision passing through the testis and the epididymis.

Penis (if indicated).—Make an opening between the upper border of the symphysis pubis and the skin. Grasp the corpora cavernosa with the urethra and, dissecting them carefully from the skin, evaginate these structures until the glans is reached. Cut the corpora at this point. The urethra can then be opened and examined.

The penis can be removed in conjunction with the prostatic urethra and the other pelvic organs by dissecting it free just as described and drawing it through an opening made below the symphysis pubis from the inside of the pelvis. The whole urethra can then be opened at one time.

(*c*) **Female Genital Organs.**—*Uterus and Vagina.*—Open the vagina by an incision along the anterior wall. Examine and inspect the cervix. With heavy scissors or a section knife, open the uterine cavity by a midsagittal section.

Ovaries.—Examine them externally and make a longitudinal incision in each.

Tubes.—Inspect and open them by cutting them from the fimbriated end.

c. Jejunum, Ileum and Colon

Palpate and dissect the mesenteric lymph nodes. Then, starting with the severed end of the jejunum, open it by cutting just to one side of the mesenteric attachment and continue down to the ileocecal valve. Pass the enterotome through the ileocecal valve and open the colon along one of the longitudinal muscle bundles down to the rectum. Wash the intestinal tract free of contents in the sink and examine.

3. En Masse Postmortem Technique

(1) Removal of the Organs "en masse"

By blunt dissection, passing the fingers retroperitoneally, separate the pelvic organs and the pelvic peritoneum from the underlying bones of the pelvis. In this way the rectum, the bladder and,

in the female, the internal genital organs are freed. These organs are grasped by the left hand and, exerting traction upward, the rectum, the vagina in the female, and the urethra are cut close to the symphysis pubis by a knife held in the right hand. The arteries, veins and nerves supplying the lower extremities must also be severed. Next, the diaphragm is cut along its entire peripheral margin. To free the remainder of the abdominal organs begin on the left. Dissect the adrenal, the kidney and the ureter from the underlying muscles, taking care not to injure any of these structures. The dissection should be continued to the midline of the vertebral column. In a similar manner the organs on the right side of the peritoneal cavity are freed. In so doing the liver must be displaced toward the midline and great care must be taken not to injure the vena cava. When these procedures are complete the organs of the abdominal cavity should be free save for their attachments to the diaphragm.

The organs of the thorax must then be freed. By blunt dissection pass the fingers beneath the origin of the great vessels, the trachea and the esophagus and tear them away from the underlying vertebral column. With traction downward by the left hand cut through these structures with a knife held in the right hand. If the pleural adhesions have already been broken or cut, the whole contents of the thorax can be stripped away from the vertebral column as far as the diaphragm. At the completion of this the contents of the thorax and the abdomen should be lifted from the body and placed in a large pan or on a dissecting board.

(2) EXAMINATION OF THE ORGANS

The organs are ready to be examined and a routine method such as that described by Saphir (1937) should be followed.

Open the vena cava.

Inspect the celiac plexus and the retroperitoneal lymph nodes.

Dissect out the adrenals and their blood vessels.

Open the aorta up to the arch and examine the azygos vein and the thoracic duct.

Dissect the abdominal aorta and the thoracic descending aorta free, leaving the latter attached to the arch.

Inspect and then remove the kidneys, the ureters, the urinary bladder, the prostate (the ovaries, the tubes, the uterus and the

vagina) and the rectum with the lower portion of the sigmoid. In this way the genito-urinary system is kept intact. Examine these organs. In the male the spermatic cord, the testis and the epididymis on each side should also be examined.

Open the esophagus, beginning at the upper end. Dissect it away from the organs of the mediastinum, but leave it attached to the stomach.

Separate the chest organs from the abdominal organs by cutting through the vena cava just above the diaphragm.

It will be noted that the thoracic and abdominal organs are now divided into the same units as the ones described under the Ghon technique, save for the fact that the thoracic portion of the esophagus is still attached to the stomach and the abdominal aorta has been freed from the abdominal organs, leaving the whole aorta intact.

These groups of organs can, therefore, be opened and examined by the procedure outlined under Ghon's technique, except for the fact that the abdominal aorta cannot be opened with the urinary system but can be examined along with the thoracic aorta.

4. Rokitansky's Autopsy Technique

(1) THORACIC ORGANS

The thorax and abdomen are opened in the usual fashion and the cavities and organs are inspected *in situ*.

The contents of the superior mediastinum, the thymus, the large arteries arising from the arch of the aorta, and the superior vena cava with its branches, are identified and inspected.

The contents of the pleural cavities having been examined and all adhesions broken or cut, the right lung is lifted out of the pleural cavity leaving the hilus intact. The lung is displaced medially with the left hand while an incision is made through the mediastinal pleura along the bodies of the thoracic vertebrae. In this way the azygos vein and the thoracic duct are laid bare and inspected.

The right lung is then rested on the external surface of the right anterior thoracic wall and is held by the left hand in such a way as to expose the anterior half of the medial surface. A section through all three lobes of the lung is made starting medially about 2 cm. below the anterior border and passing almost completely through the lung substance in a curved line between this point and

the posterior border. The lung is thus divided into two almost equal halves. The cut surface is examined and if necessary another section is made starting laterally and going to the hilus. The lung is then replaced in the thoracic cavity.

Next, the left lung is similarly lifted out of the thoracic cavity and rested on the thoracic wall adjacent to it. It is rotated to the left in such a way as to expose the whole medial surface and a cut similar to that in the right lung is made. After examination it is returned to the thoracic cavity.

The heart and the pericardial cavity are next examined. Open the pericardium by picking up a fold in the anterior wall and cutting up toward the base and down toward the apex. Grasp the apex of the heart with the left hand, examine the organ on all sides and then rotate it in such a way that the boundary between the right ventricle and the right auricle is in contact with the cut ends of the ribs on the right. The left border of the left ventricle and the left wall of the left auricle can now be seen. With a section knife (amputation knife) cut through the left border of the left ventricle beginning at the apex and continuing in a line leading to the junction of the left pulmonary veins. Do not cut through the mitral valve until the ventricle has been opened, the contents have been pulled out and the lumen of the valve has been palpated. Then the point of the knife is put in through the valve and, cutting from inside the left border of the valve, an opening is made and the incision continued through the auricular wall to the point where the left pulmonary veins empty into the auricle. The left auricle and ventricle can then be examined. The anterior and posterior papillary muscles should be intact.

The heart is again grasped by the apex, lifted up and held in such a way that the right ventricle and right auricle can be cut. This is accomplished by inserting the point of the knife through the wall of the apex of the right ventricle and cutting from the inside out in a line leading to a point halfway between the junction of the superior and inferior venae cavae. The incision is carried up to this point and then the ventricle and auricle can be opened, cleaned and examined.

Next, the tip of the apex of the right ventricle is held between the thumb and forefinger of the left hand, the thumb being in the ventricular cavity. The point of the knife is passed through the

pulmonary conus and valve to a point 3 cm. above the valve and pushed through the anterior wall of the artery. Now the pulmonary artery and the anterior wall of the left ventricle are cut in a line toward the apex of the ventricle, keeping as close to the anterior border of the ventricle as possible.

In order to open the aorta the heart is grasped once more by the apex, the fingers of the left hand holding the triangular piece of the right ventricle in place, the thumb being on the left ventricle. The heart is lifted and rotated so that the knife blade can be passed in through the left ventricle and the aortic conus into the aorta. The point of the knife is inserted through the anterior wall of the aorta about 3 cm. above the valves and the incision is carried down, cutting through the aorta, the posterior wall of the pulmonary conus, the triangular piece of the anterior wall of the right ventricle and the ventricular septum.

The aorta can also be opened by a method already described, *i. e.*, passing the blunt end of the enterotome up into the aorta and cutting across the pulmonary artery about 1 cm. above the valve and thus continuing into the aorta. By using this incision the ventricular septum is not cut but all valves may be inspected and the heart is in a more intact condition to be kept for future use as a gross specimen.

(2) ABDOMINAL ORGANS

Examine carefully all the abdominal organs and the peritoneum.

a. The Liver.—The left hand is placed between the right lobe of the liver and the diaphragm, the liver is raised out of the right hypochondrium and a deep transverse cut is made into the right and left lobes. The gallbladder is dissected free and the cystic duct is cut close to its entrance into the common bile duct. The gallbladder is removed and opened. To take out the liver grasp it with the left hand, the thumb on the under surface, the fingers in the incision which has already been made, lift it up and cut through the hepatoduodenal ligament with its blood vessels, the common bile duct, the inferior vena cava, the falciform and the coronary ligaments, and the connective tissue over the right adrenal. The liver can now be removed from the body and examined further.

In some cases it will be found advisable to leave the liver in the body until the bile ducts and the portal vein have been dissected out.

b. The Spleen.—Lift the spleen out of the left hypochondrium, rest it on the left anterior surface of the ribs and cut through the spleen from the diaphragmatic surface to the hilus. It can be removed by severing the structures at the hilus.

c. The Kidneys.—The cecum is lifted with the left hand and together with the ascending colon is freed from the attachments. It is then displaced medially exposing the right kidney. The kidney is grasped with the fingers of the left hand and by cutting posteriorly and laterally with the knife held in the right hand, the kidney and the adrenal are freed from their attachments except the blood vessels and the ureter. The blood vessels are dissected out and cut midway between their origin and their entrance into the kidney The ureter is not cut. The kidney is held in the left hand and bisected by a cut from its convexity to the hilus, passing in the midline through the calyces and the pelvis. The capsule with the surrounding fat is stripped from both halves of the kidney and then, with probe-pointed scissors, the renal pelvis and the ureter are opened as far as the brim of the pelvic cavity. Finally, the adrenal is examined by making a sagittal section through it.

The left kidney is examined in the same way. In order to do this, however, the descending colon must first be separated from it.

d. The Organs of the Pelvis.—The organs of the pelvis are freed by blunt dissection with the fingers, starting in the prevesicular space and extending laterally around the rectum. These organs are grasped with the left hand and, while traction upward is exerted, the various structures are cut close to the floor of the pelvis. The connective tissue attachments between the rectum and the coccygeal bone are dissected off, and the organs of the pelvis thus freed are lifted out of the pelvis and spread out over the symphysis pubis. The iliac veins and arteries should be left intact.

In the male the shaft of the penis can be removed with the organs of the pelvis. The primary abdominal incision is carried down to about the midportion of the dorsum of the penis, then the corpora cavernosa and the urethra are freed carefully from the investing skin and divided just posterior to the corona. Dissect these structures back to the pubic arch and free them by dissecting from within the pelvis until the shaft of the penis can be passed underneath the arch into the pelvis.

The procedures that follow differ according to sex. In both

sexes it is best first to open the rectum along its posterior wall, remove its contents and sponge off the mucosa.

In the male a midline incision is made in the anterior wall of the bladder starting on the superior surface. Probe-pointed scissors are used and the incision is carried down throughout the length of the urethra. Examine the openings of the ureters in the bladder, the ejaculatory ducts, the prostatic ducts and Cowper's glands. The testes are pulled up from the scrotum through an opening made by blunt dissection between the skin and Poupart's ligament. Each is cut in half and the section continued through the head of the epididymis. The rectum is then dissected away from the bladder, the seminal vesicles are dissected out and incised.

In the female the bladder is opened by cutting with probe-pointed scissors through the anterior wall of the urethra and continuing the cut up the midline of the bladder. The vagina and uterus are opened either by a midline incision in the anterior wall cutting the urinary bladder into halves, or in the posterior wall, having first dissected away the rectum. In the center of the fundus of the uterus the midline incision is stopped and another incision is carried from that point into each of the uterine horns. The fallopian tubes are opened with scissors beginning at their distal orifices. Each ovary is sectioned longitudinally. These organs are removed from the body together with the kidneys and the ureters.

The stomach, the intestines and the pancreas are still in the abdominal cavity.

e. The Stomach.—A 4 cm. incision is made in the anterior wall of the pylorus and the contents examined. This incision is then carried up paralliel to the lesser curvature of the stomach, about 2 cm. below it, as far as the cardia and lower portion of the esophagus.

f. The Duodenum.—The transverse colon is dissected free. Then the incision in the anterior wall of the pylorus is continued along the anterior wall of the duodenum as far as the junction with the jejunum. Next, the mucosa of the stomach and the duodenum is carefully washed off and examined. The bile duct is opened down into the duodenum.

g. The Pancreas.—Cut through the mesentery where it is attached to the stomach. The stomach is then displaced upward and the transverse colon downward exposing the pancreas. The pan-

creas is cut diagonally across, the pancreatic duct identified, probed and then opened up.

h. The Small Intestine.—Begin in the lowest portion of the ileum. Incise and cut with the enterotome along the under surface adjacent to the insertion of the mesentery. Continue this up to the duodenum, examining each portion as it is opened.

i. The Colon.—Return to the ileocecal valve. Pass the blunt end of the enterotome through the ileocecal valve into the cecum and continue cutting along the anterior muscle band until the whole colon is opened.

