Histopathologic Technic
and
Practical Histochemistry

By
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ὅταν δὲ ἔλθῃ ἐκεῖνος, τὸ πνεῦμα τῆς ἁληθείας ὀδηγήσει ἡμᾶς εἰς τὴν ἁληθείαν πᾶσαν." Ἱω. xvi-13
δίδασκε ἡμᾶς, κύριε, γνῶρις τὰ τὰν ἁληθείαν.

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Foreword

Since the previous edition was written, there has been a great deal of active investigation of histochemical procedures. I have endeavored to bring selected variants of the newer methods into this book, and to emend them so that the methods may be followed without personal instruction by one who has had previous experience, or a good deal of experimentation to find optimal times, temperatures, pH levels and reagent concentrations. This has entailed in many instances a considerable amount of experimentation in arriving at workable conditions.

I am indebted to Dr. J. H. Peers, Dr. G. Laqueur, Dr. G. Brecher, Dr. B. Highman, Dr. R. W. Mowry, Dr. J. D. Longley, Dr. E. R. Fisher and others of the staff for their suggestions and cooperation in evolving these variants, and to Miss A. Laskey and Mrs. J. Greco Henson, and Mrs. H. Burtner for active help in performance and evaluation of procedures.

I also acknowledge my indebtedness to my predecessors and colleagues from whose works I have borrowed freely. Such of these borrowings as have been taken directly from their original publications are usually so cited in the text, but many have been taken, often in modified form, from other laboratory manuals. These texts are usually cited simply by the author's name, except that in the case of Ehrlich's "Encyklopädie" I have often cited the contributor's name. This last text I have often preferred as a source of those older methods which are still used in unmodified form. The following texts have been thus used, as well as earlier editions of some of them.

General References

American Association of Textile Chemists and Colorists, Yearbooks, 1939-1949, Howes, N.Y.


Ehrlich, P.: “Encyklopädie der mikroskopischen Technik,” Berlin & Wien, Urban & Schwarzenberg, 1903


FOREWORD


R. D. LILIE

Bethesda, Md.
October, 1953
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Chapter 1

Microscopy

I do not propose to enter into any theoretical discussion of the optics concerned in the use of the compound microscope. Rather, the purpose of this chapter is to bring in certain practical points in the use of the microscope in which I have found it necessary to instruct technicians and physicians in training in pathology.

Light

The advice in older manuals about the necessity for north windows for microscopic work, the avoidance of direct sunlight, and the preferability of a white cloud as a source of illumination is still applicable for the monocular microscope. However, daylight seldom gives adequate lighting for binocular microscopes or for more than low powers; hence some form of artificial lighting generally is necessary for microscopic work. Such lighting has the further advantage of not being subject to variations in the weather.

A tungsten-filament electric lamp gives satisfactory illumination for most purposes. The slightly yellowish color of the light can be corrected by insertion of a thin blue glass disk into the microscope substage, by the use of blue glass daylight bulbs, or by the interposition of a water filter containing a weak solution of copper sulfate to which sufficient ammonia water has been added to change its color from green to blue. In form this water filter may be a cell with flat parallel sides such as the microscope-lamp manufacturers often supply, or it may be spherical, such as a 500 cc. Florence flask. The latter serves also as a converging lens.

Filament images, which give rise to uneven illumination of the microscopic field, are avoidable by the use of ground-glass disks placed in the microscope substage, by the use of frosted or milky glass bulbs, or, in more elaborate lamps, by the use of a homogeneous light source large enough to fill the field completely, such as the six-volt ribbon filament lamp.
In all lamps which do not possess a focusing or condensing device to produce parallel or converging light rays, it must be borne in mind that the intensity of the illumination is inversely proportional to the square of the distance of the light source from the object. The same law applies when a ground-glass disk is inserted in the path of a parallel or converging light beam, since this ground-glass surface acts as though it were the light source, and the available illumination diminishes with the distance from the disk to the object.

With the larger microscope lamps, which employ lens systems to focus the light accurately on the condenser of the microscope, it is advisable to mount both lamp and microscope in permanent positions on a baseboard, so that once proper optical alignment is established, it need not be disturbed. In this case it is desirable to have a cloth bag or some form of rigid cover to place over the microscope when it is not in use, in order to protect it from dust. The old-fashioned bell jar functioned well in this respect, but it was heavy and breakable. A cylinder of cellophane or similar transparent plastic, of sufficient diameter and height to cover the microscope readily and with a handle on top, makes a very satisfactory lightweight, transparent substitute which is not readily broken.

The baseboard on which the microscope and lamp are mounted may be made of sufficient thickness to support the microscope at a level such that the eyepieces are at the most convenient height for the individual observer. Among seven workers in one laboratory the most convenient height of the eyepieces above a table 76 cm. (30 in.) high varied from 33 to 40 cm. (13 to 15% in.). A swivel chair with adjustable height may also be used to bring the user's eyes to the approximate level of the eyepieces. Larger lamps with large light sources and focused beams are needed for critical work at high magnifications, for dark-field work, and, principally, for photomicrography.

The Microscope

For the average worker, the use and care of the microscope are adequately described in the booklets furnished by the manufacturers. Only a few practical points will be discussed here. As Schmorl aptly states, the microscope should be obtained from a reputable manufacturer, and from personal experience I would recommend that when possible the manufacturer's plant be in the same country as the user. Necessary repairs and adjustments are greatly expedited if it is not necessary to send instruments or lenses out of one's own country.

When practical, it is preferable to have a binocular microscope with inclined ocular tubes, so that wet mounts may be studied without standing up over the instrument. The binocular instrument furthermore lessens the fatigue of prolonged use, as compared with the monocular. By training both eyes to observe, it also guards against incapacity during temporary losses of the use of one eye.
Either achromatic or apochromatic objectives may be selected. The former are corrected for two colors only, and are considerably cheaper. They give quite satisfactory service for ordinary clinical laboratory work. The latter are corrected for three colors and are preferable for research work and for photomicrography. If color photomicrography is to be done, they are essential.

The Abbé test plate is a glass slide with a thin film of metallic leaf through which a series of parallel lines have been scored so as to leave clear lines bounded by narrow, opaque, metallic bands with jagged edges. This film is covered by a long, narrow cover slip which varies progressively in thickness from about 90 $\mu$ at one end to 230 $\mu$ at the other. At the side of this cover slip are graduations indicating the approximate cover-glass thickness at any point.

This test plate is used for testing objectives for chromatic aberration, for spherical aberration, for sharpness of definition, and for flatness of field. A complete substage with a device for oblique illumination is needed. Low-power objectives should be tested between 150 $\mu$ and 200 $\mu$ equivalent cover-glass thickness. No. 1 cover glasses average about 0.15 mm. ($= 150$ $\mu$), No. 2 about 0.21 mm. ($= 210$ $\mu$) in thickness. Test 4 mm. apochromats with correction collars at at least two points, with corresponding adjustment of the correction collar. Immersion objectives should be tested immersed in their proper immersion fluids.

With oblique illumination, achromatic objectives give relatively broad fringes of complementary colors on the edges of the metallic strips. With apochromats, these fringes are narrower—often almost inappreciable.

When sharply focused with the condenser centered and properly focused, a good objective should continue to give sharply defined points on the edges of the metallic bands when the illumination is decentered across the direction of the bands. Similar performance should be obtained in the central and peripheral portions of the field.

Relative flatness of field can be judged by the amount of focusing necessary to give sharp definition respectively in the center and at the periphery of the field. It should be borne in mind that lenses with the greatest resolving power in the center of the field ordinarily do not give as flat a field as some others inferior in resolving power. This property of flatness of field is more important with lower powers and for photographic purposes.

Resolving power may be tested on various test slides. For instance, the diatom *Pleurosigma angulatum* at 250 $\times$ should show three distinct striation systems. One runs perpendicularly to the median rib; the other two cross obliquely at an angle of about 58°. At higher magnification the striae appear as material between rounded globules which is dark at high and low focus, bright at normal focus. The wing scales of *Epinephele janira* $\varphi$ show longitudinal striation at 40 $\times$. Between these striae a fine cross striation is seen at 150 $\times$. At 800 to 1000 $\times$ the longitudinal striae are doubly contoured and
contain round granules. (The material for this paragraph was derived from Romeis. For a fuller account consult Langeron.)

The objectives to be selected for a microscope naturally vary widely with the purpose to which each is to be put. For general pathology the following seem the most desirable. An achromat of about 3-6 \( \times \) initial magnification is useful for general views of sections. Achromats or apochromats of 10 and 20 \( \times \) (16 and 8 mm.) are needed for more detailed study. A 31 \( \times \) (5.5 mm.) achromat has proved quite useful in practice. It is similar in performance to the English ½ inch objective. An immersion objective of 60 or 90 \( \times \) initial magnification (3 or 2 mm.) is required for very high magnification. These last are available in three grades: achromatic, fluorite, and apochromatic, in ascending grades of performance and cost. The second will serve almost all purposes; the last are somewhat better for photography and maximum resolution. The 4 mm. (45 \( \times \)) achromat is a useful lens for moderately high magnification when it is desired to avoid the use of immersion oil. However, the apochromat of the same designation is in practice rather unsatisfactory because of the necessity for adjustment of the correction collar for variations in thickness of cover slips and of film of the mounting medium. An oil immersion objective of 4 mm. (40-45 \( \times \)) has been found very useful for differential cell counts of leukocytes in thin blood films, because of the larger field afforded. This magnification is still adequate for identification of ordinary blood leukocytes, but for marrow films a 2 or 3 mm. (90 \( \times \) or 60 \( \times \)) objective is required.

Among eyepieces the 7.5 \( \times \), 10 \( \times \), and 12.5 \( \times \) seem the most useful. In selecting oculars it should be remembered that objectives do not give effective magnifications of over 1000 times their numerical aperture. Hence a 60 \( \times \) objective with NA 1.40 can be used with 15 \( \times \) oculars, giving about 900 diameters final magnification; but a 90 \( \times \), NA 1.30 objective will accept only a 12.5 \( \times \) eyepiece, giving 1125 diameters; or perhaps better, a 10 \( \times \) eyepiece, yielding 900 diameters. Attempts to obtain higher magnifications by use of higher oculars result in blurring of detail.

For apochromatic objectives, compensating eyepieces should be used. For achromats, the Huyghenian type is satisfactory. For fluorite objectives, eyepieces should be either compensating or of an intermediate grade designated as hyperplane or planoscopic. These last can also be used with achromatic objectives and even with apochromats, though they are not recommended for the latter.

Generally two eyepieces separated by 5 \( \times \) in magnification are adequate. For the binocular instruments, only matched pairs should be used, at a constant interpupillary distance which one may determine by trial for himself. Both should be brought to focus on some individual detail in a microscopic field by means of the focusing collar on one of the ocular tubes.

In regard to the question of parallel or converging ocular tubes, both
have their defenders and either seems to be satisfactory to the individual observer when he has become accustomed to it. Changing from one to the other is difficult. Note that the parallel design has been adopted by three of the four manufacturers whose microscopes have been commonly used in the United States.

Condensers are commonly used in the substage of microscopes to bring to bear on the object a sufficient amount of light at an adequate angular aperture to illuminate the field adequately. For work with ordinary transmitted light, the usual Abbé condenser serves for routine work with achromatic objectives. For apochromatic and fluorite objectives, an aplanatic or achromatic condenser with a wider aperture is necessary; and its numerical aperture should approximate the highest numerical aperture of the objectives likely to be used.

In order to utilize fully the wide-aperture objectives, it is necessary to fill the aperture with light. If the beam of light entering the objective from the condenser is of too narrow an angle to fill the aperture of the objective, the full resolving power of the objective is not attained. The essential condition for attainment of full resolving power is the even filling of the whole objective aperture with light. For this purpose either critical illumination or Köhler illumination is used.

For the attainment of critical illumination it is required in theory that all of the light waves reaching the object-point and forming the image at any one instant should also emanate at the same instant from a single point in the light source. This condition is fulfilled when the light source is focused on the object, as is the case when a beam of parallel rays from a very distant source or from a collimating lens on a lamp strikes the front or lower surface of the microscope condenser. For this type of illumination, the light source must be uniform over its surface to obtain even lighting. If the primary source does not cover the field, an enlarged real image can be used.

In the Köhler illumination the specimen is evenly lighted by forming an image of the light source in the lower focal plane of the substage condenser, and hence also in the back focal plane of the objective; and by forming the image of the lamp condenser in the plane of the object. In practice the image of the light source is focused on the substage iris diaphragm by the use of one or two convex lenses (or a concave mirror) in the light path between the light source and the microscope condenser. This light-source image should be just large enough to fill the aperture of the condenser. Since the microscope condenser focuses the image of the lamp condenser in the plane of the object, a diaphragm set next to the lamp condenser should be regulated to exclude all light not being used.

With the Köhler illumination, the size of the illuminated field and its aperture of illumination are easily controlled. At high magnifications, however, only a small aperture can be used at the lamp source. (From Photomicrography, 14th ed., Rochester, N. Y., Eastman Kodak Co.)
Schwind (*Blood* 5:597, 1950) gives more detailed directions for the use of Köhler illumination with immersion systems, which I summarize here:

1. Use the flat side of the substage mirror and check the centering of the substage condenser (*p. infra*).
2. Nearly close the substage diaphragm, and with the focusing device on the lamp condenser, focus the image of the lamp filament on the substage diaphragm, with the aid of a small mirror.
3. Place the slide on the stage, making immersion contact between the under surface of the slide and the top element of the condenser.
4. Focus on the object with a 16 mm. (10 ×) objective. Nearly close the field diaphragm of the lamp. Then focus the substage condenser so that the object and the image of the edge of the lamp-field diaphragm are simultaneously in focus.
5. Place a drop of oil on the cover glass, change to an immersion objective, and focus on the object. Refocus the condenser to bring the outline of the lamp-field diaphragm into focus.
6. Open the lamp-field diaphragm until its edge just disappears from the field.
7. Remove the ocular from the microscope and adjust the substage diaphragm so that its edge is just visible in the rear lens of the objective, and the rear lens is completely filled with light. If the condenser is not immersed, a dark ring is seen at the periphery. Replace the ocular.

To center the condenser, close the substage diaphragm and focus with the coarse adjustment and the substage focusing adjustment until the circle of the nearly closed diaphragm is in sharp focus. Then adjust the size of the opening of the substage diaphragm to a circle of approximately the same apparent size as the field of the microscope. Then, using the centering screws of the condenser, bring the circle of the field and that of the substage diaphragm into approximate coincidence.

One should make a practice of refocusing the condenser to give the most satisfactory illumination on each change of objectives. Generally, the highest powers require the highest focal position of the condenser to give adequately bright illumination, while low powers require a low condenser position in order to fill the field and illuminate it evenly. For very low powers, such as the 32 mm. objective, a lower-powered condenser is desirable, or one from which an upper element may be readily removed so that a larger field is evenly lighted.

Special condensers are required for dark-field and for fluorescence microscopy.

Generally the microscope can be used without eyeglasses except when the wearer's optical defect includes a considerable degree of astigmatism or is extreme in grade. So-called high point oculars are essential for persons who
find it necessary to wear their spectacles while working at the microscope. These oculars are now readily available. Or individual caps carrying small lenses of the worker’s eyeglass prescription may be procured from the microscope manufacturers and placed over the microscope eyepieces.

Objectives, eyepieces, and condensers should be cleaned by breathing on the glass and wiping with the lens paper made for that purpose. Immersion oil should be cleaned off daily at least, or when the use of that objective is finished for the time being. To do this it is well to dampen a spot on a piece of lens paper with a drop of xylene and wipe first with this damp spot, then with the remaining dry lens paper in a single movement.

For immersion objectives it is preferable to use one of the nondrying oils now made for the purpose. These are available in high and low viscosities and may be blended according to the particular need of the user. Low viscosities are better when rapid motion of the slide is to be used with short working distances, and when fresh wet preparations are being studied under a cover glass. The most practical oil for routine use may be an equal-volume mixture of the two grades. On one occasion it was found necessary to make immersion oil of a very low viscosity by mixing approximately four volumes of light mineral oil with one volume of alpha-bromonaphthalene. If it is necessary to make such mixtures, they should be checked for index of refraction with a refractometer if possible. The index of refraction should be between nA 1.515 and 1.520.

If the refractometer is not available, the proper index of refraction may be approximated by immersing a white glass slide (or a glass rod of the proper refractive index) in the mineral oil and then gradually adding alpha-bromonaphthalene until the glass can no longer be seen through the oil.

If one persists in the use of thickened cedar oil for immersion, the utmost care should be taken to have it cleaned off the immersion lenses at least daily. Dry balsam or cedar oil is probably best removed by carefully chipping off the outer portion with a knife, avoiding contact with either metal or glass, and then removing the remainder with lens paper or a soft cloth moistened with benzene or xylene. Alcohol should be avoided, since it softens the cement in which the front lenses of many older immersion objectives were mounted.

Immersion oil is conveniently removed from cover glasses covering fresh resinous or glycerol-gelatin or similar mounts by first inverting the slide on a blotter and pressing down lightly. This absorbs the bulk of the oil. The small residue is readily removed by gently wiping on a blotter wet with xylene, or by dragging a piece of lens paper moistened with xylene across the soiled area, steadying the cover glass with a finger on one corner if necessary.

Blood films may be cleaned of cedar oil by dipping repeatedly in a jar of xylene, or by dropping xylene slowly on the slanted slide just above the oil drop until it runs off the edge of the film. The nondrying mineral oils may be removed in the same manner; but it is not necessary to remove them from
blood films, since they apparently do not cause fading of Romanovsky stains, as did cedar oil. In fact, it appears that this type of oil may even act to better preserve Giemsa and Wright stains.

To use the immersion objective, one finds the desired object with a lower-power dry lens and brings it to the center of the field. The finding lens should be centered on the same point as the immersion lens. If it is not, one brings the object to such a point in the lower-power field as experience indicates will bring it within the smaller field of the immersion objective. The objective is racked up. A drop of oil is then placed on the illuminated area of the slide; the condenser is racked up to near top focus; the immersion objective is swung into position and racked down until it touches the oil drop, and then raised slightly but not enough to break immersion contact. Observing now through the eyepieces, one cautiously focuses down, using the coarse adjustment and preferably both hands (if controls are available on both sides) until the object begins to come into view.

During this focusing, one sometimes notes tiny air bubbles moving in the field. These indicate that the lens is still above focus. The coarse adjustment is recommended because it is possible to tell by feel if contact is made with the cover glass; with the fine adjustment, this contact is not perceived. Violent eddy currents in the optical field indicate that the cover glass is being pressed down by the objective. One should then inspect the preparation for undue thickness of the film of the mounting medium or for the possible presence of two cover glasses stuck together. Failure to attain immersion focus may be due to placing a slide on the microscope stage with the cover-glass surface down. Many prefer to use the fine adjustment for this focusing-down step. It is safer, though slower, since most fine adjustments exert only the pressure of a small spring when contact is made with a cover glass.

When the utmost resolution is required with lenses of a numerical aperture above 1.0, and for dark-field work, a drop of oil is also placed between condenser and slide. Usually this procedure is unnecessary and (if much movement of the slide is required) both troublesome and messy.

If after immersion contact has been made and focus attained a grayish haze appears moving in from one side to obscure the image, a tiny air bubble in the immersion fluid is probably responsible. The trouble is remedied by swinging the objective out laterally to break the immersion contact, and then back in again without changing the focus.

Oblique Illumination

Oblique illumination is used to make more prominent the lineal details transverse to the plane of oblique lighting. It is obtained by excluding light from all but one side of the under surface of the condenser. A device for this purpose is included in research model microscopes, and a similar device may be improvised for student microscopes by sliding a piece of cardboard gradually across the under surface of the condenser from the desired side. Utiliza-
tion of this type of lighting materially increases the resolving power of the objective in the plane of the oblique illumination.

**Dark-Field Illumination**

Dark-field illumination is a device of essentially similar nature, but provides oblique illumination from all sides while excluding directly transmitted light. Particulate matter in a fluid medium is thus caused to glow against a dark background, and particles materially smaller than can be discerned by direct transmitted light are thus rendered visible. Dark-field illumination can be simply achieved for low magnifications by inserting beneath the condenser a disk or stop which shuts off light from the center of the condenser while admitting it to most of the periphery. For immersion work a special dark-field condenser is needed. An immersion objective with a numerical aperture somewhat below 1.0 is required. The usual immersion objective of NA 1.25 to 1.40 may be used if a funnel stop designed for the purpose is inserted into the upper side of the objective before screwing it into the nosepiece. More convenient are immersion objectives with normal numerical apertures which are equipped with an iris diaphragm for reducing the aperture. For routine use, the objectives specially built for dark-field work are desirable. Also very convenient are dark-field illumination units which may be attached directly to the dark-field condenser. This eliminates the troublesome centering of illumination.

Both condenser and objective must be immersed. Otherwise the desired extremely oblique rays are lost by reflection from the undersurfaces of the slide and the objective.

I have recently observed a sample of one of the modified mineral-oil type of immersion oils which although perfectly satisfactory for bright-field work gave unsatisfactory dark-field illumination when used for immersion of the condenser. Focusing on the oil film revealed large numbers of minute bright particles. These were not removed by filtration. A mixture of four parts heavy mineral oil and one part alpha-bromonaphthalene proved free of these particles and was quite satisfactory.

**Polariscopy**

The use of polarized light in histology is particularly valuable in the detection and identification of certain crystalline substances and of certain lipoids, and in the study of such tissues as cross-striated muscle and myelinated nervous tissue. Polarized light may be obtained by disks of polaroid material, by interposition of series of obliquely placed thin glass plates such as cover glasses, or by the use of a Nicol prism. The polarizing device may be set at any convenient place between the light source and the object under study. In research microscopes a place is usually provided for a polarizer just beneath the condenser. For observation, a second polarizing device is re-
quired, and this is usually placed over the microscope ocular. This is called the analyzer.

The first Nicol prism or polarizer permits passage of light vibrating in a single plane. When the second prism, or analyzer, is in the optically identical position, the light which passed through the polarizer passes also through the analyzer, and the field of the microscope is bright. Now if one of the prisms is rotated 90° on its long axis relative to the other, light which passed through the first prism is unable to pass through the second, and the field is dark. In this dark field such substances as rotate the plane of polarized light are seen to glow. Such substances are referred to as anisotropic or doubly refractile. When the slide is rotated about the axis of the light beam, the bright material fades and brightens four times during a complete rotation. By inserting the mica disks supplied with polarizing outfits, color changes are substituted for the lightening and darkening. For this purpose a rotating circular stage carefully centered is almost essential, though it is possible to achieve the same results by simultaneously rotating both polarizer and analyzer in opposite directions with thumb and forefinger. This is fairly easily done with a little practice, so that the overall darkness of the field is maintained.

In using polarizing equipment in an inclined monocular tube microscope, the polarizer must be adjusted so that its polarizing effect is added to (and not neutralized by) the polarizing effect of reflection through a single prism. The optimum point is that at which the reddish cast of the darkened field changes abruptly to greenish. At that point the field is at maximum darkness.

To center a rotating mechanical stage, place on the stage the centering slide provided for that purpose, a stage micrometer, or any other section. With the eye select some conspicuous detail near the center of the field and commence rotating the stage. If the selected object describes an arc, manipulate the stage-centering screws so as to bring the apparent center of rotation to near the middle of the field. Then select some object on the edge of the field and rotate the stage. The selected object should follow around the edge of the field. Make the necessary slight adjustment with the stage-centering screws until it does.

When using polarized light it is necessary to employ appropriate preliminary technical procedures for the preservation of the material that one desires to study. Fats and lipoids must be studied in material that has not at any time been subjected to the action of fat solvents. The usual fat stain preparations from frozen sections are usable, though it may sometimes be desirable to have an unstained frozen section as well. For myelin, frozen sections are also necessary, while for striated muscle and crystals such as silica, either paraffin or frozen sections will serve. It is to be noted that a common doubly refractile material in paraffin sections is the so-called formaldehyde pigment, acid formaldehyde hematin, which is formed from hemoglobin by acid formaldehyde during fixation (see pp. 239–240).

After treatment with acetic Zenker and certain other acid aqueous fixa-
tives, red corpuscles may become doubly refractile, lightening and darkening like crystals on rotation of the stage.

Skeletal muscle, when correctly oriented to the plane of polarized light, gives sharply contrasting light and dark bands. At a 45° rotation these may be quite inconspicuous; hence rotation is necessary. Either stained or unstained, frozen or paraffin sections may be utilized. Cardiac muscle often gives only a barely discernible diffuse glow without distinct bright and dark bands, and no appreciable change is noted on rotation. Sometimes, however, distinct dark and light bands, brightening and darkening on rotation, are discernible; and on occasion it appears that insufficient illumination is the cause of the failure to show bright and dark bands. Use of azure-eosin stained sections of skeletal or cardiac muscle gives bright bands which are nearly white or bluish white and deep pink in alternating quadrants of rotation. Lint or cellulose fibers accidentally included in the mounting medium often glow brilliantly, but above the section plane.

Fluorescence Microscopy

This procedure is employed for the demonstration of substances which of themselves possess the property of emitting light of longer wave length (in the visible range) when excited by light of a shorter wave length. Also, objects stained with certain fluorescent dyes may be demonstrated by this means. The latter demonstration in its application to the auramine staining of tubercle bacilli is the commonest use of the procedure (pp. 382-383).

Usually ultraviolet light of 350 to 400 mμ wave length is employed as the exciting light. For this purpose we have used a G. E. Mazda 100 watt A4H mercury-vapor lamp with the G. E. autotransformer for 4H lamps. This gives an initial voltage of 245 to establish the arc and then drops automatically to a lower voltage. This lamp should be shielded with an adequately ventilated lamp housing and a wooden screen so placed that the light of the lamp falls only on the substage mirror of the microscope. Stray light can be very disconcerting, both in photographic work and in occasioning cutaneous or corneal burns. Lempert (Lancet 247:818, 1944) used the Merrra lamp of the British supplier, Thomson Houston, Ltd., Crown House, Aldwych, London. The glass bulb of this is made of a dark glass which cuts off most of the visible light—only dark red is seen—and 95% of the light is of the 365 mμ band of the mercury arc. With the G. E. lamp that we have used, it is necessary to interpose filters between the lamp and the condenser to screen out most of the visible light. Most of these filters also pass a certain amount of far red and infrared light. Their transmission bands in the ultraviolet vary in width, position, and total transmittancy. The width and position of the band is more important when a carbon arc lamp is used as a light source than with the mercury-vapor lamp. The latter emits a bright line at 365 mμ, with no other bright lines in the nearby portion of the spectrum.

Suitable filters are Corning's red purple corex A #9863, maximum 81%
transmittance at about 320 to 350 μm, but transmitting over 10% between
264 and 402; red purple ultra λ5970, 86% at about 360 to 370 μm, over
10% from 318 to 407; HR red purple ultra λ5874, 66% at about 362 to
368 μm, over 10% from 322 to 398; red ultra λ5840, 56% at about 356 to
363, over 10% from 320 to 386; and violet ultra λ5860, 28% at about 360,
over 10% from 340 to 379. The last is preferred for selectivity, but is of
rather low intensity. The HR red purple ultra λ5874 is probably the best
among the higher intensity group, or perhaps the red ultra λ5840.

Most of these filters transmit a considerable amount of light at the far red
end of the visible spectrum. Insertion of a thin glass water cell containing
5–10% acidulated copper sulfate solution is recommended to cut off this red
light.

These mercury-vapor lamps, both the British and the American, require
perhaps a minute to warm up after they are turned on, and when the current
is turned off, one must let them cool perhaps ten minutes before they can
be turned on again. The lamp is brought as close to the substage mirror as
possible—Lempert specified 4½ inches (114 mm.)—and no converging lens
system is used. The glass lenses of such lens systems would take out too much
light. However, if a converging light is desired to obtain greater intensity or
more critical illumination, a Florence flask or a double watchglass lens filled
with distilled water could be used, or a concave mirror placed behind the
lamp.

A condenser is necessary for adequate illumination; but for light in the
350–380 μm spectral region it need not be of quartz, though greater intensity
may thus be obtained. Similarly, quartz slides transmit more ultraviolet light
at this wave length than do glass slides. Popper tells me that he has used
quartz slides and a glass condenser; while Endicott conversely used a quartz
condenser and glass slides. The latter procedure allows greater transmission
of ultraviolet light because of the shorter light path through glass. If one has
a quartz condenser available, it is much more convenient to be able to use
ordinary glass slides. Lempert used both glass slides and glass condenser.
Metcalf and Patton (Stain Technol. 19:11, 1941) found glass slides and
condensers satisfactory. Note also that the Abbé type is satisfactory: the
aplanatic is better because of the better spherical correction and greater
concentration of light; the achromatic type occasions too much light loss. A
removable dark-field disk about 166 mm. in diameter should be inserted in
the substage below the condenser. This gives a luminous object in a perfectly
dark field. The cardioid condenser was not so satisfactory. Of the filters
mentioned, only the violet ultra λ5860 gave a black background without the
dark-field disk.

The ordinary glass objectives and eyepieces are used, and a yellow filter is
employed over the eyepiece to screen out ultraviolet light and avoid ocular
damage. Such a filter should absorb as fully as possible the 340–420 μm
region of the spectrum. For photography it is necessary to absorb also the
red end of the spectrum, which most of the ultraviolet-passing filters also transmit in addition to the 365–366 μm mercury line. Or one may use a film which is insensitive to red light.

As a yellow filter Lempert first used tartrazine-stained gelatin filters, and later obtained “suitable” glass filters which did not fluoresce in ultraviolet light. Corning’s Noviol O §3060 is the nearest to colorless of the ultraviolet cut-off filters that we have used, and is the best for judging the color of fluorescences. However, it transmits some far-violet light below 400 μm and can give headaches on continued use. The same company’s Noviol A §3389 is a quite pale yellow which transmits very little light below 425 μm; and the next, Noviol B §368, gives little below 450, but is a deeper yellow.

All of these filters give some diffuse fluorescence which occasions no difficulty visually but is quite disturbing for photographic purposes, producing a diffuse fogging of films.

Paraffin, balsam, and cedar oil fluoresce of themselves; the first must be removed from sections, and the other two avoided as mounting media. According to Metcalf and Patton (loc. cit.) a xylene or toluene solution of isobutyl methacrylate is a satisfactory mounting medium, at least for temporary mounts. Preparations can be mounted in liquid petrolatum or in glycerol. Popper found an objectionable fluorescence in glycerol in his vitamin A studies. The low-fluorescence modified mineral oils used as immersion fluids possess a higher index of refraction than unmodified heavy mineral oil (nA 1.515 vs. 1.483) and like it do not fade the sensitive Romanovsky stains. These oils and glycerol can also be used as immersion fluids for smears, though Lempert preferred to examine auramine stained smears with dry lenses.

In the fixation of tissue which is to be studied by fluorescence microscopy, formalin, alcoholic and acetic formals, and an acetic variant of Regaud’s fluid have been used; but Metcalf and Patton warn against fluids containing heavy metals (except zinc), chlorides, bromides, iodides, or nitro compounds. Picric acid is the only one of the last group commonly used in fixing fluids.

