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PATHOLOGICAL TECHNIQUE

A Practical Manual for Workers in Pathological Histology

including

Directions for the Performance of Autopsies and for Microphotography

See also S.4.4.

BY

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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

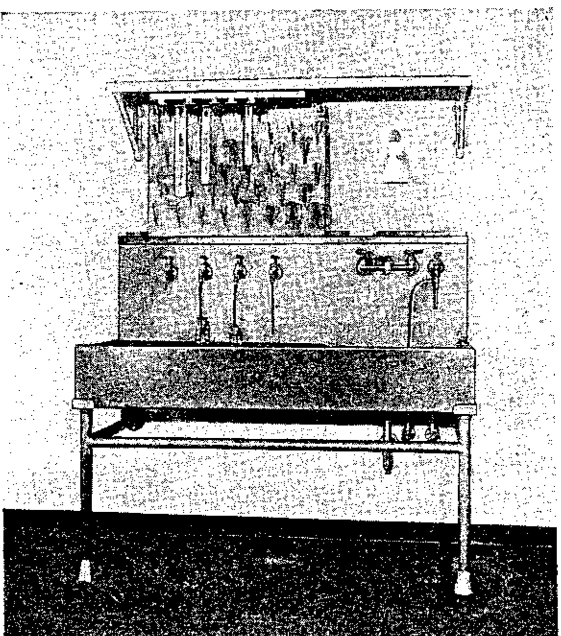

Fig. 1.—Laboratory sink showing arrangement of water outlets; also overhead light, shelf with support for inverted graduates and peg board for glassware.

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 (× 10), medium (× 20), and high (× 40) in the dry series, and much higher in the oil immersion lens (× 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.

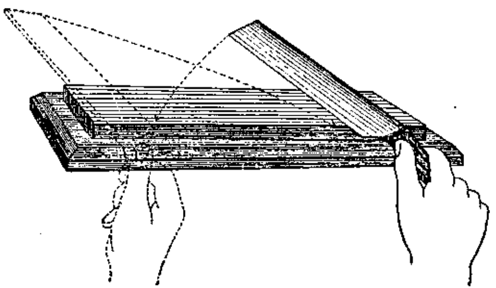

Fig. 2.—Diagram of the direction of the movements of the knife in honing.

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

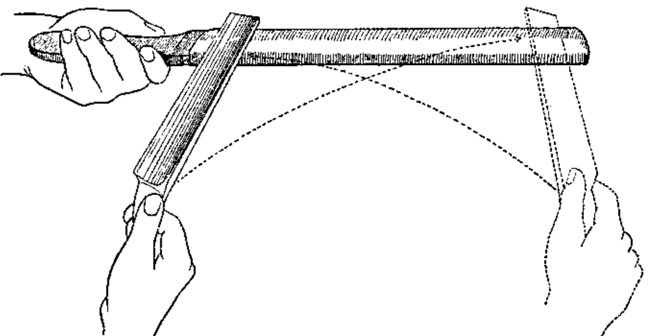

Fig. 3.—Diagram of the direction of the movements of the knife in stropping.

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.

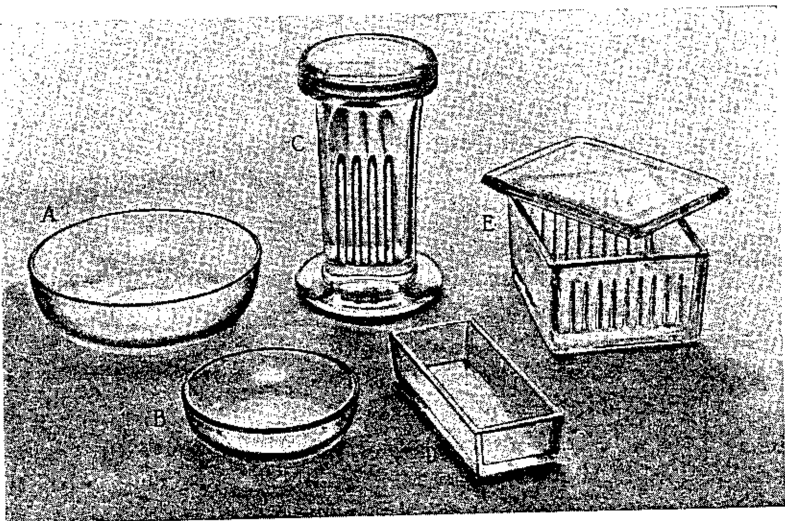

Fig. 4.—Different types of glass dishes. A, 150 cc. flat-bottomed dish for holding sections; B, 25 cc. staining dish; C, Coplin staining jar; D, McJunkin oblong staining dish; E, staining dish to hold 10 slides on side.

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 re-

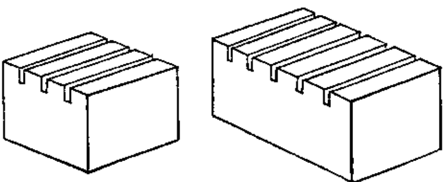

Fig. 5.—Blocks of vulcanized fiber prepared for mounting tissues embedded in celloidin.

moved 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.

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 postmortem 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)

Step	Time
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

Step	Time
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)

Step	Time
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
5. Celloidin, thick	1 or more weeks
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 difficult 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

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 253 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.

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.

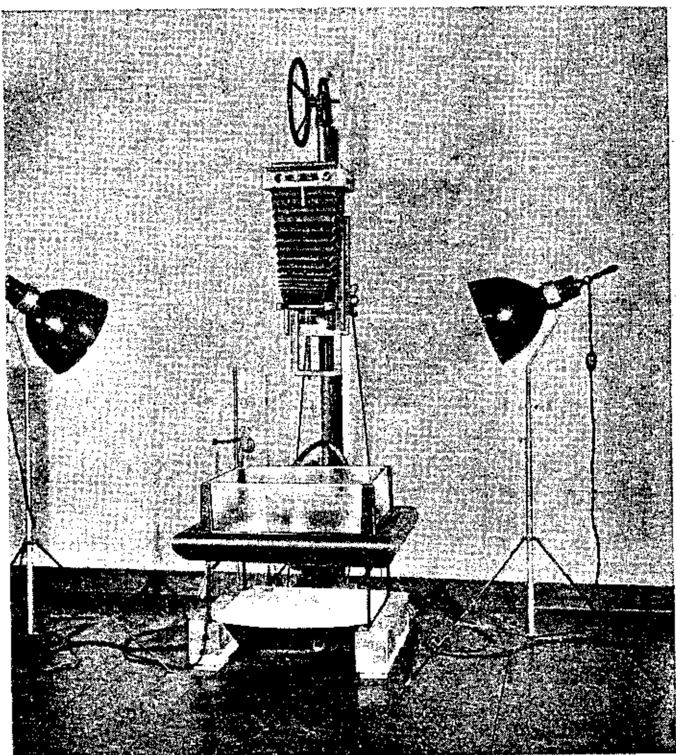

Fig. 14.—Glass tank devised by T. Bitterman for photographing gross specimens under water. The illustration shows the necessary arrangement of camera, lights and background.

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.