This method has the advantage that the intestine remains attached to its mesenteries and any abnormalities in the blood or lymph supply of the gut can be traced.

Finally, the appendix is opened by entering through the cecum and cutting into the lumen with probe-pointed scissors.

The stomach and the intestines, together with the pancreas, are then removed from the body.

The inferior vena cava, the iliac veins and the abdominal aorta with its branches are dissected and opened. Also, the lymphatics and the nerve plexuses of the abdomen can be dissected out.

Next, examine the diaphragm which should still be intact, and finally the vertebral column and the pelvis.

5. Advantages and Disadvantages of the Different Autopsy Methods

The Virchow technique is excellent for demonstrating pathological changes in individual organs, but the relationships between the various organs are sometimes hard to interpret. It has the advantage that, in cases where the chief interest centers about one organ, this organ can be immediately removed and examined.

The "en masse" technique undoubtedly preserves relationships more satisfactorily than the other methods. It has perhaps the disadvantage that it is a somewhat lengthy one. It is also difficult to carry out without the aid of an assistant. It cannot be done in cases that are limited to small incisions.

The "en bloc" technique is a sort of compromise between the two methods already mentioned. It preserves relationships very satisfactorily. It is somewhat simpler for one person to execute. It has the disadvantage that the aorta and the esophagus are cut

at the diaphragm. Therefore, in cases of cirrhosis of the liver where esophageal varices are of importance, and in lesions of the aorta such as dissecting aneurysms, some other technique should be followed. The method of opening the gastro-intestinal tract while still attached to the mesentery is considered by many to be too time-consuming to be worth while. The solution for this is, probably, that this step in the technique need be employed only when a gastro-intestinal lesion is present of a type in which the blood supply and the lymphatic drainage are important.

The Rokitansky technique is used but little in this country. As a routine procedure it has no great advantages over the others. A knowledge of it, however, enables the prosector to examine and open any of the thoracic and abdominal organs without removing them from the body. This is sometimes an advantage when speed is necessary or when autopsies are done in the home.

6. Postmortem Examination of the Central Nervous System and of the Head

(1) Removal of the brain

The incision into the scalp (Fig. 9, A) should begin from 1 to 2 cm. behind the right ear, near its lower border, and extend over the vertex of the skull to a corresponding point behind the left ear. The cut is most easily made by thrusting a small narrow-bladed scalpel, with its back toward the calvarium and its point toward the vertex, through the skin behind the ear and carrying it along in the desired direction. By making the incision in this manner the hair is not cut, but simply parted. The anterior flap should be stripped nearly as far forward as the orbits from the calvarium and the temporal muscles, by stretching the flap and dividing the loose connective tissue holding it with sweeping strokes of the scalpel. After a part of the flap has been freed it is often possible to strip the rest without using the scalpel. For the posterior flap, which should be removed back as far as the occipital protuberance, the scalpel nearly always has to be used.

If the hair is long, the anterior portion can be rolled into the anterior flap over the face and thus protected. The posterior portion is gathered at the nape of the neck, and then a towel is wrapped tightly around the head and neck, extending from the line where the

flaps are reflected down to the shoulders, and is pinned over the lower part of the forehead. In this manner the hair is protected from being soiled and ample room is left for work.

There are two methods commonly used for opening the skull. One is to make a circular incision around the skull starting about 5 cm. above the orbits and continuing in a line passing about 2 cm. above the external auditory meatus on each side. This gives an opening large enough for the removal of the brain but presents the difficulty that when the calvarium is replaced at the end of the autopsy it is difficult to keep it in place. To obviate this to a certain extent the following procedure is used. Two holes are bored into the cut edge of the base, one in the center of the frontal area

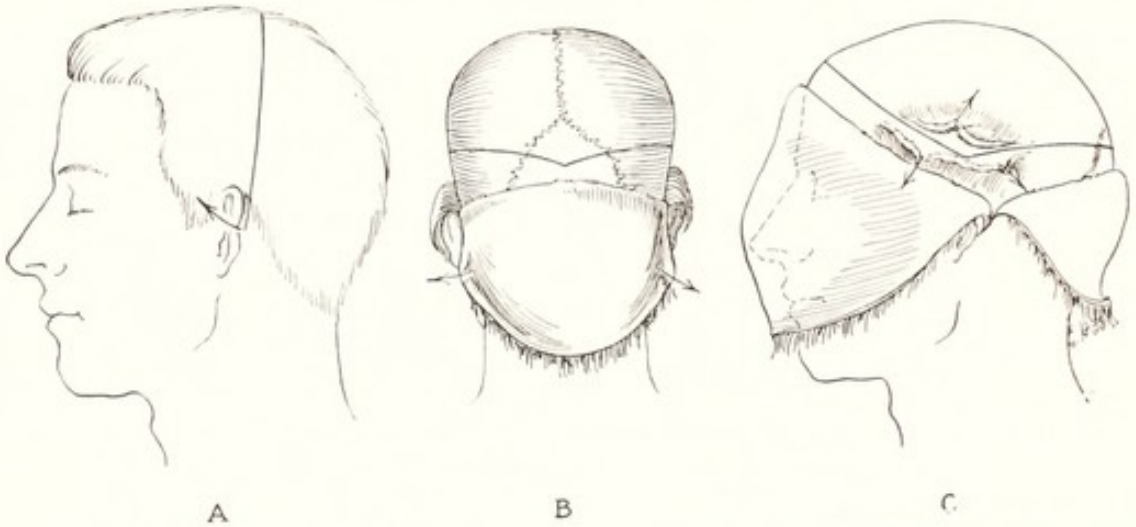

Fig. 9.—Method of opening the head. Diagram of incisions followed in removal of the brain. A, Incision in scalp; B, posterior view showing saw cuts in the skull; C, lateral view showing saw cuts in the skull.

and the other over the occiput. Steel or brass pins are driven into these holes. Then corresponding holes are drilled in the corresponding portions of the calvarium and it is then put in place by driving the free end of the pins into these holes.

A method that is much more frequently used in this country and is probably simpler consists of removing the calvarium by means of several saw cuts (Fig. 9, B and C), as a result of which an irregular, wedge shaped lower edge is produced which automatically holds the calvarium in position when replaced. The wedges are made by three cuts, which should be outlined on the periosteum of the skull with a scalpel. The first cut begins just above and behind the left ear, and is carried over the forehead just back of the edge

of the hair or over the frontal eminences to a corresponding point above and behind the right ear. The two other cuts begin at each end of the first incision, forming there an obtuse angle, and are carried back to meet in the median line behind at an angle of about 160° a little in front of the occipital protuberance. After thus outlining the cuts the temporal muscle on each side is severed along the line of the incision and dissected out of the way of the saw without cutting either the upper or the lower attachment. The holder for the head, if one is used, is attached with a foot in each obtuse angle in the temporal region. If a holder is not employed, the head is best steadied by hands on the calvarium and face. Use towels or a cloth to prevent slipping.

Start the incision with the saw at the forehead and extend it back along the lines marked out. It is best not to carry the incision clear through the inner table of the bone for two reasons: first, on account of the danger of injuring the brain substance; secondly, because if the inner table or a part of it is cracked through with a chisel and hammer, it can be done without injuring the underlying tissue, and the irregular overlapping fragments of bone thereby formed serve afterward for holding the calvarium firmly and steadily in place.

After the sawing is completed, insert a chisel in the frontal region and with a quick, sharp blow crack through the rest of the inner table. In like manner insert the chisel in the middle of the other cuts and free the calvarium posteriorly. To remove the loosened calvarium insert the chisel end of the hammer in the incision in the frontal region, and press down with the left hand while swinging the handle around in a horizontal plane. By means of the powerful purchase thus obtained, any remaining bony attachments of the calvarium are easily broken. Then, by catching the hook of the hammer over the calvarium, it may be freed by prying it off with the point of a closed enterotome. The calvarium should be examined after it has been removed.

In young children, and sometimes in old people, it is necessary to remove the dura with the calvarium. To do this, cut through the dura with the point of a scalpel along the lines of the incision in the skull; then cut the falx cerebri in the median line both anteriorly and posteriorly.

The opening of the skull and the examination of the brain in

newborn infants and very young children is best carried out by a special technique. The details of this will be found on pages 366–371.

In cases of fracture of the skull no cracking with the hammer and chisel is permissible; the calvarium must be freed entirely by sawing.

The next step after the removal of the calvarium is the inspection of the dura. Under normal conditions it is not tense in the frontal region, but can be picked up with forceps or fingers. If the dura is not thickened the convolutions normally should be visible through it. The superior sagittal sinus is opened with a knife or scissors and its contents examined. Pacchionian granulations are not infrequently found projecting into it.

To remove the dura, cut through it with scissors or a knife along the same lines in which the calvarium was sawed. Turn back each half of the dura and examine the surface of the convolutions and the inner surface of the dura. The convolutions should be distinct and rounded, not flattened with obliteration of the gyri as occurs when there is internal pressure. There may be apparent adhesions between the dura and pia due to veins passing from one to the other.

The dura is still further freed by seizing it anteriorly and lifting it until the falx is tense at its insertion into the crista galli of the ethmoid. Pass a knife, with the edge forward, parallel to the falx on the left side, in as far as the cribriform plate; turn it to the right and cut until the falx yields. Withdraw the knife in the same manner in which it was inserted. Next, draw the dura back. It is usually more or less attached along the longitudinal fissure by pacchionian granulations and by blood vessels. These structures may be torn or cut through. Do not cut the dura posteriorly, but let it hang down.

To remove the brain, insert the two forefingers or the first and second fingers of the left hand anteriorly between the dura and the frontal lobes, one on each side of the falx cerebri, and draw the brain gently back until the optic nerves are visible. Ordinarily, the olfactory nerves come away from the cribriform plate without trouble, but sometimes they have to be freed with the point of a knife. With a long, slender-bladed knife divide the optic nerves as far forward as possible while holding the brain back with the left hand. Continue to draw the brain carefully back and divide the cranial nerves and the internal carotid arteries. Then draw forward

first the left, then the right temporal lobe, and cut the tentorium cerebelli close to its attachment to the petrous portion of the temporal bone, using the tip of the knife with a sawing motion. Insert the knife laterally close to the inner squamous portion of the occipital bone, and cut from there in toward the foramen magnum. Then sever the nerves given off from the medulla oblongata while supporting the convexity of the brain in the left hand.

Lastly, carry the knife as far as possible into the spinal canal and divide the cervical cord by an oblique incision from each side, severing the vertebral arteries with the same stroke. A myelotome is better than a knife, because it gives a cross-section of the cord and allows more of it to be removed.

The brain can now be taken out by passing the first and second fingers of the right hand in on either side of the cord, and everting the brain while still supporting it posteriorly with the left hand.

Before proceeding with the opening of the brain it is best to examine the base of the skull, particularly the dura, of which the sinuses should be incised, and the pituitary gland.

If a fracture at the base is suspected, strip off the dura, in order to give a better opportunity for an examination of the bone.

The brain should be weighed before it is dissected.

(2) EXTERNAL EXAMINATION OF THE BRAIN

Place the brain with the base uppermost and with the cerebellum toward the prosector. Examine first the pia mater and the cranial nerves, then the arteries, especially the middle cerebral arteries and their branches running in the fissure of Sylvius, for it is here that emboli most frequently lodge. The pia bridging the fissure of Sylvius can sometimes be torn through, but usually has to be cut.

It is important, particularly in cases of obscure cerebral symptoms, to feel gently with the fingertips all over the surface of the brain for any areas of increased or decreased density, because patches of sclerosis and softening may in that way be found which might otherwise be overlooked.

By stripping off the pia mater, a procedure not often advisable, adhesions over pathological areas can sometimes be found pointing to the lesions beneath, but the pia should not be stripped from those portions which are to be examined microscopically. To remove the

pia an incision is made in it the length of the median surface of each hemisphere just above the corpus callosum, and the pia stripped back first from the median and then from the convex surface. The stripping is done by means of the fingers, with occasional aid from forceps.