The same authors list a number of dyes with their Colour Index numbers, fluorescence colors, and general properties or uses. The Colour Index lists many of these dyes as giving fluorescence colors also by daylight, and as a matter of interest I have added these daylight fluorescence colors. The general dyes, some of which are also used for bacteria and protozoa, include acridine orange NO (C. I. No. 788), a basic dye with orange-red fluorescence color (the daylight fluorescence is green); the basic yellow dye acridine yellow (C. I. No. 785), which possesses a green fluorescence both by daylight and in ultraviolet light; the basic yellow dye acriflavine (C. I. No. 790) which gives a yellow fluorescence in ultraviolet, and a green fluorescence by daylight; the basic orange-yellow dye coriphosphine O (C. I. No. 787), a yellow fluorescence in ultraviolet, and a yellowish green fluorescence by daylight; the pale-yellow acid dye primuline (C. I. No. 812) which fluoresces
blue to blue-violet both in ultraviolet and in daylight; the acid golden yellow dye thioflavine S (C. I. No. 816) which gives a blue-green to green fluorescence in ultraviolet and in daylight; and the substances rivanol and berberine sulfate, giving respectively green-yellow and yellow fluorescence colors in ultraviolet. The last two are listed for staining of protozoan parasites.

As counterstains—presumably plasma stains—Metcalf and Patton list eosin Y (C. I. No. 768), which is pink with yellow fluorescence; fluorescein (C. I. No. 766), yellow with green fluorescence; acid fuchsin (C. I. No. 692), red without notable fluorescence by daylight, and a red color in ultraviolet; and Congo red (C. I. No. 370), brownish red without fluorescence by daylight, dull red in ultraviolet. These are all acid dyes.

For staining of fats Metcalf and Patton list three basic dyes: phosphine (C. I. No. 793), yellow with a green fluorescence by daylight, but yellow in ultraviolet; rhodamine B (C. I. No. 749), strongly fluorescent bluish red by daylight, orange-red in ultraviolet; and magdala red echt (C. I. No. 857), red with orange fluorescence both in ultraviolet and in daylight, only slightly soluble in water, but more so in alcohol.

They list for the same purpose also two acid dyes, titan yellow (C. I. No. 813), a yellow dye without fluorescence by daylight, but giving a blue color in ultraviolet; and thiazine red R (C. I. No. 225), a crimson dye without fluorescence in daylight, violet in ultraviolet. No oil soluble dyes are included among their fat stains.

The basic dye auramine (C. I. No. 655) is listed only for the staining of tubercle bacilli, to which it imparts a brilliant yellow fluorescence, though its yellow solutions do not fluoresce in daylight.

A number of substances possess natural fluorescence, including carotenes and vitamin A, chlorophyll, porphyrins, ceroid, riboflavin, atabrine, and a number of alkaloids. These possess each its own fluorescence colors and spectra. The latter may be observed with Amici prisms or with the Jelley microspectroscope.

According to Popper (Arch. Path. 31:766, 1941) vitamin A yields a green fluorescence which fades fairly promptly; small quantities perhaps in 10 seconds; normal amounts in the liver in about 45 seconds. Glycerol gave a disturbing fluorescence of its own; so sections were studied in water (oil dissolves vitamin A). Malaria pigment, hemosiderin, and bile pigment did not fluoresce. Hepatic lipofuscin gave a brown fluorescence which was stable under continued ultraviolet irradiation. After alcohol extraction the lipofuscin fluorescence changed to red, while the extract gave the green labile vitamin A fluorescence. Similar brown-red fluorescence was given by adrenal lipofuscin. Brown fluorescence is given by a brown acetone soluble pigment in the testicular Leydig cells; and there was an alcohol insoluble lipofuscin with brown-red fluorescence in the testicular germinal epithelium; as well as brown-fluorescing lipid droplets, and, in involuting testes, granules of ultraviolet-stable bright-yellow fluorescent material. Lutein cells yield a faint
brown fluorescence after the green vitamin-A fluorescence has faded. Heart-muscle lipofuscin gives red fluorescence. Yellow-fluorescent granules are seen in the epithelium of sweat glands and of the prostate. Amyloid gives a dim blue fluorescence. Ceroid, according to Endicott and to Popper, yields golden brown fluorescence in paraffin sections, in frozen sections greenish yellow slowly changing to yellowish white. This is given by both paraffin sections and frozen sections in water, dry, or in paraffin; but not in xylene or xylene Clarite (Am. J. Path. 20:149, 1944; Arch. Path. 37:161, 1944). Riboflavin (vitamin B₂) gives a green fluorescence like that of vitamin A, but is quickly reduced and rendered nonfluorescent by saturated aqueous sodium hydro­sulfite (Na₂S₂O₄·2 H₂O) solutions. Thiochrome, the oxidation product of thiamin (vitamin B₁), has a bluish fluorescence. Chlorophyll, used sometimes as a fat stain, gives a fiery red, quickly fading fluorescence. Atabrine gives an intense green fluorescence, prontosil a red, penicillin a green, sodium salicyl­ate a blue.

Heating of preparations to 170–200° C. for 3–5 minutes alters the natural blue and green fluoroscences of muscle and of certain drugs to give contrast­ing colors (Helander, Nature 155:109, 1945). Thus, heating to 170° C. for five minutes, gives yellow sulfathiazole and blue muscle fluorescence. Heating of sulfanilamide, sulfapyridine, papaverine, or inulin to 200° C. for three minutes gives yellow drug and blue muscle fluorescence.

Absorption Spectroscopy of Tissues

Two rather dissimilar technics have been applied. In the one, undispersed light is passed through the condenser, object, and objective-ocular magnifying system, and then through a dispersing prism or grating system so located as to receive the light from only a small portion of the microscope field. The resulting band spectra are photographed and compared with similar spectra taken from unoccupied areas in the object plane. Of necessity this method lacks precise cytologic localization, but gives a general view of the total spectrum of the tissue component studied.

In the second technic, the light source is a monochromatic beam proceeding from a monochromator through the usual quartz or glass optics of the microscope. The results may be registered qualitatively by photomicrographs taken at selected wave lengths; or quantitatively by means of electropho­tometric cells placed in the projection plane to receive light only from the area under study. Since the actual size of the sensitive surface of the phot­electric cell is constant in any given apparatus, variation in object area is achieved through changes in the lens system and the bellows extension. Again it is necessary to compare the light intensity received through the structure under study with that received (preferably simultaneous) through unoc­cupied areas of the object field. And again only the average absorption re­ceived over the total sensitive surface of the photometric cell can be recorded.

For spectroscopic studies with ultraviolet light between 200 and about 350
m\(_\mu\), quartz condensers, objectives, and oculars are required. Objectives may be corrected for a wave length between 500 and 550 m\(_\mu\) (green) as well as for the ultraviolet range desired, so that the microscope may be focused visually, and the monochromator then shifted to the desired wave length. Or fluorescent screens may be employed for focusing, and materials selected which emit fluorescence in the visible spectrum when excited by the required ultraviolet bands.

The new reflecting microscope used by Mellors (Science 111:627, 1950) would appear to lend itself not only to the studies in the ultraviolet range for which he used it, but also for work in the infrared and visible ranges. Since it is without the chromatic aberrations inherent in refracting systems, objects are in focus at the same objective plane throughout the utilizable spectrum.

Mellors used Kodak 103-0 UV spectroscopic plates for high sensitivity and speed in photography of living tissue cultures, and Kodak 1372 (35 mm. film) for high resolution with diminished speed and sensitivity. Corning Vycor No. 791 ultraviolet-transmitting cover glasses were used. The tissue-culture fragment was enclosed in an aqueous film between two such cover glasses. Mellors washed the nutrient medium off the hanging-drop culture with a suitable Locke type of saline solution before enclosing it with the second cover glass for photography. Focusing was done with the green mercury line and exposure to the 253.7, 265.2, 275.3, 280.4, 312.6, and 334.1 lines kept to the minimum required for photographic exposure.

Phase Microscopy

The refractive index, "nA," is a constant, characteristic of each transparent substance, and represents the ratio of the velocity of sodium light in a vacuum to the velocity of light in the substance in question. It is the ratio of the sine of the angle of incidence to the sine of the angle of refraction in the medium.

It follows that when two transparent substances of the same or nearly the same refractive index abut on each other there is no refraction at the interface, or very little; and the two substances are not visually distinguishable in the unstained state.

In the case of the relation of the refractive indices of mounting or examination media to the index of the included tissues, it is possible to vary greatly the refractive index of the including medium by suitable selection of its constituents. This problem is considered in more detail in Chapter 7, pp. 106–107, 108–111.

In the case of interfaces between the various transparent substances within tissues, small differences in refractive index may be exaggerated by advancing or retarding by \(\frac{1}{4}\) wave length, the phase of the diffracted rays relative to that of the direct rays passing through an object and forming an image at the rear focal plane of the objective. This phase change is accomplished by depositing under high-vacuum thermal evaporation on a phase plate or directly
on an objective lens, usually in an annular pattern, a film of glass of sufficient thickness to alter the phase of green light by \( \frac{1}{4} \) wave length.

In order to compensate for the greater intensity of the undiffracted light, a metal absorbing film is deposited in the area of the phase plate annulus to equalize the intensity of the diffracted and undiffracted light.

An annular aperture diaphragm is placed in the front or lower focal plane of the substage condenser, and is illuminated by the Köhler method (p. 5) to form a secondary source of light. In accord with the principle of Köhler illumination, the lighted circle of the annular aperture forms in the rear focal plane of the objective an image which should coincide accurately with the phase-altering annulus. Thus the oblique light which passes directly through the object without diffraction passes also through the altering annulus of the phase plate while the diffracted light which has been deviated from its direct path by encountering interfaces of differing refractive indices passes through the remaining area of the objective phase plate without the advancement or retardation of \( \frac{1}{4} \) wave length produced by the phase plate annulus. When these diffracted rays are brought to focus along with the undiffracted light passing through the same object area, they differ in phase by \( \frac{1}{4} \) wave length and consequently interfere. The positive phase plate accelerates the undiffracted light, and the interference produces dark areas in a bright background; the negative phase plate retards the undiffracted light, and the interference pattern gives brighter spots on a darker background.

Specially designed objectives covering the usual range of magnifications are available for positive and negative phase contrast, and annular aperture substage diaphragms are provided for each objective.

A telescope ocular is provided for insertion in place of the usual observation oculars for use during accurate centering of the image of the substage annulus on the objective or phase plate annulus.

The method of phase microscopy appears to offer its greatest advantages in the observation of surviving cells in warm-stage preparations, tissue cultures, and the like.

**Micrometry**

In order to measure the absolute size of microscopic objects, one uses either projection or an eyepiece micrometer. The projection method is convenient when many measurements are to be made in the same field. This is accomplished by first projecting the image of the stage-micrometer ruling on the ground-glass screen of a bellows camera, and then adjusting the magnification by varying the bellows length so that a simple magnification factor is reached. The rulings of the usual stage micrometer are at 10 micron intervals and cover a total space of 1 mm. The image on the ground glass is readily measured with a millimeter rule. If the total 1 mm. ruling covers a space of 10 cm. on the screen, it is evident that one has a magnification of 100 diameters.
After thus setting up the projection system at an appropriate magnification, the desired field is then substituted for the stage micrometer and photographed or measured directly on the ground-glass screen with a millimeter rule. Division of the measurements in millimeters by the magnification factor yields the true measurements.

More often the virtual image of a tiny scale engraved on a clear glass disk is used as a standard of comparison. This disk is inserted into the desired eyepiece by unscrewing its top lens, inserting the disk on the shelf within the eyepiece so that the engraved figures are erect and not reversed, and replacing the top lens of the eyepiece. The scale on this disk is brought directly over or beside the object to be measured, and the number of divisions on the scale noted.

The value of each single division of the eyepiece micrometer scale, as well as that of the whole 100 or 200 such divisions comprised in the total ruling, in terms of absolute measurement, should be determined in advance by direct comparison of the eyepiece micrometer scale with that of the stage micrometer. It is necessary to record these values for each objective that is used with the microscope, and if the micrometer disk is used with different eyepieces, for each eyepiece as well.

Each worker should possess one of these eyepiece micrometer disks and should have a card beside his microscope carrying the value of the eyepiece micrometer divisions in micra for each lens combination that he uses. One stage micrometer should suffice for a group, as this is used only occasionally. If no stage micrometer is available, the rulings of a good hemocytometer may be used instead. The small squares in the central area are $\frac{1}{400}$ sq. mm., and hence measure $\frac{1}{200}$ mm. or 50 $\mu$ each way. The group of 25 small squares measures 250 $\mu$ each way, as do the 16 subdivisions of the outer 8 squares of 1 sq. mm. each.

**Mechanical Stages**

Although it is quite possible to explore a section adequately by freehand movement of the slide on a plain stage, one can never be sure that every area has been seen by this method, especially when higher magnifications are being used. For systematic search of a preparation, the mechanical stage is invaluable. Generally the types which form an integral part of the microscope stage are superior to the detachable accessory type, since the former are less apt to present lost motion, and do not give the distressing variation in vernier readings which is contingent on removing and replacing a stage of the detachable accessory type.

Periodically the mechanical stage should be thoroughly cleaned with xylene or benzene or other appropriate solvent, and then regreased with petrolatum. This facilitates free movement and reduces wear.

Verniers are usually placed on mechanical stages and serve to permit the reading of tenths of a millimeter. The small vernier scale presents 10 divi-
sions covering a space of 9 mm. When the 0 on the vernier scale is directly opposite one of the markings on the stage scale, read the whole number of millimeters indicated. When the 0 on the vernier scale lies between two markings on the stage scale, read the whole number of millimeters next less than the point opposite the 0 on the vernier, and then count the number of vernier divisions to the point where one of these is directly opposite one of the divisions on the stage scale. The number of vernier divisions thus counted is the number of tenths of a millimeter to be added to the whole number of millimeters just below the zero point on the vernier.
Chapter 2

Equipment

Microtome Knives

These should be of the type recommended by the manufacturer for freezing, paraffin, and celloidin microtomes respectively. Commonly these knives are provided with detachable backs for use during sharpening. The purpose of these backs is to insure the correct bevel in the edge of the knife. Handles are also generally provided which can be screwed into the base of the knife.

The process of sharpening can be divided into three stages: the removal of gross nicks in the edge on a fine carborundum stone or on a glass plate with emery powder; the honing proper on a fine water hone or on a glass plate with diamantine powder; and the stropping on a leather strop mounted on a board or on a glass plate with rouge powder. When these procedures are done by hand, in the first two the edge of the knife is moved forward, and the blade drawn toward the operator in a diagonal stroke. In the stropping step the back of the knife is drawn forward and toward the operator, with the edge following.

When several technicians can be served it is a true economy to purchase a machine-operated knife sharpener, even though the price appears to be high. Sharpening by hand takes an average of more than an hour daily for a histologic technician, and even two or three hundred dollars can soon be saved in technicians' time. Further, the machine sharpening is more uniform, and will grind the full length of the knife evenly rather than produce the familiar concave edge of the hand-sharpened knife.

Devices for holding safety-razor blades are quite valuable for paraffin and frozen sections, since these blades are readily replaced when they become dull, and they cut nearly as good sections on most material as microtome knives. Mallory recommended them particularly for partially calcified material.
Paraffin Oven

An incubator of bacteriological pattern that can be regulated to 55-60° C. is quite satisfactory. Incubators with water jackets maintain a more even temperature. Sufficient space should be available for paraffin to be filtered within the oven. Beakers holding between 30 and 50 cc. are useful containers for the paraffin infiltration of individual specimens, and a two-liter beaker forms a good reservoir for the stock of melted paraffin. A vacuum chamber installed within the oven or a specially built vacuum oven is useful for quick infiltration of tissues, particularly of lungs. Such a vacuum chamber can be improvised from a domestic pressure cooker of appropriate size. The services of a plumber are required to make the necessary connections.

I have made an even simpler model by fitting a porcelain electric light socket to the inside of the lid of the pressure cooker, and setting the cooker itself in a wooden box packed around the cooker with sawdust covered with plaster of Paris. The temperature was regulated by changing the wattages of the electric bulbs until one was found which kept the temperature at 55-60° C.

The small paraffin units on the Technicon tissue changer are also quite satisfactory. These expedite infiltration by a continuous gentle rotation which brings fresh paraffin continually to the surface of the tissue and removes the solvent as it diffuses out.

For field use I have improvised a paraffin oven by using a flat tin for a paraffin container, a larger tall tin with a hole cut in the side at the bottom so that the paraffin container could be inserted for part of its length, and an electric light bulb set in the lid of the tall tin as a heat source. The position of the paraffin container, partly under the light, should be so adjusted that paraffin remains unmelted at its outer end. Tissues for infiltration are placed in the melted paraffin immediately adjacent to the remaining unmelted paraffin.

For work where the preservation of undenatured proteins (such as enzymes) is important, the vacuum type unit is highly recommended.

Mechanical Tissue Changes

Electrically operated devices are now available which will transfer tissues from one fluid to another at prescribed intervals. Tissues may thus be carried through a succession of fixing, washing, dehydrating, and clearing baths, and even through paraffin infiltration if desired. One such device not only transfers tissues from one fluid to another by the clock, but also continually agitates the fluid by rotation of the specimen carrier in it, so that diffusion and fluid interchange are materially accelerated. Such machines can be set for a schedule of 24 hours or of 48 hours or longer by cutting clock disks appropriately. By substitution of a shorter-period clock and a slide carrier, the device can be altered to a routine slide stainer, carrying slides through de-
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paraffinization, hydration, staining, washing, counterstaining, dehydration, and clearing. At present there is a limit of 12 steps, but the usual routine technics can be adapted readily to such a schedule.

To remove paraffin from metal imbedding molds, Technicon tissue carriers, and the like (including the nylon-plastic carriers furnished by the Technicon Company) boil them for 5–10 minutes completely immersed in a tall metal vessel containing about 10–12 Gm. (a level tablespoonful or 16 cc.) of powdered Oakite, Calgonite, or other technical sodium phosphate detergent in about a liter of water. Then cool until the paraffin can be removed as a solid cake; rinse and dry. The greasy film left by xylene cleaning is absent with this method, and the danger of working with an inflammable solvent is eliminated (Peers, Am. J. Clin. Path. 21:794, 1951).

Other Incubators

Besides the paraffin oven, which is kept regulated to a temperature 2–5° C. above the melting point of the paraffin used, it is often necessary to maintain other temperatures over fairly long periods. Small thermostatically controlled incubators that can be regulated at 37° C. and at 45–50° C. are valuable for enzyme digestions, chemical extractions, metallic impregnations, enzyme localization technics, and other methods.

At least one such incubator should be available, and preferably two, if such incubation technics are in frequent use.

Water Bath

Of even greater value than incubators for maintenance of temperature during various staining and incubation procedures is a thermostatically regulated serologic water bath. The contents of Coplin jars quite promptly reach a temperature, within 1–2° C., of the surrounding bath; and temperature maintenance seems to be more steady than in incubators. It is valuable to have a well fitting but easily removable cover to aid in maintaining temperatures above 60° C. and to retard evaporation of the bath.

Extraction with Hot Solvents

A reflux extraction apparatus for treatment of slides with boiling acetone, ether, xylene, and the like is readily improvised by selecting a beaker large enough to contain a metal or glass slide rack, and a Florence flask of such diameter that it will fit the top of the beaker quite closely, but will not quite go down into it. The Florence flask is fitted with a two-hole rubber stopper, and a current of cold water is run through the Florence flask while the contents of the subjacent beaker are kept gently boiling on an electric hot plate. I have boiled acetone in such an apparatus for several hours without having to replenish it.
Cryostat

Coons, Leduc, and Kaplan (J. Exper. Med. 93:173, 1951) describe a cryostat modified from that of Linderström-Lang which facilitates the preparation of frozen sections by the Adamstone-Taylor technic. This cryostat has a capacity of 6 cu. ft. (about 170 liters). It is cooled by coils attached to the walls, which are lighted with a fluorescent bulb. Air circulation within the box is provided by a small fan, and must be interrupted during section handling to avoid blowing the sections away. Gloved armholes provide access to the microtome and other apparatus within the cryostat.

The microtome is equipped with a glass guide which, when swung into position, is maintained parallel to the knife edge at a distance of 50 µ. This prevents section rolling. Thus the hard-frozen sections may be transferred to slides without the difficulty of unrolling. After the section is cut and lies on the knife blade under the glass guide, the latter is then rotated forward and down to permit removal of the section. The device is hinged at the lower edge of the knife. The supports consist of two clamps set laterally over the edge of the knife supporting the frame; and two screws to regulate the angle made by the glass plate and its supporting hinge with the knife blade, so as to keep the plate parallel to the level of the knife edge. The plate rests laterally at the level of the knife edge on two strips of cellophane that give the required approximate 50 µ space interval over the cutting area.

The usual freezing microtome is not especially suitable for this method when done at room temperature. A large celloidin microtome equipped with a CO₂ freezing attachment can be used.

Instruments

Instruments should include surgeon's knives, scissors, thumb forceps, scissors type forceps (both with and without mouse tooth tips), a hacksaw with spare blades for cutting bone specimens, section lifters of flat metal for celloidin sections, dissecting needles for handling frozen sections, thin double-edged brain knives, safety razor blades, and some glass syringes for injecting. A slab of paraffin affords a convenient surface on which to cut tissue blocks for later imbedding. Its surface is readily cleaned and smoothed from time to time by flaming or by melting and recasting.

Staining Dishes and Carriers

For frozen sections, small, flat, covered glass dishes in which a few cubic centimeters of stain can be used for staining are desirable. For celloidin sections stained before affixing to slides, similar dishes can be used. For paraffin and other sections mounted on slides, one needs slotted glass dishes in which slides can be stood on end singly or in pairs back to back or zigzag fashion so that the second slide is in contact with the first one at one side and with the third at the other. Monel metal carriers in which a number of slides can
be racked simultaneously can be used for staining in round or square jars of appropriate size, and are very convenient when considerable numbers of sections are to be stained in the same way. Copper carriers can be used for hematoxylin eosin and iron hematoxylin Van Gieson technics, but interfere with azure eosin staining.

For many procedures slides are stained face up with small amounts of reagent. For this purpose parallel glass rods joined together at one end at about a 5 cm. (2 in.) interval are convenient. These should rest over a trough or large bowl or sink to catch spilled and discarded reagents. For this purpose we have used for years large copper trays with a drain at one corner to empty into a small sink, a bucket, or other convenient receptacle. These trays measure 25 x 50 x 5 cm. (10 x 20 x 2 in.). Fastened in each, near one corner, is a smaller and deeper container—say 15 x 10 cm. and 10 cm. deep (6 x 4 x 4 in.)—in which running water is received. This serves for washing slides in running water. On one edge is cut a shallow V-shaped depression to allow overflow of water. It is convenient to place this V on the side opposite the drain so that the overflowing water will traverse most of the area of the large pan before reaching the outlet.

Very convenient for dispensing small quantities of stain and reagents on individual slides are dropping bottles which possess glass stoppers with slots on each side to match slots cut on the inside of the bottle neck. A 90° turn closes the bottle tightly, and the stopper is equipped with an overhanging point opposite one of the slots, from which the drops fall.

Serologic pipets of 1 cc., 5 cc., and 10 cc. capacities are invaluable for dispensing measured quantities of stains and reagents on individual slides. Graduated cylinders, both stoppered and open, with capacities of 10, 25, 50, and 100 cc. should be available, as well as a few larger open cylinders, with capacities of say 250, 500, and 1000 cc.

A balance of perhaps 1–2 mg. sensitivity and 100 gm. capacity serves well for most stains and reagents in the histologic laboratory. It is well to have access also to quantitative analytic balances and to scales of larger capacity. A pair of 50 cc. burets with buret stand may sometimes be useful.

In heating equipment, Bunsen burners, a one-burner gas plate for heating large amounts of water, an electric hot plate with covered elements for heating inflammable fluids, thermostatically controllable warm plates which may be set at 35–40° C. for spreading paraffin sections, or at 70–80° C. for heating to steaming during staining, are accessories which will prove their value.

However, I have done very well with a long copper plate which can be heated at one end with an alcohol lamp.

Cleaning of Slides and Cover Slips

New slides and cover slips may often be satisfactorily cleaned by simply immersing them in alcohol and carefully polishing with a soft cloth. Usually we have found it preferable to wash them in warm soapy water first and
EQUIPMENT

rinse them in several changes of warm water before putting in alcohol for polishing. In this procedure, if a 1% aqueous solution of acetic acid is substituted for the alcohol, slides take a brilliant polish. Some writers recommend acid alcohol from which to polish slides.

In cleaning used slides on which blood films or unstained paraffin sections remain, slides should first be boiled in an aqueous solution of sodium carbonate or trisodium phosphate or a commercial detergent powder of similar nature. Remnants of sections are then readily wiped off, as is glass-marking ink. The usual soapy water, water, alcohol, or acetic sequence follows.

Labeling

Labeling of slides before staining is a necessity in a pathology laboratory. The traditional diamond pencil can be used, but slides so marked are quite prone to breakage during and after staining. For several years now I have used a black glass-marking ink* which resists all ordinary reagents and even serves in place of paper labels when the slides are filed after examination. Such inks are removed by alkali, especially when fresh.

Labeling of paraffin blocks is readily accomplished by affixing with a hot iron on one side a small paper label inscribed with India ink.

Labeling of specimen bottles can be conveniently accomplished by inserting within the bottle a label first written with India ink on paper and then immersed briefly in smoking hot paraffin. The paper should promptly lose all its contained air and water as bubbles and should have a translucent appearance. Opaque looking paper labels are not adequately waterproofed and tend to go to pieces. Similar labels may often profitably be prepared and affixed with a hot iron to the outside of reagent bottles, especially those containing reagents which tend to destroy ordinary gummed labels.

Specimen Bottles

Specimen bottles, both for storage of specimens and for their collection, fixation, dehydration, and clearing, should be procured in a variety of sizes ranging from perhaps 25 cc. or 1 oz. up to brain jars containing some 4 liters for the fixation of whole human brains. They should be wide mouthed and furnished with covers. In the case of storage bottles the covers should fit closely to prevent evaporation. We have found that 25 cc. bottles are most often required for storage, 50 and 100 cc. bottles for fixation and dehydration procedures. The bottles used commercially for mayonnaise are quite convenient. These have a paraffined paper inside seal and a screw cap which may be removed by a quarter turn. These are available in quart, pint, half pint, and quarter pint sizes, or about 960, 480, 240, and 120 cc.

* Gold Seal black ink for glass and porcelain, of the Clay Adams Co., 44 E. 23rd St., N.Y.C. The Fisher Co. lists a white ink of similar properties. Neither of these is a ceramic ink, and they need not be fired.
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<table>
<thead>
<tr>
<th>Compound</th>
<th>Point</th>
<th>Value</th>
<th>Refractive Index</th>
<th>Value</th>
<th>Value</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene</td>
<td></td>
<td>-95</td>
<td>110.8</td>
<td>1.4955</td>
<td>1.921</td>
<td>0.866</td>
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<tr>
<td>Trichloroethylene</td>
<td></td>
<td>-73</td>
<td>87.2</td>
<td>1.4777</td>
<td>3.232</td>
<td>1.466</td>
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<tr>
<td>Trimethylbenzene</td>
<td>-25/—45</td>
<td>165/176</td>
<td>1.4931*</td>
<td>1.898</td>
<td>0.861*</td>
<td>1.08</td>
</tr>
<tr>
<td>Water</td>
<td>0.0</td>
<td>100.0</td>
<td>1.3330</td>
<td>2.205</td>
<td>0.9982</td>
<td>nominal</td>
</tr>
<tr>
<td>Xylene</td>
<td>13/—47</td>
<td>138/144</td>
<td>1.4966*</td>
<td>1.903</td>
<td>0.863*</td>
<td>1.41</td>
</tr>
</tbody>
</table>

*Melting and boiling points, densities, and refractive indices determined by Greco, Pathology Laboratory, National Institute of Health.*

† Boiling point of tetracosane.

/ Melting and boiling range of compounds in the commercial mixtures. To judge by its melting point, the paraffin seems to be chiefly penta-

cosane.

‡ Tax free.

a Approximate nA of octanes of the same density.
b Refractive index of pentacosane at 80°.
c nA for mixture of pentane and isopentane.
d nA of hexane.
e nA of heptane.

To find cost per liter, multiply the price per pint by 2.113; or the price per gallon by 0.264; or the price per pound by the pounds-per-
liter factor given above; or the price per kilogram by the density in kilograms per liter.
Reagents

Reagents should be purchased from reputable manufacturers under appropriate specifications for the purpose for which they are to be used. There is no point in paying for reagent grades when ordinary technical grades are perfectly satisfactory.

Dyes are unstable organic chemicals and should be bought in such quantities as are likely to be used up within two or three years. It is good practice to stamp the date of receipt on each bottle of dye purchased. Unless one is prepared to test his own dyes for quality he should require certification by the Biological Stain Commission (pp. 74–75).

Inorganic chemicals of stable nature may be bought in larger quantities, especially if considerable amounts are apt to be required suddenly.

Solvents are often interchangeable one with another as far as results are concerned, and cost may be a material item in the selection of a dehydrating agent or a paraffin solvent. Here prices per unit weight are deceiving, since these solvents are used by volume. Carbon tetrachloride, for example, is nearly twice as heavy per unit volume as toluene, and at 17 cents a pound is actually the same price as the latter at 30 cents a pound. For convenience a table of conversion factors for common solvents is appended.
Chapter 3

Fixation

The greatest handicap to the pathologic histologist is improper preservation of material. Several factors enter into this, among which are delay in fixation, postmortem decomposition, drying of tissues, inadequate quantity of fixing fluid, poor penetration of fixing fluid, fixing fluids improper for the material, prolongation of fixation beyond the proper interval, improper storage fluids for prolonged storage, and poor dehydration, imbedding, and sectioning technics.

Tissues should be fixed as promptly as possible after cessation of circulation. Autopsies should be made as soon after death as possible; and when this is not immediately possible, prompt refrigeration is of material advantage. Surgical specimens should preferably be fixed as soon as removed. The practice of keeping tissues unfixed until an operation is concluded often results in a distorting dehydration of surface layers, and the practice of keeping them in physiologic saline at operating-room temperatures permits autolysis to progress often to a confusing extent. Animal autopsies are preferably made on animals killed or dying immediately before dissection. Complete evisceration by a trained attendant and fixation of the entire visceral mass is a procedure preferable to storage on ice, as far as histologic detail is concerned; but when bacteriologic investigation is an essential part of the autopsy, prompt refrigeration should be the rule when immediate autopsy is not possible.

Tissue blocks should be cut of such thickness that the fixing fluid readily penetrates throughout in a reasonably short period of time. This time varies with the fixative, and inversely with the fixation temperature. While low temperatures retard fixation, they also stop autolytic changes; so that the best fixation with many fixatives may be attained by prolonged fixation at temperatures approaching the freezing point of the mixture. With mixed fixatives, it must be recalled that the rates of penetration may vary for the dif-
ferent constituents. For example, mixtures of acetic acid and mercuric chloride, such as sublimate acetic, susa, Zenker, and the like, may show practically pure acetic acid effect in the deeper parts of the block, with dissolution of albuminous granules and the like, while in the outer zone these structures are preserved.

The volume of fixing fluid employed should be 15 or 20 times that of the tissue to be fixed. The length and breadth of blocks to be fixed should be such that they are not bent or folded by the container in which tissue is fixed. It is well to open hollow viscera or fill them with fixing fluid. Lungs of small animals may be conveniently fixed by filling them by intratracheal injection, taking care to allow the fluid to escape freely around the injecting needle so as to avoid overdistension.

Fixation by intravascular perfusion must be preceded by washing out blood with an indifferent fluid such as Ringer's or Locke's solution or 0.85% saline. Here it is important so to regulate the injection pressure that it does not exceed the blood pressure. Otherwise, fixation artefacts are produced. This method has the disadvantage that the blood content of the vessels is lost. Further, the method is not possible when postmortem clotting of the blood in the vessels has occurred.

From the point of view of the histologist, the practice of hardening an entire human brain without perfusion, by immersion in dilute formaldehyde solution or other fixative before dissection, can only be condemned. It seems preferable, when topographic study is contemplated and perfusion cannot be done, to divide the brain stem first by a single transverse section just anterior to the oculomotor roots and the anterior margins of the anterior colliculi, thus separating the cerebrum from the mid- and hindbrain. Then make a series of transverse sections through the brain stem and attached cerebellum at 5–10 mm. intervals, leaving part of the meninges unsevered so as to keep the slices in sequence. Then separate the two cerebral hemispheres by a sagittal section, perhaps better slightly to one side of the median plane, or through the third ventricle. Then on the sagittal surface identify dorsal and ventral points through which sections should pass so that the brain sections will agree in plane with one of the standard cross or frontal section atlases. Make the first section through these points perpendicular to the sagittal surface. Then section the rest of the brain at perhaps 10 mm. intervals, cutting parallel to the first plane. This greatly facilitates identification of various areas for the anatomist or pathologist to whom the human brain is an occasional object of study.

(For these sections a long thin-bladed knife is essential. We find a 2 gallon (8 liter) brain jar suitable for thus fixing a human brain.

In general, solid viscera should be cut in slices perpendicular to the surface, in such wise as to expose their anatomic structure to best advantage. For instance, kidney sections should show cortex, medulla, pelvis, and pyramid. Adrenal sections should show cortex and medulla. Sections of tumors
should show adjoining tissue sufficient to identify the blocks anatomically and to give the relation of the expanding tumor margin to preexistent tissue. Abscess walls should also show adjacent, relatively uninvolved tissue. The margin as well as the center of a pneumonic focus is often instructive. Digits and other skin covered objects should be opened so as to admit fixative to the significant areas; skin is almost waterproof. Small bones should be largely stripped free of muscle if their marrow is of interest.

Friedenwald (The Pathology of the Eye, New York, The Macmillan Company, 1929) condemned the practice of freezing the eye and bisecting it before fixation because of the damage resulting from the formation of ice crystals and because of immediate collapse of the tissues and wrinkling when they are placed in fixative after freezing; yet I have had excellent results from the very rapid freezing obtained by immersion in petroleum ether with solid carbon dioxide, followed by immediate axial or paralenticular anteroposterior section and immersion in fixatives while still frozen. Even with Carnoy’s fluid the rods and cones remain fully expanded and clearly delineated, and the retina generally remains in contact with the chorioid. A sharp razor blade must be used, and care taken not to fracture the frozen tissues.

### Fixing Fluids

**Formaldehyde**

The most widely used fixing agent for pathologic histology is formaldehyde. Not only is it used as the sole or principal active agent in fixing fluids, but it also enters into many fixing mixtures. Formaldehyde is ordinarily available commercially as an approximately saturated solution of the gas in water. Such solutions contain from 37-40% by weight of formaldehyde gas and are commonly called “formalin,” or better, “strong formalin.”

In designating the strength of aqueous formaldehyde solutions it is common practice to denote a dilution of one volume of 40% formaldehyde solution with four or nine volumes of water as 20% or 10% formalin. Others designate the same solutions as 8% or 4% formaldehyde, or perhaps when

<table>
<thead>
<tr>
<th>% by Weight</th>
<th>Specific Gravity</th>
<th>Grams per 100 cc.</th>
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</thead>
<tbody>
<tr>
<td>40 . .</td>
<td>1.124</td>
<td>44.96</td>
</tr>
<tr>
<td>39 . .</td>
<td>1.120</td>
<td>43.67</td>
</tr>
<tr>
<td>38 . .</td>
<td>1.116</td>
<td>42.39</td>
</tr>
<tr>
<td>37 . .</td>
<td>1.111</td>
<td>41.12</td>
</tr>
<tr>
<td>36 . .</td>
<td>1.107</td>
<td>39.86</td>
</tr>
<tr>
<td>35 . .</td>
<td>1.103</td>
<td>38.605</td>
</tr>
</tbody>
</table>
the formaldehyde concentration of the stock solution is lower than 40%, as
7.4% or 3.7% for example. Actually this last practice is erroneous. Concentrated formaldehyde solution of 40% designated strength and specific gravity of 1.124 contains approximately 45 Gm. in 100 cc.; and 37% solutions, with specific gravity of 1.111, contain 41 Gm. Hence 20% and 10% dilutions contain actually 8.2-9% and 4.1-4.5% formaldehyde, respectively.

Solutions of formaldehyde diluted with distilled water are commonly acid, owing to the presence of small amounts of formic acid either as an impurity remaining during manufacture or as a result of oxidation of part of the formaldehyde. (For certain silver-impregnation technics this natural acidity may be desirable, but for azure-eosin methods and for study of possibly iron-containing pigments, it is essential to correct it. A common practice is to shake the diluted formaldehyde solution with calcium carbonate and store it over a layer of this salt. This gives only approximate neutrality; Romeis cites pH levels of 6.3 to 6.5. Others have used magnesium carbonate, attaining pH levels of about 7.5. With either of these methods, formaldehyde solution drawn from the storage reservoir and used for fixation very promptly becomes more acid as the tissue is fixed. Levels of pH 5.7 to 6.0 are not uncommon after calcium carbonate treatment.

This shift in pH is avoided by using a soluble buffer in the dilute formaldehyde solution used for fixation. Addition of 4 Gm. monohydrated acid sodium phosphate (or of the anhydrous acid potassium phosphate) and 6.5 Gm. anhydrous disodium phosphate per liter gives approximately pH 7.0 and a total salt content of the two sodium salts of about 1%, dry weight. Other sodium and potassium phosphates may be substituted, as shown in the accompanying table.

Table 3

<table>
<thead>
<tr>
<th>Acid Component</th>
<th>Basic Component</th>
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</thead>
<tbody>
<tr>
<td>NaH₂PO₄ (anhydr.)</td>
<td>Na₂HPO₄ (anhydr.)</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>Na₂HPO₄·12H₂O</td>
</tr>
<tr>
<td>K₂HPO₄ (anhydr.)</td>
<td>K₂HPO₄ (anhydr.)</td>
</tr>
</tbody>
</table>

The quantities given are grams per liter. The higher, parenthesized quantities are those required to yield the same quantity of metallic sodium per liter as does 0.85% NaCl solution, or corresponding molar quantities of potassium.

Substitution of this fluid for fixation in certain toxicologic studies resulted in a definite increase in frequency of demonstrability of ferric iron in blood pigments and it almost entirely prevents formation of the so-called formalin pigment.

Fixation in formaldehyde is influenced by the concentration of the reagent and by temperature just as are other chemical reactions. A 4.1%-4.5%
formaldehyde solution (10% formalin) fixes adequately in 48 hours at 20-25° C. (68-77° F.), in 24 hours at 35° C. (95° F.), and an 8% to 9% formaldehyde solution (20% formalin) hardens in 3 hours at 55° C. However, autolysis is also hastened by higher temperatures, so that better fixation is attained with longer exposures at lower temperatures; some writers have recommended fixation at 0-5° C. The use of higher formaldehyde concentrations also tends to overharden outer tissue layers and to affect staining adversely, especially with azure eosinates. With alcoholic formaldehyde fixations the above times may be reduced by 50%.

The use of temperatures above 60° C. involves the factors of heat coagulation and of loss of formaldehyde through volatilization. Small pieces of tissue up to 5 mm. thick may be hardened throughout by two minutes' boiling in 0.85% sodium chloride solution alone, and an egg may be boiled hard in 10 minutes.

Substitution of alcohol as the diluent of formaldehyde solution results in faster fixation, greater hardening, loss of fats and lipoids, better preservation of glycogen, poorer preservation of iron bearing pigments, and sometimes partial lysis of red corpuscles.

The use of 0.85% sodium chloride in dilute formaldehyde solutions has been recommended. On routine material this addition makes little discernible difference.

Ramón y Cajal’s ammonium bromide formalin is often prescribed for central nervous tissues, especially when silver impregnations are contemplated. Baker’s calcium formalin is 10% formalin to which is added 1% calcium chloride, and his calcium cadmium formalin contains 1% each of calcium chloride and of cadmium chloride (Quart. J. Microscop. Sci. 85:1, 1944). These fluids were recommended especially for the fixation and storage of material for the study of phospholipids (p. 322). For the purpose of combining a calcium formalin with a buffered neutral formalin, I have added instead of Baker’s chloride, 2% of calcium acetate. This gives approximately pH 7.0, and is about as effective against additions of formic acid as the neutral phosphate formalin given below.

Formaldehyde alone is a rather soft fixative, and often does not harden certain cytoplasmic structures adequately for paraffin imbedding. Brush borders of renal epithelium are often frayed and radial striation partially obscured in paraffin sections, while at least the latter feature is plainly discernible in frozen sections of the same material. Also, cross striations of heart muscle are better defined in frozen than in paraffin sections of material fixed with formaldehyde. Pyramidal cells in the cerebral cortex often are surrounded by clear spaces due to shrinkage in paraffin sections of formaldehyde fixed brain.

To remedy this soft fixation one may substitute for or add to formaldehyde various other reagents such as mercuric salts, chromic acid and its salts,
osmium tetroxide, picric acid, alcohol, or various other less commonly used reagents. Likewise, treatment with chromate, picric acid, and mercuric chloride solutions may be used after a previous formalin fixation (p. 95).

Formulae for Formaldehyde Solutions

1. **10% formalin**
   - Concentrated formaldehyde solution (37–40%)
   - Tap water
   - 100 cc.
   - 900 cc.

2. **Formol saline**
   - 37–40% formaldehyde solution
   - Sodium chloride
   - Tap or distilled water
   - 100 cc.
   - 8.5 Gm.
   - 900 cc.

3. **Neutral 10% formalin**
   - 37–40% formaldehyde solution
   - Water
   - Calcium or magnesium carbonate to excess
   - 100 cc.
   - 900 cc.

4. **Neutral buffered formaldehyde solution (pH 7.0)**
   - 37–40% formaldehyde solution
   - Water
   - Acid sodium phosphate, monohydrate
   - Anhydrous disodium phosphate
   - 100 cc.
   - 900 cc.
   - 4 Gm.
   - 6.5 Gm.

5. **Calcium acetate formalin**
   - 37–40% formaldehyde solution
   - Distilled water
   - Calcium acetate (monohydrate)
   - 100 cc.
   - 900 cc.
   - 20 Gm.

6. **Ramón y Cajal's formol ammonium bromide, FAB**
   - 37–40% formaldehyde solution
   - Ammonium bromide
   - Distilled water
   - 140 cc.
   - 20 Gm.
   - 1000 cc.
   - 150 cc.
   - 20 Gm.
   - 850 cc.

7. **Alcoholic formalin**
   - 37–40% formaldehyde solution
   - 95% alcohol
   - If desired, one may add 0.5 Gm. calcium acetate to this formula, to insure neutrality.
8. Acetic alcohol formalin

<table>
<thead>
<tr>
<th></th>
<th>Tellyesniczky*</th>
<th>Fekete†</th>
<th>Opie &amp; Lavin‡</th>
<th>Lillie’s AAP§</th>
</tr>
</thead>
<tbody>
<tr>
<td>37-40% formaldehyde</td>
<td>5 cc.</td>
<td>10 cc.</td>
<td>5 cc.</td>
<td>10 cc.</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>5 cc.</td>
<td>5 cc.</td>
<td>5 cc.</td>
<td>5 cc.</td>
</tr>
<tr>
<td>Alcohol</td>
<td>concentration</td>
<td>70%</td>
<td>70%</td>
<td>80%</td>
</tr>
<tr>
<td></td>
<td>amount</td>
<td>100 cc.</td>
<td>100 cc.</td>
<td>90 cc.</td>
</tr>
</tbody>
</table>

*Arch. mikroskop Anat. 52:202, 1898.
‡J. Exper. Med. 84:107, 1946.

Alcoholic formalin and acetic alcohol formalin are excellent for glycogen preservation and are good cytoplasmic fixatives when ribonucleic acid digestion tests are to be performed. In the latter case it is well to fix at 0–5° C. or lower for 24 hours only. Tellyesniczky recommended a similar fluid to the last, containing 50 cc. each of formalin and acetic acid and 1000 cc. of alcohol. This fluid is to be distinguished from the same author’s acetic bichromate formula. Both are often referred to as Tellyesniczky’s fluid. Mallory, Langeron, and Romeis note only the acetic bichromate fluid, Cowdry gives only the acetic alcohol formalin, and Lee gives both.

Traditionally, material fixed in aqueous formalin solutions is stored indefinitely in the same fluid. For this purpose the buffered solution is superior to the unbuffered, even if solid calcium carbonate is included in the storage bottle. On storage in formalin, gradual decrease in basophilia of cytoplasm and nuclei occurs, as well as a progressive loss in reactivity of myelin to Weigert’s iron hematoxylin method.

Buffered formalin retards the loss of basophilia, but storage in 70% alcohol or in 10–20% diethylene glycol in water appears to be better. Ethylene glycol and triethylene glycol probably can be used in the same way, but have not been tested as exhaustively.

After alcoholic formalin fixation, storage in 70% alcohol would appear to be superior to leaving the tissue in the formalin solution.

Alcohol

Alone, alcohol has only limited application as a fixing fluid. Absolute alcohol is often used for preservation of glycogen, for fixation of pigments, and for fixation of blood and tissue films and smears. For the last purpose methyl alcohol is usually preferred and is effective in 80–100% concentration. Lower percentages hemolyze red cells and inadequately preserve leukocytes. Preservation of hemosiderin is less adequate with ethyl alcohol fixation than with buffered formaldehyde solutions. In 60–80% concentration at low temperature (−20° to −25° C.) alcohol is a useful fixative for preserving certain proteins and enzymes in relatively undenatured state.
In fixation of tissue, absolute or 100% alcohol alone penetrates rather slowly. In combination with formalin at a 9:1 ratio it fixes adequately in 18–24 hours or less. In the Carnoy mixtures fixation may be completed in 1–2 hours. Thin (3–5 mm.) slices of tissue should be used. With a Carnoy mixture of 60 cc. 100% alcohol, 30 cc. chloroform, and 10 cc. glacial acetic acid, fix for 1–2 hours. This mixture is the one usually referred to as Carnoy in pathologic usage, and is the only fluid given under Carnoy’s name alone by Langeron, Romeis, Schmorl, Mallory, and Cowdry. Fixation at about 0° C. for 18 hours is sometimes advantageous. Wash in two changes of 100% alcohol of 30–60 minutes each, and store in thin cedar oil. For prolonged storage one may transfer the slices into liquid petrolatum or imbed them in paraffin.

Or if immediate imbedding is desired, carry the slices from the second 100% alcohol into a mixture of equal volumes of 100% alcohol and benzene for 20 minutes, and thence through two 30-minute changes of benzene into paraffin.

Carnoy fixatives hemolyze, occasion a considerable shrinkage, and dissolve acid-soluble cell granules and pigments. They do not decalcify appreciably in the 2–3 hours required for fixation. They give excellent nuclear fixation. Nissl granules are well preserved, and many cytoplasmic structures are adequately fixed. Myelin is lost. Glycogen is apparently better preserved than with 100% alcohol and I prefer Carnoy for this purpose. Alcoholic 10% formalin, with or without 5% of glacial acetic acid, is perhaps superior to Carnoy for glycogen. Fix 12–24 hours or longer.

Newcomer’s (Science 118:161, 1953) fluid contains 60 cc. isopropanol, 30 cc. propionic acid, 10 cc. petroleum ether, 10 cc. acetone, and 10 cc. dioxane. It is recommended as a substitute for Carnoy’s fluid for the fixation of chromosomes. Fix 12–24 hours, and store at 3° C. in a fresh portion of the same fluid. It is said to preserve Feulgen stainability of chromatin better than chloroform-containing fluids.

Acetone

Though long used as a rapid fixative of brain tissue for rabies diagnosis, acetone has only recently come into more extended use as a fixative for the study of tissue enzymes, notably the phosphatases and lipases (pp. 200–219). These latter technics prescribe 24 hour fixation at about 0° C. Acetone is apparently superior to 100% alcohol for the fixation of phosphatases, and this in turn is better than formalin. Later workers have preferred 80% alcohol, followed by alcohol dehydration, to acetone. Whether a similar superiority in the preservation of oxidases would be evident is not known; but these are fairly well preserved with brief formalin fixation. Dopamelnase appears to be preserved. Glycogen was not preserved in one series of tests in which acetone was tried as the fixing agent.
Mercuric Salts

Usually the chloride $\text{HgCl}_2$ is used, and this most often in saturated (5-7%) solution in water. Mercuric chloride rapidly hardens the outer layers of tissues into a quite hard, white mass. It penetrates poorly after the first 3 or 4 mm.; consequently, tissue should be cut in thin slices, not more than 5 mm. in thickness. It hardens cytoplasm well, increases its affinity for acid dyes, and decreases the affinity of cellular, bacterial, and rickettsial chromatin for basic anilin dyes. Nuclear chromatin tends to appear in finer particles with mercuric chloride than with formaldehyde. Because of the increased affinity of cytoplasm for acid dyes, the differences in basophilia and oxyphilia of regenerating, mature, and necrosing cells are less well shown than with formaldehyde. Cytoplasmic structure of renal epithelium, fibrin, connective tissue, cross striae of muscle, and other features may be better shown.

Because of its poor penetration and the shrinkage of tissues produced by it, mercuric chloride is seldom used alone. It is combined with acetic and other acids, with chromates, with formaldehyde, with alcohol, and with various mixtures.

Except for the susa mixtures, material fixed in mercuric chloride fixatives generally requires treatment with iodine to remove granular black precipitates which are distributed throughout the tissues. This may be done by soaking blocks of unimbedded tissue in 70% alcohol containing enough tincture of iodine to color it a fairly deep reddish brown or port wine color (about 0.5% iodine). It is necessary to inspect daily and add a few more drops of tincture of iodine to restore the color, until the alcohol is no longer decolorized.

However, in pathologic practice (particularly when conservation of time is important) it is customary to imbed and section without iodizing and to remove the precipitates from the deparaffinized sections before staining. Sections are treated 5-10 minutes in 0.3-0.5% iodine in 70-80% alcohol (a 1:15 or 1:20 dilution of the U.S.P. Tincture of Iodine), rinsed in water, decolorized 1-5 minutes in 5% sodium thiosulfate, and washed 5-10 minutes in running water before staining.

Lee preferred the practice of iodization before imbedding, and stated that serious artefacts were produced during imbedding if the mercury was not first removed. Mallory condemned this process, preferring to iodize after sectioning, and stated that prolonged iodization of blocks impaired the staining quality of cells. I have used both methods and can see little important difference. Generally I prefer to iodize after sectioning, because of the time factor.

After mercuric chloride fixation the excess fixative should be washed out, preferably with 70-80% alcohol, except when combined with chromates.
In the latter case washing overnight in running water is recommended. Storage in 70% alcohol is usually recommended.

On long storage in alcohol the material becomes quite hard and brittle. Hence it seems better to complete the dehydration of material not wanted at once and either clear and imbed it in paraffin or store it in thin cedar oil. This fluid keeps the tissue reasonably soft and seems to improve its quality for sectioning.

Formulae

1. Saturated Aqueous Mercuric Chloride (5.6% at 15° C.; use 6–7 Gm. per 100 cc.).
   Lee recommended addition of 1% of glacial acetic acid. Spuler (Ehrlich’s *Encyklopädie*) recommended addition of 0.5–0.75% of sodium chloride, by which means the concentration of mercuric chloride is increased to about 9%.

2. Saturated Alcoholic Mercuric Chloride (33% at 15° C.). This coagulates tissues very rapidly.

   Saturated aqueous mercuric chloride 2 parts
   Alcohol (absolute) 1 part
   Fix 24 hours, replace fixative with fresh solution and continue fixation another 24 hours. Then transfer to 70% alcohol.

I consider the cytoplasmic fixation achieved with this fluid inferior to that achieved with Zenker’s fluid. Giemsa recommended it for wet-smear fixation, and the fixative has often been attributed to him.

4. Ohlmacher’s Fluid (Ehrlich’s *Encyklopädie*) (a), Carnoy and Lebrun’s Fluid (Cowdry, McGlung, Lee) (b).
   100% alcohol (a) 32 cc. (b) 15 cc. may be kept
   Chloroform (a) 6 cc. (b) 15 cc. as stock solution
   Glacial acetic acid (a) 2 cc. (b) 15 cc.

Before using, add 8 Gm. mercuric chloride to the above 40-cc. mixture of Ohlmacher’s fluid. Lebrun’s is saturated with mercuric chloride. Add 4 Gm., which is an excess. These mixtures fix very rapidly. Ohlmacher’s fluid penetrates about 1 mm. in the first 10–15 minutes, 2.5 mm. in 2–3 hours. Blocks should be cut quite thin before fixation. Handle while in the fluid with instruments previously dipped in hot paraffin to avoid contact of the mercurial solution with metal. Wash in two changes of 100% alcohol, about one hour each; clear in cedar oil, and store surplus tissue in this fluid. Ohlmacher’s fluid gives comparatively little mercurial precipitate, and there is little difference in appearance between material fixed 30 minutes and that fixed 150 minutes. It preserves glycogen poorly in comparison with Carnoy.
5. Heidenhain's Susa Fluid (Romeis) consisted of 4.5 Gm. mercuric chloride (HgCl₂), 0.5 Gm. sodium chloride, 20 cc. formalin, 80 cc. distilled water, 4 cc. glacial acetic acid, 2 Gm. trichloroacetic acid. Fixation for 12 hours is followed by washing in 95% alcohol. This fluid is said not to give mercury precipitates. (I have not tried it extensively.) It does not preserve glycogen.

6. Zenker (a), Helly (b), Spuler or Maximow (c), Zenker Formic (d):

   Stock solution "Zenker without acetic"
   Mercuric chloride to saturation     7 Gm.
   Sodium sulfate (often omitted)     1 Gm.
   Potassium bichromate               2.5 Gm.
   Distilled water                    100 cc.

   The stock solution is stable. In Zenker's solution (Ehrlich's Encyklopädie) add 5 cc. glacial acetic acid, traditionally just before using. However, with modern C. P. grade acetic acid the mixture seems quite stable and will keep at least some months at room temperature. It withstands heating at 58° C. for 3 months. Generally an excellent fixative, it hemolyzes erythrocytes (at least in part) and removes a considerable amount of the iron component of hemosiderin. It is fairly acid (pH 2.3) on mixing, with or without the sodium sulfate.

   Helly added 5 cc., Spuler and Maximow 10 cc. of 40% formaldehyde solution just before using, in place of Zenker's acetic acid.

   These mixtures become turbid and darken in color in a day or two, depositing a dark brown precipitate. Hence they must be mixed immediately before using. I can recommend these variants highly for blood-forming organs, especially the Spuler-Maximow fluid; but do not like them for demonstration of rickettsiae, bacteria, acute cytoplasmic degeneration, necrosis, and regeneration. They tend to increase cytoplasmic oxyphilia and decrease the basophilia of gland and epithelial cells. These fluids are often called Zenker-formol and are usually attributed to Helly (Ztschr. f. wiss. Mikroskop. 20:413, 1904), though Spuler included his variant as his own in Ehrlich's Encyklopädie (1903). This last fluid is often called Maximow's (Ztschr. f. wiss. Mikroskop. 26:177, 1909), for he used it extensively in his Studien über Blut und Bindegewebe. These variants are less acid (pH 3.4) than Zenker's fluid.

   The formic acid variant contains 5 cc. 90% formic acid in place of the acetic acid of Zenker's, and is an active decalcifying agent. The fixation effect is similar to that of the traditional Zenker's fluid. This variant was attributed to Mary J. Guthrie in Lee's Microtomist's Vade-Mecum, 9th ed. (1928), and was re devised in this laboratory about 1943. However, formic acid reduces aqueous mercuric chloride solutions (a white deposit of calomel starts to appear in about three hours) and also reduces potassium bichromate so that the color changes from the usual orange-yellow to dark red-brown in
about 48 hours. Without mercuric chloride, bichromate formic acid mixtures yield no precipitate in that time.

For all these Zenker variants, cut blocks 3–5 mm. in thickness. Fixation times vary from 6–18 hours with thickness and temperature. Wash overnight in running water, or in 2–3 changes of water, 1–2 hours each on the Technicon; then store in 70% alcohol, or proceed at once with dehydration, clearing and imbedding.


Mercuric chloride 4 Gm.
Sodium sulfate 0.8 Gm.
Potassium bichromate 2 Gm.
Distilled water 80 cc.

At time of using add 20 cc. neutral 40% formaldehyde solution. Fix 8–16 hours in the dark.

Bencosme prescribed 24 hours or more washing in running water. Embryonic and very young animal tissues were then dehydrated in successive two-hour changes of 40%, 50%, 60%, 70% alcohol; three hours in 90% alcohol; and ten hours in 95% alcohol; the last two containing 0.25% iodine. Dehydration was completed with three- and five-hour changes of 100% alcohol. Tissues from older animals were transferred directly from the wash water to 0.25% iodine in 95% alcohol for 24 hours, followed by four changes of 100% alcohol, the first two of which were used, the last two fresh.

Embryonic tissues were cleared through 1:1 and 3:1 toluene:100% alcohol mixtures (2 hours each), and four changes of toluene (3, 10, 2, and 4 hours); adult tissues directly from 100% alcohol in four changes of toluene (8, 16, 8, 16 hours). Used toluene was employed for the first two changes, fresh for the last two changes in both cases.

Bencosme infiltrated with a mixture of 9 parts 50–52° paraffin and 1 part yellow beeswax. Embryonic tissues were infiltrated 2 hours at room temperature in equal parts of toluene and paraffin, and then transferred to the melted beeswax paraffin at 55° C. With adult tissues this step was omitted, and the tissues were infiltrated directly in beeswax paraffin. Three ovens were required, and tissues were kept in them for 8 hours, 16 hours and 1–5 days respectively. It is claimed that the toluene is not adequately eliminated unless three separate ovens are employed.

I cite this schedule as an example of the elaborateness which cytologists can achieve. Because of the high concentration of formalin in this mixture, I would advise very thin blocks. Otherwise considerable differences of fixation of surface and inner zones are apt to be observed. The solvent toluene can be eliminated from the tissue far more expeditiously, and probably more
effectively, by infiltration in vacuo (10 mm. mercury) for 30–60 minutes. The process can be hastened still more by substituting a low-boiling petroleum ether for the toluene. The latter is also less toxic than toluene.

8. Acetic mercuric chloride formalin (pH 1.9–2.0)

- Mercuric chloride 6 Gm.
- Glacial acetic acid 5 cc.
- 40% formaldehyde solution 10 cc.
- Distilled water 85 cc.

Add the acetic acid and formalin to the mercuric-chloride solution at time of using.

9. Sodium acetate mercuric chloride formalin (“B–5”) (pH 5.8–5.9)

- Distilled water 90 cc.
- Mercuric chloride 6 Gm.
- Sodium acetate (anhydrous) 1.25 Gm.
- At time of using add 40% formaldehyde 10 cc.

If the salt available is the trihydrate NaCO₂CH₃·3H₂O use 2.074 Gm. Calcium acetate as monohydrate, 2 Gm., has been substituted; but it may be necessary to filter to remove the slight turbidity resulting probably from the presence of a small amount of calcium hydroxide in the salt.

Fixation with these mercuric chloride formalin mixtures appears to be adequate in 12–24 hours, but even rodent livers are not overhardened in 6–7 days. Tissues should be transferred directly to and stored in 70% or 80% alcohol.

Spuler (Ehrlich’s Encyklopädie) recommended for blood a strong mercuric chloride solution to which he added 1% acetic acid and 10% formalin. W. H. Cox (Anat. Hefte 10:99, 1898) employed for nerve cell (Nissl) granules a mixture of 30 cc. saturated aqueous mercuric chloride solution, 10 cc. formalin and 5 cc. glacial acetic acid. Stowell (Arch. Path. 46:164, 1948) recommended Stieve’s fluid in studies of liver regeneration. This consists of 76 cc. saturated aqueous mercuric chloride solution, 20 cc. formalin and 4 cc. glacial acetic acid. Dawson and Friedgood (Stain Technol. 13:17, 1938) in studies of the anterior lobe of the hypophysis used a mixture of 90 cc. of physiological saline solution saturated with mercuric chloride and 10 cc. formalin. I have used mixtures of 10 cc. formalin, 5 cc. glacial acetic acid, 6 Gm. mercuric chloride and 85 cc. water and of 10 cc. formalin, 2 Gm. calcium or sodium acetate, 6 Gm. mercuric chloride and 90 cc. water. G. Brecher in this laboratory has found the last mixture (B–5) excellent for differentiation of pancreatic islet cells.

Unbuffered mercuric chloride formalin mixtures are quite acid (ca. pH 2.9); the acetate formulas much less so (pH 5.9, 5.85 for sodium and calcium formulas); but the acetic formula is slightly more acid (pH 1.9). The stock solutions appear to be quite stable, but the formaldehyde mixtures are
unstable and should be freshly prepared. Formaldehyde reduces mercuric chloride to calomel and metallic mercury, evident as a white to gray or black-flecked precipitate.

Mann (Ztschr. wiss. Mikroskop. 11:479, 1894) gives two mercuric chloride formulae which are still sometimes used. His osmic-sublimate is composed of equal volumes of 1% aqueous osmium tetroxide and of 0.75% aqueous sodium chloride solution saturated with mercuric chloride. In his tannin-picro-sublimate he adds 1 Gm. each of picric acid and of tannin to 100 cc. of Heidenhain’s 0.75% aqueous sodium chloride solution saturated with mercuric chloride. These fluids were originally used for the study of nerve cells. Lee as early as 1896 changed the solvent to physiological salt solution, and Cowdry prescribes 0.85% sodium chloride.