(3) SECTIONING OF THE BRAIN

To examine and section a brain in a way that will be satisfactory both to the general pathologist and to the more specialized neuropathologist is difficult. This is due to the fact that the brain is a very complicated organ and may be affected by a great variety of lesions both focal and diffuse. Furthermore, its consistence when fresh is such that it is somewhat difficult to handle without distortion.

There are two extremes of opinion as to which is the best method of procedure. One group, mostly neurologists and some neuropathologists, feels that the brain, after an external examination, should be fixed and hardened by suspending it in a solution of 10 per cent formalin for 2 to 3 weeks before it is sectioned. After this is done the brain is cut with a brain knife into a series of frontal sections each from 0.5 to 1 cm. in thickness. The obvious advantage of this method is that the sections are easily cut and the exact location and extent of any gross lesions can be readily identified. There are, however, rather serious disadvantages. The delay before any pathological diagnosis can be made is discouraging to both clinician and pathologist. The brain tissue is by necessity put into only one fixative, *i. e.*, formalin, thus limiting the number of stains that can be done on it. Finally and perhaps most important, the formalin penetrates slowly into the central portions of the brain substance, as a result of which these portions lose all their finer histological detail before they are properly fixed.

The other extreme is represented by the group of pathologists who use the old Virchow technique for opening the brain. This method consists essentially of sectioning the cerebrum by a series of partial, more or less sagittal, cuts parallel to the external wall of each lateral ventricle. This is done on fresh brain tissue. It will uncover immediately any gross lesions and at the same time histological material can be obtained and put into different fixatives. This procedure also has disadvantages. Except in the hands of the

very experienced the exact location of the lesions is hard to identify. The brain substance is so mutilated by the sectioning that subsequent fixation of the remaining tissue is unsatisfactory and the identification of various anatomical landmarks almost impossible. Good gross specimens are difficult to obtain when this method is used.

The details of the Virchow technique, which is now rarely used, will be given later.

a. Procedure for Making Frontal Sections

In general, a method by which a series of frontal sections is made through the cerebrum while it is still unfixed is the most satisfactory one. The areas through which these sections should pass may be determined by the clinical history of the case or by the general findings of the autopsy. In many cases in which a large area of softening or hemorrhage is present, this can be located by palpation and the first cut should then be made through its center. Its extent can be traced by incisions on each side of the original cut. In cases in which the location of the lesion is unknown a routine series of frontal sections should be cut. In doing this three things are of importance. In the first place the brain should be left as intact as possible; secondly, no attempt should be made to cut the slices of fresh tissue thinner than 1.5 to 2 cm. The section knife should be moistened in water (1.8 per cent saline should be used to avoid distortion of the surface tissue) before trying to cut the brain tissue. In the third place a definite routine should be followed in making the sections. The location of the sections should be so chosen that they will pass through important areas and areas where the anatomical landmarks can be easily recognized.

Immediately after the external examination of the brain it is sectioned as a whole, in order to obtain a satisfactory comparison between the same portions of the two halves of the brain. A series of six frontal sections parallel to the fissure of Rolando should be made. The names of these sections, as described by Pitres (Bourneville and Bricon, 1885) and the important parts they show (Fig. 10) are as follows:

1. The prefrontal section (1–1) through the frontal lobe, 5 cm. anterior to and parallel to the fissure of Rolando, shows the gray and white substance of the frontal convolutions.

2. The pediculofrontal section (2–2) through the posterior portions of the three frontal convolutions, 2 cm. in front of the fissure of Rolando, shows the frontal convolutions, the anterior extremity of the insula, the posterior extremity of the orbital convolutions, the lenticular and the caudate nuclei, and the internal capsule.

3. The frontal section (3–3) through the anterior central convolution, parallel to the fissure of Rolando, shows the anterior central convolution, the optic thalamus, the lenticular and the caudate nuclei, the claustrum, the external and the internal capsules, the anterior portion of the descending horn of the lateral ventricle, and the lobule of the insula.

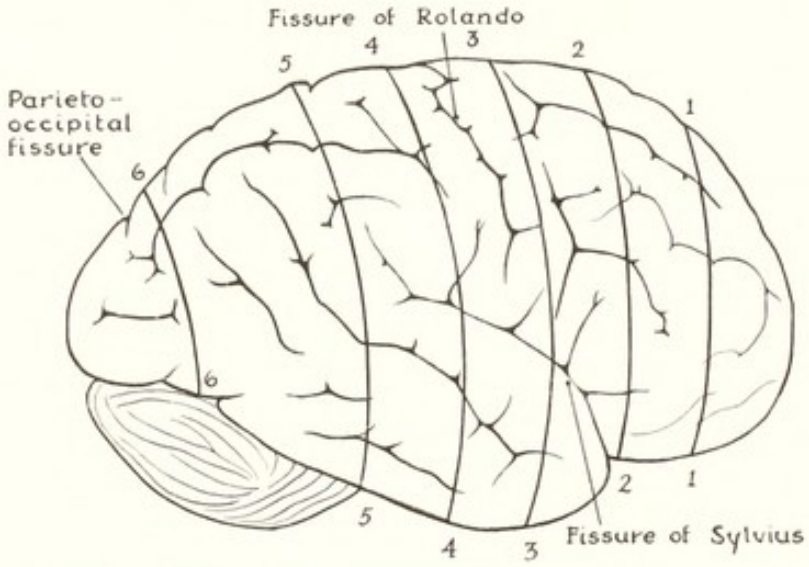

Fig. 10.—Dorsolateral surface of the cerebrum showing the position of the cuts made in sectioning the brain.

4. The parietal section (4–4) through the posterior central convolution shows portions of the same structures as the preceding, and a transverse view of the hippocampus.

5. The pediculoparietal section (5–5) through the parietal lobe, 3 cm. posterior to the fissure of Rolando, shows the tail of the caudate nucleus in two places and the posterior portion of the optic thalamus.

6. The occipital section (6–6) through the occipital lobe, 1 cm. in front of the parieto-occipital sulcus, shows simply the white and gray matter of the occipital lobe, especially the area calcarina.

The cerebellum and medulla oblongata are examined next.

The pediculoparietal section (5–5) severs the cerebellum and medulla together with the occipital lobes from the rest of the

cerebrum. A series of two or three frontal sections (slightly thinner than those of the cerebrum) are then made through the cerebellum, midbrain and medulla and examined for lesions. If it is necessary to search for small lesions, a series of sagittal sections may be cut through each half of the cerebellum.

Upon completion of the examination of the fresh gross sections of the brain, certain representative areas are selected from which small pieces (25 mm. by 35 mm., unless the size of the lesion indicates a larger piece), not more than 0.5 cm. in thickness, are to be cut for immediate fixation.

Where focal gross lesions exist, pieces should be taken from both the lesions and the adjacent areas.

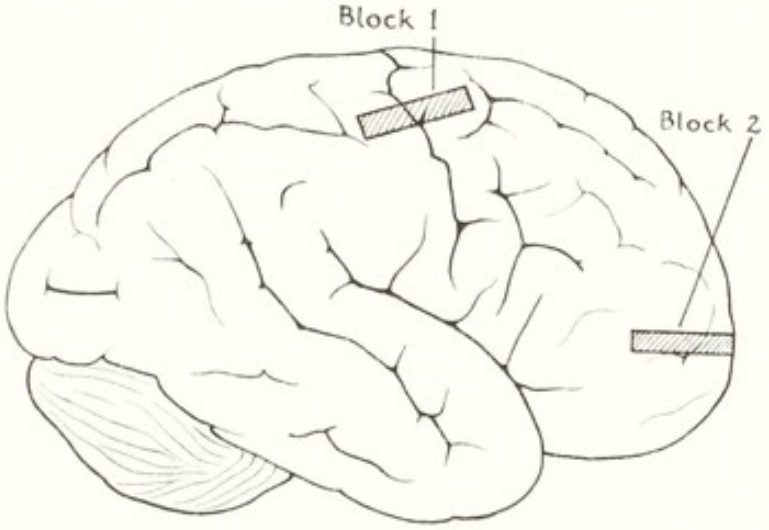

Fig. 11.—Dorsolateral surface of the right cerebral hemisphere indicating the position of the principal blocks to be removed from that aspect of the brain for fixation.

Whenever no gross lesions are present a fairly wide sampling is recommended. A choice of areas which has gained favor in a number of laboratories is as follows:

1. Anterior and posterior central convolutions (precentral and postcentral gyri).—A block (Fig. 11, Block 1) is taken from the motor and the sensory area on either side of the fissure of Rolando high up on the convexity of the hemisphere near the interhemispheric cleft. The motor portion controls the lower trunk and thigh muscles and the Betz cells here are of large size. The sensory portion is the area subserving the skin, joint and muscle senses.

2. Frontal lobe.—A section (Fig. 11, Block 2) is taken from the interhemispheric margin at the frontal pole. This is the so-called anterior association zone.

3. Hippocampus.—Blocks (Fig. 12, Block 3) are taken from the inner edge of the temporal lobe opposite the crura cerebri, near the uncus, and represent the area of olfactory function and its more complicated associations. This area is selected on account of its vulnerability, which makes it a sort of indicator for the adequacy or inadequacy of the cerebral circulation, not on account of the rather insignificant functions, at least in humans, which are localized there.

4. Occipital lobe.—From the occipital pole adjacent to the calcarine fissure a sample (Fig. 12, Block 4) is taken which represents the area striata which can be identified with the naked eye because of the prominent white line parallel to the cortex surface

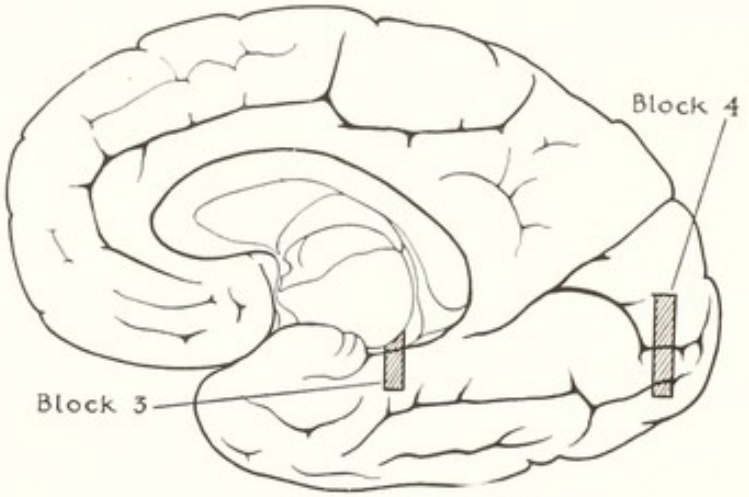

Fig. 12.—Medial surface of the right cerebral hemisphere indicating the position of the principal blocks to be removed from that aspect of the brain for fixation.

and about midway in its depth. This is the "arrival platform" subserving vision.

5. Hypothalamic region.—A sample (Fig. 13, Block 5) is taken from the surface exposed by the third frontal section and should include the hypothalamus, the mammillary body and the adjacent thalamus.

6. Basal ganglia.—A second block (Fig. 13, Block 6) is taken from the surface exposed by the third frontal section in the region of the caudate nucleus, the internal capsule, the lentiform nucleus and the thalamus.

7. Medulla oblongata.—A section (Fig. 13, Block 7) of the medulla which includes a portion of the inferior olive and the nucleus of the vagus is chosen for fixation.

8. Cerebellum.—A representative piece (Fig. 13, Block 8) including cerebellar cortex, white matter and dentate nucleus is removed for histological examination.