**Lead**

Sylven (Exper. Cell Research 1:582, 1950), Bunting (Arch. Path. 49:590, 1950) and others have used simple 4% aqueous solutions of basic lead acetate for fixation of the connective-tissue mucins. Sylven prescribed one day in the lead solution, followed by one day in 5% formaldehyde.

I have used various lead nitrate and lead nitrate formalin solutions, aqueous and in 80% alcohol. The following alcoholic lead nitrate formalin can be recommended for umbilical cord and vitreous.

**Lillie’s alcoholic lead nitrate formalin**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lead nitrate</td>
<td>8 Gm.</td>
</tr>
<tr>
<td>40% formaldehyde</td>
<td>10 cc.</td>
</tr>
<tr>
<td>Water</td>
<td>10 cc.</td>
</tr>
<tr>
<td>100% alcohol</td>
<td>80 cc.</td>
</tr>
<tr>
<td>Fix</td>
<td>24 hours at 25–30° C., or longer at 0–5° C. (2–3 days) or —25° C. (10–14 days).</td>
</tr>
</tbody>
</table>

Similar solutions in water are also quite effective, and good fixation is obtained with the alcoholic solution from which the formalin is omitted.

The pH of 4% lead subacetate is 3.8–4.2; that of 8% lead nitrate solutions lies between 3.6 and 4.0. Formaldehyde has little effect on the pH level. With the addition of 2% sodium acetate \((\text{NaCO}_2\text{CH}_3\cdot3\text{H}_2\text{O})\) the pH of 8% \(\text{Pb}(\text{NO}_3)_2\) rises to 5.15.

Lead subacetate apparently varies considerably in its basicity and solubility. I have had to add appreciable amounts of glacial acetic acid to some samples in order to achieve anything like a 4% solution. For example one 100 cc. lot required 2 cc. glacial acetic to produce a clear solution of pH 4.0. With 1.5 cc. the solution was cloudy at pH 4.3.

**Chromates**

The most commonly used primary chromate fixatives are Orth’s mixture and Möller’s (Regaud’s) mixture. The former I prefer for routine work. It is
equal to formaldehyde for the study of early degenerative processes and necrosis. It is perhaps superior to formaldehyde for demonstrating rickettsiae and bacteria. Chromaffin takes its characteristic brown tone and its basophilia is well brought out with Orth’s fluid. Myelin is better preserved, and the pericellular shrinkage seen about the pyramidal cells after the use of formalin is avoided. Hemosiderin is less well preserved with Orth than with buffered neutral formalin. The fixation period for Orth’s fluid should be between 36 and 72 hours. Human tissues are usually given 48 hours; brain is perhaps better with 72 hours, though 48 hours is adequate. Mouse tissues appear to be overhardened in 48 hours, and 24 hours is often adequate. Rat and guinea pig tissues should be fixed longer, perhaps 36–48 hours. After fixation, tissues are washed overnight in running water. The traditional storage fluid is 70% alcohol; but tissues may be kept at least a year in 10% formalin, so that material for fat stains can continue to be available. Glycogen is well shown by the Bauer method after Orth fixation.

Romeis’ acetic variant of Orth’s fluid gives similar pictures, except that partial hemolysis occurs, more iron pigment is lost, and nuclear chromatin is more sharply defined. This variant was used routinely by Ophüls for many years. He commonly fixed in it for 3–4 days. Tellyesniczky used an acetic bichromate mixture without formalin. This mixture appears to be stable.

I have employed Kose’s variant of Orth’s fluid, which contains 3 Gm. potassium bichromate in place of 2.5 Gm. per 100 cc., and find that it gives greater hardening in the same period of time, without appreciable change in the overall picture. This fluid, like Zenker’s, renders ribonucleic acid relatively highly resistant to specific enzyme digestion. This effect is less evident when 2% calcium or sodium acetate is added to Kose’s fluid or to Spuler’s formalin Zenker mixture.

Möller’s or Regaud’s fluid is similar in general effect to Orth’s, but hardens more rapidly and to a harder final consistency. With this 20% formalin bichromate mixture, as with aqueous 20% formalin and with Bouin fluids containing 20–25% formalin, there is produced an outer zone of hyperoxyphilia of tissues. In this zone nuclear staining is apt to be poor, and cytoplasm will stain more strongly with acid dyes there than in the center of the block.

Sometimes it is desirable to modify the naturally quite acid pH level (3.8–3.95) of potassium bichromate solutions. For example, iron-containing pigments are better preserved at higher pH levels. Lower pH levels seem necessary to elicit the chromaffin reaction of the enterochromaffin cells. Addition of 5% of acetic acid to a 3% potassium bichromate solution lowers the pH to 2.9. This solution is stable for months, but the further addition of formaldehyde quite promptly causes the usual reduction changes: first darkening to dark brown, then to brownish green. Hence formalin should not be added to acidified bichromate mixtures until the time of using. Formulas for the buffering of bichromate solutions appear in Table 4. Addition of 10 parts
of 40% formaldehyde to 90% bichromate produces little change of pH level: perhaps a rise of .05 below pH 4; none above. The upper pH levels do not darken on addition of formaldehyde; but at pH 6.3 some dark-green precipitate is formed in 18-24 hours, the solution remaining clear chromate yellow and smelling strongly of formaldehyde. The more acid pH 5.5 acetate solution shows no precipitation.

Table 4
Buffered Bichromate Solutions

<table>
<thead>
<tr>
<th>N/I HCl cc.</th>
<th>M/5 KH₂PO₄ cc.</th>
<th>M/5 Na₂HPO₄ cc.</th>
<th>M/5 Acetic cc</th>
<th>M/5 NaAcet cc</th>
<th>6% K₂Cr₂O₇ cc</th>
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<td></td>
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<td>22.0</td>
<td>50</td>
<td></td>
<td></td>
<td>6.27</td>
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</tbody>
</table>

Formulae

1. Müller's Fluid (Ehrlich's *Encyklopädie*). Dissolve 2.5 Gm. potassium bichromate and 1 Gm. sodium sulfate in 100 cc. distilled water.

2. Orth's Fluid (*Berl. klin. Wchnschr.* 33:273, 1896). To 100 cc. of Müller's fluid add 10 cc. 37-40% formaldehyde solution at the time of using. I regularly use 2.5% potassium bichromate in place of Müller's fluid; Mallory used 2-2.5%. Romeis and Schmorl reduced the Müller's fluid to 90 cc. Fix 1-4 days, usually 2. Wash overnight in running water.

3. Romeis' and Ophüls' Variants. Romeis used 85 cc. 3% potassium bichromate, 10 cc. formalin, and 5 cc. glacial acetic acid. Ophüls used 100 cc. Müller's fluid; I use 100 cc. 2.5% potassium bichromate in place of Romeis' 85 cc. 3%. The acid and formalin were added immediately before using. The mixture darkens rapidly. Fix 6-24 hours according to Romeis, 2-4 days according to Ophüls. Wash overnight in running water.

Vereins “Lotos,” Prag, 46 (N.F. 18):224, 1898). To 90 cc. 3% potassium bichromate add 10 cc. 37-40% formaldehyde solution at time of using (pH 3.6-3.9). Fix 1-3 days. Wash 16 hours in running water.

5. Buffered Orth. Dissolve 25 or 30 Gm. potassium bichromate and 16.2 Gm. sodium acetate crystals in 1000 cc. water. To 90 cc. of this fluid add 10 cc. 40% formaldehyde solution at time of using (pH 5.5). Fix 1-3 days. Wash 16 hours in running water.

6. Telyesniczky’s Acetic Bichromate (Arch. mikroskop. Anat. 52:202, 1898). To 100 cc. 3% potassium bichromate add 5 cc. glacial acetic acid at time of using. (The mixture seems to keep well after mixing.) Fix 1-2 days. If simultaneous decalcification is desired, substitute 5 cc. 90% formic acid for the acetic.

7. Möller’s or Regaud’s Fluid. To 80 cc. 3% potassium bichromate add 20 cc. 40% formaldehyde solution at time of using. Fix 1-2 days. Möller (Ztschr. f. wiss. Zool., 66:69, 1899) prescribed one-day fixation, followed by 3-4 days in 3% potassium bichromate. Regaud (Arch. d’anat. micro., 11:291, 1910) recommended four days, and followed it with eight days’ further chromation in 3% potassium bichromate for mitochondria. This seems excessive for general purposes. In my experience the prolonged chromation interferes with nuclear staining. However, certain methods for Golgi substance and for mitochondria call for the long chromate treatment. Ehrlich’s Encyklopädie (1903) cites this fluid as Möller’s; Mallory, Langeron, Romeis, Conn and Darrow, Lee, and Cowdry all call it Regaud’s; while Regaud himself stated that the fluid used by him was the same as Möller’s.


- 5% aqueous potassium bichromate solution 20 cc.
- 10% formalin 20 cc.
- Glacial acetic acid 5 cc.
- 50% trichloracetic acid 5 cc.
- Saturated aqueous (10%) uranyl acetate 5 cc.

Fix 24 hours, wash six hours in water or 25% alcohol, followed by six-hour baths in ascending alcohols: 35%, 50%, 70%, 95%, 100%.

9. Held’s fluid: To 100 cc. 3% potassium bichromate solution add 4 cc. 40% formaldehyde and 5 cc. glacial acetic acid and allow to ripen for some weeks until the solution is green in color. In this fluid the formaldehyde is partly oxidized to formic acid; the chromic acid partly reduced to chromic salts; and the pH rises from an initial level of about 3.0 to about 3.8 (Romeis).

Osmium Tetroxide

Osmium tetroxide (“osmic acid”) mixtures are little used in pathology because of their high cost, poor penetration, and interference with various
staining methods. Since the development of modern frozen section methods for fat and degenerating myelin, they have less value than formerly. Osmium tetroxide is especially valuable for fixation of cytoplasmic structures; but nuclei are poorly stainable, and when its action is prolonged, unsaturated fats reduce it to form black masses.

The vapor of 1–2% aqueous solution may be used for the fixation of blood and tissue films. This requires 30–60 seconds or more according to the thickness of the film. Slides are placed face down over a small flat dish containing a thin layer of the solution.

1. Flemming’s strong solution (Ehrlich’s Encyklopädie and all modern texts) contained 20 cc. 2% osmium tetroxide, 75 cc. 1% chromic acid and 5 cc. glacial acetic acid. Fix 1–3 days; wash 6–24 hours in running water; store in 80% alcohol.

2. Hermann’s fluid (Ehrlich’s Encyklopädie) substituted 75 cc. 1% platinum chloride for the chromic acid in Flemming’s, with the same 20 cc. 2% osmium tetroxide and 5 cc. glacial acetic acid. It was used in the same manner.

3. Marchi’s fluid consists of 1 part 1% osmium tetroxide and 2 parts Müller’s fluid (p. 44). This composition is that given in all modern texts. Its main use is for demonstration of degenerating myelin.


Picric Acid

Picric acid gives a quite hard fixation which is excellent for many purposes. Anilin blue methods of the Mallory type, with its Heidenhain and Masson variants, do well after this fixation. Unless very thorough washing of sections is done, it interferes with azure eosinate stains. It penetrates well and fixes small objects rapidly.

The most popular picric acid fixative is Bouin’s fluid. Lee recommended not more than 18 hours’ fixation, Masson up to 3 days, Cowdry 24 hours, and I have found 1–2 days satisfactory.

This fluid contains 75 cc. 1.2% (saturated) aqueous picric acid solution, 25 cc. formalin (40% formaldehyde) and 5 cc. glacial acetic acid (Ehrlich’s Encyklopädie). Allen’s B-15 modification contains in addition 2 Gm. urea and 1.5 Gm. chromic acid, while in his PFA3 modification the formalin is reduced to 15 cc., the acetic acid increased to 10 cc., and 1 Gm. urea only is added (Lee). I have found that the superficial hyperoxyphilia seen in Bouin fixed tissues when 20–25% formalin is included may be obviated by decreasing the formalin content to 10%. Substitution of 90–95% formic acid for the glacial acetic will decalcify small bones in 24 hours during fixation. This PFF fluid (J. Tech. Methods 24:35, 1944) contains 85 cc. 1.2% picric acid, 10 cc. 40% formaldehyde, and 5 cc. 90–95% formic acid.

Bouin fixatives generally take red cells and remove the demonstrable ferric
iron from blood pigments. They render ribonucleic acid relatively resistant to ribonuclease digestion.

Lee recommends extraction with 50% and 70% alcohol until most of the yellow color is removed. Masson (J. Tech. Methods 12:75, 1929) transferred tissues from the fixative to water, using enough to cover them and leaving them in it until ready to imbed them. I find that alcohol extraction of blocks may take many days with daily changes of alcohol, and prefer to imbed and section after no more than 2–3 days in 70–80% alcohol. It is far easier to wash the excess picric acid out of the sections after removal of paraffin. Surplus tissue is to be stored in 70% alcohol.

Two alcoholic picric acid fluids which have enjoyed some popularity as glycogen fixatives are Rossman’s and Gendre’s fluids.

**Rossman’s fluid** (Am. J. Anat. 66:342, 1940) consists of 90 volumes of 100% alcohol saturated with picric acid and 10 volumes of neutralized commercial formalin. Fix for 12–24 hours and wash for several days in 95% alcohol.

**Gendre’s fluid** (Bull. d’histol. appliq. à la physiol. 14:262, 1937) is composed of 80 volumes of 90% (by weight, equals 95% by volume) alcohol saturated with picric acid, 15 volumes of 40% formaldehyde solution and 5 volumes of glacial acetic acid. Fix 1–4 hours in this mixture and wash in several changes of 80% alcohol. I find that four hours at 25°C, gives excellent fixation of glycogen in liver tissue. Two changes each of 80%, 95%, and 100% alcohol give adequate washing and dehydration.

The picrosublimate acetic fluid sometimes attributed to Tellyesniczky (Arch. f. mikroskop. Anat. 52:202, 1898) was derived by him from vom Rath (Anat. Anz. 11:286, 1895). This was a mixture of equal volumes of mercuric chloride saturated in warm sodium chloride solution and of filtered cold saturated picric acid solution. Vom Rath added, either 0.5% or 1% glacial acetic acid to the mixture. Tellyesniczky used the latter proportion and condemned the mixture both for resting nuclei and for cytoplasm.


**Formula:** In 100 cc. distilled water dissolve successively without heating 2.5 Gm. copper acetate, 4 Gm. picric acid, 10 cc. 40% formaldehyde, and 1.5 cc. glacial acetic acid. The fluid keeps well and will decalcify small bones in a week or less.

**Heat**

I have occasionally fixed small objects, up to 5–10 mm. in thickness, by boiling in physiologic (0.85–0.90%) sodium chloride solution for 2–3 minutes. This method may be of value where it is desired to avoid the introduction of alien chemical substances. Some shrinkage is produced. The method
has the further advantage of speed. Frozen sections may be completed in a
matter of minutes after removal of tissue from the body. I see no advantage
in the substitution of formalin solutions, either concentrated or dilute, when
boiling is to be employed.

It is well known that boiling water dissolves starch and glycogen (Bull.
boiling distilled water destroys red corpuscles, but, if brief, leaves a good deal
of glycogen in liver cells, and preserves nuclear and cytoplasmic detail quite
well. Collagen is not greatly altered.

Unfixed Tissue

Formerly a great deal of histologic work was done with this material, but
its use is now principally restricted to the study of various enzyme systems,
notably the labile cellular oxidases. Dopamylanase, for which this method
was recommended, has been found to resist cold acetone fixation.

A variant of this procedure is the Altman-Gersh freezing-drying procedure.
In this procedure small tissue fragments are immersed in isopentane chilled
with liquid air or liquid nitrogen (which is safer) to $-130^\circ$ or $-140^\circ$ C.
and promptly frozen. They are then desiccated at $-30^\circ$ C. in high vacuum
($10^{-4}$ to $10^{-6}$ mm. mercury) and when thought to be dry (which may be a
matter of several days for what are ordinarily regarded as moderate-sized
blocks) they are infiltrated directly in paraffin without breaking the vacuum.
This is accomplished by depositing the frozen tissue out of the isopentane
directly on the surface of the solid paraffin in a small container, to which
external heat can be applied without opening the vacuum chamber. As the
paraffin melts the tissue sinks in and promptly infiltrates, since there is no
fluid to be displaced. (I wish to thank Dr. Gersh at this point for the detailed
demonstration of the apparatus in his laboratory.)

The practice of preceding infiltration by water-miscible polyethylene
glycol 400 (M.P. $5^\circ$ C.) suggested by Hack (Proc. Histochem. Soc. 1951) or
by Carbowax 4000, with the freezing-drying procedure seems to be a compli-
cated and unnecessary elaboration, since these imbedding media are already
miscible with water and will replace it by diffusion at appropriate tempera-
tures without the elaborate and expensive apparatus. Simple immersion of
tissue (quickly frozen with solid carbon dioxide and petroleum ether in glycol
at $-5^\circ$ to $-10^\circ$ C. to dehydrate the frozen tissue; or with polyethylene glycol
400 at $+10^\circ$ C., which is low enough to stop autolytic enzyme action) would
seem to serve, if indeed a water-soluble imbedding mass actually improves
sectioning on the freezing microtome.
Chapter 4

Sectioning

Freehand Sectioning

Freehand sectioning with a sharp razor is sometimes employed in making sections thin enough to be translucent, either of unfixed or of fixed tissue. This method was employed by Terry (J. Tech. Methods 12:127, 1929) for his rapid-diagnosis method. Following the preparation of the thin sections they are placed upon a glass slide, and a drop of dilute stain is carefully placed on one surface and rinsed off after a few seconds. With practice it is readily possible to regulate this staining interval so that only one or two surface layers of cells are stained. For this purpose Terry recommended a neutralized polychrome methylene blue made by boiling with alkali for variable periods. It is probably better to use 1% toluidin blue, thionin, azure C, or azure A for this purpose. After rinsing put on a cover glass and examine at once.

Aside from this procedure, almost all sectioning is done with microtomes. With these instruments sections may be prepared which are much thinner and more uniform than those prepared by freehand methods. Microtomy requires a firmer consistence than is present in raw or fixed tissue. This consistence is attained by freezing or by infiltration with imbedding masses in a fluid state, followed by solidification of the imbedding medium. The commonly used imbedding media are paraffin and other waxes, soaps, gelatin, agar, and nitrocellulose.

Frozen Sections

The freezing microtome is used for preparation of sections for rapid diagnosis, and for the study of fatty and lipoid substances which would be lost if paraffin or nitrocellulose methods were employed, and for many metallic impregnation methods.
Place a few drops of water on the object holder of the freezing microtome, lay a 3 to 5 mm. slice of tissue in this water, freeze rapidly until the surface of the tissue appears dry, wait a few seconds, and then try cutting a section at a time at short intervals until satisfactory coherent sections at 10–15 μ are obtained. The sections as cut remain on the edge of the microtome knife. They are conveniently transferred to small dishes of water by first moistening the outer side of the left little finger with water, and then wiping the sections off the knife edge with the moistened area and dipping the finger with sections into the dish of water. The sections float off; first as shreds, when the block is too hard; then as coherent sections which fold freely in the water as they float. When this point is reached, quickly cut a dozen or more sections, removing three or four at a time to the water. Store sections from each case in an individual small covered dish with a small slip of paper bearing the case number in the water with the sections. The number can be written in pencil; but if prolonged storage is contemplated, write labels with India ink and then dip them in smoking hot paraffin. For prolonged storage use 10% diethylene glycol by preference; otherwise 5–10% formalin. Vials holding 10–15 cc. are convenient for this purpose. The India ink label is put inside with the sections.

In selecting sections for staining, complete sections which fold and unfold freely in water are to be chosen. Sections which float rigidly are too thick. For rapid diagnosis such frozen sections may be stained in toluidin blue, thionin, or one of the azures. Mallory recommended 0.5% thionin or toluidin blue in 20% alcohol for 30–60 seconds. For fixed tissue the addition of 0.5–1% of glacial acetic acid sharpens the stain and makes it more selective for nuclei. Sections may be stained thus by immersion in a small amount of stain in a watch glass, then fished out with a needle after the required time, rinsed in a large container of water, and floated onto a slide. Bring the edge of the section on the slanted slide carefully to just above the surface of the water. Tease the remainder of the section half floating in the water into a reasonably flat position, and gradually raise the slide out of the water. If any portion of the section is not flat, dip that side again under the water so as to float it smooth, and again withdraw. Blot dry with hard, smooth filter paper; dehydrate with a few drops of acetone; clear with acetone and xylene, then xylene; and mount in 60% clarite in xylene.

Some prefer first to float sections onto slides as above, then blot them down with hard filter paper, immerse them briefly in 0.5–2% collodion solution in ether and 100% alcohol, drain for 30–60 seconds, and harden in chloroform, 80% alcohol, or water before staining. This method is to be preferred if sections are to be heated or treated with alkaline solutions. Such sections may then be handled as are paraffin or attached nitrocellulose or celloidin sections (pp. 93–99).

Sections stained for demonstration of fats with oil-soluble dyes (pp. 301–306) are to be floated out on slides and blotted flat as above, but all dehydrat-
ing agents or fat solvents are to be avoided. Instead, mount directly in a gum arabic, gelatin glycerol, syrup, or other aqueous mounting medium; or temporarily in pure glycerol, water, or other indifferent fluid (pp. 112-113). Gelatin media require melting before use and may be very tenacious of air bubbles. Gum-arabic media are fluid at room temperature and most of them dry hard. Fructose syrup will dry hard if the humidity is not too high, but, like Arlex gelatin, will remain sticky for some time in hot, humid weather. Such syrup mounts may be sealed by painting the edges of the cover glasses with a polystyrene solution.

The Adamstone-Taylor Technic for Frozen Sections

In this procedure (Stain Technol. 23:109, 1948) fresh tissue is quickly frozen in slices of 2 mms. thickness or less, either on the freezing block of the microtome, or by immersion in liquid air or in isopentane chilled with liquid air or in petroleum ether containing chunks of solid carbon dioxide. By using one of the latter procedures a number of blocks may be quickly frozen and then stored in a Deepfreeze compartment at -25° C. until sectioning becomes convenient. Since the essence of the technic is the avoidance of thawing until sections are finally attached to slides, it is necessary to chill the knife of the microtome by fastening to it on each side of the cutting area blocks of solid carbon dioxide. Use Scotch tape or thin strips of sheet metal cut from scrap copper or tin plate.

When the knife is chilled, the tissue blocks are placed on the freezing head and frozen to the block. As the sections are cut, a small camel’s-hair brush is used to hold them flat as they come onto the knife blade. Then Adamstone and Taylor used a small, hollow scoop containing dry ice chips to transfer the flat sections to slides, and pressed them down. As the sections soften, but before complete melting, they must be immersed in the chosen fixative. If the objective is microincineration for demonstration of soluble salts, quick heating to coagulate tissue protein would seem preferable to exposure to the solvent action of any liquid fixative.

The authors warn that the procedure is much more difficult in a warm or humid atmosphere. The whole process might be much easier (though less comfortable to the operator) if it could be carried out in a refrigerator room at a temperature somewhat below 0° C.

For this purpose the use of a Linderstrøm-Lang cryostat (p. 23) is suggested. In this type of apparatus the sections can be kept for a time in the frozen state, without the necessity of immediate processing required by the open-air Adamstone-Taylor method. Coons’s procedure is given on p. 23.

The recent Bush-Hewitt (Am. J. Path. 28:863, 1952) technic for preparation of thin frozen sections, though it is perhaps too cumbersome for routine diagnostic use, would appear to have some real value for procedures in which frozen sections of fresh, unfixed, and unheated tissue are demanded. The procedure would seem to require less elaborate equipment than the Coons
and Linderström-Lang procedures, and to be perhaps easier to accomplish than the Adamstone-Taylor procedure.

Tissue blocks should presumably be quickly frozen by solid carbon dioxide, either by direct contact or by brief immersion in petroleum ether containing a number of fragments of solid CO₂. After freezing they may be kept frozen hard with dry ice (solid CO₂) or in a Deepfreeze compartment at -20° to -25° C. until sectioning.

Then affix the block to the freezing head of a clinical microtome or of a celloidin microtome equipped for frozen sections, and affix a block of solid carbon dioxide to the knife as in the Adamstone-Taylor method (p. 51). Cut off sections from the surface of the block until the desired depth in the block is reached, maintaining the block in a hard-frozen condition so that unsupported sections crumple (about -15° C.). Then moisten the gelatin surface of a strip of stripping film with water for a few seconds, and apply the film, wet gelatin surface down, to the frozen block. It immediately freezes onto the block. Then support the free edge of the stripping film with a pair of thumb forceps. Cut the next section and transfer it directly to a slide, section side up. The section melts at once. Two or three minutes should be allowed at this point for the gelatin to absorb the excess water from the section film interface, and to ensure adhesion of the section to the film.

It would seem to be required that staining procedures be performed with the slides flat and face up, since there is no provision for causing the cellulose acetate base to adhere to the glass.

Suitable stripping film with a cellulose acetate base 15 or 24 μ thick and a soft or slightly hardened gelatin film 10 μ thick may be procured, mounted on heavier cellulose acetate, from the Industrial Photographic Sales Division of the Eastman Kodak Co., Rochester, N. Y.

Though Bush and Hewitt suggest the use of stripping film prestained with toluidine blue for transfer staining, the greatest value of this procedure would seem to lie in those areas of histochemistry and enzyme localization where the use of fresh sections untreated with aqueous or lipid solvents is demanded.

Imbedding Methods

The other commonly used sectioning methods require infiltration with imbedding masses. Of these the Carbowax, gelatin, agar, and soap masses are soluble in water, and tissue may be infiltrated directly. Nitrocellulose is soluble in a mixture of 100% alcohol and ether, paraffin in various fat solvents; and tissues must first be freed of water (dehydration) and brought into the appropriate solvent before infiltration.

Carbowax. Solid polyethylene glycols (Carbowaxes of the Carbide & Carbon Chemicals Corp.) have been recommended as imbedding media in place of paraffin and celloidin. As they are readily soluble in water, infiltration is done without preliminary treatment with fat-solvent dehydrating agents. Blocks are of a hard, paraffinlike consistency and are cut on a paraffin micro-
tome in ribbons. Although floating the sections on water promptly dissolves the wax, this procedure is often used for sections of solid organs. Rather violent diffusion currents are created, and noncoherent sections literally explode. Blank and McCarthy (J. Lab. & Clin. Med. 36:776, 1950) use a weak solution of gelatin and potassium bichromate (0.02% each) at room temperature for floating the sections, either on the slide or in a dish. The wax dissolves at once and the section is picked up on a slide and dried thoroughly. After floating it directly on the slide, a second immersion in the gelatin bichromate solution may be needed to remove excess wax. The gelatin potassium bichromate solution is prepared by boiling for five minutes in daylight. It is then cooled and filtered.

Other surface tension depressants have been used more or less successfully to combat the diffusion currents. A little Carbowax itself works well and seems the least objectionable of these, in that it introduces no additional chemical reagent.

Floating on metallic mercury has been suggested. Firminger tells me that it is necessary to heat the mercury nearly to the melting point of the wax to spread the ribbons. A scum of oxide, Carbowax, and (probably) albumen forms on the surface of the mercury. The sections often pick up minute droplets of mercury which are difficult to get rid of. Though the procedure offers a method of smoothing out Carbowax ribbons, the difficulties noted would seem to militate against its routine use. I am indebted to H. I. Firminger (then at the National Institutes of Health) for the foregoing notes.

Blank and McCarthy (loc. cit.) infiltrated blocks directly from water in a 9:1 mixture of Carbowax 4000 and Carbowax 1500 at 55-60° C., in the paraffin oven or in a thermostatically controlled bath. They allowed 30 minutes for a block 1 mm. thick, 80 minutes for a 2 mm. block, and proportionately longer for thicker blocks. Even four days' exposure to molten Carbowax is said to give little evident distortion. After infiltration, imbed the blocks in fresh Carbowax mixture. Chill them 5-10 minutes in a refrigerator, avoiding contact with ice. Attach them to a block carrier as in the case of paraffin blocks, and section them on a rotary microtome at 2-10 μ at room temperature.

Firminger (J. Nat. Cancer Inst. 10:1350, 1950), in discussion, stated that pure Carbowax 4000 was preferable for summer use (with temperatures of 30-36° C.). He found that the ribboning consistency of Carbowax was improved by first heating each lot to 175° C. for about 30 seconds.

By strictly controlling the temperature of melted Carbowax 4000 (M.P. 50° C.) to one or two degrees above its melting point, McLane (Stain Technol. 26:63, 1951) was able to preserve the enzymatic activities in plant tissues which hydrogenate tetrazolium compounds to formazans. Since freezing destroys these enzyme systems, excessive chilling of the blocks is avoided.

Mellors warns (J. Nat. Cancer Inst. 10:1351, 1950) that though glycols and polyethylene glycols do not dissolve neutral fats, they do have a consider-
able solvent capacity for aromatic compounds such as steroids, especially those of the adrenal. Rinehart (A.M.A. Arch. Path. 51:666, 1951) prefers Carbowax 1000 because the infiltration is more rapid than that obtained with Carbowax 4000. Like Firminger, Rinehart heats the fresh supply of Carbowax to smoking temperature before using. Infiltration is done in 70%, 90%, and pure Carbowax at 48-52° C. for 30, 45, and 60 minutes respectively. Sections may be cut (at San Francisco temperature) as thin as 2 μ. He floats sections on a 10% formalin solution containing 40% diethylene glycol.

A glycerol gelatin fixative (10 Gm. gelatin, 60 cc. distilled water, 50 cc. glycerol, 1 Gm. phenol) is thinly smeared on slides. Sections may be dried at room temperature as long as five days before staining. The cut surface of blocks should be dipped in melted Carbowax before putting them away in a cool dry place.

Wade (Stain Technol. 27:71, 1952) uses a 15:85 mixture of Carbowax 1540 and 4000, and infiltrates skin blocks six hours in two changes of the wax. He adds 5 mg. Turgitol 7 to 100 cc. distilled water to reduce the disruptive effect of water on the Carbowax sections. He finds the Carbowax method especially valuable for conservation of the acid-fast material in lepra bacilli.

Hack (J. Nat. Cancer Inst. 12:225, 1951), for the study of plasmal lipids, first freezes in isopentane with liquid air or nitrogen and desiccates in vacuo by the Altmann-Gersh procedure. He then infiltrates at 15° C. in polyethylene glycol 400 (M.P. 5° C.) and sections on a freezing microtome at 10-20 μ, handling thereafter as is usual for frozen sections.

In place of the foregoing rather elaborate procedure, blocks may be infiltrated directly at 5° C. in a strong aqueous solution of Carbowax 4000, and the water can then be evaporated in vacuo until the Carbowax is quite hard. For this suggestion I am indebted to H. Blank in a discussion of Hack's paper at the Histochemical Society meeting of 1951 (J. Nat. Cancer Inst. 12:226).