In most cases it is not necessary to take pieces for fixation routinely from all the regions mentioned above. Whenever there is no evidence of any gross pathological changes and no clinical symptoms have been recorded, Blocks 2 and 5 should suffice. If

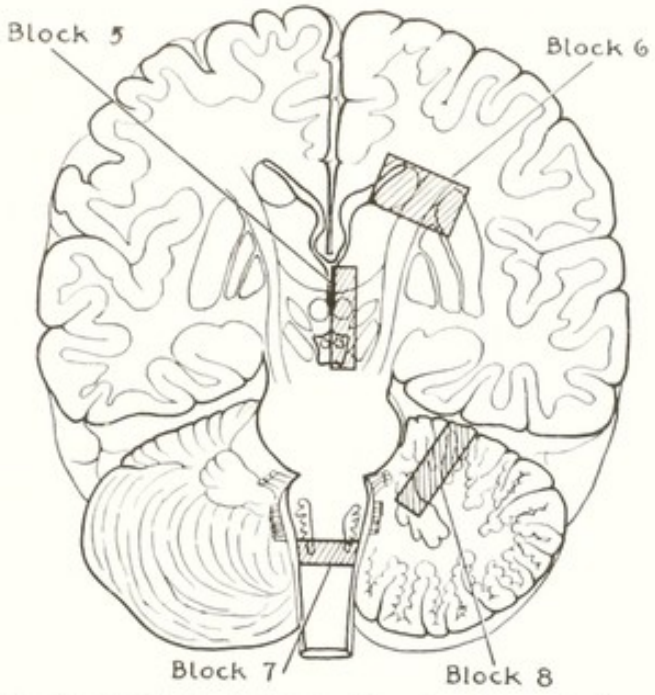

Fig. 13.—Section through the brain in the direction of the cerebral peduncles viewed anteriorly. In the left half of the cerebellum a second cut has been made parallel to the first but further dorsalward. The position of the principal blocks to be removed from this section of the brain for fixation is indicated.

slight clinical symptoms, such as drowsiness, have been noted and no gross pathological changes are present, Blocks 1, 2, 3, and 5 should be taken. In cases with a record of severe, generalized clinical symptoms and no gross pathological findings, samples from all regions described above should be fixed for histological study.

b. Virchow's Method

The brain is placed on its base, the cerebellum toward the prosector. Press the hemispheres apart a little so as to expose the corpus callosum. Hold the left half of the cerebrum in the left hand with the fingers on the lateral aspect and the thumb in the longitudinal fissure. Then make an almost vertical incision with a long slender knife through the roof of the left lateral ventricle in

its middle third, 2 to 3 mm. from the median raphé of the corpus callosum. The roof of the ventricle is slightly raised vertically by the thumb, so that the incision, which must not be too deep, does not injure the basal ganglia. The incision should be continued into the anterior and posterior cornua. Then make a long incision from the one end of the above cut to the other, passing just outside of the basal ganglia at an angle of about 45°. Repeat the process on the right side, turning the brain half around. Next, seize what remains of the corpus callosum and the fornix in the middle, lift them and cut through from below up, passing the knife through the foramen of Monro. The parts are then turned back, exposing the tela choroidea of the third ventricle and the choroid plexuses. By drawing back the tela choroidea the third ventricle is uncovered.

The corpora quadrigemina are exposed by cutting transversely the right posterior pillar of the fornix and the adjoining brain substance and carrying them over to the left. Each ventricle, as it is opened, should be carefully inspected and any abnormal condition of its ependyma noted. The cortex is further divided on one side, and then on the other, by holding it in the left hand and making vertical straight sections from the upper angle of the previous cut into the convex cortex, allowing the sections to fall apart, in order to avoid touching and soiling the surface with the knife or fingers. Each portion thus cut represents a prism. The incisions should go well into the cortex, but not so far as to separate the different pieces. The basal ganglia are examined by means of a number of frontal sections. For this purpose the left hand is placed palm upward underneath the brain, so that as each section is made over the tips of the fingers by one long stroke of the knife it falls forward, exposing a clean surface, the two halves of which can be compared. An incision is next carried through the middle of the pineal body, the corpora quadrigemina and the vermiform process of the cerebellum, opening the aqueduct of Sylvius and the fourth ventricle.

Each half of the cerebellum is divided by a median horizontal section into halves, and these portions are still further subdivided by a series of cuts radiating from the peduncles.

In order to make sections of the pons and medulla the brain is folded together and turned over. Several cross sections are then made with the left hand placed beneath as in sectioning the basal ganglia.

(4) REMOVAL AND SECTIONING OF THE SPINAL CORD

The body is placed face downward, with the head over the end of the table and a block under the chest. An incision is made over the spinous processes from the occiput to the sacrum. Dissect the skin and muscles back on each side, in order to leave the vertebral laminae as bare as possible. The laminae may be cut through by means of several instruments, of which the double-bladed saw, Luer's rachiotome, is perhaps the safest, at least for beginners. The single-bladed saw with rounded end is also very useful and can be recommended. The operation can be done most rapidly by biting off the spinous processes with heavy bone forceps and cutting through the laminae with the chisel and hammer, but there is greater danger of injuring the cord.

The laminae should be sawed nearly or entirely through in a line with the roots of the transverse processes from the third or fourth lumbar vertebra to the cervical region. The arches of the cervical vertebrae are best divided with a heavy bone cutter, because they cannot be easily sawed, and there is sufficient space for the point of the bone cutter without danger of pressing on the cord.

It is important to strike the outside limits of the spinal canal, in order to get as much room as possible for the removal of the cord. Test whether the sawing is deep enough by the mobility of the spinous processes. If necessary, they can be freed by means of the hatchet-chisel and a hammer in the same way in which the calvarium is loosened.

As the cord reaches only to the second lumbar vertebra, cut through between the third and fourth vertebrae, free with the heavy bone cutter the lower end of the row of spinous processes, which are held together by their ligaments, and strip them up to the neck; then cut through the cervical arches with the bone cutter, taking care that the point within the canal does not come in contact with the cord.

The nerve roots are divided with a sharp scalpel by means of a long cut on each side of the cord. Then cut across the dura and the nerve roots at the lower end of the exposed canal and, while holding the dura with forceps, carefully free the cord from below upward with scissors or a scalpel, taking care all the time not to pull or bend the cord, because in either way artefacts may be produced. Cut the cord squarely across as high in the cervical canal

as possible, so that the remaining portion may be easily removed with the brain.

Place the cord after removal on a flat surface and incise the dura longitudinally, first posteriorly and then anteriorly. A series of cross sections, usually 1 to 2 cm. apart, is made through the cord while supported on the fingers during the cutting, so that the cut surfaces will fall apart. The different segments should ordinarily be left attached to the dura, so that their position in the cord can easily be determined.

A diagnosis from the fresh, macroscopic appearance of the cord is often very difficult to make.

For fixation, thinner sections 2 to 4 mm. in thickness should be removed and placed in different fixatives; the remaining cord can be preserved in 10 per cent formalin.

(5) THE EYE

The contents of the orbit, including the posterior part of the eye, can be readily examined by chiseling off the roof of the orbit. The posterior half of the eye can be removed by cutting around the eyeball with sharp scissors, keeping a firm hold on the sclera with forceps. If done quickly, the retina remains quite well spread out. The anterior half of the eyeball can be propped in place by a plug of cotton dipped in ink or in a solution of potassium permanganate.

(6) THE EAR

The middle ear can be exposed by chipping off with a chisel or heavy bone cutters its roof, which lies in the middle of the petrous portion of the temporal bone. If, however, it is desirable to examine the ear more carefully by means of a section through the external meatus and the middle ear, it will be necessary to remove the whole of the petrous bone. For this purpose the incision behind the ear must be carried back along the anterior edge of the trapezius muscle halfway down the neck. Then the skin-flaps, including the external ear and the underlying tissues, must be dissected back for some distance on each side of the incision. A wedge shaped piece is then sawed out of the skull, the anterior cut of the saw passing through the root of the zygomatic arch, the posterior just back of the sigmoid sinus (the portion of the transverse sinus which occupies the groove on the mastoid part of the temporal bone), so

as to come together at the apex of the pyramid of the petrous bone or, better still, to meet in the foramen magnum. An ordinary chisel and a hammer or mallet will be found very convenient for freeing the petrous bone after the incisions have been sawed.

In the examination of the petrous bone, after it has been removed, the first step is to chisel off the tegmen tympani in order to get a view of the middle ear. Next, remove the lower wall of the external meatus, so as to expose the outer surface of the tympanic membrane. Finally, divide the petrous bone with a fine hair saw by an incision, starting in at the styloid process and coming out at the carotid canal, parallel to the crest of the petrous bone.

This incision divides the tympanic cavity into halves. In the lateral half can be seen the tympanic membrane with the malleus and the anterior half of the mastoid cells. In the median half are the labyrinthic wall of the tympanic cavity with the stapes and the posterior half of the mastoid cells. It is best to remove the incus before sawing through the bone. The eustachian tube can be easily exposed by starting from its termination in the middle ear.

(7) THE NASOPHARYNX

Although a fair view of the nares and pharynx can be obtained by chiseling off the portion of the base of the skull lying over them, this method does not begin to offer the satisfactory view that can be obtained by the one described by Harke (1892). His method is not so difficult as it might seem at first sight; it consists in halving the base of the skull by a longitudinal cut after the cranium has been opened and the brain taken out. To do this the original incision in the scalp must be extended on each side over the mastoid processes and along the anterior edge of the trapezius muscle to a point below the middle of the neck. Then the posterior flap and the underlying muscles must be freed from the occipital bone and the upper portion of the cervical vertebrae. In like manner the anterior flap must be dissected from over the root of the nose and the upper edge of the orbits, and be drawn over the face. Then flex the head strongly forward and saw through the occipital bone and the base of the skull, dividing the occipital and frontal bones into equal halves. Anteriorly, it is well to go a little to the left or right, lest the nasal septum be injured.

The next step is to cut the dura mater and the occipito-axoid

ligaments between the anterior edge of the foramen magnum and the odontoid process, as well as the inner side of the atlanto-occipital joint from within. Then the two halves of the skull are drawn forcibly apart. The nasal bones, the hard palate, and the alveolar process of the upper jaw break, and the two halves of the base of the skull open like a book, revolving around an axis which passes through the joint of the lower jaw and the atlanto-occipital joint.

If the foramen magnum offers too much resistance, break through it with a chisel, and also if necessary through the anterior and posterior arches of the atlas.

It is now possible to inspect the nasal septum, the frontal and sphenoidal sinuses and to open the ethmoidal cells. The nasal passages should be examined; the middle nasal conchae are taken off with scissors and the infundibulum thus freed. To examine the opening of the nasolacrimal duct, cut off the inferior nasal conchae. The maxillary sinus is easily opened with forceps and a pair of bone shears.

After the operation the two halves of the base of the skull are brought together, and wired if necessary, and the skin-flaps are replaced.

7. Postmortem Examination of Newborn and Very Young Children

Postmortem examinations on infants present a certain number of special problems which are best handled by alterations in the routine technique.

1. An infant's skull and that of very young children is best opened after reflecting the scalp in the usual way by cutting with a pair of scissors through the dura along the unossified sutures (in the longitudinal fissure cut on each side of the falx) well down to the floor of the skull. This procedure gives five bone-flaps which may be turned out like the petals of a flower, leaving the brain uninjured. It is often necessary to cut half of the base of each flap in a horizontal line to aid its being turned out. The falx cerebri must, of course, be divided anteriorly and drawn back before the brain is removed. In sewing up, the bone flaps are turned in over plaster of paris filling the cranial cavity, and are kept perfectly in place by the skin.

2. The skull of the newborn is opened in a manner similar to

that given above. However, in view of the prominent place occupied by intracranial hemorrhages among the causes of death in the newborn (approximately 35 per cent), it is desirable that the method of postmortem examination of the skull and its contents be such that an adequate examination may be made macroscopically, without at the same time causing postmortem injury which might obscure or be confused with antemortem lesions. Therefore, more detailed instructions for examining the head of newborn infants based on the original method of Beneke (1910) and compiled by Dr. Donald Munro are given below.

An incision is made through the scalp down to the pericranium or periosteum, extending from just behind one ear to just behind the other across the vertex. The scalp is then peeled forward to the supra-orbital ridges, and back to the superior curved line of the occiput. Hematomas in the scalp, subperiosteal hematomas (the so-called cephalhematomas) or other evidence of gross injury to the skull or its coverings are easily identified at this point.

Having completed the examination of the scalp and pericranium the skull should be opened as follows: At the right anterior border of the anterior fontanelle a sharp-pointed scalpel is inserted through the fused periosteum and dura at their junction with the mesial edge of the right frontal bone. The point of the scalpel is directed laterally and is kept close to the inner surface of the right frontal bone in such a way that it penetrates between the bone and the dura, especial care being taken to prevent injury to the dura. A longitudinal slit in the suture line sufficiently large to admit the handle of the scalpel is made in this plane. The handle of the scalpel is then inserted through this slit between the bone and the dura and the two are gently and easily separated as far as is possible.

The right frontal bone is then separated from the adjacent left frontal and right parietal bones by extending the original incision along the anterior portion of the sagittal suture to the level of the floor of the anterior fossa, and also along the frontoparietal suture to the superior edge of the temporal bone. The right frontal bone, thus isolated, is turned outward and forward and broken along its base. A similar procedure is carried out with the right parietal bone, the underlying dura being separated as described above and the bone being isolated by incisions along the posterior portion of

the sagittal suture, the parieto-occipital suture, and the parietal edge of the frontoparietal suture. The parietal bone is turned outward and broken at its base.

The removal of the frontal and parietal bones thus exposes intact the dura covering the right cerebral hemisphere. Epidural hemorrhages may be seen and, should the question of an antemortem rupture of the middle meningeal artery be raised, it is simple to expose this artery by removing the squamous portion of the right temporal bone according to the technique outlined above, following which, retraction of the intact dura mesially will expose the entire artery as far as its entrance into the skull through the foramen spinosum. The superior sagittal sinus should not have been opened.

Having inspected this portion of the dura while intact, it should be incised longitudinally slightly to the right of the midline and in such a way that the superior sagittal sinus is not injured. The flap of dura is then turned outward, vertical incisions being made at the anterior and posterior portions of the flap if necessary, and the right cerebral cortex exposed to view. Subarachnoid, subdural or subpial collections of blood on this side should be noted, as should also the condition of the cerebral veins in relation to the superior sagittal sinus and the parieto-occipital (lambdoidal) suture. Antemortem rupture of these veins is difficult if not impossible to identify positively, but a diagnosis of probable antemortem rupture may at times be made.

The gloved fingertip is next inserted between the mesial surface of the right cerebral hemisphere and the filmy layer of tissue which makes up the body of the falx, care being taken to avoid tearing the latter. The corpus callosum is thus exposed. This should be carefully incised longitudinally in the midline and the cavity of the third ventricle be thus exposed to view. If this has been done with care the two internal cerebral veins and their point of junction to form the great cerebral vein of Galen will be plainly visible. With a rupture of the great cerebral vein the two internal cerebral veins are collapsed and difficult to see, otherwise they are plainly visible and often are noticeably congested. The presence of intraventricular hemorrhage should be ascertained by observation of the contents of the third ventricle.

Further incision through the floor of the third ventricle and the subthalamic region should be carried out at this point although the

internal cerebral veins are thus destroyed. A lateral horizontal incision through the right cerebral peduncle and section of the right optic tract now frees the right cerebral hemisphere, which with a little urging will fall out into the operator's hand. The hemisphere may be examined at once or laid aside in some suitable solution to harden prior to later sectioning and examination.

Removal of the right cerebral hemisphere in this manner exposes the entire falx cerebri and its junction with the tentorium cerebelli, as well as the right half of the latter.

The body of the falx should first be inspected for tears, hematomas or other lesions. This is best and in fact can only be accomplished by lifting it free from the mesial wall of the left cerebral hemisphere on a scalpel handle or other suitable instrument. The lower free border containing the inferior sagittal sinus should next be inspected for ruptures. The junction of the falx with the tentorium should be observed with care, especially if the two internal cerebral veins have been found collapsed, thus indicating a rupture of the great cerebral vein. Should this have occurred the posterior end of the ruptured vein may be present, still attached by its junction with the straight sinus or with the inferior sagittal sinus.

The right half of the tentorium should now be inspected. Tears of the free margin are frequent at or near its junction with the falx. Ruptures in any portion of the tentorium may or may not involve the lateral sinus, the straight sinus or even the confluence of the sinuses (torcular Herophili), and may present themselves as frank holes, from which blood can be expressed, should they communicate with the cavity of a sinus; or they may be indicated by an adherent antemortem clot or may be present only as tears without hemorrhage.

The superior sagittal sinus should be opened throughout its entire length and inspected for tears from its internal aspect. This will also expose the internal aspect of the confluence of the sinuses.

The right lateral sinus may now be split along its course, together with the superior and inferior petrosal sinuses, and the connection between the lacerations in the tentorium, thought to have extended into the cavity of one of these sinuses, thus be verified.

The falx should next be freed along its upper border as far as may be convenient, turned to one side out of the way, the left cerebral peduncle incised horizontally, the left optic tract sectioned,

and the left cerebral hemisphere allowed to drop out without further removal of bone.

The dura which previously covered this hemisphere may now be inspected from the inside, and the left meningeal artery observed. The left cerebral veins cannot be inspected except from the inner aspect of the superior sagittal sinus, and subarachnoid and subdural hemorrhages in this portion of the cerebrum will be evident chiefly as collections of blood on the cerebral cortex itself.

The anterior and middle fossae with their contents may be searched for fractures, hemorrhages or other lesions.

The left half of the tentorium cerebelli should be examined for evidence of injury either with or without hemorrhage, and the left lateral sinus split throughout its length just as was done on the right side.

The tentorium should be split in the midline toward the internal occipital protuberance and the two halves turned one to either side to expose the cerebellum and brain stem.

The cerebellum and brain stem are best removed in one piece in the following manner: The anterior border of the midbrain is pushed gently backward off the body of the sphenoid bone and the cranial nerves are exposed, identified and sectioned pair by pair. In this way also the basilar and the two vertebral arteries are brought into view and their continuity verified. The posterior cerebral and the posterior communicating arteries, as well as the anterior half of the circle of Willis, cannot be saved.

Having freed the brain stem by section of all the cranial nerves, a thin-bladed scalpel is inserted along the anterior aspect of the medulla through the foramen magnum as far down the cervical cord as possible (usually not more than one or two segments) and the cord is sectioned at this point. Occasionally it will be necessary, in addition, to bring the knife blade to the lateral aspects of the cord in order that the upper cervical roots may be cut before the cord will be found to be entirely free.

The cerebellum, brain stem and what little of the cervical cord it is possible to get may then be removed in one piece. If this is done with care, the roof of the fourth ventricle and the great basal cisterna overlying it may be kept intact and later, after being suitably preserved and hardened, may be inspected for meningitis, obstruction of the foramens or other lesions.

The posterior fossa and the foramen magnum are exposed together with the occipital sinus which should be split, in its long axis, up to its junction with the confluence of the sinuses.

After completing the examination, the skull should be filled with some appropriate material, the right frontal and parietal bones replaced, the scalp pulled back over them and the original incision sewed up.

3. According to Nauwerck (1921), the spinal canal can be opened by dividing the vertebral arches with strong scissors.

4. The umbilical cord, if present, and the umbilical arteries demand close attention in children who have lived a few days or weeks, for the purpose of determining if infection has taken place at that point. Nauwerck advises a modification of the primary long incision. A little above the umbilicus it should divide into two diverging incisions running to the pubes. In this way a triangular flap is left containing the umbilical arteries, while from the upper end is given off the umbilical vein. The vessels may be ligated or opened at any point that seems advisable.

5. Anomalies of circulation should be looked for in all "blue babies." The closure or non-closure of the ductus arteriosus is best determined *in situ* by dissecting off the thymus and opening up the pulmonary artery in the middle of its anterior surface. The cut may be extended downward, if desired, through the pulmonary valve and the wall of the right ventricle. The duct lies in the median line of the pulmonary artery, a little above the division into its two main branches. A small probe can be passed through it into the aorta. The condition of the foramen ovale between the auricles is easily examined.

For other anomalies of the circulation it will usually be found most satisfactory to remove the thoracic organs "en masse," so as to be able to open up the heart and the vessels given off from it before any of the vessels have been severed from their connections.

6. In medicolegal cases, especially, it is important to determine whether or not a child has breathed. The main steps of the process are as follows:

Locate the position of the diaphragm before the chest is opened. When the lungs are fully distended it is at the fifth or sixth rib on the right and at the sixth rib on the left. When the lungs contain no air or are but partially distended the diaphragm reaches to the fourth rib.

Ligate the trachea above the sternum before opening the thorax.

After examining the heart and the other structures, sever the trachea above the ligature and remove the thoracic organs in one piece.

Dissect off the thymus gland and the heart, and place the lungs in a large dish of clear cold water to see whether they will float or not.

Incise the lungs and notice if they are crepitant; squeeze the lung tissue gently and see if bubbles of air mingle with the blood on the surface, or squeeze the lung under water and observe if bubbles of air rise to the surface. Decomposition may give rise to gas in the lungs.

Divide the lungs into lobes and then into small pieces, and determine if any of them will float.

7. The long bones should be incised so as to expose the epiphyseal line which should be examined for evidences of congenital syphilis or rickets. The ends of the femur and tibia at the knee are usually chosen. For making the incision a fine hair-saw is preferable to a knife, because the latter often causes the bone to break apart at the epiphyseal line.

8. The age of the fetus in months can be determined after the fifth month by dividing the length of the fetus in centimeters by 5.

8. Restitution of the Body

After a postmortem examination is finished the body should be put in such a condition that no evidence of the autopsy will be noticed except on careful inspection. Every practical means should be employed to facilitate embalming for the undertakers. All fluid should be sponged from the cavities. The rectal and vaginal openings should be closed with sutures or plugged with absorbent material packed into the pelvis. The carotid and femoral arteries should be ligated as far proximally as possible. All organs, including the brain, which are not required for further examination should be replaced in the body cavity. The gastro-intestinal tract should be carefully cleansed of all its contents before it is returned to the body. In the cranial cavity the internal carotid and vertebral arteries should be tied. Leakage is best prevented by filling the base of the skull with plaster of paris. It can also be packed with oakum. The sternum is best sutured back in position.

If part of the vertebral column has been removed, a stick or heavy iron rod should be run into the spinal canal above and below, in order to stiffen the body and hold it in position while it is filled about half full of plaster of paris. After this has set there is little danger of the body losing its form.

In sewing up the body cavity begin at the neck. Use a piece of twine a little over one and a half times the length of the incision. Take one stitch and fasten the end with a knot. Turn the loose end in under the skin. Hold the attached end of the twine taut with the left hand about 8 to 10 cm. from the line of incision. The needle is then passed from within outward through the edge of the flap and in a diagonal line from below upward. The stitches should be from 1 to 2 cm. apart, and about the same distance from the edge of the flap. The object of keeping the end of the twine taut is to keep the sutures tight and the edges of the flaps up so that the needle can be thrust in easily.

On reaching the lower end of the incision, take two button-hole stitches and draw them tight. Then take a long stitch off to one side and cut the twine close to the skin, so as to bury the end of it deeply and securely.

If in removing the calvarium the precaution is taken to crack at least a part of the inner table with the chisel and hammer, projecting pieces of bone are usually left which interlock and hold the calvarium in position when it is replaced. It is further fastened by sutures on each side through the fascia of the temporal muscle. It is always more difficult to sew up the incision in the scalp than the one in the body, especially when the hair is long. Care should be taken to bury the ends of the sutures securely.

9. Autopsy Bacteriology

In order to obtain satisfactory results with cultures taken at autopsy it is essential that great care be exercised in the manner in which these cultures are taken, because of the ever present danger of contaminating organisms. When searing the surface of an organ be sure to do so until the seared surface is dry and be sure to keep it dry.

Heart's Blood.—The culture is best taken from the right auricle. The heart is lifted by the apex and the auricular wall just above the inferior vena cava is seared with a hot iron. The area seared should

be at least 2 cm. in diameter. No contaminated material should be allowed to come in contact with this area until the culture has been taken. The culture is best taken by forcing the end of a sterile capillary pipette through the center of the seared area and sucking 1 or 2 cc. of blood into the pipette. It will be found convenient to keep a supply of capillary pipettes in the autopsy room. Each should be in a sterile test tube and have a rubber bulb on the upper end to exert suction.

Inflammatory Exudates and Infectious Lesions.—Two swabs should be made from each lesion, one for a culture, one for a smear. When the culture is contaminated the predominant organism can often be recognized from the smear.

Cultures of Organs.—Sear the outside surface of the organ and force a swab through the seared area into the underlying uncontaminated tissue.

Vegetations of Heart Valves.—Snip off a small portion of the vegetation and place it in a test tube. The vegetation is then dipped in 80 per cent alcohol for a few seconds to kill superficial contaminating organisms. It is now ground up in a mortar with sterile sand and this ground up material is cultured in the usual way.

10. Tables of Weights and Measurements

TABLE I

NORMAL LIMITS OF MEASUREMENTS OF HEART VALVES

(Dana and Reidy, 1936)

Tricuspid Valve	Pulmonary Valve	Mitral Valve	Aortic Valve
10–12.5 cm.	6–7.8 cm.	8–10.5 cm.	6–7.5 cm.