Carsten (Arch. Path. 44:96, 1947) fixed eyes by a Bouin-formalin sequence, and infiltrated at 37° C. in one change of 50% Carbowax 1000 and four changes of pure Carbowax 1000; then, at 45° C., in a 50:50 mixture of Carbowax 1000 and Carbowax 1540 distearate, and in four changes of the distearate, embedding in the last. The sections were dehydrated with alcohol, collodionized, detached from the slides, and handled as celloidin sections (p. 98). This procedure, which takes two to three days, seems to have little to commend it. Rapid nitrocellulose technics (p. 70) are even briefer and yield sections just as thin, already in celloidin. The Carbowax 1000 seems to serve simply as a dehydrating agent. Since formaldehyde fixatives are used, myelin is preserved; but it is unlikely that more soluble lipoids would be preserved, since the distearate is essentially a fat. The rationale of floating the wax sections on sucrose syrup is not at all clear, since the distearate is quite insoluble in water.

Goland, Jason, and Berry (personal communication, 1953) find that tissues may be infiltrated (6-12 hours) directly from water with polyethylene
glycols or their water-dispersible esters (laurate, oleate, stearates) and then transferred directly to melted paraffin for imbedding. They have used as short a period as 30 minutes in hard paraffin. By this means sectioning is considerably facilitated, ribbons may be prepared, and sections can be floated on water on the slides without the violence of dispersion which occurs with plain Carbowax sections. Sections prepared in this way have been stained for fats successfully. The outer rim of paraffin apparently floats off in the aqueous reagents. It appears that paraffin does not penetrate appreciably.

The method sounds interesting, but for lipoids I would suggest that only the polyethylene glycols (and not their esters) be used for the primary infiltration of 6–12 hours at 60° C., and that the infiltrated tissues be simply rinsed in molten paraffin and imbedded immediately.

Gelatin. Zwemer (Anat. Rec. 57:41, 1933) recommends washing tissues four hours or more in water, followed by 24 hours' infiltration at 37° C. in 5% gelatin and 12–16 hours further in 10% gelatin. The tissues are then oriented and imbedded in 10% gelatin which is allowed to harden by cooling at 0–5° C.

Blocks may then be sectioned by the freezing method and mounted before staining. The gelatin may be removed with warm water.

Usually it is preferable to harden the gelatin first by a day or more of fixation in 10% formalin. Zwemer prescribed several hours. Blocks may be sectioned on the freezing microtome or may be dehydrated and imbedded in paraffin.

The advantage of the method lies in making coherent sections of friable or fragmented tissues. Such material as uterine curettings may thus be handled as a single block. The disadvantage is that the gelatin stains rather strongly with basic anilin dyes and the stained mass may be confusing. I have found the method helpful, on occasion, in outlining individual cells in masses that were otherwise apparently syncytial in nature.

Baker (Quart. J. Microscop. Sci. 85:1, 1944) infiltrated in 25% gelatin at 37° C. for 20–24 hours. Ordinarily he then cooled, blocked, and hardened the sections in his calcium cadmium formalin (p. 33). If thin sections were required, he infiltrated instead for 30 hours in an open container over anhydrous calcium chloride in a vacuum desiccator at 37° C. Concentration was stopped best just before the gelatin sol set to a gel. The solution was then cooled, blocked, and hardened as before.

In both instances sections were cut on the freezing microtome. With the vacuum concentration technic, 5 μ sections were prepared by this author.

Nicholas (Bibliogr. Anat., Paris, 1896, p. 274) practically perfected the gelatin infiltration method for preparing frozen sections of eyes. Oakley (J. Path. & Bact. 44:365, 1937) fixed first for four days in 10% formalin saline (p. 34). He recommended postchroming in Müller's fluid (p. 44) for six weeks in the incubator (47° C.), or in Perdrau's fluid (K₂Cr₂O₇ 5, CrF₃ 2.5, water 100), or in Perdrau's fluid half strength for four days at room
temperature. Large eyes are then windowed before washing for 24 hours. After washing, cut the eyes in half and infiltrate them at 37°C overnight in 12.5% gelatin and in 25% gelatin for 24 hours. Use gelatin not more than twice, and add 1% phenol to prevent mold growth. Imbed cut surface down in 25% gelatin, and harden by cooling to not below 0°C. Trim the blocks, and harden them 2–3 days in 10% formalin. Wash them for 15 minutes in water before sectioning. Sections of 5–10 μ are claimed for this method. Since the gelatin tolerates dehydration poorly, sections should be mounted in glycerol gelatin.

**Agar.** I have occasionally infiltrated tissues from water in melted 2% agar at 55–60°C, for 2–4 hours. This mass becomes quite stiff on cooling and may be cut on the freezing microtome. Its value is for holding exudates and friable tissues in place. I have used it for the study of fat content of loose pulmonary alveolar exudates. It does not stain appreciably with the usual dyes.

Friedland (*Am. J. Clin. Path.* 21:797, 1951) accelerates this procedure by transferring tissues previously fixed briefly in boiling formalin to melted agar, and boiling gently for one minute. The agar-infiltrated tissue is then sectioned on the freezing microtome. This variant is recommended for friable and partially necrotic tissues, to improve coherence.

**Paraffin.** In order to infiltrate tissues with paraffin it is necessary to displace from them the aqueous or alcoholic fixing fluids, or the water or alcohol used to wash these out, and to replace with a fluid miscible with paraffin. This is usually accomplished by diffusion of the containing fluid out of the tissue block into a surrounding bath of another fluid, which in turn diffuses into and replaces the original fluid content.

Since most water-miscible fluids are not paraffin solvents, it is usually necessary to dehydrate first with a water miscible fluid, and then to replace the dehydrating agent with a paraffin solvent which is at the same time miscible with the dehydrating agent used.

One fluid—1,4-diethylene dioxide or dioxane—is freely miscible with water on the one hand and with melted paraffin on the other. Dioxane is becoming increasingly popular, especially in hospital laboratories. Lee (10th ed.) warns against the cumulative toxicity of this compound and speaks of a lack of warning odor. Actually there is a distinctive odor, but this is no longer noticed after a short time. This text recommends the interposition of a bath of equal volumes of dioxane and paraffin at 56°C after dehydration. Mallory recommended the schedule of Graupner and Weisberger, which included successively three changes of dioxane and three of melted paraffin.

The greatest disadvantage of dioxane is the very low tolerance of its mixtures with paraffin solvents or with paraffin for small amounts of water (Table 5). A slight excess of water carried into the dioxane-paraffin bath will occasion stratification with a lower dioxane-water layer and an upper dioxane-paraffin layer. To combat this water intolerance Tannenberg (*Am.
J. Clin. Path. 19:1061, 1949) adopts an automatic siphonage chamber into which dioxane drips at a fairly rapid rate (3.75 liters per hour) and which drains down to a level just above the specimens by means of a siphon as often as the fluid level rises to the top of the siphon tube, 2–3 cm. above the tissue. Adequate dehydration of blocks 2 mm. thick in two hours is claimed, and of curettings in one hour. The used dioxane is again dehydrated with anhydrous CaCl₂ or CaO for 18–22 hours before using it again. The re-use of the dioxane is required from its high initial cost (currently about seven to eight times that of tax-free 100% alcohol) and from the large volume used in this procedure.

Tannenberg then transferred tissues directly to melted paraffin (two changes of 30–60 minutes each). He suggested further shortening of the total time required by use of vacuum infiltration (p. 60). The boiling point of 1,4-dioxane is 101.1°C and its volatilization at reduced pressure would be much slower than that of some of the more volatile solvents.

The traditional dehydrating agent is ethyl alcohol. It is usual to transfer tissues from water into 70% alcohol, thence to 80% or 85%, then to 95% and to 100% (absolute) alcohol. Two changes daily may be made, ending with at least two or three changes covering 2–4 hours in 100% alcohol. Some omit the first step and start directly with 80% alcohol.

For cytologic work and for dehydration of embryos, eyes, and other large or delicate objects, longer intervals in each grade of alcohol and a greater number of gradations are used, and it is advisable to start as low as 50% or 60% alcohol.

By use of continued mechanical agitation and multiple changes of each grade of alcohol, and by cutting thin blocks for embedding, the intervals may be cut down to two or three hours in each grade of alcohol.

Isopropyl alcohol, 99%, is probably the best all-round substitute for ethyl alcohol, and is now procurable at a quite modest price even when compared with tax-free ethyl alcohol. The tolerance of its mixtures with various paraffin solvents for small amounts of water at least equals on the average that of corresponding ethyl alcohol mixtures (Table 5). As seen in the same table the tolerance of corresponding acetone and dioxane mixtures for water is quite low, that of methanol mixtures intermediate.

According to Hauser (Mikrosk. 7:208, 1952), tissues may be infiltrated in paraffin directly from isopropanol or from a warm (50°C) mixture of paraffin and isopropanol.

Nevertheless, in spite of its avidity for atmospheric water vapor and the low tolerance of its fat-solvent mixtures for water, acetone is often a very effective dehydrating agent when rapidity of action is desirable. This can be obtained for about $2.40 a gallon (53¢ a liter) in technical grade. No fewer than four changes of acetone of 40 minutes each should be used as a matter of routine. Of these only the fourth need be fresh acetone. For the third change use the acetone which has previously been used once for the last
**Table 5**

**Percentage of Added Water Required to Render Turbid Equal-Volume Mixtures of Dehydrating and Clearing Agents**

<table>
<thead>
<tr>
<th>Dehydrant</th>
<th>Benzene</th>
<th>Xylene</th>
<th>Chloroform</th>
<th>Carbon tetrachloride</th>
<th>Petroleum ether B</th>
<th>100-octane gasoline</th>
<th>Methyl salicylate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol, absolute</td>
<td>6.5–7</td>
<td>3–3.5</td>
<td>17.5–18</td>
<td>4.5–5</td>
<td>1</td>
<td>immisc.</td>
<td>6.25</td>
</tr>
<tr>
<td>Ethanol, absolute</td>
<td>12–14</td>
<td>7–8</td>
<td>13–14</td>
<td>10–11</td>
<td>4.5</td>
<td>3.5–4</td>
<td>13.25</td>
</tr>
<tr>
<td>Isopropanol, 99%</td>
<td>9.5–10</td>
<td>10</td>
<td>7–7.5</td>
<td>7.5–8.5</td>
<td>11–11.5</td>
<td>8.5–9</td>
<td>17.</td>
</tr>
<tr>
<td>Acetone, technical</td>
<td>1.75–3</td>
<td>1.5–2</td>
<td>2–2.2</td>
<td>1.5–1.6</td>
<td>0.5–1</td>
<td>1.5–1.6</td>
<td>4.75</td>
</tr>
<tr>
<td>Dioxane, pure</td>
<td>1.5–2</td>
<td>1–1.5</td>
<td>1.5–2</td>
<td>1–1.5</td>
<td>1.5</td>
<td>1–1.5</td>
<td>3.75</td>
</tr>
</tbody>
</table>
change, for the second use that previously used twice, and for the first that used for three previous changes. The acetone used four times can be saved for cleaning paint brushes or for redistillation, or it may be discarded.

Even this process may be expedited by using four changes of fresh acetone for 20 minutes each.

Following dehydration with alcohol or acetone, blocks should be transferred to a paraffin solvent which is miscible with the dehydrating agent. An intermediate bath composed of equal parts of the dehydrating agent and of the paraffin solvent or even two such baths, the first of a 2:1, the second of a 1:2 mixture, are to be recommended, particularly when working in very humid atmospheres.

**Paraffin Solvents**

The best paraffin solvents are benzene, toluene, xylene, petroleum ether, carbon bisulfide, chloroform, carbon tetrachloride, and cedar oil. All of these are miscible with paraffin at 56° C. The disagreeable odor and the toxicity of the fumes of carbon bisulfide usually operate to exclude it as a paraffin solvent, although it is said to give excellent results (Lee).

As far as I can determine on direct comparison of blocks of the same tissues, chloroform (B. P. 61° C., vapor pressure 160–248 mm. at 20°–30° C.) has no definite superiority over the far cheaper and probably less toxic carbon tetrachloride (B. P. 77° C., vapor pressure 91–143 mm. at 20°–30° C.). (The lethal dose of carbon tetrachloride is perhaps double that of chloroform in mice.)

Benzene also seems at least as good as its more expensive and less volatile homologs toluene and xylene, on the same basis of direct comparison.

For many years I used with great satisfaction a high-test gasoline, designated *aviation gasoline, lead-free*, which is essentially a petroleum ether. (Caution: lead tetraethyl is dangerously toxic.)

From the data in Table 5, methyl salicylate would seem to have very interesting possibilities as a clearing agent with a relatively high tolerance for water. Its rather powerful odor deters from routine use of it, but it should be useful when anhydrous alcohol is unavailable.

Of the fluids cited, cedar oil, gasoline, and petroleum ethers occasion the least hardening of tissues. Overnight immersion in gasoline and petroleum ether does not render tissues brittle. Chloroform and xylene appear to make tissues more brittle, but this does not interfere especially with sectioning. Thin cedar oil is an excellent dealcoholization or clearing agent and is to be highly recommended for such objects as human skin, uterus, thick masses of smooth muscle and tendon and the like, since it appears to improve their consistency for cutting. It requires multiple changes of paraffin to remove it from the tissue. I usually prefer to interpose two or three baths of 20–40 minutes each in gasoline, xylene, or benzene. Tissues may be left in cedar oil for months without harm. After use for clearing for some time, cedar oil may
be restored by filtering and then heating to 60° C. in vacuo for 30–60 minutes to remove alcohol, acetone, and water.

Occasionally, after use for clearing of material fixed in acetic-alcohol fixatives, cedar oil may crystallize into a firm mass melting at around 35° C. A small quantity of this altered oil will cause a whole bottleful to crystallize. Heating to 200° C. restores the normal behavior of the oil.

Popham (Stain Technol. 25:112, 1950) recommends, instead of heating, the addition of a little (1:80) xylene to reliquify the altered cedar oil.

Except for cedar oil, I recommend at least two changes of the paraffin solvent between the dehydration and the paraffin infiltration.

The use of saturated solutions of paraffin in the solvent has been recommended as an intermediate step between clearing and paraffin infiltration. It is unnecessary for routine work, and I have not used it for years. It may be necessary, however, for such difficult objects as parasitic worms, fleas, and ticks, on account of their chitinous exoskeleton.

Paraffin infiltration is done in an oven regulated to a temperature a few degrees above the melting point of the paraffin used. Ordinarily paraffin is quite satisfactory as obtained from the refiners. It may be obtained in approximately 5 kg. blocks directly from the refiners in lots of eight blocks at a materially lower cost than when bought in pound blocks from biological supply houses.

Ribboning consistency is sometimes improved by addition of 10% or 20% beeswax or by addition of 3–5% Halowax. Soft, or 52°, paraffin will dissolve 15% by weight (but not 20%) of this Halowax (M.P. 115–125° C.) with a resultant fall of the conglomeration point to about 50.5° C., and quite pronounced hardening of the paraffin. Paraffins of even lower melting point (40–42° C.) are available, and may be similarly hardened. Beeswax makes the mixture more sticky than pure paraffin so that sections stick together better.

Paraffins recommended for sectioning range in melting point from 45° to over 60° C. For our laboratory conditions I find 45° and 50° paraffin too soft. Some recommend a mixture of 50° and 55° paraffin and some prescribe infiltration first by a soft, then by a hard paraffin. To my mind this practice simply prolongs the heating period unnecessarily. For ordinary work I find a paraffin of 55–56° melting point satisfactory with laboratory temperature ranging from about 22° C. in winter to 30–35° C. in summer.

The usual practice is to use three or four changes of melted paraffin of 30–60 minutes each, in order to infiltrate tissue thoroughly and rid it of traces of the solvent which might unduly soften the paraffin within the tissue.

Such prolonged heating inevitably shrinks tissues to a considerable extent. For some 15 years I have used a vacuum chamber within the oven for infiltration in vacuo. With the use of a volatile solvent, 10 (or better, 15) minutes' infiltration at an absolute pressure of 25 mm. mercury is adequate, and pressures as high as 175–200 mm. can be used (≈ -585 to -560 mm. or
23–22 in. of vacuum). Vacuum infiltration furthermore removes air and gases from cavities within the tissues, notably the pulmonary alveoli, and permits their filling with paraffin.

Dr. Peers now uses a three-day Technicon schedule for whole human brain stems (Table 7, p. 64, schedule G); also for cerebellum, large blocks of cerebral cortex, whole hemispheres of dog, cat, and monkey brains, and the like. Blocks are cut transversely about 6 mm. thick after fixation in 10% formalin for some days or even weeks. They are then hardened for three days in 2.5% aqueous potassium bichromate solution and washed six hours in running water. On the Technicon they then pass through 12 six-hour baths for dehydration, clearing, and paraffin infiltration. Sections are floated out on 70% alcohol warmed to 40–45° C., dipped out on large slides, immediately blotted down with hard, smooth filter paper, and then dried 1–18 hours at 40–45° C.

I have included also in Table 7 (p. 64) Technicon schedules for fresh and formalin-fixed surgical tissues (A, B, C) and for autopsy tissues (D, E, F). Schedules D, E, and F are designed to use the same time-disk and solutions. Tissues fixed in chromate fixatives (including Zenker variants) are started at Step 1 on the schedule; mercuric chloride tissues, which are normally transferred directly to 70% or 80% alcohol, start at Step 3; and tissues fixed in acetic alcohol fluids, such as Carnoy, acetic alcohol formalin, etc., start at Step 5. The calcium carbonate in the 95% alcohol of Step 5 is intended to neutralize acetic acid which is carried over from the fixing fluid.

In working with Zenker variants it is necessary to use plastic capsules for tissue and to have the metal parts completely protected by first coating them with smoking hot paraffin. It is probably best to put tissues into the Technicon only after the fixation period is completed.

Enzyme demonstration in tissues is materially facilitated by the use of brief vacuum infiltration in paraffin. Substitution for the 56° paraffin of the 15% Halowax, 52° paraffin mixture (p. 60), which remains liquid at 51° C., and infiltration at 55° C. instead of 60° C. further improves demonstration. In this process petroleum ether is recommended as the paraffin solvent. (See also p. 203.)

Perhaps Steedman's ester wax (p. 68), which melts at 46–48° C., with vacuum infiltration directly from an acetone mixture at not over 50° C., would serve even better for this purpose.

Schedule H on Table 7 (p. 64) is designed for Technicon handling of a considerable number of blocks for alkaline phosphatase demonstration. Gasoline is substituted for the usually preferable petroleum ether, because of its much lower evaporation losses and its higher flash point. With vacuum infiltration at 10 mm. pressure the solvent-evaporation time is almost as brief as that of petroleum ether.

I must warn at this point that tissues for enzyme demonstration must not be processed in the Technicon with or following tissues fixed in other possibly
Table 6
SCHEDULES* FOR DEHYDRATION, CLEARING, AND PARAFFIN INFILTRATION

<table>
<thead>
<tr>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>VIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td>water</td>
<td>water</td>
<td>water</td>
<td>water</td>
<td>water</td>
<td>water</td>
<td>Carnoy's or similar fluid</td>
</tr>
<tr>
<td>70% alcohol</td>
<td>16 hr.</td>
<td>acetone (used 3x)</td>
<td>40 min.</td>
<td>acetone (fresh)</td>
<td>20 min.</td>
<td>acetone (used)</td>
<td>40 min.</td>
</tr>
<tr>
<td>05% alcohol</td>
<td>80% alcohol</td>
<td>acetone (used 2x)</td>
<td>40 min.</td>
<td>acetone (fresh)</td>
<td>20 min.</td>
<td>acetone (used)</td>
<td>40 min.</td>
</tr>
<tr>
<td>95% alcohol</td>
<td>95% alcohol</td>
<td>acetone (used 1x)</td>
<td>40 min.</td>
<td>acetone (fresh)</td>
<td>20 min.</td>
<td>acetone (used)</td>
<td>40 min.</td>
</tr>
<tr>
<td>100% alcohol</td>
<td>100% alcohol</td>
<td>acetone (fresh)</td>
<td>40 min.</td>
<td>acetone (fresh)</td>
<td>20 min.</td>
<td>acetone (fresh)</td>
<td>40 min.</td>
</tr>
<tr>
<td>100% alcohol</td>
<td>1 hr.</td>
<td>acetone + benzene</td>
<td>40 min.</td>
<td>acetone + benzene</td>
<td>40 min.</td>
<td>cedar oil</td>
<td>16 hr.</td>
</tr>
<tr>
<td>benzene</td>
<td>30 min.</td>
<td>benzene</td>
<td>40 min.</td>
<td>benzene</td>
<td>20 min.</td>
<td>benzene</td>
<td>30 min.</td>
</tr>
<tr>
<td>benzene</td>
<td>30 min.</td>
<td>benzene</td>
<td>40 min.</td>
<td>benzene</td>
<td>20 min.</td>
<td>benzene</td>
<td>30 min.</td>
</tr>
<tr>
<td>55° paraffin</td>
<td>30 min.</td>
<td>O R</td>
<td>paraffin (55°)</td>
<td>30 min.</td>
<td>in vacuo at</td>
<td>25 mm. Hg</td>
<td>15 min.</td>
</tr>
<tr>
<td>55° paraffin</td>
<td>30 min.</td>
<td>paraffin (55°)</td>
<td>30 min.</td>
<td>paraffin</td>
<td>30 min.</td>
<td>paraffin</td>
<td>15 min.</td>
</tr>
</tbody>
</table>

*M Schedules I and III are routine alcohol and acetone schedules; II and IV accelerated schedules; V is a special schedule for very fatty tissues; VI a cedar oil schedule for skin, muscle, uterus, and other difficult objects; VII is a dioxane schedule; and VIII a very fast schedule for fresh unfixed tissue.

Labels written on paper with India ink and dipped in smoking hot paraffin should be carried through the solvents with the tissue and finally attached to the paraffin block.

†Other paraffin solvents, such as carbon tetrachloride, gasoline, petroleum ether, chloroform, toluene, and the like may be substituted for benzene.
injurious fixatives. Traces of mercuric chloride left in the Technicon baths from a previous lot of Zenker fixed tissues, or diffusing from simultaneously included blocks, can completely inactivate the tissue enzymes.

Incomplete dehydration is a common cause of the shrinkage and hardening of tissues within paraffin blocks after they have been cut from and put away. It is to be avoided by the use of sufficient changes of pure dehydrating agent to dehydrate the tissues completely before clearing and paraffin infiltration.

Imbedding. During the process of imbedding the tissue blocks must be oriented so that sections will be cut in the desired plane. Generally it is convenient to place the surface of the block from which sections are desired next to and parallel to the bottom of the imbedding container.

A convenient imbedding container may be made from a 5 x 7.5 cm. (2 x 3 in.) oblong of fairly heavy (2-3 mm. thick) sheet metal and two L-shaped cross sections of angle iron about 2-3 cm. high. Angle iron may be obtained in various sizes from 3 to 5 or more cm. arm length, and cut with a hacksaw into desired lengths. The two L-shaped pieces are laid on the metal plate so as to enclose a rectangular or square space. This is then partly filled with paraffin and the tissue placed in it in the desired position and pressed firmly against the bottom. The container is then filled with melted paraffin and allowed to cool until reasonably firm. Then it is further chilled with cold water and the metal blocks are removed by dropping sharply on a table. Usually the three metal pieces and the paraffin block separate cleanly at this point.

Trimming of Blocks and Preparation for Sectioning. The upper curved surface which is to form the back of the block is then trimmed off flat with a fairly stout knife. The four sides are then cut down square to within 1 or 2 mm. of the edge of the tissue block on the cutting surface and sloping outwardly toward the back, so as to form a truncated pyramid. When ribbons are to be cut it is convenient to cut narrow triangular wedges from the four corners of the block, so that in the ribbon formed by the coherent sections V-shaped notches are present on both sides between the individual sections. When the block is trimmed the number should be affixed to one side with a hot spatula.

For attachment to the microtome it is necessary to fasten the paraffin blocks to wooden or fiber blocks or to metal object holders. The last are the most convenient, as they may simply be heated to above the melting point of paraffin and then pressed firmly against the back of the paraffin block. The block and object holder are then immersed in cold water. The object holder should be hot enough to melt its way 1-2 mm. into the back of the block, but not further.

For attachment to wooden or fiber blocks, melt the back surface of the block with a hot metal spatula and press it immediately against the wood or fiber and immerse at once in cold water.
<table>
<thead>
<tr>
<th>Surgical Schedules</th>
<th>Autopsy Tissue Schedules</th>
<th>Peer's Brain Schedule</th>
<th>Alkaline Phosphatase Schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh Tissues</td>
<td>Prefixed Tissues</td>
<td>48 hr. formalin</td>
<td>24 hr. sublimate formalin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>chromate fixatives</td>
<td>acetic fixatives</td>
</tr>
<tr>
<td>Alcohollic</td>
<td>Zenker's fluid</td>
<td>80% alc. 1 hr.</td>
<td>80% alc. 2 hr.</td>
</tr>
<tr>
<td>formalin</td>
<td>8 hr.</td>
<td>water 2 hr.</td>
<td>water 2 hr.</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>80% alc. 1 hr.</td>
<td>80% alc. 2 hr.</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>water 2 hr.</td>
<td>80% alc. 2 hr.</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>95% alc. 1 hr.</td>
<td>80% alc. 16 hr.</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>80% alc. 16 hr.</td>
<td>95% alc. 6 hr.</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>80% alc. 16 hr.</td>
<td>80% alc. 1 hr.</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>80% alc. 1 hr.</td>
<td>80% alc. 1 hr.</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>80% alc. 1 hr.</td>
<td>80% alc. 1 hr.</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>80% alc. 1 hr.</td>
<td>80% alc. 1 hr.</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>80% alc. 1 hr.</td>
<td>80% alc. 1 hr.</td>
</tr>
<tr>
<td></td>
<td>J</td>
<td>80% alc. 1 hr.</td>
<td>80% alc. 1 hr.</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>80% alc. 1 hr.</td>
<td>80% alc. 1 hr.</td>
</tr>
<tr>
<td></td>
<td>acetone</td>
<td>methylsalicylate</td>
<td>xylene</td>
</tr>
<tr>
<td>-----</td>
<td>---------</td>
<td>------------------</td>
<td>--------</td>
</tr>
<tr>
<td>8.</td>
<td>1 hr.</td>
<td>xylene 1 hr.</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>1 hr.</td>
<td>xylene 1 hr.</td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>1 hr.</td>
<td>xylene 2 hr.</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>1 1/2 hr.</td>
<td>paraffin 2 hr.</td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>1 1/2 hr.</td>
<td>paraffin 2 hr.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18 hr.</td>
<td>22 hr.</td>
<td></td>
</tr>
<tr>
<td>IMBED</td>
<td>IMBED</td>
<td>IMBED</td>
<td>IMBED</td>
</tr>
</tbody>
</table>
**Sectioning.** The wooden or fiber block or metal object holder is then inserted into the object carrier of the microtome and clamped firmly in place. The object carrier is then oriented so that the surface of the block is parallel to the knife edge. While adjusting the microtome, its motion should be locked to avoid accidental cuts either to the specimen or to the hands of the operator.

One then cuts off paraffin in rather thick sections by operating the microtome in the usual manner, until the tissue is reached. It may be necessary to readjust the orientation of the block at this point, so as to obtain complete sections in the desired plane.

Sections may then be removed individually by a small dampened scalpel; or by holding the end section with the same implement at a slight tension, a number of sections may be cut in series so as to form a ribbon. This is necessary when serial sections are required. Convenient lengths of ribbon are transferred to a smooth board or cardboard surface and cut into segments of the desired number of sections with a sharp knife. They adhere to the cardboard along the line of cut and are loosened from it by passing the edge of the knife obliquely along the line of adhesion. Sections are then lifted singly or in strips onto the surface of a pan of water heated to about 38° C., or onto water on a 25 x 75 mm. glass slide which is then warmed on a metal plate. In preparation of serial sections, a number of rows of sections may thus be arranged in proper order on a single slide. The warming causes the wrinkles in the section to flatten out, and the whole section or ribbon segment elongates during the process. Persistent wrinkles or rolls may be flattened out by stretching the section with a pair of small knives or needles. From the pan the sections are then floated onto clean glass slides and removed from the water, holding one edge of the section in place with a needle or knife. If floated on slides, the excess water is drained off to one side, holding the section in place as before. When preparing serial sections of several rows to a slide, it is well to place a length of blank paraffin ribbon at each margin, so that sections will not lie under the edge of the cover glass when stained.

It is preferable to use fresh distilled water for floating out sections. If rapid drying is required one may use 30% ethyl alcohol instead.

Many workers prefer to spread a minimal quantity of Mayer's albumen glycerol fixative on the surface of the slide before floating the section onto it. A very small drop is placed on one end of the slide, the previously thoroughly cleansed ball of the little finger is used to spread it over the whole surface of the slide, and any excess is then wiped off on the hypothenar portion (little finger side) of the palm of the hand.

Traditionally (Ehrlich's *Encyklopädie*) Mayer's albumen glycerol is made by thoroughly mixing one volume of the whites of perfectly fresh eggs with an equal volume of pure glycerol. The mixture is then filtered through absorbent cotton or relatively coarse filter paper at 55–58° C. (paraffin-oven temperature). A small lump of thymol or camphor is added to prevent growth of molds.
SECTIONING

We (Stain Technol. 20:99, 1945) have successfully substituted a 5% solution of dried egg white made by shaking gently at intervals for one day in 0.5% sodium chloride solution. Avoid frothing when shaking, and filter on a Buchner funnel with vacuum. At least 90–95% of the solution should be recovered as a clear filtrate. To 50 cc. of this filtrate add 50 cc. glycerol and 0.5 cc. 1:10,000 merthiolate (sodium ethylmercurithiosalicylate).

Since 2 or 3 cc. distilled water is used to float out a section on a slide thinly coated with this fixative, and the constituents of the fixative are readily soluble in water, it is difficult to imagine that any of the "fixative" remains on the slide to fasten a section to it. It seems probable instead that it functions as a surface tension depressant, and thereby secures closer capillary adhesion of the section to the slide.

Ordinarily such paraffin sections are dried overnight before deparaffinizing and staining. With the aid of gentle heat and an air current they may often be dried sufficiently for staining in an hour or so. By using dilute alcohol for floating the sections onto the slides, and heat for drying, I have stained sections without undue losses in 30 minutes.

Hard paraffin is more apt to curl when sections are cut than soft; but soft paraffin permits more lateral compression of tissues along the path of the knife. Paraffin blocks may be made harder for cutting by preliminary immersion in ice water, and it may be advisable to chill the microtome knife as well. An electric light bulb near the object carrier of the microtome tends to make the blocks softer.

Sometimes during ribbon sectioning the ribbon acquires a charge of static electricity which causes it to move about violently and to adhere to various adjacent objects. Breathing on the ribbon and block surface may remedy this difficulty.

Tissues containing much blood or yolk (and certain other substances, including bone and cartilage) are apt to be quite brittle and shatter under the microtome knife instead of yielding coherent sections. Painting the surface of the block before cutting each section with a 0.5% solution of collodion in absolute alcohol and ether or acetone will prevent their crumbling. The sections smooth out better if floated on 80% alcohol, but if the collodion film is thin enough this is often not necessary.