TABLE II

RATIO OF OTHER VALVES TO AORTIC VALVE

(Dana and Reidy, 1936)

$$\frac{\text{Tricuspid Valve}}{\text{Aortic Valve}} = 1.68$$

$$\frac{\text{Pulmonary Valve}}{\text{Aortic Valve}} = 1.05$$

$$\frac{\text{Mitral Valve}}{\text{Aortic Valve}} = 1.34$$

TABLE III

Average Normal Weights of Viscera from Adults

(Ross, 1928)

Adrenals (2)	5 – 6	gm.
Bladder	30 – 60	"
Brain		
Female	1250 –1275	"
Male	1365 –1450	"
Heart		
Female	250 – 280	"
Male	270 – 360	"
Kidneys		
Left	150	"
Right	140	"
Liver	1440 –1680	"
Lungs		
Right	480 – 680	"
Left	420 – 600	"
Ovary (1)	4 – 8	"
Pancreas	60 – 135	"
Parathyroids* (4)	0.12– 0.18	"
Pineal	0.2	"
Pituitary	0.3 – 0.6	"
Prostate	22	"
Spleen	155 – 195	"
Testis (1)	20 – 25	"
Thymus		
At birth	13.7	"
At end of 2nd year	26.2	"
Thyroid	30 – 40	"
Uterus	40 – 70	"

* The figures for the weight of the parathyroid glands were supplied by Dr. T. B. Mallory.

TABLE IV

Weight and Length of the Fetus at Each Month of Gestation

(Nauwerck, 1921)

Time *mos.*	Weight *gm.*	Length *cm.*
2	4	2.5– 3
3	5–20	7 – 9
4	120	10 –17
5	284	18 –27
6	434	28 –34
7	1218	35 –38
8	1549	39 –41
9	1971	42 –44
10	2334	45 –47

TABLE V

Organ Weights of Infants and Children

(Coppoletta and Wolbach, 1933)

Age	Body length	Heart	Lung		Spleen	Liver	Kidney		Brain
			Right	Left			Right	Left	
	cm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Birth—3 days	49	17	21	18	8	78	13	14	335
3–7 "	49	18	24	22	9	96	14	14	358
1–3 wks.	52	19	29	26	10	123	15	15	382
3–5 "	52	20	31	27	12	127	16	16	413
5–7 "	53	21	32	28	13	133	19	18	422
7–9 "	55	23	32	29	13	136	19	18	489
2–3 mos.	56	23	35	30	14	140	20	19	516
4 "	59	27	37	33	16	160	22	21	540
5 "	61	29	38	35	16	188	25	25	644
6 "	62	31	42	39	17	200	26	25	660
7 "	65	34	49	41	19	227	30	30	691
8 "	65	37	52	45	20	254	31	30	714
9 "	67	37	53	47	20	260	31	30	750
10 "	69	39	54	51	22	274	32	31	809
11 "	70	40	59	53	25	277	34	33	852
12 "	73	44	64	57	26	288	36	35	925
14 "	74	45	66	60	26	304	36	35	944
16 "	77	48	72	64	28	331	39	39	1010
18 "	78	52	72	65	30	345	40	43	1042
20 "	79	56	83	74	30	370	43	44	1050
22 "	82	56	80	75	33	380	44	44	1059
24 "	84	56	88	76	33	394	47	46	1064
3 yrs.	88	59	89	77	37	418	48	49	1141
4 "	99	73	90	85	39	516	58	56	1191
5 "	106	85	107	104	47	596	65	64	1237
6 "	109	94	121	122	58	642	68	67	1243
7 "	113	100	130	123	66	680	69	70	1263
8 "	119	110	150	140	69	736	74	75	1273
9 "	125	115	174	152	73	756	82	83	1275
10 "	130	116	177	166	85	852	92	95	1290
11 "	135	122	201	190	87	909	94	95	1320
12 "	139	124	...	...	93	936	95	96	1351

CHAPTER XVII

PRESERVATION OF GROSS SPECIMENS

1. PREPARATION

CARE and planning at the time of the autopsy or when the surgical specimen is received is one of the first essentials of obtaining good gross specimens. Too many times museum preparations are an afterthought when the specimen is already too much cut up to be of any value for preservation. The following suggestions may be useful in this respect:

If the routine autopsy technique will tend to spoil the specimen, plan some method that will not.

Cut surfaces should be smooth and even. To obtain this result the section knife must be sharp and a long continuous cut made rather than numerous saw-like ones.

Trim all superfluous tissue from the specimen.

With large organs, such as the liver, it is usually better to save a slice 2 to 3 cm. in thickness rather than the whole organ. This may be cut tangential to the surface or in cross section through the organ, depending on what one is attempting to show.

Many specimens are ruined by taking samples for histological examination from the center of the lesion. A compromise is frequently necessary. Usually satisfactory material for histology can be taken from the sides or back of the specimen without injuring its gross appearance.

Drying or staining with bile will quickly spoil a specimen. It should be carefully washed with salt solution and put in fixative as soon as possible. When delay is necessary it should be kept covered with a cloth dampened in saline.

2. PRESERVATION

(1) GENERAL CONSIDERATIONS

No matter what method of fixation is used several general rules are important. They are the following:

Specimens should be fixed separately or the colors are apt to be spoiled. Bile-stained organs in particular will stain other organs preserved in the same jar.

A specimen should not be allowed to come in contact with another specimen or with the bottom or sides of the jar as this prevents proper fixation. Placing a layer of absorbent cotton in the bottom of the jar or suspending the specimen in the center of the jar by string will prevent this. Cotton or a piece of cloth should also be put on top to prevent the specimen from floating on the surface and drying. Keep the cut surfaces of the same specimen separated.

Light causes the colors to fade. Therefore, opaque jars made of porcelain or enamel ware should be used or the fixation process carried out in a dark room.

The type of specimen, the method of preparation and preservation are dependent to a certain extent on what organ one is dealing with. The more important organs are, therefore, listed with the various problems associated with them.

The *heart,* once it is opened, will fix completely. When it is fixed it becomes somewhat rigid. Because of this, if the important lesion is in one chamber or on one valve, the heart should be opened and put in fixative in such a way that the lesion can be seen.

The *lungs* are easily penetrated by fixative and therefore a whole lung can be saved. Usually, however, a hemisection makes a better specimen. One of the greatest dangers to be avoided is their floating at the surface of the fixative, as a result of which they become partially dry and discolored. To prevent this attach a glass weight heavy enough to keep them below the surface of the fluid.

The *spleen* fixes very poorly. Sections not more than 2 cm. in thickness make the best specimens. If whole spleens are to be kept they should be injected with fixative through the blood vessels.

The *liver* is also a difficult organ to preserve in its entirety. Usually 2 to 3 cm. thick slices cut tangentially or in cross section are most satisfactory as specimens.

It is frequently better to preserve one-half of one, or one-half of both *kidneys,* than one whole kidney. In this way both capsular and cut surfaces can be shown.

With the *gastro-intestinal tract* the greatest danger is that the specimen becomes distorted in the fixative. This is best avoided by opening the specimen and fixing it stretched open in a flat dish or pan. Even better results may be obtained by sewing it to a glass frame in the position desired. The simpler method of pinning the specimen to a board is somewhat dangerous, as in the fixative

all pins, nails or thumb tacks rust and as a result stain the specimen. If it is desired to maintain the tubular form of the gastro-intestinal tract, this can be done, if it has not been opened, by tying off the upper and lower ends and injecting fluid into the lumen with a syringe and needle.

If only the surface of the *brain* is important, as in meningitis or subarachnoid hemorrhage, fixation *in toto* is satisfactory. With internal lesions, however, cutting of the brain while fresh, and the preservation of sections 1 to 3 cm. in thickness, are much more satisfactory. Good fixation of the whole brain can be obtained by injection of fixative through the blood vessels.

(2) Fixing Solutions

A great variety of solutions have been devised for the preservation of the color in gross specimens. In our experience the methods of Kaiserling and Jores have proved the most satisfactory and these will be given in detail.

a. **Kaiserling's Solutions.**—The Kaiserling (1897) method consists of the use of three different solutions of the following compositions:

Kaiserling Solution No. I

Formalin	400	cc.
Water, tap	2000	cc.
Potassium nitrate	30	gm.
Potassium acetate	60	gm.

Kaiserling Solution No. II

Alcohol, ethyl, 80 per cent

Kaiserling Solution No. III

Glycerin	500	cc.
Arsenious acid, 1 per cent aqueous solution	200	cc.
Water, tap	2300	cc.
Potassium acetate	250	gm.
Thymol	2.5	gm.

The arsenious acid water and the thymol are added to prevent growth of molds. Some difficulty may be encountered in getting the arsenious acid into solution. This can be easily done if a 1 per cent solution of arsenic trioxide (As_2O_3) is made up in water and

the flask containing this solution is placed in an Arnold steam sterilizer for 6 hours. The thymol crystals are ground up in a mortar and allowed to float at the surface of the solution.

Solution No. I is for fixation. The specimens must be left in it from 1 day to 2 weeks, depending on their size. Large specimens can be fixed more rapidly by injecting fixative through the circulatory system, or by impregnation of the center by use of a long needle and syringe.

After fixation the specimen is washed for 12 hours in running water and placed in Solution No. II. The purpose of this is to bring back the maximum amount of color. The time necessary varies from 10 minutes to 1 hour. During this process the specimen should be watched, as after the optimum change has occurred the colors begin to fade and cannot again be restored.

The specimen is next rinsed off in water and put in Solution No. III, where it can remain indefinitely.

b. **Jores' Solutions.**—The method of Jores (1913) is similar but makes use of only two solutions. They are made up in the following manner:

1. Fixing Solution

Chloral hydrate	50 gm.
Carlsbad salts, artificial	50 gm.
Formalin	100 cc.
Water, tap	1000 cc.

The artificial Carlsbad salts (Klotz and Maclachlan, 1915) have the following composition:

Sodium sulfate	22 gm.
Sodium bicarbonate	20 gm.
Sodium chloride	18 gm.
Potassium nitrate	38 gm.
Potassium sulfate	2 gm.

2. Preserving Solution

Potassium acetate	300 gm.
Glycerin	600 cc.
Water, distilled	1000 cc.

Specimens are fixed in the first solution for 2 days to 2 weeks, according to size, washed for 12 hours in running water and then

placed in the preserving solution. No alcohol is necessary. In our laboratory instead of the preserving solution of Jores we prefer to use Kaiserling's Solution No. III, following fixation in Jores' fixing solution.

An additional method of producing good colors is one that was originally described by A. Schultz (1931). It makes use of carbon monoxide obtained from illuminating gas, to change the blood pigment present in the specimen to CO-hemoglobin. Either the Kaiserling or Jores fixing solutions may be used, but the solution chosen is saturated with carbon monoxide by bubbling illuminating gas through it. The specimen is then fixed in this solution in the same way as described above. No alcohol treatment is necessary.

The colors produced by this process are excellent but the method is somewhat dangerous due to the chances of poisoning and explosion. It should be carried out either in a well ventilated chemical hood, or in the special apparatus described by Robertson and Lundquist (1934).

3. LABELING

During the process of fixation a string long enough to reach outside of the jar is attached to the specimen. A tag is tied to the string with the autopsy or surgical number written on it. Excellent tags for this purpose can be made by sawing wooden tongue depressors in half and boring a hole in the rounded end, so that the string can be inserted. Pencil rather than ink should be used for numbering.

After the specimen has been carried through the preserving fluids a smaller permanent label should be attached. Sheets of white celluloid about 0.5 mm. in thickness are cut up in sizes convenient for labeling and a small hole punched in either end. The number of the specimen is written on the tag in ordinary ink and the tag is dipped in acetone for a moment. It is then allowed to dry. This process fixes the labeling on the tag and makes it permanent. The tag can now be sewed on the specimen. Care should be taken to sew it to as fibrous a portion of the specimen as can be found and yet not have it too conspicuous. This method has proved very satisfactory in our experience and labels have lasted well.

4. MOUNTING

Although unmounted specimens are in many ways more satisfactory for teaching, they soon lose their colors and, if fragile, are broken. Therefore, any unusual or valuable specimens should be mounted in separate glass containers. The most common type of container used is a rectangular glass museum jar. These can be obtained in a great variety of sizes.

Success in mounting specimens depends to a certain degree upon the ingenuity of the person doing it. There are quite a variety of methods that can be used. The three most common are the following:

1. A frame, which will fit in the jar that has been chosen, is made by bending a glass rod. The specimen is sewed to the frame in such a way that important lesions can easily be seen through the jar. The glass bending, while simple for the expert, is difficult for the beginner. Museum jars can be bought with glass frames already made to fit them. These frames hang in slots made in the upper wall of the jar. They are very convenient.