Lendrum (Stain Technol. 19:143, 1944) recommends that when tough and hard tissues fail to give satisfactory paraffin sections, the paraffin block be soaked face down in a mixture of 9 parts glycerol and 1 part aniline for 1–3 days. This probably serves best with incompletely dehydrated tissues, as it seems to make little difference when care has been taken to completely dehydrate before paraffin infiltration.

After sectioning from a paraffin block I have found it good practice to dip the cut surface briefly into rather hot (70–80° C.) paraffin before putting it away. This helps to prevent drying out and shrinkage of perhaps imperfectly dehydrated tissues. With adequate dehydration this practice should not be
necessary, but it takes little time and also helps protect tissue from roaches. I have seen unprotected tissue completely eaten out of the paraffin blocks.

Paraffin blocks should be stored in a reasonably cool place. I have found small pasteboard boxes containing the blocks of one or more consecutive cases convenient. These are packed consecutively in drawers of slightly greater depth than the greatest diameter of the boxes, standing on end, with the exposed end bearing the case number. The drawer bears the first and last case numbers on its exposed end.

Ester wax. Steedman’s ester wax (Quart. J. Microscop. Sci. 88:123, 1947) is highly recommended by some workers as an imbedding medium of lower melting point which has sectioning and ribboning properties similar to those of paraffin. It is now commercially available as 250 Gm. tablets.* For the formula and details of the manufacture see the original reference. It is a translucent waxlike substance melting at 46-48° C. Section and ribboning ranges of 2-20 μ and 2-15 μ are claimed. The wax is miscible with ethanol at 43° C., with n-butanol or isopropanol at 38° C., with Cellosolve at 38° C., with glycol monobutyl ether at 34° C., with acetone at 33° C., and with anilin at 18° C., and is soluble in the usual paraffin solvents. Cellosolve, dioxane, and cedar oil are especially recommended. A mixture of solvent and ester wax should be used for infiltration between the solvent and the wax. Infiltration in the wax requires three changes and apparently somewhat longer intervals than with paraffin. The temperature was not specified by Steedman. He mentioned a 24 hour infiltration time for cat pylorus and 60 hours for a brain block 10 mm. thick.

For imbedding, the ester wax should be heated to about 70° C. and poured into cold metal molds. The tissue is then placed close to one angle at the bottom and the block chilled rapidly.

The wax is much harder than paraffin, resembling celloidin in compressibility. Sections should be cut slowly on a heavy, rigidly secured microtome knife. Sections are floated on water at 35-40° C. to be flattened, and slides are dried at the same temperature. If they are dried at room temperature, the water taken up during flotation causes crumpling of the sections. After dewaxing in xylene, sections are stained as is usual for paraffin sections. For staining schedules for undewaxed sections see the original reference.

Celloidin and Low Viscosity Nitrocellulose Imbedding. The lower viscosity of low viscosity nitrocellulose permits more rapid infiltration with solutions of higher concentration. Consequently, harder blocks may be obtained in less time and thinner sections can be cut.

In pathology this method is utilized principally for eyes and for the study of bones and their surrounding tissues in situ, and when it is essential to avoid shrinkage and the creation of artificial spaces. For such tissues a rather slow dehydration and a gradual infiltration are required.

The following schedule can be recommended for eyes. After 10% formalin fixation for at least 48 hours, dehydrate the eye with successive 24 hour baths of 35%, 50%, 65%, 80% alcohol. Open the eye by cutting off the upper and lower portions of the posterior chamber with two cuts passing horizontally respectively through the upper and lower margin of the anterior chamber, and including the nerve head and the fovea centralis between them. Return it to 80% alcohol for 3–4 hours; then give it a 24 hour bath in 95% alcohol; then 24 hours in two changes of 100% alcohol, and a six hour bath in equal volumes of 100% alcohol and ether. Infiltrate 5–7 days in 10% nitrocellulose in 100% alcohol and ether, and a similar period in 20% nitrocellulose, in a tightly stoppered container.

Imbed in a fairly deep, cylindrical glass dish in which the 20% nitrocellulose, sufficient to cover the eye, reaches about halfway up the side. Place under a bell jar and examine daily. When the surface is solid and the deeper portion still somewhat soft, flood with chloroform and let stand 16–24 hours. Pour off the chloroform and let dry until the solid nitrocellulose block can easily be dislodged from the dish. Trim the block into octagonal form, leaving 1–2 mm. of nitrocellulose on all sides. Dip the back surface of the block into 20% nitrocellulose and mount it on a fiber or wooden block with a scored or incised surface. Fasten an identifying number to the side of the block with a little 20% nitrocellulose. Let dry a few minutes and return the mounted block to chloroform for several hours. Transfer through successive 24 hour baths of 3:1 and 1:1 chloroform and cedar oil mixtures to pure cedar oil. Keep in cedar oil at least one day before cutting.

Drain, and fasten the fiber or wooden block in the object clamp of the celloidin microtome. Cut sections and store them in 80% alcohol.

The technic for decalcified bone is similar. Sections have been cut as thin as 5μ by this technic. Ordinarily 10μ sections will serve.

After susa fixation for 12 hours, transfer eyes directly to 95% alcohol. Treat with iodine until the alcohol is no longer decolorized by adding tincture drop by drop with constant agitation. Let the brown solution stand 30 minutes. Wash out the excess iodine with three or four changes of 95% alcohol. Let stand overnight in 95% alcohol. Open the eye as described above. Complete dehydration, infiltration, and imbedding are as above. When sectioning is completed, cut the nitrocellulose or celloidin blocks off the fiber or wooden block and store them in cedar oil or mineral oil. The latter is cheaper and does not dry or evaporate.

Chesterman and Leach (Quart. J. Microscop. Sci. 90:431, 1949) used three baths of low viscosity nitrocellulose, at 5%, 10%, and 20% in an ether-alcohol mixture, allowing 3–5, 1–2, and 1–5 days respectively. They then
imbedded in a solution of 140 Gm. “industrial nitrocellulose dampened with 7:3 butyl alcohol. HX.30/50,” in 210 cc. 100% alcohol and 250 cc. ether, adding 5 cc. tricresyl phosphate as a plasticizer to prevent cracking and separation from the tissue blocks. This solution is designated “20%.” Apparently the nitrocellulose contains 98 Gm. (or 70%) of the principal ingredient; and the 20% is by weight of the final mixture. Blocks are allowed to set in a loosely closed space for 1–3 days until there is formed a moderately stiff but still flexible gel. They are then hardened for 1–3 days in two or more changes of 70% alcohol. Such blocks become quite hard and may be cut dry on a celloidin or paraffin microtome. Sections of 15\(\mu\) from half a cat brain, or 5–7\(\mu\) from blocks 5 mm. square are reported.

**Rapid Nitrocellulose Imbedding.** Koneff and Lyons *(Stain Technol. 12:57, 1937)* recommend the following rapid schedule for low viscosity nitrocellulose imbedding. I have not tried this method.

1. Fix one hour at 56° C. or 2–5 hours at 37° C. in 10% neutral formaldehyde, Bouin, susa, or Carnoy II (which last is a chloroform formula, probably that given on p. 36, but not here more closely defined). Use screw-capped bottles.

2. Wash in several one-hour changes of distilled water, except Carnoy material, which is extracted two hours at 56° C. in 3–5 changes of 100% alcohol.

3. Dehydrate (except Carnoy material) in two thirty-minute changes each of 70%, 80%, 95%, and 100% alcohol at 56° C. Use iodized 70% alcohol for the first step if fixation was in susa.

4. 100% alcohol and ether one hour at 56° C.

5. Infiltrate one hour at 56° C. in 10 Gm. low viscosity nitrocellulose dissolved in 50 cc. 100% alcohol and 50 cc. ether.

6. Infiltrate overnight in 25 Gm. nitrocellulose in 45 cc. 100% alcohol and 55 cc. ether.

7. Infiltrate 2–3 hours at 56° C. in 50 Gm. low viscosity nitrocellulose in 40 cc. 100% alcohol and 60 cc. ether.

8. Imbed on a fiber block in 50% nitrocellulose as usual.

9. Harden the blocks one hour in two changes of chloroform.

10. Transfer to 80% alcohol, three changes, 20 minutes each.
11. Section the blocks while wet with 80% alcohol, and store blocks and sections in the same.

I suggest the cedar oil procedure (given on p. 69) from "Imbed" on as an alternative, since this method appears to give thinner sections than sectioning from alcohol.

For squirrel and lizard eyes Wall (Stain Technol. 13:69, 1938) recommends fixation by immersion in Kolmer's fluid for 24 hours; followed by successive 6 to 18 hour baths in water, 35%, 50%, 70%, and 95% alcohols; and infiltration by a hot nitrocellulose process (p. 70). For demonstration of the ellipsoids particularly, Heidenhain's iron hematoxylin and phloxine are recommended.

For thinner sections of retina, Wall recommends removal of the lens before imbedding. In any case it is necessary to cut a window through the sclera with a sharp razor blade before nitrocellulose infiltration.

Nitrocellulose and paraffin imbedding. For difficult and fragile objects a combined celloidin or low viscosity nitrocellulose infiltration and paraffin infiltration and imbedding process has been recommended. One infiltrates with the nitrocellulose as usual, clears with chloroform and cedar oil as above, and carries the blocks thence through two changes of benzene or chloroform into paraffin as usual.

I have used this process with a variable degree of success. The sections do not flatten well when floated on water, and are prone to curl and separate from the slides. They flatten better if floated on clean slides with 95% alcohol. This softens but does not dissolve the nitrocellulose. If sections still curl, it may be necessary to soften them with ether vapor, blot down with filter paper, and soak in 0.5–1% collodion before deparaffinizing. In any case deparaffinize the section and simultaneously harden the nitrocellulose in chloroform, transferring thence directly to 95% alcohol.

For sectioning bones without decalcification, Arnold (Science 114:178, 1951) dehydrates in acetone and infiltrates in a mixture of 55 Gm. air dried ½-cosec nitrocellulose, 45 Gm. diamyl or dibutyl phthalate and 65 to 100 Gm. anhydrous acetone for several days. The acetone dehydrated tissues are placed in a separatory funnel and the air is evacuated down to 10 mm. mercury with a vacuum pump attached to the upper end of the funnel. The lower end is then immersed in the nitrocellulose solution and the stopcock is opened slowly to allow the nitrocellulose to enter and cover the specimens. When five or ten times the volume of the tissue has entered, the vacuum line is opened and the pressure is allowed to rise to atmospheric pressure. Tissues are then transferred to closed containers and infiltration is continued at 58° C. under three atmospheres of pressure. (So long as the pressure is sufficient to prevent evaporation of the solvent, it is difficult to understand why the author prescribed that it be so high.)

Imbedding is then completed by filling a paper container to 4 or 5 cm.
height with the nitrocellulose-plastic solution, orienting the specimen in the bottom, and allowing evaporation at 20° C. to a height of about 1 cm. to produce a puttylike consistency. Then cut out the specimen, reorient and cement it to a regular fiber block with the same solution. Let it dry for a week at 25° C. until quite hard. Cut the hard tissues at 5-8 μ, wetting the block and sections in a 1% aqueous Aerosol solution. Use a heavy sliding microtome and a specially hardened knife blade.

For cutting thin sections for electron microscopy, Baker and Warren (Nature 169:421, 1952) recommend infiltration (after the usual dehydration and clearing) with a 1:2 mixture of white beeswax with Harleco synthetic resin. They first infiltrate blocks of 200μ thickness for one hour in molten beeswax (M.P. 61–62°), then for one hour in the mixture. The thin sections are caught on water in a trough affixed to the microtome knife. A 0.2% collodion solution is used to form the film for placing sections on the electron microscope screens. The imbedding medium may be removed by treatment with carbon tetrachloride to dissolve the beeswax, followed by benzene or toluene to remove the resin.
Chapter 5

Stains and Staining

The examination of unstained material often has considerable value. However, it is usually restricted to the study of surviving cells and tissues, examinations with polarized light, fluorescence microscopy, phase microscopy, ultraviolet photography, microincineration procedures, and the study of pigments.

The purpose of staining is to make more evident various tissue and cell constituents and extrinsic materials. Some stains are strictly solution phenomena, such as the staining of neutral fats with oil soluble dyes; others are strictly chemical, such as the formation of Prussian blue by hemosiderin in the presence of acid and ferrocyanide; others depend on the presence of mordants, as hematoxylin depends on the presence of ferric or aluminum or other metallic salts; in others an adsorption phenomenon may be responsible.

Generally, sequences or combinations of stains are employed, to render two or more tissue elements conspicuous in contrasting colors. No process has been devised which will show all tissue elements to the best advantage; but procedures exist which will stain differentially as many as four elements, such as the elastin-collagen procedures which show cell nuclei and cytoplasmic structures as well as elastic and collagen fibers. Some procedures are best adapted to the general study of cell nuclei and cytoplasm, and these are often employed as general oversight stains for primary examination of tissues.

Certain stain solutions are employed in a variety of procedures. To prevent duplication it seems well to describe these first. Others, used only in certain single or related procedures, are best described in connection with the special methods in question. Simple solutions of dyes in single solvents need no special description and will be referred to as such in the procedures concerned, though they may be used in a variety of procedures.

The dyes used in staining may be classed in various ways: according to origin as natural and synthetic; according to chemical class, as azo, tri-
Origin is of little significance, and certain dyes, such as the indigos and orcein, may be made in either way. Each of the chemical classes contains dyes used for diverse purposes, and chemically dissimilar dyes may be used for the same purpose. For example, the disazo dye Sudan IV and the anthraquinone dye coccine red impart nearly identical colors to neutral fats; hematoxylin and certain oxazine dyes are good iron-mordant nuclear stains. Two of the best collagen stains are the triphenylmethane dye methyl blue and the disazo dye naphthol blue black.

For practical purposes a classification based on use seems best and will be followed in this work.

Dyes are generally complex organic chemicals whose behavior depends on their precise chemical constitution to a variable extent. They may often be mixtures of homologs varying by differences of one or more methyl, ethyl, or phenyl groups, by the number of sulfonic acid or carboxylic acid radicals included, or by the degree of oxidation or reduction. Many are quite stable in the dry state and in solution; others alter spontaneously in solution or even in the dry state. These alterations are generally accelerated by heat and light, and it is well to store dyestuffs in a cool dark place.

On purchasing dyes—particularly unusual ones or those whose names closely resemble those of other perhaps dissimilar dyes—it is well to specify the Colour Index Number (abbreviated C. I. No.) of the dye in question; or if it has no C. I. No., its chemical constitution or the precise designation given by the manufacturer, including the manufacturer’s name. The Colour Index is a publication of the British Society of Dyers and Colourists in 1924 (with a 1928 supplement) which lists some 1300 synthetic and natural dyestuffs, giving their chemical constitution, often the method of manufacture, and a list of the various synonyms applied to each.

In regard to nomenclature of dyes, I have generally followed that adopted in the Biological Stain Commission’s publication, Biological Stains (H. J. Conn et al., editors).

The Biological Stain Commission is a nonprofit corporation whose trustees are for the most part representatives of the various American scientific societies in those fields which use stains. The function of the Commission is to test stains and to supply and disseminate information regarding their constitution, behavior, and uses.

Because of the variability of performance of various lots of dyestuffs, it is well to purchase only dyestuffs which have been tested for the purpose for which they are to be used. Many of Grubler’s dyes were so tested, but the tests employed were never made public. In this country such testing is done on most dyes employed in biological staining procedures by the Biological Stain Commission in accord with the tests given in the latest edition of the Commission’s book, Biological Stains; and dyes bearing the Commission’s
certificate are generally reliable for the purposes specified by the Commiss­
ion, provided they have not decomposed since the date of certification.
Dates of certification may be determined approximately by consulting the
quarterly lists of certifications published in the Stain Commission's journal,
Stain Technology. It is good practice to stamp or write the date of receipt on
each bottle of dye purchased.

Statements regarding the identity and chemistry of dyestuffs are derived
generally from Conn's Biological Stains, from the Colour Index, from the
Yearbooks of the American Association of Textile Chemists and Colorists,
from the literature, and from our own files; and generally are made without
specific reference. For further details the first three references above are
recommended.

On the following pages the abbreviation (C&B) refers to the Coleman
and Bell Co., (HL) to the Hartman Leddon Co., (NAC) to the National
Aniline Division of the Allied Chemical and Dye Corp., (duP) to the E. I.
duPont de Nemours Co., (Ciba) to the Ciba Co., and other abbreviations as
used in the Yearbooks and the Colour Index for dye manufacturers.

Nuclear Stains

These include hematoxylin, carmine, and a large number of basic anilin
dyes. Besides staining the nucleic acids, they often stain other acid compo­
nents of tissues, such as chondroitin sulfuric acid and other basophilic sub­
stances. The term basophilic means simply that the substances so described
are stainable with basic dyes; the converse is oxyphilic or acidophilic, posses­
sing affinity for acid dyes. The term sudanophilic denotes stainability with
oil-soluble dyes, of which the first and most widely used are Sudan III and
IV.

Hematoxylin (C. I. No. 1246)

Most hematoxylin stains depend on the presence in the solutions of its oxi­
dation product hematein. This substance is formed gradually in solution or
in the solid state in contact with air, and more rapidly in the presence of
heat. It may be formed at once by certain oxidizing agents such as peroxides,
iodates, permanganates, perchlorates, mercuric oxide, and ferric salts. When
solutions are made from old samples of hematoxylin it is well to test these for
staining capacity before adding an oxidizing agent. I have seen samples that
gave good alum hematoxylin stains at once, and these were ruined by addi­
tion of the customary quantity of oxidant.

Alum Hematoxylin. The most used hematoxylin solutions are those em­
ploying aluminum and iron salts as mordants. The former stain nuclei blue,
and to a variable extent other substances as well. The selectivity for nuclei
is increased by the presence of an excess of aluminum salts or, better, by the
presence of acids in the solution. The same purpose may be accomplished
by first staining with relatively neutral solutions and then removing the stain.
<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Mayer Hemalum</th>
<th>Mallory Aqueous</th>
<th>Harris</th>
<th>Böchmer-Schmorl</th>
<th>Apáthy</th>
<th>Littke-Mayer</th>
<th>Delafield</th>
<th>Ehrlich Acid</th>
<th>Bullard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematoxylin</td>
<td>1</td>
<td>2.5</td>
<td>5</td>
<td>5</td>
<td>3.3</td>
<td>5</td>
<td>6.4</td>
<td>6.4</td>
<td>8</td>
</tr>
<tr>
<td>Ammonium alum</td>
<td>50</td>
<td>50</td>
<td>100</td>
<td>100</td>
<td>30</td>
<td>50</td>
<td>60</td>
<td>(in excess)</td>
<td></td>
</tr>
<tr>
<td>Ethyl alcohol</td>
<td></td>
<td></td>
<td>50</td>
<td>60</td>
<td>233</td>
<td></td>
<td></td>
<td>200†</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td></td>
<td></td>
<td>1,000</td>
<td></td>
<td>333</td>
<td></td>
<td></td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>1,000</td>
<td>1,000</td>
<td>1,000</td>
<td></td>
<td>423</td>
<td>700</td>
<td></td>
<td>640</td>
<td></td>
</tr>
<tr>
<td>Normal ripening agent</td>
<td>0.2 NaIO₃</td>
<td>None</td>
<td>2.5 HgO</td>
<td>None</td>
<td>None</td>
<td>0.5 NaIO₃</td>
<td>None</td>
<td>None</td>
<td>8 HgO</td>
</tr>
<tr>
<td>Time and temperature</td>
<td>25° C., inst.</td>
<td>25° C., 10 d.</td>
<td>100° C., few min.</td>
<td>25° C., 8 d.</td>
<td>25° C., 6 wk.</td>
<td>25° C., inst.</td>
<td>25° C., 6–8 wk.</td>
<td>25° C., 6–8 wk.</td>
<td>100° few min.</td>
</tr>
<tr>
<td>Alternate ripening</td>
<td></td>
<td>0.44 KMnO₄</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.64 NaIO₃</td>
<td></td>
</tr>
<tr>
<td>Time and temperature</td>
<td></td>
<td>25° C., inst.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10% acetic</td>
<td>2% acetic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Acetic 10</td>
<td>Acetic 20</td>
<td>None</td>
<td>Acetic 32</td>
<td>Acetic 34</td>
</tr>
<tr>
<td>Variant</td>
<td>1 citric or 20 acetic</td>
<td>40 acetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stated life</td>
<td>2–3 months</td>
<td>2–3 months</td>
<td>Months to years</td>
<td>Months to years</td>
<td>Months to years</td>
<td>Months to years</td>
<td>Years</td>
<td>Years</td>
<td>indef.</td>
</tr>
<tr>
<td>Preservative</td>
<td>50 chloral hydrate</td>
<td>2.5 thymol</td>
<td></td>
<td></td>
<td>0.33 acid salicylic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* In general it is prescribed that glycerol and acid be added after ripening is well along. In case of iodate ripening the sequence of combination of ingredients appears to make no difference. The statements regarding spontaneous ripening in periods of 8–10 days are not mine, and seem to me unduly optimistic. 25° C. is adopted as average room temperature. Quantities are in grams for solids, in cubic centimeters for liquids. "inst." = instantaneous.

† Originally 40 cc. ethyl and 160 cc. methyl alcohol.
from other constituents with acid or other differentiating agents. The latter method also demonstrates a number of substances related to the myelins which stain intensively with basic dyes and retain them longer than all other tissue elements on treatment with acids and other differentiating agents.

With these properties in mind, it makes little difference how the hematein is formed. Solutions vary in vigor of staining according to the amount of dye used in their preparation, and in selectivity on progressive staining according to their acidity. The function of alcohol seems to be principally preservative against molds; that of glycerol to stabilize the solution against overoxidation. In fact, I have seen deliberately overoxidized 30% glycerol solutions regain their staining capacity to a considerable extent on standing.

The accompanying table gives a number of the more usual formulae on a per liter basis. Some methods prescribe dissolving the hematoxylin first in alcohol or water and oxidizing either by exposure to air or by addition of an oxidizing agent. On trial it seems to make no difference whether the alum, glycerol, water, acetic acid, and other constituents are added to the hematoxylin solution before or after oxidation, and the method of oxidation is similarly unimportant.

Delafield's formula is cited from Mayer in Ehrlich's Encykl.ädie. I have set the amount of alum at 60 Gm., which is a slight excess over the amount demanded by the 1:11 solution described as saturated by Mayer. Mallory called for a 15% alum solution and used ethyl alcohol throughout. The latter recommendation seems logical.

Both Mallory's and Schmorl's formulae are essentially modifications of the vague original Boehmer formula (Encykl.ädie) and are cited from the texts of the two authors.

Mayer's formula (Ztschr. wiss. Mikr. 20:409, 1904) is cited in essentially the form given by nearly all texts subsequent to the Encykl.ädie. My modification (Stain Technol., 16:1, 1941, 17:89, 1942) resembles Mayer's glycerol hemalum in its solvent, but contains 0.5% hematoxylin in place of the 0.4% hematein of that mixture (Ehrlich's Encykl.ädie). Langeron cites a similar, weaker formula with 0.1% hematoxylin, 0.02% sodium iodate, 20% glycerol, and 5% alum, which was devised by Carazzi (Ztschr. wiss. Mikr. 28:273, 1911).

Apáthy's formula is cited from Lee in modified form. The (1%) hematoxylin was first ripened in 70% alcohol, and this then mixed with equal volumes of glycerol and 3% acetic, 9% alum solution.

Harris' formula and Ehrlich's mixture I have cited from the Encykl.ädie. I have set the amount of alum in the latter at 4%, which is more than sufficient for saturation at 30° C. Iodate ripening of Ehrlich's formula was suggested by Mallory, and I have used it successfully.

Bullard's hematoxylin is the most concentrated formula that I have encountered. Dr. Bullard tells me (April 1949) that the formula was devised for the relatively crude, dark-colored American hematoxylin available in
1915–18. It was originally published by MacCallum in Laboratory Methods of the U. S. Army, 2nd ed. Philadelphia and N. Y., Lea & Febiger, 1919, p. 69. Bullard directs: Dissolve 8 Gm. hematoxylin in 144 cc. 50% alcohol, add 16 cc. glacial acetic acid and a (heated) solution of 20 Gm. ammonia alum in 250 cc. water. Heat to boiling and add slowly (to avoid frothing) 8 Gm. red mercuric oxide. Cool quickly, filter and add 275 cc. 95% alcohol, 330 cc. glycerol, 18 cc. glacial acetic acid, and 40 Gm. ammonia alum.

If spontaneous ripening of hematoxylin solutions is preferred to one of the chemical means, it is necessary to anticipate one's needs by several months, and keep a supply ahead in various stages of ripening.

I prefer the solutions containing glycerol because of their greater stability and those containing 0.5% or higher of hematoxylin because of their greater vigor of staining and capacity for staining larger numbers of sections before exhaustion. Acidified hematoxylin solutions are preferable for routine nuclear staining, but the nonacidified have valuable properties as well. Alum percentages generally approach saturation, and are lower in the partly alcoholic and glycerol solutions than in the aqueous. The alum prescribed is always ammonium aluminum sulfate, \( (\text{NH}_4)_2\text{SO}_4 \cdot \text{Al}_2(\text{SO}_4)_3 \cdot 24\text{H}_2\text{O} \).

Though the traditional amounts, 177 mg. potassium permanganate, 200 mg. sodium iodate and 500 mg. mercuric oxide per gram hematoxylin work well in practice, recent work indicates that considerably smaller quantities of some may be used with good immediate ripening and probably a longer useful life of the solution. Quantities recommended per gram hematoxylin are: sodium iodate \( \text{NaIO}_3 \) 40 to 100 mg., potassium permanganate 175 mg., potassium periodate \( \text{KIO}_4 \) 50 mg., mercuric oxide \( \text{HgO} \) 100 mg. Of these the first three are used cold, while the fourth is boiled.

Iron Hematoxylin. These fall into overstaining and regressive differentiation methods, divided into sequence methods and combined solution methods, and into progressive methods with acid or an excess of iron salt to prevent overstaining.

The sequence methods depend on a preliminary mordanting with an aqueous solution of a ferric salt, staining with an aqueous solution of hematoxylin until black, and then differentiating with an acid or a ferric salt solution until the desired grade of differentiation is evident on microscopic examination. These methods are widely used by cytologists and protozoologists for the study of chromatin, chromosomes, spindles, centrosomes, and other cytologic details. They are too cumbersome for routine use in pathology because of the excessive amount of time required for each slide.

Hematoxylin solutions for these techniques are variously prescribed as fresh or as ripened, usually by standing at room temperatures for several weeks to months. Rawlins and Takahashi (Stain Technol. 22:99, 1947) ripened with aeration for two hours by bubbling an air stream through the solution, and found the product satisfactory for the Heidenhain method.

Short exposures to mordant and to hematoxylin tend to yield blue-
**Table 9**

**Comparative Schedules for Sequence Iron Hematoxylin Staining**

| Mordant solution | Benda | Heidenhain | Heidenhain | Regaud
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liq. Fer. Sulph. Oxid. P.G. in 50–33% dilution</td>
<td>1.5–4% iron alum in water</td>
<td>2.5% iron alum</td>
<td>5% iron alum</td>
</tr>
<tr>
<td>Time and temperature</td>
<td>20° C., 24 hr.</td>
<td>20° C., ½–3 hr.</td>
<td>20° C., 3–12 hr.</td>
<td>45–50° C., 5–10 min.</td>
</tr>
<tr>
<td>Washing</td>
<td>Dist., then tap water</td>
<td>Water</td>
<td>Water</td>
<td>Thorough, water</td>
</tr>
<tr>
<td>Hematoxylin</td>
<td>1% aq.</td>
<td>0.5% aq.</td>
<td>0.5% aq.</td>
<td>1%</td>
</tr>
<tr>
<td>Ripened?</td>
<td>No</td>
<td>No</td>
<td>Yes, 4–6 wk.</td>
<td>No</td>
</tr>
<tr>
<td>Time and temperature</td>
<td>20° C., until black</td>
<td>20° C., 30 min.</td>
<td>20° C., 24–36 hr.</td>
<td>45–50° C., 5–10 min.</td>
</tr>
<tr>
<td>Differentiating agent</td>
<td>5–30% acetic, or weaker acetic, or 1:30 Liq. Fer. Sulph. Oxid. P.G.</td>
<td>1.5–4% iron alum</td>
<td>2.5% iron alum</td>
<td>$\frac{1}{2}$ saturated (6%) alcoholic picric acid</td>
</tr>
<tr>
<td></td>
<td>Note</td>
<td>Note</td>
<td>Note</td>
<td>0.25% ferric chloride</td>
</tr>
</tbody>
</table>

Liquor Ferri Sulphurici Oxidat P. G. consisted of 80 Gm. iron sulfate, 40 Gm. water, 15 Gm. sulfuric acid, and 18 Gm. nitric acid, and contained 10% metallic iron. Iron alum is Fe$_2$(SO$_4$)$_3$·(NH$_4$)$_2$SO$_4$·24H$_2$O. 20° C. is taken as average German laboratory temperature, 25° C. as American. Saturated alcoholic picric acid solution is approximately 9% according to Conn’s *Biological Stains*, 6th ed. Heidenhain directs periodic transfer to water for microscopic examination during differentiation.

Benda's formula and the second formula of Heidenhain above are cited from Ehrlich's *Encyklopädie*; the first formula of Heidenhain is from Lee; the Regaud formula is from Masson (*J. Tech. Methods* 12:75, 1929); and Mallory’s is from his 1938 text.

Iron alum solutions should be freshly prepared from clean violet crystals.
black preparations; longer treatments and aged hematoxylin, black (Lee).

Benda's method, two of Heidenhain's, one of Regaud's used by Masson, and one of Mallory's are included in the accompanying table (p. 79).

Goldman (Am. J. Clin. Path. 21:198, 1951) presents an interesting variant of the traditional iron hematoxylin methods for protozoa. In brief, he prescribes Zenker, formalin, or Bouin fixation. Section in paraffin and deparaffinize and hydrate as usual, inserting the iodine and thiosulfate sequence for the Zenker material. Then mordant 30 minutes or longer, up to 24 hours, in a solution of 4 Gm. iron alum, 1 cc. glacial acetic acid, 0.12 cc. concentrated sulfuric acid and 100 cc. distilled water. Wash ten minutes in running water. Soak for three hours or more in saturated aqueous picric solution containing 0.1 cc. concentrated sulfuric acid per 100 cc. Rinse one to two minutes in distilled water. Stain in ripened (0.5%) aqueous hematoxylin (p. 77) for one hour. Wash 15 minutes in running water. Blue for 15 minutes in 70% alcohol containing five drops saturated aqueous lithium carbonate (per 100 cc.?). If desired, counterstain with 0.5% eosin in alcohol. Dehydrate with alcohols, clear with xylene, mount in a resinous medium.

Results: Nuclei stain black; basophil cytoplasm, gray. After Bouin fixation, staining is less intense. Goldman states that overstaining does not occur unless sections are left in the hematoxylin for many hours. The time in picric acid cannot be reduced without causing overstaining, unless the bath is heated to 58° C.; in that case 30 minutes suffices, but Goldman complains of section losses when the warm picric acid is used.