2. When only one surface of the specimen is important, this surface can be pressed against the side of the jar by packing the remaining space of the jar with absorbent cotton saturated with preserving fluid. This is easily done by the use of long surgical dressing forceps. Care should be taken to get rid of all air bubbles.

3. An old *x*-ray film can be cleaned of its emulsion by soaking it in hot water. It is then cut to a size that will fit a jar and the specimen sewed to it. The celluloid film is not injured by the preservatives and is practically invisible when the jar is filled with fluid.

Before cementing the cover on a jar it should be filled with fluid to within about 5 mm. of the top. No portion of the specimen or the cotton should extend above the fluid. It is a good practice to sprinkle a small amount of thymol crystals on the surface of the fluid as this prevents the growth of molds. Before attempting to cement on the top, clean the contact edges of the jar with alcohol and allow them to dry.

Almost every museum curator has his own formula for a cement for museum jars. The main ingredient of most of these is asphalt and this is usually diluted with a small amount of paraffin. The formula used by us is the following:

Battery-seal asphalt	2 parts
Trinidad asphalt	1 part

These are melted and mixed by heating on an electric stove or sand bath. If the resulting mixture proves too brittle a little paraffin is added. To use this cement it must be melted and applied while hot to the top of the jar. It should harden in 12 to 24 hours. It is wise to put some sort of weight on top of the jar until the cement is hard.

A simpler and, in our opinion, more satisfactory method of sealing museum jars is to use the ordinary type of household cement that is sold at hardware stores for mending china. We use a du Pont preparation called "Duco." One needs only to apply the substance to the top of the jar and put the cover on the jar carefully so that the cement distributes itself evenly over the contact surfaces. It will harden in 8 to 12 hours.

In most museum jar tops a small hole is present in one corner, so that fluid may be added. The jar should never be filled completely, an air space of 1 to 2 mm. must be left to allow for the expansion of the preserving fluid with variations in temperature. This hole may be closed by filling it with soft paraffin or putty.

Another method of mounting small specimens is the so-called watchglass method. In this the specimen is trimmed so that it will fit in a watchglass or Petri dish. The watchglass is then filled with preservative and cemented to a glass plate. Details of this method may be obtained by reading the descriptions given by Warren (1933) or by Larson and Levin (1937).

The method has the advantages that the materials used are cheap and the space needed for the storage of finished specimens is small. It has the disadvantage that it is somewhat time-consuming and difficult technically for the beginner.

CHAPTER XVIII

PHOTOGRAPHY

1. PHOTOGRAPHY OF GROSS PATHOLOGICAL SPECIMENS

(1) Apparatus

a. Camera.—The type most frequently used is a bellows camera taking 5 by 7 inch plates. It should have a tripod or stand so that it can be used in a vertical, oblique, or horizontal position. A fast lens or a shutter is of no advantage as practically all photographs will need time exposure.

b. Illumination.—Artificial illumination is in general more satisfactory than daylight, because it is always available and constant in quantity. It is best provided by two portable lights with 500 to 1000 watt bulbs and effective reflectors. The specially prepared photoflood bulbs are cheap and very good.

c. Tank for Specimens.—Most specimens should be taken under water. For small specimens a flat-bottomed glass dish can be used. The tank (Fig. 14) devised by T. Bitterman (1922) with a glass bottom and sides can be highly recommended. Its dimensions are: length 25 inches (63.5 cm.), width 16 inches (40.6 cm.), height 10 inches (25 cm.). It is elevated on legs 10 inches (25 cm.) high in order that shadows cast by the specimen do not reach the background which is put on the floor beneath. The tank has a small hole drilled through one corner of the bottom to facilitate the removal of the water.

d. Plates.—A great variety of plates and films are used. In general it can be said that panchromatic emulsions should be employed, that on the whole emulsions that will give contrast are better than soft ones, and that there is no advantage in using very rapid plates or films. It is undoubtedly true that for the non-professional photographer it is better to use consistently one type of plate and become thoroughly acquainted with its possibilities rather than to change plates constantly. When panchromatic emulsions are used, color filters are usually unnecessary. Sometimes a Wratten K_3 filter can be used to advantage.

(2) Photographs

When possible it is more satisfactory to fix the specimens in the Kaiserling or Jores fixatives before attempting to photograph them. Fresh material can be taken, however, and fair results obtained. A knowledge of the general principles of photography is very helpful, but if the following suggestions are carried out good photographs should be obtained.

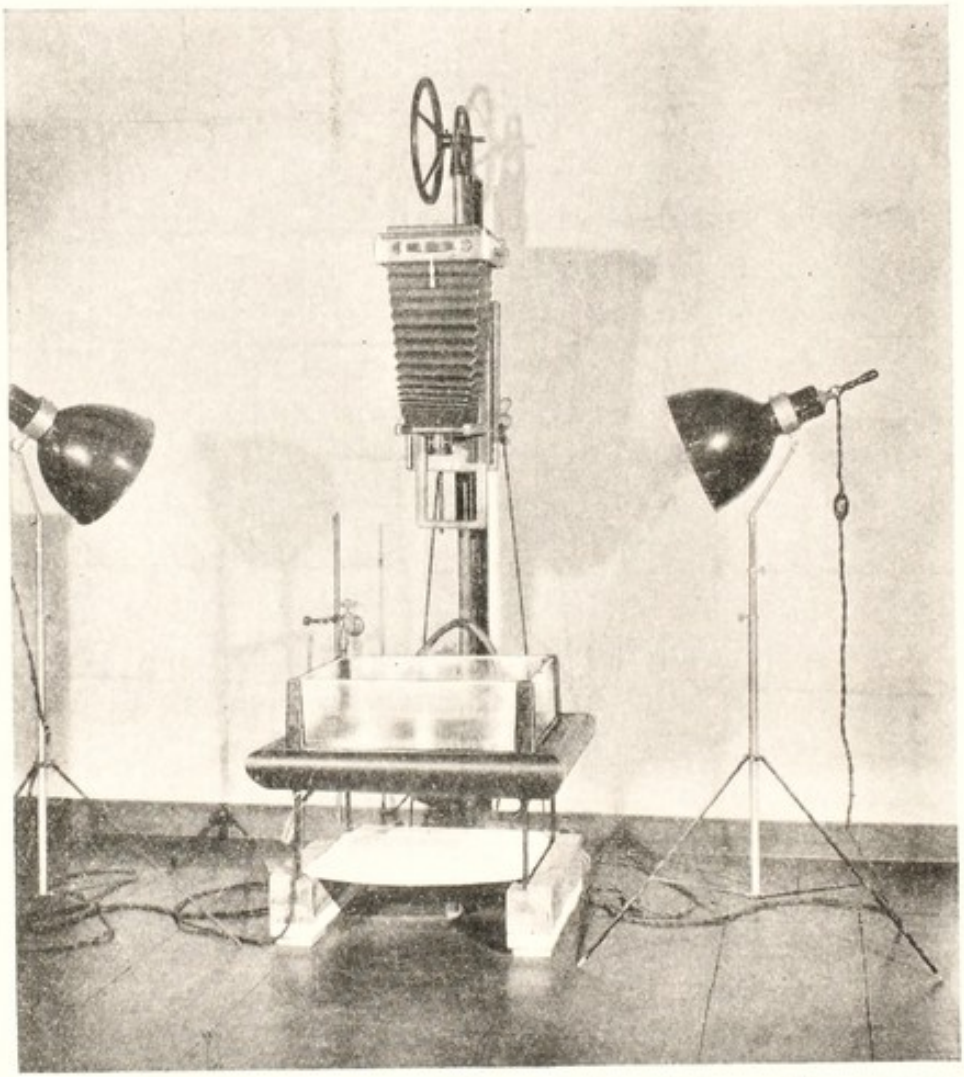

Fig. 14.—Glass tank devised by T. Bitterman for photographing gross specimens under water. The illustration shows the necessary arrangement of camera, lights and background.

The first and often most important step is the preparation of the specimen. It is obvious that it should be placed in such a position that the most important side, or surface, faces the camera. Frequently, by careful dissection important lesions can be brought into sight. A piece of glass rod can be used to spread open a cavity or the lumen of a blood vessel, or the preparation can be pinned to

a piece of cork or wood. When the specimen is to be photographed under water the same result can be accomplished by sewing a weight to the back surface. For this purpose variously sized pieces of sheet lead about ⅛ inch in thickness are convenient. If numerous perforations are bored in these, sewing is made easier.

Most specimens, either fresh or fixed, will be wet and have shiny surfaces. Because of this they will produce annoying high lights in the photograph. This can be avoided to a certain extent by drying the specimen carefully and arranging the lights in such a manner that no high lights are visible when the image is seen in the ground glass of the camera. A better method of obtaining the same result is to photograph the specimen while it is just, but completely, covered with water. This can be done even with fresh material if all free blood is first carefully washed away.

The specimen should be illuminated chiefly from one side and somewhat obliquely. Too even an illumination without shadows leads to a flat photograph without perspective. Shadows around the object, however, are to be avoided when possible. This is best accomplished by the use of a tank with a glass bottom.

Use a white, gray or dark background, according to the color of the tissue to be photographed. The specimen should stand out against it in sharp contrast.

Focus on the surface of the specimen. If no sharp details are present place a printed card or celluloid ruler on the highest point and focus on that. Then close the diaphragm to a small opening (F-32 to F-64). This procedure greatly lengthens the time of exposure required, but gives sharp detail and great depth of focus.

Some indication of size is often important in a photograph. A celluloid ruler, if put at the side of the specimen and photographed at the same magnification, is of value for this purpose.

An exposure meter is a great convenience and, when properly used, will avoid much over- and underexposure. Two factors must, however, be kept in mind. In the first place the reading should be taken from the specimen itself and should not include too great a proportion of background. In the second place, since most gross specimens are photographed with the camera relatively close to the object, in most instances the reading obtained with the meter must be corrected by multiplying it by a factor which varies considerably with the distance. This factor can be calculated by the use of

somewhat complicated formulas, but can be easily and accurately enough figured out from the table given below. The formulas used to calculate the exposure factor are:

$$T = \frac{D^2}{F^2}$$

$$D = \frac{B \times F}{B - F}$$

T = the exposure factor
D = the distance between the lens and the image
F = the focal length of the lens
B = the distance between the object and the lens

To avoid the calculations necessitated by the use of these formulas the following table will be found convenient and sufficiently accurate.

TABLE VI

1. Magnification	Very small	0.054	0.11	0.20	0.25	0.33	0.50	0.66	1.0	1.50	1.75	2.0
2. Distance from lens to object measured in focal lengths B/F	>20	20	10	6	5	4	3	2.5	2	1.7	1.6	1.5
3. Exposure factor T	1	1.10	1.23	1.44	1.55	1.78	2.25	2.75	4	5.86	7	9

The table is used in the following manner:

1. Focus the camera at the desired magnification or reduction.

2. Measure the distance between the lens and the specimen.

3. Divide this measurement by the focal length of the lens. This gives the distance between the lens and the object measured in focal lengths of the lens (Table VI, 2).

4. Apply this measurement to the table and read the corresponding exposure factor (Table VI, 3).

5. Multiply the reading of the exposure meter by this factor and the proper time of exposure will be obtained.

6. Table VI, 1, gives the actual magnification obtained for each B/F.

2. MICROPHOTOGRAPHY

(1) APPARATUS

a. Microphotographic Apparatus.—A great variety of microphotographic apparatus is manufactured by the various optical

apparatus companies but it is not within the scope of this book to recommend or deprecate any particular type. The following general statements can, however, be made.

The better the lenses of the microscope used, the better will be the photographs that can be taken. Apochromatic lenses are undoubtedly better than achromatic ones but good photographs can be taken with the latter.

A stand that is rigid and a location that is free from vibration are both necessary. It is also indispensable that the apparatus should always be kept in good alignment, with the light properly centered.

Particularly for low power microphotographs an especially designed group of condensers is more satisfactory than trying to use those usually provided in the ordinary microscope.

A brilliant but constant source of illumination is to be desired. An arc light with automatic feed is fairly satisfactory but somewhat variable in intensity. A powerful ribbon filament bulb (18 amp., 6 volt, T 10 projection lamp, burn base down) is more efficient.

b. Color Screens.—We have found the following liquid filter most satisfactory, and it works perfectly well with all the staining methods mentioned below.