I have found the usual Weigert iron chloride hematoxylin, Van Gieson picrofuchsin sequence (p. 346) quite satisfactory for demonstration of Entamoeba histolytica in tissues, and far less time consuming.

More generally useful in pathology are the premixed ferric salt hematoxyllins. These fall into two groups: those mixed with an optimal amount of iron for dense staining and used for regressive staining (principally in the myelin and related methods) and those made more or less selective for nuclei by addition of acid or by an excess of ferric salt.

About 0.5 Gm. metallic iron in the ferric state is required for each gram of hematoxylin for maximal staining (Lillie and Earle). This corresponds to 3.91 cc. of the official solution of iron chloride or to 4.32 Gm. iron alum. Doubling the quantity of ferric iron in the mixed solution prevents overstaining.

The two commonest mixed iron hematoxyllins used for regressive staining are Weigert's and Well's. Both prescribed the mixture of an equal volume of an aged and “ripened” 1% alcoholic hematoxylin with an aqueous solution of a ferric salt. Weigert used 4 cc. of the official (P. G.) iron chloride solution and 96 cc. of water; Well used 4% iron alum. With Well's hematoxylin I find that hematoxylin solution only 1–5 days old is superior to the solution several months old prescribed by Weil, and also is readily available at all times. Inasmuch as ferric salts oxidize hematoxylin promptly, it is difficult
to see the necessity for previously oxidized hematoxylin. For these stains I prefer 0.5% iron alum for differentiation, and leave recent, properly chromated material an hour in the differentiating bath. Myelin requires treatment with a bath of 1% borax and 2.5% potassium ferricyanide for 5–10 minutes after the iron alum differentiation.

Satisfactory formulae for progressive staining of nuclei without overstaining are those of Janssen and of Weigert. Both stain nuclei in 3–5 minutes and do not overstain in 10–30 minutes, and color effects are the same.

Janssen (Cellule 14:203, 1898) dissolved 2 Gm. hematoxylin in 60 cc. methanol, and 20 Gm. iron alum in 200 cc. water, mixed the two solutions, and added 60 cc. glycerol. The solution becomes blue black at once, then purplish, and in about an hour brownish black. It remains usable perhaps 4–5 weeks.

Weigert's (Ztschr. wiss. Mikr. 21:1, 1904) solution is less stable, lasting only 2–3 weeks, and if large numbers of sections are being stained, it may have to be made fresh twice weekly. It is the easiest to prepare, requiring only one weighing and no glycerol. Mix 100 cc. fresh 1% hematoxylin in 95% alcohol with 95 cc. distilled water, 1 cc. concentrated hydrochloric acid, and 4 cc. of the official (U.S.P. XI = P. G.) solution of iron chloride. This hematoxylin undergoes the same color changes as Janssen's formula, but even more rapidly.

Doubling of the amount of hydrochloric acid and reduction of the hematoxylin content to half appears to make the Weigert formula more selective for nuclei; and addition of 4.44 Gm. ferrous sulfate stabilizes it so that with occasional use the solution remains active for several weeks. Thus: Mix 100 cc. fresh 1% alcoholic hematoxylin with 288 cc. distilled water, 4 cc. official ferric chloride solution, 8 cc. concentrated hydrochloric acid (Sp. Gr. 1.19) and 4.44 Gm. ferrous sulfate (FeSO₄·7H₂O). When large numbers of sections are being stained, depletion and dilution become more important than oxidation. Under these circumstances the simpler Weigert formula is preferred.

If, after an alum hematoxylin stain, one first rinses the slides in water and then dips them for 30 seconds in a 1/25 dilution of U.S.P. iron chloride solution, nuclear staining is converted from blue to blue black.

Use of alum hematoxylin in Van Gieson stains and in periodic acid Schiff methods with picric acid plasma stains yields a deep red-brown nuclear stain. If one rinses the slides in water after the hematoxylin, and then immerses them for 30 seconds in 4 cc. U.S.P. iron chloride solution plus 96 cc. water, the nuclear stain after picric acid or picrofuchsin counterstains is converted to black.

The official solution of iron chloride contains 10% by weight of metallic iron, or 29.1% FeCl₃. It is essentially the same in the German Pharmacopoeias of 1884 to 1900 (when Weigert introduced it into histologic technic) as in the U. S. Pharmacopoeia XI and the present Dispensatory. Because its
specific gravity is 1.280 to 1.283, each 100 cc. contains 12.8 Gm. iron, or 37.2 Gm. FeCl₃.

The British official (1932) solution of ferric chloride contains 15 Gm. FeCl₃ per 100 cc., or 5.17 Gm. Fe. The French (1905) solution was 26% FeCl₃ by weight, 8.95% Fe, containing 32.4 Gm. FeCl₃ per 100 cc. or 11.17 Gm. Fe. The Spanish solution (1905) was 27% FeCl₃ by weight, corresponding to 33.95 Gm. per 100 cc. and 11.7 Gm. Fe per 100 cc. The Netherlands (1905) Pharmacopoeia called for 75 Gm. FeCl₃ crystals and 25 cc. water, and 1.47 to 1.482 specific gravity, corresponding to 45% by weight of the anhydrous salt, or 66.8 Gm. per 100 cc. of FeCl₃ and 19.4 Gm. Fe per 100 cc. Corresponding corrections of formulas should be made: 128 mg. Fe = 1 cc. U.S.P., P.G. = 2.48 cc. Br. = 1.15 cc. Fr. = 1.09 cc. Sp. = 0.66 cc. Neth.

The official solution of ferric sulfate, used by Benda in his sequence iron hematoxylin method also contained 10% iron by weight, according to both the 1884 German Pharmacopoeia and the present U. S. Dispensatory. Its specific gravity of 1.43 indicates a content of 14.3 Gm. iron or 71.9 Gm. ferric-sulfate crystals, Fe₂(SO₄)₃·9H₂O per 100 cc.

The common iron alum (NH₄)₂SO₄·Fe₂(SO₄)₃·24H₂O used in histology contains 11.58% metallic iron.

Since the active agent in each of these three preparations is ferric iron, they are readily interchangeable in equivalent quantities. A table of equivalents is offered, Table 10, p. 83.

**Hematoxylin Substitutes**

A number of other dyestuffs have been substituted for hematoxylin in forming iron and aluminum mordant stains. Besides the closely related carmine and brazilin, there are gallamin blue (C. I. No. 894, p. 85), gallo-cyanin (C. I. No. 883, p. 85, 141), celestin blue B (C. I. No. 900), and anthracene blue (C. I. No. 1062, p. 115). All these dyestuffs possess o-diphenolic groupings or quinonoid oxidation products thereof. It is worthy of note that good gray to black nuclear stains may be obtained by immersing sections in fresh mixtures of ferric chloride solution with aqueous solutions of catechol, pyrogallol, or hydroxyhydroquinone (1,2-, 1,2,3-, and 1,2,4-hydroxybenzene, respectively); but not with phenol, resorcinol, hydroquinone, or phloroglucinol, which do not possess vicinal dihydroxy groupings. The iron o-diphenol mixtures darken quite rapidly and are probably unstable. It is possible that Salazar's "tannin-iron" technic (Stain Technol. 19:131, 1944; 21:149, 1946; 22:3, 1947) depends on this same action. Tannin is m-galloylgallic acid, with vicinal hydroxyl groups on both benzene rings. The effect of the open carboxyl group would probably be to make the iron complex less selective, since it could unite with basophilic elements by this group as well as by the iron mordant action. The other diphenols and diphenolic dyestuffs which form iron lakes do not possess acid groups and without mordants generally do not function as basic dyes.
Table 10

**EQUIVALENTS IN IRON CONTENT OF CERTAIN FERRIC SALTS AND THEIR OFFICIAL PHARMACOPEIAL SOLUTIONS**

<table>
<thead>
<tr>
<th></th>
<th>Metallic Iron Gm.</th>
<th>Iron Alum Gm.</th>
<th>Ferric Chloride Gm.</th>
<th>Solution Ferric Chloride cc.</th>
<th>Ferric Sulfate Gm.</th>
<th>Ferric Sulfate Cryst. Gm.</th>
<th>Solution Ferric Sulfate cc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 Gm. Iron alum</td>
<td>11.58</td>
<td>100.0</td>
<td>33.63</td>
<td>90.34</td>
<td>41.45</td>
<td>58.25</td>
<td>80.96</td>
</tr>
<tr>
<td>100 cc. Sol. FeCl₃</td>
<td>12.815</td>
<td>110.69</td>
<td>37.22</td>
<td>100.0</td>
<td>45.88</td>
<td>64.48</td>
<td>89.41</td>
</tr>
<tr>
<td>100 cc. Sol. Fe₂(SO₄)₃</td>
<td>14.30</td>
<td>123.52</td>
<td>41.52</td>
<td>111.84</td>
<td>51.195</td>
<td>71.95</td>
<td>100.0</td>
</tr>
<tr>
<td>100 Gm. Fe₂(SO₄)₃ • 9 H₂O</td>
<td>19.87</td>
<td>171.67</td>
<td>57.72</td>
<td>155.09</td>
<td>71.15</td>
<td>100.00</td>
<td>138.98</td>
</tr>
<tr>
<td>100 Gm. FeCl₃</td>
<td>27.92</td>
<td>241.27</td>
<td>81.32</td>
<td>217.96</td>
<td>100.0</td>
<td>140.54</td>
<td>195.33</td>
</tr>
<tr>
<td></td>
<td>34.43</td>
<td>297.38</td>
<td>100.0</td>
<td>268.66</td>
<td>123.26</td>
<td>173.23</td>
<td>240.87</td>
</tr>
<tr>
<td>100 Gm. Fe</td>
<td>100.0</td>
<td>863.78</td>
<td>290.45</td>
<td>780.33</td>
<td>358.01</td>
<td>503.16</td>
<td>699.30</td>
</tr>
<tr>
<td>M Fe</td>
<td>55.85</td>
<td>482.41</td>
<td>162.22</td>
<td>435.81</td>
<td>199.95</td>
<td>281.09</td>
<td>390.55</td>
</tr>
<tr>
<td>M Fe₂</td>
<td>111.70</td>
<td>964.82</td>
<td>324.44</td>
<td>871.62</td>
<td>999.90</td>
<td>562.18</td>
<td>781.10</td>
</tr>
<tr>
<td>4 Gm. Iron alum</td>
<td>.463</td>
<td>4.0</td>
<td>1.346</td>
<td>3.615</td>
<td>1.658</td>
<td>2.340</td>
<td>3.24</td>
</tr>
<tr>
<td>4 cc. Sol. FeCl₃</td>
<td>.513</td>
<td>4.43</td>
<td>1.488</td>
<td>4.0</td>
<td>1.850</td>
<td>2.582</td>
<td>3.58</td>
</tr>
</tbody>
</table>

Recalculated in accordance with 1949 atomic weights (Lange).
Several other alizarin dyes could probably be used in this way, since they have the required catechol structure: alizarin, alizarin red S, purpurin, quinalizarin, acid alizarin blue, alizarin cyanine R, alizarin cyanine black, alizarin green S, alizarin black S (C. I. Nos. 1027, 1034, 1037, 1063, 1064, 1065, 1068, 1070) and other similar dyestuffs.

Carmine (C. I. No. 1239)

Formerly the most important dyestuff in the histologist's armamentarium, carmine is less and less used today. I have used it as a nuclear stain in such procedures as Weigert's fibrin stain, and occasionally in other methods; but now no longer use it in any procedure except the Best carmine stain for glycogen, and for coloring injection masses red. Formulae for these will be given under the special methods.

Orth's formula (Mayer in Ehrlich's Encyklopädie) called for 2.5-5 Gm. carmine, dissolved by boiling 15 minutes in 100 cc. saturated aqueous lithium carbonate solution. After cooling, 1 Gm. thymol was added as a preservative. Sections were stained 2-5 minutes and differentiated in a 1:100 dilution of concentrated hydrochloric acid in 70% alcohol.

In Grenacher's formula Mayer (Ehrlich's Encyklopädie) recommended 2 Gm. carmine dissolved by boiling for one hour in 100 cc. 5% ammonium alum, restoring water to maintain volume. After cooling Mallory added 1 Gm. thymol. Grenacher originally used 0.5-1% carmine and 1-5% alum.

Mayer's (Ehrlich's Encyklopädie) alcoholic carmine ("paracarmine") may be used when it is desired to avoid aqueous solutions. Dissolve 1 Gm. carminic acid, 0.5 Gm. aluminum chloride, and 4 Gm. calcium chloride (crystals?) in 100 cc. 70% alcohol. Filter before using. Stain sections 15-30 minutes and wash with 70% alcohol, adding 2.5% glacial acetic acid if a more purely nuclear stain is desired.

Basic Aniline Dyes

The thiazins, thionin (C. I. No. 920) and its mono-, di-, tri-, and tetramethyl derivatives azure C, azure A, azure B (comprised under C. I. No. 923) and methylene blue (C. I. No. 922); its relatives toluidin blue (C. I. No. 925) and new methylene blue (C. I. No. 927) are valuable nuclear stains and exhibit in varying measure the property of metachromasia, or of staining cartilage matrix, mucin, and the granules of mast cells in a more violet or redder tone than they do nuclei. Of these, new methylene blue is the reddest, then thionin, azure C, azure A, toluidin blue, azure B, and methylene blue in order. The middle three members of the series afford perhaps the greatest color contrast between nuclei and cytoplasm on the one hand and cartilage and mucus on the other.

They are much used as bacterial stains and (in combination with dyes of the eosin group) as general tissue stains, especially for blood and blood-forming tissues.
The higher homologs, methylene blue and azure B, readily undergo oxidation with loss of methyl groups and formation of formaldehyde, and give rise to lower homologs and to certain decamethylized oxidation products (known as thiazoles) which are relatively insoluble in water and have been thought to contribute to the polychromasia of blood stains. The substance Bernthsen's methylene violet appears to be a mixture of these. This oxidation of methylene blue to azure and thiazoles is known as polychroming, and mixtures of methylene blue with these products are called polychrome methylene blue.

This process of polychroming occurs freely in alkaline solutions without added oxidants, and is expedited by rise in pH level above 8.0 and by heat. It is inhibited in acid solution. It may be induced by deliberate oxidation in acid solution by addition of chromic acid, but in this instance only azures appear to be formed, and the amount of alteration is strictly proportional to the amount of available oxygen furnished. Thus, use of 250 mg. potassium bichromate per Gm. 88% methylene blue produces a product which is spectroscopically and tinctorially chiefly azure B, while double that amount produces chiefly azure A. The reactions are thus: K$_2$Cr$_2$O$_7$ furnishes 3 O, Cr$_2$O$_3$ and K$_2$O, the metallic oxides being promptly converted into salts by the excess of acid present. One mol of methylene blue C$_{16}$H$_{18}$N$_5$SCl + 1 O yields 1 mol azure B C$_{15}$H$_{16}$N$_5$SCl + 1 mol formaldehyde HCHO.

A similar decomposition of methylene blue and the higher azures appears to go on even in the dry dyes, and occurs readily also in methyl alcohol solutions of their eosinates and more slowly in glycerol methanol solutions. Also in these solutions the change is accelerated by higher temperatures and by presence of alkali and retarded by acid. Particularly annoying is its occurrence during the drying of eosinate precipitates. Here thorough washing with distilled water, rigid adherence to temperatures below 40° C. for drying, and the use of alcohol or vacuum to accelerate drying are of help.

The oxazine dyes brilliant cresyl blue (C. I. No. 877) and cresyl violet are used, the one chiefly for supravital staining, the other for staining of metachromatic substances. Three others of this group, gallocyanin (C. I. No. 883), gallamin blue (C. I. No. 894), and celestin blue B (C. I. No. 900), have been used as iron lakes for nuclear staining in place of hematoxylin (cf. p. 82). The iron lakes are prepared by boiling 0.5% dye in 5% iron alum solution for 3–5 minutes. They stain nuclei blue to black in 3–5 minutes, leaving cytoplasm colorless. The picric acid of Van Gieson mixtures changes this to green. Proescher and Arkush (Stain Technol. 3:28, 1928) preferred celestin blue B to the other two. I have not tried these stains. Gallocyanin with chrome alum in acid solution is an excellent nuclear and tigroid stain (p. 141).

The remaining commonly used member of this group is Nile blue A (C. I. No. 913). This dye is used extensively for staining of fatty acids and fats. On boiling with sulfuric acid it forms a minor quantity of a red, oil-soluble (oxazine) dye, thus coloring neutral fats pink and fatty acids blue.
The fat stain is at best a feeble one and I have replaced it with others such as oil red O, oil blue N, and coccinel red (no C. I. Nos.). I was never well satisfied with the specificity of the fatty-acid stain. Nile blue A stains all basophilic substances about the same shade of blue, varying only in intensity. As Feshay (J. Lab. & Clin. Med., 17:193, 1931) notes, it stains B. tularense well. However, it is not differential, staining other bacteria, nuclear débris, and certain cell granules the same color. In fat stains mounted in syrup media, it diffuses badly, even after acid differentiation.

Of the rosanilin group of basic dyes, fuchsin (C. I. No. 677), which is a mixture of rosanilin and pararosanilin (C. I. No. 676); new fuchsin (C. I. No. 678), the tritolyl homolog of pararosanilin; methyl violet (C. I. No. 680) and crystal violet (C. I. No. 681), which are methyl derivatives of pararosanilin, are the principal ones used in histology. Their principal use is for the demonstration of bacteria in the tissues. Fuchsin and new fuchsin are used in the Ziehl-Neelsen acid-fast and related technics for tubercle bacilli and other acid-fast substances; in combination with iron salts in elastic-tissue methods; as general nuclear stains when red nuclei are desired in contrast to the ferrocyanide reaction, the Gram stain and other procedures; and in the Schiff reaction for aldehydes (p. 154) as used for nuclei in the Feulgen method and for glycogen in the Bauer technic.

Crystal violet is used chiefly in the Gram and Weigert technics for bacteria and fibrin; methyl violet for amyloid; both sometimes as nuclear stains in neutral stain combinations. The related dye iodine green (C. I. No. 686) is sometimes used for amyloid; and methyl green, which is usually ethyl hexamethyl pararosanilin (C. I. No. 685) or ethyl green rather than true methyl green (C. I. No. 684), is a relatively pure chromatin stain. As such it is used also in certain bacterial technics such as the methyl green pyronin stain. Another dye of this group sometimes used as a basic stain in alcoholic solution is spirit blue (C. I. No. 689), also called alcohol-soluble anilin blue, Lyon blue, Paris blue, and other synonyms.

A related yellow basic dye is auramine. This is used principally for the staining of tubercle bacilli for fluorescence microscopy (pp. 382-383).

Safranin O (C. I. No. 841), safranin 6B (C. I. No. 843), and a few related azin dyes are valuable and precise nuclear stains, either when staining progressively from acid solution, or reggressively with acid differentiation. Safranin O also exhibits a valuable orange metachromasia.

The principal use of the azin dye neutral red (C. I. No. 825) is in vital staining, in tigroid staining, and in such neutral stains as Twort's light-green neutral red.

Three weakly basic red dyes of the xanthene group are the pyronins Y and B (C. I. Nos. 739 and 741), and rhodamine B (C. I. No. 749). I have occasionally used these as red nuclear stains, but find other dyes better, notably the fuchsins and the safranins.

All three have been used as basic stains for cytoplasm, basophil granules,
and bacteria in contrast to a nuclear stain with methyl green. The pyronins are used for this purpose in the Unna-Pappenheim-Saathof methods (p. 133), and are also employed as counterstains in the alphanaphthol oxidase methods (p. 227). Rhodamine B has also been employed in the supravital staining of mitochondria (p. 182), and as a fat stain in aqueous solution for fluorescence microscopy.

Basic acridine dyes used in fluorescence microscopy as general stains include acridine yellow, coriphosphine O, acridine orange NO, and acriflavine (C. I. Nos. 785, 787, 788, and 790).

The basic azo dyes Janus green B (C. I. No. 133) and the Bismarck browns (C. I. Nos. 331, 332) are valuable nuclear stains in contrast-staining for fats, as they possess the valuable property of relative permanence and lack of diffusion in aqueous syrup media. Janus green B seems also to excel methylene blue as a nuclear counterstain for the acid-fast method for tubercle bacilli in tissues; but for permanence alum hematoxylin is to be preferred for this purpose. The Bismarck browns possess a metachromasia in yellower tone, and stain mucus and cartilage well. Janus green B, Janus black I (a mixture of Janus green B and some brown dye), and the related Janus blue are widely used in the vital staining of mitochondria.

**Plasma Stains**

This group is composed chiefly of alkali salts of sulfonic and carboxylic acid aniline dyes, together with a few nitro and nitroso dyestuffs. Functionally they fall into two groups: plasma stains proper and those selective for collagen and reticulum fibers, bone and cartilage matrix, and other basic extracellular substances. However, many individual acid dyestuffs may be used for either purpose.

One nitroso dye, naphthol green B (C. I. No. 5), has been used as a plasma stain in various connective tissue procedures, and, with an iron mordant, as a collagen stain (J. T och. Meth. 25:32, 1945).

Picric acid (C. I. No. 7) or its ammonium salt are widely used as plasma stains in contrast to acid fuchsin, anilin blue, methyl blue, and indigocarmine collagen stains, usually as mixtures, but also in sequence methods; also in contrast to basic fuchsin in certain tubercle-bacillus methods. If ammonium picrate is not available, it may be made by adding ammonium hydroxide solution gradually to boiling water containing 10% to 15% picric acid, until the steam a minute after the last addition is alkaline to moist litmus or nitrazine paper. Then continue boiling until the steam is no longer alkaline.

Acid azo dyes used principally as plasma stains include C. I. No. 27 orange G, No. 29 chromotrope 2R, No. 79 Ponceau 2R (xylidene ponceau), No. 88 Bordeaux red, No. 138 metanil yellow, No. 153 azofuchsin G, No. 225 thiazine red R, No. 252 brilliant croceine, No. 280 Biebrich scarlet (Ponceau 3RB), No. 370 Congo red, No. 375 Congo Corinth G (Erie
Garnet B), No. 454 brilliant purpurin R, and No. 581 chlorazol black E. Of these I regard orange G, Biebrich scarlet, and brilliant purpurin R as possibly the most useful. The last is a brown dye.

Congo red has also been used as a plasma stain in fluorescence microscopy. A considerable number of acid disazo dyes have been used as vital stains for the reticuloendothelial system. These include trypan blue (C.I. No. 477), vital red (C.I. No. 456), trypan red (C.I. No. 438), Evans blue (Conn's D56), vital new red (Conn's D60), dianil blue 2R (C.I. No. 465), and probably other sulfonated disazo dyes with benzidine and tolidine nuclei.

In the azo group only naphthol blue black (C.I. No. 246) has any outstanding value as a collagen-fiber stain. It gives a dark-green color to collagen in picric acid, combined plasma stain and picric acid, phosphomolybdic acid, and hydrochloric acid technics; and is fully the equal of anilin blue and methyl blue in its selectivity and precision. Recently the red disazo dye Ponceau S (C.I. No. 282) has shown promising results as a collagen stain from picric acid mixtures.

The di- and triamino-triphenyl- and diphenynaphthylmethane dyes include light green SF (C.I. No. 670), fast green FCF, acid fuchsin (C.I. No. 692), methyl blue (C.I. No. 706), anilin blue (C.I. No. 707), wool green S (C.I. No. 737), and violamine R (C.I. No. 758). Most of these are good collagen stains in the various technics noted above, and the two reds and the three greens are also good plasma stains. Blue plasma stains I consider too dense, and have not used these dyes in that way. Acid fuchsin has been used as a contrast stain in fluorescence microscopy. Eriocyanine A (C.I. No. 699) and other acid violets are used as cytoplasmic-granule stains in neutral combinations with safranin O.

The eosins, including eosin Y (C.I. No. 768), eosin B (C.I. No. 771), erythrosin B (C.I. No. 773), and phloxin B (C.I. No. 778) are among the most widely used and popular plasma stains. The first two function well in simultaneous azure eosinate stains, the latter two are used only in sequence staining. Eosin B is a deeper red than eosin Y; phloxin B is the deepest red. None of these have any special value as collagen stains. Eosin Y has been used in fluorescence microscopy, but fluorescein (C.I. No. 766) appears better for this purpose, and apparently acts as a supravital indicator.

The azocarmines (C.I. Nos. 828 and 829) were highly recommended as plasma stains in the complicated Heidenhain modification of Mallory's anilin-blue connective-tissue method.

Alizarin red S, alizarin SX or anthropurpurin, and purpurin (C.I. Nos. 1034, 1040, and 1037) have their principal use in pathology in the demonstration of calcium deposits. The first may be used also for staining recently deposited bone during life.

Indigocarmine (C.I. No. 1180), though inferior for that purpose, is still widely used as a collagen stain in picric acid mixtures. Orcein (C.I. No. 1242 includes this dye as well as litmus and other lichen products), either
the natural or the synthetic product, is quite valuable as an elastic-tissue stain, and many workers prefer it to the iron fuchsin methods.

Two acid thiazol dyes, primulin (C. I. No. 812) and thioflavine S (C. I. No. 816), have been used in fluorescence microscopy as general stains and for bacteria and viruses.

**Fat Stains**

The oldest member of this group, osmium tetroxide, is not a dye at all, but an unstable oxide which is reduced to a black substance by unsaturated fats and fatty acids, by olefin, and by other substances. Osmium tetroxide, commonly called osmic acid, now finds its principal use in pathology in the Marchi technic for degenerating myelin. I have discarded it even for that purpose, preferring frozen-section technics which permit combination of degenerating-myelin methods with methods for other structures. It still finds frequent use in cytology in the study of various lipoid and related substances.

The oil-soluble dyes comprise, in addition to the first used, Sudan III (C. I. No. 248), its close chemical relatives Sudan IV (C. I. No. 258) and oil red O. These are betanaphthol disazo dyes in which respectively two benzene, toluene, and xylene rings are united to the naphthol by azo linkages. A similar dye is oil red 4B or EGN, xyleneazotolueneazobetanaphthol. These give orange to deep orange-red stains, Sudan III the lightest, oil red O the darkest.

The monoazo dyes Sudan II (C. I. No. 73), Sudan brown (C. I. No. 81), and Sudan R (C. I. No. 113) are respectively bright orange-yellow, deep reddish brown, and yellowish orange in color. The first two are excellent fat stains. Sudan II affords good contrasts to the blue black of Weigert myelin preparations on frozen sections of degenerating myelinated nervous tissue.

The anthraquinone dyes oil blue N (1,4-bisamylaminoanthraquinone), coccinel red (1,5-diamylaminoanthraquinone) and carycinel red (1-amylaminoanthraquinone) are respectively deep blue, deep orange-scarlet, and deep pure red in color. They stain well from lower concentrations of isopropyl alcohol than do any of the azo oil reds, and consequently occasion less loss of fat during staining. Carycinel red shows a slight tendency to diffusion; the other two are preferable, giving sharp, clean stains.

Sudan black B has been widely used by cytologists for the localization of lipid substances, and the impression is gained that it demonstrates more of these than the popular red oil-soluble dyes. In common with the amylaminoanthraquinone dyes, coccinel red, carycinel red and oil blue N (which also seem to be more powerful fat stains than the azo dyes) Sudan black B also possesses amino groups and should be capable of functioning as a basic dye. Cohen (Stain Technol. 24:177, 1949) has actually used solutions of it in 20% aqueous organic acid solutions as a stain for chromosomes. These stain brown rather than the blue black imparted to lipids by the alcoholic solutions of the dye base.
Acetylated and benzoylated oil red O, acetylated Sudan IV, and acetylated Sudan black B have recently been used by Lillie and Burtner (p. 304) as oil-soluble dyes which do not react readily as naphthols and naphthylamines.

In fluorescence microscopy several acid and basic dyes have been used in simple aqueous solution as fat stains. Of these phosphine (C. I. No. 793), a basic acridine dyestuff, seems to be one of the best (p. 14). Also used are methylene blue (C. I. No. 922), rhodamine B (C. I. No. 749), magdala red echt (C. I. No. 857), all basic dyes, and the acid dyes titan yellow (C. I. No. 813) and thiazine red R (C. I. No. 225).

**Diazonium Salts**

Stabilized diazonium and tetrazonium salts, now commercially available, are being substituted for the freshly diazotized aryl amines in an increasing number of procedures. The delay incident to the completion of the diazotization process is thereby eliminated and the salts may be simply dissolved in water and the appropriate alkaline or acid buffer added at the moment of using.

Conditions of relatively mild alkalinity (pH 7.5 to 8.0) appear to be adequate to allow coupling of the diazonium salts with phenols, naphthols, etc.; though Danielli (p. 149) recommends pH 9 for coupling with histidine, tryptophane, tyrosine, purines, and pyrimidines.

A series of diazonium salts from one manufacturer gave pH levels ranging from 7.45 to 7.9 when 0.2% solutions were mixed with an equal volume of 0.2% disodium phosphate (Na$_2$HPO$_4$). The diazonium salts are generally stabilized with varying amounts of zinc chloride, aluminum chloride, or other secret reagents, which in case of necessity may be inquired about from the manufacturers. Where zinc or aluminum salts are found to interfere (as in certain enzyme localization procedures) suitable stable diazonium salts without these metals may generally be procured.

Diazonium salts generally yield colorless or light-yellow solutions, and are named for the colors which they produce on coupling, generally with a naphthol. Most of them are relatively simple compounds, often containing an anisidine as the diazotizable base. They are commercially available in mixtures representing 16–20% of the original aryl amine base.

Since information about the nature of the commercial products may not be readily available, I give the following data regarding a number of the presently available salts.

The zinc chloride double salt of $p$-chloro-$o$-nitroaniline diazo salt is available as Red 3GS Salt (NAC) which contains 20% of the original base (M.W. 172.5). A mixture of 0.2% Red 3GS Salt with 0.2% Na$_2$HPO$_4$ gave pH 7.45.

Several anisidine derivatives are included. The diazo salt of 4-nitro-$2$-aminoanisole is available as a zinc chloride double salt. Scarlet RS Salt
(NAC) contains 20% of the original base (M.W. 168). The mixture of its 0.2% solution with 0.2% Na₂HPO₄ gave pH 7.6.

The diazo salt of 3-nitro-4-aminoanisole is available as a zinc chloride double salt. Bordeaux GPS Salt (NAC) contains 20% of the original base (M.W. 168). The mixture of its 0.2% solution with 0.2% Na₂HPO₄ gave pH 7.8.

The diazo salt of 5-nitro-2-aminomethoxybenzene is known as Echrotsalz B (Holborn) and as Fast Red B Salt NAC. The latter is a zinc chloride double salt containing 20% of the original base (M.W. 168). Gurr supplies this compound under the names Fast Red Salt B, Naphthanil Diazoo Red B, Kernchrot Salz B and Nuclear Fast Red Salt B. The nature of the stabilizing agent in the Gurr products is not stated.