Copper sulfate	175 gm.
Potassium bichromate	17 gm.
Sulfuric acid, concentrated	2 cc.
Water, tap	up to 500 cc.

It is used in a flat glass cell, 1 cm. in thickness. If the cell is too thick the solution must be diluted.

The commercial, dyed celluloid filters are also very useful.

c. Plates.—Plates with a moderately rapid panchromatic emulsion should be used.

(2) The Essentials of Microphotography

The first and greatest secret of good microphotographs is a perfect section perfectly stained. If one has this the rest of the process is comparatively simple.

The second important point is choice of field. This is a time-consuming process but it is absolutely necessary if the picture is to

tell its story. No artefacts of any sort, such as scratches or foreign bodies, should be tolerated. The selected fields should be circled either by means of a diamond marker or with a pen dipped in methyl violet shellac or India ink. When it comes to photographing the desired fields the slide should be rotated by means of the movable stage until the field represents a well balanced appearance with the main lines running horizontally or vertically and with the most important feature in the center of the field.

It is a frequent mistake to expect too much of a microphotograph as far as depth of focus is concerned. One cannot satisfactorily get more in a photograph than one can see with the same combination of lenses without changing the fine adjustment. As a rule, low power lenses have greater depth of focus than those of high power. If a whole cell or fiber can be brought into focus with a low power lens, but not with a high power, it is possible to take the photograph with the lower power and to enlarge the negative in printing. This, however, is not considered good photography as the enlargement of the negative can produce no greater detail than is present in the original negative.

In photographing a series of slides it is advisable to do at one time all the fields requiring the same magnification. By this method the timing of the exposures is simplified and rendered a certainty and the changes of objectives, oculars and condensers reduced to a minimum. It will be found that, as a rule, it is much easier to take good high power pictures than those of low magnification.

The following staining methods all give good results photographically. An intense, sharp stain is desired but it should not be too deep except when very low magnification is wanted:

Phloxine and methylene blue after Zenker fixation; the best general stain and the one most commonly useful. Under this heading should be included the Giemsa and Romanowsky stains.

Alum hematoxylin and phloxine or eosin; used chiefly for celloidin sections. The resulting prints and lantern slides have a soft attractive quality. The hematoxylin alone is useful for bringing out the nuclei in Gram-Weigert and tubercle bacillus stains.

Phosphotungstic acid hematoxylin; excellent for tumors, especially when mitotic figures and neuroglia, fibroglia or myoglia fibrils are present.

Weigert's and Verhoeff's elastic tissue stains; the latter is especially designed for tissues fixed in Zenker's fluid.

Gram-Weigert and tubercle bacillus staining methods.

A working knowledge of photography is a desirable asset before undertaking microphotography, but with persistence and experience any one can in time expect to get fairly good results.

3. DEVELOPING, FIXING, PRINTING, MAKING OF LANTERN SLIDES

(1) Developing

Developer for Plates.—The following developer has proved very satisfactory for quite a variety of plates but it is not necessarily better than the type recommended by the manufacturers of the plate used. Make up and keep Solutions A and B separate.

Solution A

Water, tap, cold	1000	cc.
Pyrogallic acid	20	gm.
Potassium metabisulfite	2.5	gm.

Solution B

Water, tap, warm	1000	cc.
Sodium sulfite, anhydrous	100	gm.
Sodium carbonate, anhydrous	40	gm.

Just before use, mix equal parts of the two solutions. Add 10 to 12 drops of a 10 per cent aqueous solution of potassium bromide to 120 cc. of the mixture. Develop 3 minutes at 68° F.

This is a strongly alkaline pyrogallic mixture. A properly exposed plate is fully developed by it in 3 minutes. As soon as the developer is poured over the plate, brush the surface gently with a wad of cotton to remove all air bubbles. Go over the surface in the same way after the plate is washed and just before it is put to dry in order to remove any particles of dirt or iron rust in the running water which may have been deposited on it.

(2) Fixing

The fixative is made up in two parts, A and B; when all salts are dissolved, combine the two solutions and keep as a single stock solution.

Fixatives for Plates and Prints.—

Solution A

Water, tap	3840 cc.
Sodium thiosulfate	960 gm.
Sodium sulfite, anhydrous	90 gm.

Solution B

Water, tap	960 cc.
Chrome alum, powdered (chromium ammonium sulfate)	60 gm.
Sulfuric acid, concentrated	15 cc.

Pour Solution B into Solution A while stirring well. Fix plates about 20 minutes.

This is the acid chrome alum fixing solution in general use by professional photographers. It keeps indefinitely in stock solution. Other fixing baths may be used if desired.

(3) Printing

Printing Paper.—A glossy, moderately contrasting paper is recommended for general use. Best results can be obtained by keeping paper of three or more degrees of contrast on hand to try when the print on the usual paper is not satisfactory.

Developer for Prints

Metol	1 gm.
Hydroquinone	3.5 gm.
Sodium sulfite, anhydrous	24 gm.
Sodium carbonate, anhydrous	24 gm.
Potassium bromide	1.3 gm.
Water, tap	1000 cc.

The ingredients must be dissolved in the following manner using warm tap water of about 125° F.

To 600 cc. water add the metol, one-half the sodium sulfite, the hydroquinone, and then the second half of the sodium sulfite. Each must be in solution before the next is added. In a separate container add the sodium carbonate to 400 cc. warm water. When this is in solution the contents of the two containers may be mixed and the potassium bromide added.

As is well known, the metol sometimes causes eczema of the fingers but with a little care or by the use of rubber cots this can generally be avoided.

Squeegeeing.—After the prints have been fixed and washed they are squeegeed onto the surface of ferrotype plates.

To prevent sticking, a few drops of a saturated solution of white beeswax or paraffin in turpentine or in xylol are poured on the plate, and then rubbed over the surface with a soft towel until it is highly polished.

As prints prepared in this way often show a moth-eaten appearance on the surface where particles of air have prevented intimate contact with the plate we have found it advisable to dip the prints in 25 per cent alcohol just before squeegeeing them.

Old prints can be treated in the same way if they are first soaked in water. As soon as the prints come off the plates they should be put under pressure (between the leaves of a book will do) to prevent curling. If they have curled at all badly, hold them over a steaming dish of hot water for a moment to soften the surface and then run the prints over the edges of a desk or table, with the print surface uppermost to reverse the curl; then put under pressure again until perfectly dry.

(4) Making of Lantern Slides

Lantern Slides.—We have used various makes and have found most of them satisfactory.

Developer for Lantern Slides

Water, tap	1000	cc.
Hydroquinone	6.25	gm.
Sodium sulfite	11.25	gm.
Citric acid	0.7	gm.
Potassium bromide	0.7	gm.
Sodium carbonate, anhydrous	50	gm.

Dissolve in the order listed. Develops slowly at first, rapidly later. Good results can also be obtained with the metol-hydroquinone developer recommended for prints. Do not overexpose; develop up full; make exposures in an enlarging and reducing camera, not by direct contact.

CHAPTER XIX

ADDENDA

1. METHOD FOR BLACKENING TABLE TOPS

WHITEWOOD is preferable for table tops as it is cheaper and takes the stain better than pine. Oak and birch also give good results. If the table tops are blotched with paraffin, wax or resin, remove these before applying the stain. It is not necessary to remove stains made by dyes. Paint the top surfaces and edges with the following solution and proceed as given below.

Solution A

Copper sulfate	125 gm.
Potassium chlorate	125 gm.
Water	1000 cc.

Boil until dissolved.

Solution B

Aniline hydrochloride	150 cc.
Water	1000 cc.

1. Cover the floor completely under and around the tables with papers and with a brush apply two coats of Solution A while hot, the second as soon as the first is dry.

2. When dry, apply in the same way two coats of Solution B and let them dry thoroughly.

3. Rub in a thin coat of raw linseed oil and polish by rubbing with a cloth.

4. When dry, wash thoroughly with hot soapsuds and rub again with raw linseed oil.

5. Rub with cotton waste over and over again until the black stain no longer comes off.

6. Once a week apply liquid paraffin to keep the tops in perfect condition.

2. METHOD FOR CLEANING GLASSWARE

For ordinary purposes new slides and coverslips are cleaned by dipping in alcohol and wiping dry with a soft linen cloth. They

may also be cleaned by dipping in acid alcohol, washing thoroughly in water and wiping dry from 95 per cent alcohol. Keep in a tightly covered glass dish or in a box.

For cleaning glassware of all kinds, especially for use in silver stains, a better method is to leave the glassware 1 hour or longer in the following cleaning solution:

Potassium bichromate	160 gm.
Water	2000 cc.
Sulfuric acid	240 cc.

Dissolve the potassium bichromate in the water, which should be heated, and then add the sulfuric acid slowly while stirring constantly.

A similar solution, much stronger and omitting the water, can also be used.

Potassium bichromate	100–150 gm.
Sulfuric acid	2500 cc.

Place in a large pyrex flask and dissolve the potassium bichromate carefully by heating on an electric stove.

These solutions remove any alkaline silicates that may have formed on the surface of the glass.

After treating with one of the cleaning solutions, wash glassware thoroughly in tap water and rinse in distilled water. For silver stains it is essential that the glassware shall not be handled.

To clean old slide preparations, heat until the balsam softens so that coverslips and slides can be drawn apart. The slides and coverslips are then treated separately.

The coverslips are soaked first in xylol and then in alcohol for several days to remove the balsam, left overnight in a strong solution of one of the powdered soaps (do not boil), washed thoroughly in water, dipped in dilute acid or acid alcohol, washed again in water and wiped dry from 95 per cent alcohol.

The slides are put into jars of waste alcohol, or of xylol followed by alcohol, for several days to remove the balsam, and are then treated in the same manner as the coverslips.

3. METHYL VIOLET SHELLAC FOR MARKING

Shellac, best white	10 gm.
Alcohol, 95 per cent	20–25 cc.
Methyl violet	0.1 gm.

This solution will be found very convenient for marking important fields in mounted sections. It may be used with the circular markers made for this purpose, but a pen is just as convenient and less liable to cause injury to the preparation by pressure. The desired field is readily outlined under the low power of the microscope by a series of dots or a continuous line. The solution after drying is insoluble in xylol or water.

4. HAND LOTION

Gum tragacanth, powdered	5–10 gm.
Boric acid	50 gm.
Glycerin	200 cc.
Water	up to 1000 cc.
Oil of rose geranium	1 cc.

Mix the first four ingredients in a bottle and set in a warm place. Shake occasionally. In about a week the gum tragacanth will be thoroughly softened and diffused in the water. The lotion will be smoother if filtered or squeezed through fine cheesecloth or a towel. Dissolve the oil of geranium in a little alcohol and add directly to the mixture. The amount of glycerin can be increased if desired.

5. PHYSIOLOGICAL SOLUTIONS

These solutions are used for perfusing organs, suspending tissue cells for supravital staining, and in tissue culture methods. The solutions most commonly used are those of Ringer, Locke and Tyrode.

(1) Ringer's Solution

Sodium chloride (NaCl)	7 gm.
Potassium chloride (KCl)	0.3 gm.
Calcium chloride ($CaCl_2$)	0.25 gm.
Water, distilled	1000 cc.

The exact composition of this solution has been varied by different workers. The one given here is that used by Ringer for cold-blooded animals. For mammalian tissue Locke's solution is better.

Ringer's solution may be sterilized by autoclaving.

(2) Locke's Solution

Sodium chloride (NaCl)	9	gm.
Calcium chloride ($CaCl_2$)	0.24	gm.
Potassium chloride (KCl)	0.42	gm.
Sodium bicarbonate ($NaHCO_3$)	0.2	gm.
Dextrose	0.1–2.5	gm.
Water, distilled	1000	cc.

In making up this solution add the calcium chloride last. The solution does not keep long and should not be boiled. Sterilize with a Berkefeld filter.

(3) Tyrode's Solution

Sodium chloride (NaCl)	8	gm.
Potassium chloride (KCl)	0.2	gm.
Calcium chloride ($CaCl_2$)	0.2	gm.
Magnesium chloride ($MgCl_2$)	0.1	gm.
Sodium bicarbonate ($NaHCO_3$)	1	gm.
Monosodium acid phosphate (NaH_2PO_4)	0.05	gm.
Glucose	1	gm.
Water, distilled	up to 1000	cc.

In making up this solution the monosodium acid phosphate must be added last. Sterilizing is done through a Berkefeld filter.

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