The diazo salt of aminoazotoluene is called Fast Garnet Salt G.B.C. (Gurr) and Garnet G.B.C. Salt (NAC). The latter contains no zinc and is standardized at 18% of the original base (M.W. 225).

The salt formed by diazotization of the product of coupling of diazotized p-nitroaniline into aminohydroquinone dimethyl ether is known as Fast Black Salt K (Gurr) and as Black K Salt NAC. The latter is a zinc chloride double salt containing 20% of the base (M.W. 302).

The diazo salt of 6-benzoylamino-4-methoxy-3-aminotoluene is Violet BN Salt (NAC), which is a zinc chloride double salt containing 20% of the original base (M.W. 256) and yielded pH 7.85 on mixture of its 0.2% solution with 0.2% Na₂HPO₄.

The diazo salt of 1-aminoanthraquinone is called Naphthanil Diazoo Red AL (duP), Red ALS Salt (NAC), and Fast Red Salt V (Ciba). The first contains 5% zinc chloride and 12% aluminum sulfate, according to Seligman and Mannheimer (J. Nat. Cancer Inst. 9:427, 1949). The second is available as a zinc chloride double salt containing 20% of the base (M.W. 223) and the mixture of its 0.2% solution with 0.2% Na₂HPO₄ gives pH 7.6. The zinc-free form prepared according to Seligman and Mannheimer is available from Dajac (cf. p. 208).

Tetrazotized o-dianisidine is called Diazoo Blue B (duP), which contains 5% ZnCl₂ and 20% Al₂(SO₄)₃, and Blue BNS Salt (NAC) which is a 1:1 molar precipitate with ZnCl₂. Gurr's Naphthanil Blue Salt B or Fast Blue Salt B is thought to be the same as the latter. Gomori identifies Fast Blue Salt BN (G) as this diazo salt. The mixture of the 0.2% solution of the NAC product with 0.2% Na₂HPO₄ gave pH 7.55. Nachlas and Seligman (J. Nat. Cancer Inst. 9:415, 1949) used Diazoo Blue B (duP) in their esterase technic.

The same authors also recommended a stabilized α-naphthyl diazonium-naphthalene-1,5-disulfonate, available from Dajac, to yield a red color with the liberated β-naphthol (p. 216).

Tetrazotized 4,4-diaminodiphenylamine, precipitated in 1:1 molar proportion with zinc chloride, is standardized to contain the equivalent of 16% of the original diaminodiphenylamine (Black BS Salt, NAC). I have used
this compound and Blue BNS Salt (NAC) in the Seligman-Ashbel carbonyl technic (p. 314). The 1:1 mixture of the 0.2% solution with 0.2% Na$_2$HPO$_4$ yielded pH 7.75.

Gomori also employs Naphthanil Diazo Red B (duP), Red B Salt (NAC) and Fast Red Salt V (Ciba) as diazo reagents for demonstration of enterochromaffin cells. They give an intense rusty-red color. Red B Salt NAC is C. I. No. 117, a stabilized p-nitro-o-anisidine, which yields deep pink colors on coupling with β-naphthol, which is at least partly soluble in toluene. The other two salts Gomori did not identify.

**Iodine**

Probably the oldest of all stains, used by Caventou (*Ann. de Chim. 31:358, 1826*) in the study of the structure of the starch granule, the element iodine is still widely used in a variety of tests, which are detailed in the appropriate sections. It gives reactions with amyloid, cellulose, chitin, starch, carotenes, and glycogen. It is used as a reagent to alter crystal and methyl violet so that they are retained by certain bacteria and fibrin. It may serve as an oxidizing agent. It is widely used for the removal of mercurial fixation artefacts.

It is used in the form of dilute alcoholic solutions and in various modifications of Lugol's solution. The term *Lugol's solution* has been quite variously used by different writers, as shown in Table 11. Many writers simply refer to it by name without hint as to which formula was used.

*Table 11

**Composition of Lugol's Iodine Solution***

<table>
<thead>
<tr>
<th></th>
<th>Iodine</th>
<th>Potassium Iodide</th>
<th>Distilled Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td>Lugol's &quot;rubefacient solution&quot; 1830</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>(B)</td>
<td>U.S.P. 1870 to XIV, A.M.A. editors, Army Techn. Manual</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>(C)</td>
<td>Lee 1890-1937, Cowdry 1943, 1948</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>(D)</td>
<td>Mosse in <em>Enzykl.,</em> Schmorl 1907, 1929 Roulet 1948, Lillie 1948, Langeron's &quot;double&quot; 1934, 1949*</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>(E)</td>
<td>Langeron 1934, 1949</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>(F)</td>
<td>Romeis 1932-1948, Roulet 1948 Gomori (v.p. 240)†</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

* Commonly called Weigert's or Gram-Weigert's iodine.
† Commonly called Gram's iodine.

For all these formulas, I recommend that the potassium iodide be first dissolved in one to two times its weight of water. The iodine then dissolves easily in this concentrated solution. When solution is complete, add the rest of the prescribed volume of water.
After sectioning, the prepared sections must be brought into appropriate solvents for the staining procedure to be used; and when the staining procedure has been completed the sections must then be brought without loss of the desired stain into media suitable for examination and temporary or permanent preservation.

The technics employed for these purposes vary with the method of sectioning and with staining and mounting methods.

Paraffin sections are generally first warmed to just above the melting point of paraffin and then immersed in xylene to dissolve out the paraffin. If immersion in xylene is prolonged for some minutes, heating is unnecessary. A second change of xylene is necessary to prevent carrying paraffin into succeeding reagents. The xylene is then removed from the sections in two successive changes of 100% alcohol. They turn white at this point. Two changes each of 95% and 80% alcohol follow. These reagents should be changed at intervals, and the second change of each may be moved over so as to form the first change and fresh reagents then replace the second changes.

Nedzel's observations (Quart. J. Microscop. Sci. 92:343, 1951) indicate that the usual practice of immersing unheated sections in cold xylene may be inadequate to remove paraffin from sections. Birefringent crystals with the same melting point as the paraffin employed may often be found in cell nuclei. These may be stained with oil red O heated to above the melting point of the paraffin. These birefringent crystals are more apt to persist after brief acetone dehydration than after alcohol-xylene sequences. The use of hot xylene at either the deparaffinization or the clearing stage, or the practice of melting the paraffin before immersion in xylene, will tend to eliminate
these artefacts; and heating of sections mounted in resins to 60° or 65° C. will cause most of them to disappear permanently.

_Torn or ragged sections_, or those which tend to become detached from the slides during staining or metallic impregnation procedures may be attached more securely to the slides by immersion in 0.5–1% ether alcohol solution of collodion for five or more minutes. This step is inserted immediately after the 100% alcohol step. After soaking in collodion drain the sections for about one minute and then harden for five minutes or as much longer as is convenient in 80% alcohol. When it is also necessary to iodize the sections for removal of mercury precipitates, it is advantageous to combine the hardening of the collodion with the iodine treatment by using a 0.5% solution of iodine in 80% alcohol for both purposes. It may be necessary to prolong staining intervals to as much as double the time usual in technics not devised for collodionized paraffin or attached celloidin and nitrocellulose sections. This procedure, like celloidin imbedding, tends to diminish shrinkage and creation of artificial spaces during staining. Before acetylation or benzoylation procedures which employ pyridine as the solvent, collodionization is futile. Pyridine dissolves collodion. If later portions of the procedure require collodionization, wash the sections with 100% alcohol after the pyridine and then collodionize as usual.

Collodion films prevent the access of enzymes to their substrates in the sections. Therefore, if the procedure is necessary for later parts of the technic, dehydrate and collodionize after the completion of digestion.

Goetsch, Reynolds, and Bunting (Proc. Soc. Exper. Biol. & Med. 80:71, 1952) report successful staining of undeparaffinized paraffin sections by direct 18 hour immersions in aqueous or alcoholic 3% eosin solutions, Van Gieson's stain, 2% anilin blue, 0.25% light green, 0.05% toluidine blue, 2% methylene blue, 0.5% methyl green, or Bullard's hematoxylin.

Less cytologic distortion than in deparaffinized sections is claimed for this method. After staining, the sections are washed in water or alcohol to remove the excess; then dried and deparaffinized and mounted. The paraffin, unlike collodion, does not prevent access of amylases to glycogen. (But see p. 275.)

In using undeparaffinized sections for staining, avoid heating to near the melting point of the paraffin. Even momentary melting forms a virtually impenetrable film. Ordinary staining procedures may require 6 to 12 times as long as in deparaffinized sections; enzyme digestions 30 to 60 times as long.

_Iodizing and Hydration_. When material fixed with mercury salts is being handled, substitute a five minute bath in 0.5% iodine in 80% alcohol for the usual second 80% alcohol step. The usual prescription calls for 0.3–0.5% in 70–95% alcohol. Mallory used 0.5% in 95% alcohol. After the iodine, rinse the material in water and immerse it for a few seconds in 5% sodium thiosulfate (Na₈S₂O₃·5H₂O). Mallory used a five minute bath in 0.5% solution. Then wash five minutes in running water.

With material fixed with nonmercurial fixatives the foregoing steps are
omitted and the sections are transferred either directly to the staining solution (if this is in a hydroalcoholic solvent) or to water. However, if both types of fixations—mercurial and nonmercurial—are included in the material being stained, the iodine thiosulfate procedure seems to be harmless to the latter in most instances.

Postmordanting

Often one desires to use some special staining method for which the author has prescribed a fixation method other than that used on the tissue in question. Although in many instances the methods will work quite as well after other fixations than that prescribed, it is still often desirable to have the prescribed fixation or to be able to modify the tissues by some pretreatment in lieu of the prescribed fixation.

In lieu of Bouin or Zenker fixations for Masson trichrome methods, I have found that mordanting for two minutes in a saturated (6-8%) alcoholic picric acid solution in place of Bouin’s fluid, (cf. pp. 240, 350–351), or with saturated aqueous mercuric chloride solution for five minutes in place of Zenker’s fluid, will serve (Stain Technol. 15:17, 1940).

Peers reported that three hours’ mordanting in saturated aqueous mercuric chloride solution than 58° C. was required for formalin fixed material in place of the primary Zenker fixation prescribed by Mallory for his phosphotungstic acid hematoxylin stain (Arch. Path. 32:446, 1941). Earle, however, was able to stain successfully after fixation in buffered neutral formalin (personal communication).

I have long used a 48 hour mordanting of brain tissue in 2.5% potassium bichromate after primary formalin fixation, before dehydration and embedding, in lieu of a primary Orth fixation, to prevent pericellular shrinkage during embedding. Apparently this procedure does not serve to preserve the characteristic staining of chromaffin in the adrenal.

In place of primary fixation in Ramón y Cajal’s formalin ammonium-bromide for metallic-impregnation methods on brain tissue, Globus (Arch. Neurol. & Psych., 18:263, 1927) prescribes soaking frozen sections of old formalin-fixed material in a 1:10 dilution of 28% ammonia water for 24 hours. Then rinse rapidly in two changes of distilled water and immerse for 2–4 hours in a 1:10 dilution of 40% hydrobromic acid. Then rinse in two changes of 1:2,000 dilution (Conn and Darrow) of 28% ammonia water and proceed.

Arcadi (Stain Technol. 23:77, 1948) modifies the foregoing procedure by placing the sections in concentrated (28%) ammonia water and then washing slowly with a 1 mm. stream of water for 24 hours, repeating this procedure for a second 24 hour period, and ending the ammonia treatment with a seven minute bath in concentrated ammonia water. From this, the sections are transferred directly to a ½ dilution of 40% hydrobromic acid and incubated at 38° C. for one hour. This is followed by four washes of four
minutes each in distilled water. Arcadi used this method for preparation of old formalin material of monkey brains for a modified Del Rio-Hortega-Penfield method for oligodendroglia.

Dehydration

After staining, paraffin sections are usually dehydrated with two changes of 95% alcohol followed by two of 100% alcohol. Then follows a mixture of equal volumes of 100% alcohol and xylene, and then two changes of xylene. In place of alcohol two or three changes of acetone or of 99% isopropyl alcohol may be used, and these are followed by an equal-volume mixture of the dehydrating agent and xylene, and then two changes of xylene as before. Some have used toluene in place of xylene as a clearing agent; but there is more hazard of drying, and more evaporation from the clearing jars; and it is at least no better.

The choice of a dehydrating agent depends on several factors. Alcohol extracts methylene blue and other thiazin dyes from the sections; acetone does not. Isopropyl alcohol also fails to extract thiazins appreciably. Acetone dissolves collodion films from sections; isopropyl alcohol does not; and ethyl alcohol does partly. Ethyl alcohol aids in the differentiation of certain stains.

Mounting

Paraffin sections stained with oil-soluble dyes for the demonstration of certain lipid substances which are not lost in the dehydration, clearing, embedding, deparaffinization, and hydration procedures must be mounted in media which are not fat solvents. For this purpose it is sufficient to drain off the excess water and mount in gum syrup, glychrogel, glycerol gelatin, or the like.

Mounting in Resinous Media. After sections stained by ordinary methods have been cleared in xylene, they are mounted preferably in some resinous medium. Formerly xylene and chloroform solutions of Canada balsam or gum dammar, thickened cedar oil, euparal, and the like were preferred as mounting media. Canada balsam is quite useful for hematoxylin eosin stains. For anilin blue and acid fuchsin connective tissue stains Curtis (Arch. de méd. expér. et d'anat. path. 17:603, 1905) first prescribed acidifying with salicylic acid. A quantity of xylene balsam of thin syrupy consistency is saturated with salicylic acid crystals, filtered through filter paper, and mixed with an equal quantity of xylene balsam. The use of the fully saturated solution as a mounting medium is apt to produce visible crystals in the preparations. All of the above resins are unsatisfactory for Romanovsky stains, causing progressive fading of the blue component. The best medium for preservation of these stains is heavy mineral oil, but preparations must be sealed with a gelatin or pyroxylin cement and inevitably leak. Langeron suggests Apáthy's gum syrup as a cement.

Actual mounting of resin and gum-syrup mounts is accomplished in this
wise. Select cover glasses of appropriate size to cover the section. We use 22 x 22, 22 x 32, and 22 x 44 mm. sizes. Deposit in the center of a square cover a moderate-sized drop of thin syrupy xylene resin or gum syrup; with long covers, two drops. The cover glasses lie flat on a paper towel or blotter on the table. As many as six cover glasses may be thus laid out at one time for xylene resin mounts; but with gum-syrup mounts of frozen sections, one at a time is better. The stained section is taken from xylene or water, as the case may be, drained, and placed face down with one long edge of the slide in contact with the blotter adjacent to the edge of the selected cover glass, and with the other edge perhaps 1 cm. up from the table. The slide is then gradually rotated downward with the edge in contact with the blotter as an axis until the section comes in contact with the drop of xylene resin or gum syrup. The drop spreads to the edges of the cover glass, picking it up from the table surface. If air bubbles chance to be included under the cover glass, they may be coaxed out by gentle pressure on the cover glass with a needle point, or by slightly raising one edge of the cover glass. Sometimes it is necessary to add more mounting medium. One then blots the two lateral edges of the cover glass, holding the slide face down at an angle of about 30° to the table top, to remove excess mounting medium. Slides are then laid flat on trays in a warm place to dry. Should any resinous medium get onto the upper surface of the cover glass, it may be best removed by steadying one corner of the cover glass with a finger tip while dragging a sheet of lens paper moistened with xylene across the cover glass with the other hand. No pressure is put on the lens paper, which should adhere to the cover glass only by capillary attraction.

In mounting large sections for topographic study of whole human breasts, cerebral hemispheres, bones, embryos, and the like, air spaces are quite apt to form between the base and covering glasses after mounting has been accomplished with apparent success. The use of thicker resin solutions, warmed to lower the viscosity, is suggested to avoid aspiration of air after mounting. The polystyrenes are unsuitable for this purpose because of their very high viscosity at relatively low solution concentrations. Natural balsam, the relatively neutral ester gums, and some of the cycloparaffin and terpene resins are suggested; if warmed somewhat they can be used at 60–80% concentration in xylene.

Opaque or cloudy areas in mounted stained sections are often due to incomplete dehydration. Microscopic examination reveals numerous fine droplets of water in and above the section. Such areas tend to show severe fading of stains. The remedy is simple. Remove the cover slip, wash off the synthetic resin or balsam with xylene, and again dehydrate with the appropriate agent (100% alcohol, acetone, or isopropyl alcohol); again clear through the appropriate xylene mixture and two or three changes of fresh xylene, and remount.

Restaining. If fading has already occurred, it may be necessary to re-stain.
In this case, after soaking off the cover glass in xylene (and it may take several hours to loosen it) pass through 100% alcohol and succeeding reagents just as with a fresh section.

The same technic may be employed when only a stained section is available and some other staining procedure is desired than that originally employed. In this case one may need to decolorize the previous stain with acid alcohol; then wash thoroughly with water and proceed with the new technic. Ordinarily sections previously stained with iron hematoxylin are not suitable for the iron reaction with potassium ferrocyanide, since the iron lake reacts.

Where there were two sections of the same block on a slide, I have sometimes restained one of them by another method, leaving the other covered by the cover glass during the new staining procedure.

Nitrocellulose sections may be affixed to slides either before or after staining. They are floated out in a vessel of appropriate size onto the surface of clean slides and manipulated on the slanted, partly immersed slide until they lie smooth. They are then blotted firmly with filter paper.

If not already stained, one should tilt an open bottle of ether so that the heavy vapors can be seen descending from the mouth of the bottle onto the surface of the slide. This softens the nitrocellulose so that it adheres firmly to the slide. Or one may dip the slide into 0.5% nitrocellulose in ether alcohol mixture for a few seconds, drain, wipe the back of the slide clean, and harden the film by immersing the slide in chloroform for 5–10 minutes. Sections are then carried into successive baths of 95% alcohol, 80% alcohol (0.5% iodine in 70% alcohol 10–15 minutes, 5% sodium thiosulfate 5–10 minutes for mercury fixations only) and water; or directly into stain if this is in a hydroalcoholic solution. After staining, wash free of excess stain as prescribed in the method used.

Loose Nitrocellulose Sections. Some prefer to stain celloidin and nitrocellulose sections before attaching them to slides. This is particularly convenient when large numbers of sections from the same block are to be prepared for class use.

In this case sections are transferred on the slightly curved spatulae known as section lifters, perhaps with the aid of a needle, from the 80% alcohol in which they are stored, through 70% and 50% alcohols and water, or through 0.5% iodine in 70% alcohol if it is necessary to remove mercury precipitates; and thence to sodium thiosulfate solution and to water, or directly into alcoholic staining solutions.

Dehydration and Clearing of Nitrocellulose Sections. After staining, loose sections are dehydrated and cleared by transfer through dishes of the same successive reagents used for attached nitrocellulose sections. Attached sections are handled much as are paraffin sections, and the same technics apply to collodionized paraffin sections.

The more resistant stains may be dehydrated with 95% alcohol, cleared in Weigert’s (Ehrlich’s Encyklopädie) “carbol-xylene” which is a mixture of
one volume of melted phenol crystals with three volumes of xylene, washed in
two to four changes of xylene and mounted as described for paraffin sections
in xylene clarite, balsam, or other suitable resin.

**Destaining of Nitrocellulose.** If the staining of the nitrocellulose with basic
anilin dyes is objectionable, it may be removed in 2% colophonium alcohol,
and dehydration and clearing completed as above or by one of the following
methods.

**Clearing of Sections Stained by Easily Extracted Dyes.** For basic anilin
dyes which are extracted by his carbol-xylene, Weigert (Ehrlich's *Enzyklo-
pédie*) suggested clearing by repeated application of xylene and blotting
with filter paper between applications.

Many writers have used various essential oils for clearing, among which the
best seems to have been the Cretan origanum oil. Sections stained with basic
anilin dyes, especially with azure eosinates, may be dehydrated with two or
three changes of 99% isopropyl alcohol, which does not dissolve nitrocellu-
lose and does not extract the azure either from the section or from the sur-
rounding nitrocellulose; and then passed through a mixture of equal parts of
isopropyl alcohol and xylene and two or three changes of xylene. Or they
may be differentiated with a 2% solution of colophonium in 95% alcohol
until the nitrocellulose is colorless, and then dehydrated and cleared by the
isopropyl alcohol xylene sequence just described. Or after the colophonium
alcohol one may blot and flood with xylene alternately for two or three times
until the sections are clear.

**Frozen sections stained for fats** are floated out smooth on clean slides.
One brings one edge of a section in contact with the partially immersed,
obliquely held slide, raises the slide gradually so that the section settles
smoothly on the surface of the glass, smoothing out folds by varying the angle
of immersion so that the folded portion is floated loose while the rest remains
above water on the slide. Needle manipulation may occasionally be neces-
sary, especially with irregular or torn sections. When the section is satis-
factorily smoothed out, drain and blot it with smooth, hard filter paper by
running the tip of one finger along the slide on top of the paper while one
end of the paper and slide are firmly held down with the other thumb. Then
mount as described above (p. 97) in a suitable aqueous medium. Glycerol
can be used for temporary mounts. These may be sealed by carefully drying
the border zone of the slide and cover glass and painting with xylene or
toluene Clarite or with a pyroxylin cement.

To seal sticky glycerol gelatin or Arlex gelatin mounts, I often dip a glass
rod in 20% polystyrene in xylene, and run it along each edge of the cover
glass. It dries to nonstickiness in an hour and can be packed away in direct
contact with other slides on the next morning, without fear of the slides
sticking together.

Lanolin rosin is recommended by Romeis as the best of the sealing media
for aqueous mounts. Dry 20 Gm. anhydrous lanolin in a hot porcelain evapo-
rating dish, stirring it for 15 or 20 minutes. Then add, bit by bit, 80 Gm. rosin, stirring the while until a clear, light-brown fluid results. It is best to heat it on a closed electric hot plate to avoid the chance of fire. Pour it out into small molds and let it harden. To use, heat a glass rod or metal spatula quite hot, melt off a few drops from a block of the lanolin rosin, and fix the corners of the cover glass in place. Then apply more hot, melted rosin to complete the sealing.

For permanent mounts, media containing gum arabic or gelatin are usually employed. The first dry hard after a time; the second have to be melted for use, and set on cooling. Formulae for several usually successful media are described on p. 109, and tabulated in Table 13, p. 111.

Resinous Mounting Media

These are composed in general of a solid natural or synthetic resin dissolved in a suitable solvent to lower the viscosity to a point where the solution will readily enter tissue interstices, flow between slide and cover glass to fill the space completely and quickly release entrapped air bubbles. The solvent (either that in which a natural resin is dissolved as it comes from its source or a suitable added solvent, usually an aromatic hydrocarbon; but sometimes an alcohol or a chlorinated hydrocarbon) must be sufficiently volatile to allow fairly prompt drying of the resin to a hard state, and at the same time not so volatile as to dry prematurely during mounting.

It has been observed that highly volatile solvents, such as benzene and to a less extent toluene, are prone to produce air spaces under cover glasses by their excessive evaporation. This fault may be assigned also to low concentration of resin in the solvent. However, higher-boiling solvents, such as xylene (B.P. 138–144° C.), trimethylbenzene (B.P. 165–176° C.) and diethylbenzene (B.P. 181–184° C.) are less likely to give rise to air bubbles. Indeed, trimethylbenzene solution mounts remain liquid internally for a long time.

The rate of drying and the tendency of some mounting media to aspirate air into the mounting space have importance in relation to the conditions of study. Prompt drying to nonstickiness and to nondisplaceability of cover glasses is of great importance to the surgical pathologist; the late aspiration of air bubbles poses problems only if it is necessary to refer back to the same section. On the other hand, with 50–100 μ sections, and mounts of membranes, eggs, and small embryos, time is of less consequence, and it becomes highly important that air should not enter the preparations.

The refractive indices of mounting media have been much discussed by microscopists, some of whom strongly advocate media with low indices, whereas others insist on high. Without going into theoretical optics, the practical effect seems to be that in media of refractive indices ranging from 1.44 to 1.50 much detail is apparent microscopically, even in unstained and uncolored objects, by reason of the difference in average refractive index of
tissues (1.530 to 1.540) from that of these mounting media. As the index of refraction of the mounting medium approaches that of the tissue, the latter becomes more and more transparent, and unstained objects may be extremely difficult or quite impossible to discern. I have seen partly faded Nissl preparations in which one had great difficulty in even finding the section, either grossly or microscopically. When the critical range (about 1.535) is passed, unstained objects again become evident by reason of their now lower refractive index, and media with refractive indices above 1.60 may be quite useful.

Much stress has been laid by some writers, notably Groat, on the refractive index of the dry resin as opposed to that of the solution used for mounting. Groat agrees with me (personal correspondence, 1950) that both indices are important. Obviously the refractive index of a resin solution will lie between that of the solvent and that of the dry resin; and as the solvent evaporates, the refractive index of the medium surrounding the specimen gradually approaches that of the dry resin.

It appears from the foregoing discussion that no single mounting medium will serve all purposes equally well. For this reason data regarding refractive indices of mounting solutions and of dry resins are presented in Table 12.

Natural Resins

Balsams are deep-amber or yellow resins composed to a considerable extent of terpenes and their carboxylic acids such as abietic acid (pine resin), laevopimaric acid (French fir), both of which are said to contain two carbon double bonds (Karrer).

Canada balsam is derived from the liquid rosin of the Canadian fir Abies balsamea. Its acid number ranges from 88 to 106, saponification number from 105 to 116 (Lange). When prepared by heating with water until the evolution of steam ceases, it solidifies to a clear yellow, hard, brittle resin which is freely soluble in xylene, toluene, benzene, chloroform, etc. Xylene solutions of 60–65% by weight of the hard resin correspond in consistency to the syrupy solutions commonly recommended. The resin sets slowly and takes many months to dry hard enough so that slides will not stick together if warmed to summer room temperatures.

Hematoxylin and eosin stains are well preserved in Canada balsam, though there is a gradual differentiation of the eosin so that after a year or two muscle, connective tissue fibers, cytoplasms, and oxyphil inclusion bodies present a considerably greater difference in intensity of staining than was evident when the preparations were fresh. I have found renal intranuclear inclusion bodies much more conspicuous in such aged preparations than by most other methods. Canada balsam is superior to many of the synthetic media for preservation of the cobalt sulfide deposits in alkaline phosphatase preparations. Basic aniline dyes are poorly preserved and Prussian blue fades fairly soon. Acid fuchsin in Van Gieson stains fades, but this fading may be retarded by half saturating the balsam with salicylic acid. This procedure I
have modified from Curtis (Arch. de méd. expér. et d'anat. path. 17:603, 1905). Its rationale is obscure.

Natural syrupy Oregon fir balsam possesses a refractive index of 1.5271. On heating to 200° C. it does not boil, but with an air stream it loses about 18% of its weight and solidifies on cooling. Its refractive index then reads 1.5407. This resin sets slowly and takes months to dry hard. It fades basic aniline dyes, bleaches Prussian blue promptly, and conserves cobalt sulfide well. Thus its properties are closely similar to those of the usual xylene solutions of Canada balsam.

Dammar is a common resin used in the varnish and lacquer industries, derived from various East Indian trees of the genus Shorea. It softens at 75° C. and melts at 100° C. Its acid and saponification numbers are 35 and 39 respectively (Lange), and it contains unsaturated compounds (iodine number 64–112). It is dissolved in aromatic hydrocarbons, chloroform, ether, and the like. It is commonly used as a xylene solution of about 60–75% resin content by weight.

Since dammar is often dirty, and its 60–75% solutions are practically impossible to filter because of their viscosity, the usual practice is to dissolve it in a much larger volume of benzene or xylene, filter, and then evaporate down to the required viscosity or to the predetermined weight of solution. Evaporation of xylene is greatly expedited by passing a fairly rapid air stream over the surface of the solution while heating it on a closed electric hot plate under a chemical hood.

Rosin or colophony, obtained from various pines, is composed largely of abietic acid. It is a dark brown, brittle resin with a melting point of 120–135° C. Acid number is 155–175, saponification number 167–194, iodine number 80–220. This rosin is sometimes used as a 1% alcoholic solution for acid differentiation of azure stains, occasionally as the xylene solution for mounting, but most often as a constituent of varnishes and cements.

Cedar oil when used as a mounting medium is usually concentrated to the point where its refractive index has risen from the initial 1.503 to about 1.5150. On heating in an oil bath, the native solvent boils off at 168–187° C., and the oil loses about 52% of its initial weight as the thin cedar oil for clearing. The refractive index of the residual resin is 1.5262, and the resin is solid at room temperatures.

Cedar-oil mounts set slowly and take months to dry hard. Basic aniline dyes fade gradually. Wolbach used this action to differentiate his Giemsa stain for rickettsiae (p. 385). Prussian blue fades completely in this medium, but cobalt sulfide is well preserved.

**Semisynthetic Mixtures**

**Euparal** is composed of gum sandarac dissolved in a mixture of eucalyptol and paraldehyde with a liquid mixture of camphor and phenyl salicylate of unstated proportions and a refractive index of 1.53576, according to Gilson.
Gum sandarac contains 85% sandaracolic acid and 10% callitrolic acid. Its acid number is 140–154, saponification number 142–174, iodine number 66–160 (Lange), and these characteristics of acidity and unsaturation place this mixture in the same general group as the natural resins. The carbonyl group of the paraldehyde offers a possible additional reducing agent.

The refractive index of Euparal is usually given as 1.483. At 20° C. we got 1.4776. On concentration in partial vacuum at 60° C. it rose to 1.5174, and the resin solidified on cooling. Romeis states that the solid resin has a refractive index of 1.535.

Euparal sometimes causes discoloration and fading of hematoxylin stains (Lee). The green or vert variant contains a small amount of a copper salt to prevent this action. Basic aniline dyes and Romanovsky stains are fairly well preserved, though not as well as in some synthetic resins. Prussian blue is reduced and bleaches. Cobalt sulfide is well preserved.

Diaphane is composed of a juniper-gum base with certain natural and synthetic phenols, according to its manufacturers, the Will Corporation. No formula has been published. The refractive index of Diaphane and of green Diaphane is quoted at 1.483; at 20° C. we found 1.4777 and 1.4792. On concentration in vacuo at 60° C., Diaphane lost 59.6% of its original weight and solidified on cooling. Its refractive index was 1.5486.

Like Euparal vert, green Diaphane contains a little copper to intensify and conserve hematoxylin stains. Basic aniline dyes and Romanovsky stains are fairly well preserved, Prussian blue is decolorized, and cobalt sulfide is excellently conserved. Some fading of fuchsin and of acid fuchsin is observed, which I am inclined to attribute to reduction to leuco dyes rather than to acid action.

**Ester Gums**

Attempts at neutralizing the acid of the natural balsams with small amounts of sodium or calcium carbonate in the cold have been largely futile, since the resins themselves consist largely of carboxylic acids.

Esterification of abietic acid from pine rosin with glycols or glycerol has resulted in the formation of essentially neutral esters, called generally ester gums and used widely in the varnish industry. The class of these which has proved most useful in microscopy is the neutral or low-acid class, with acid numbers below 8. Clear, amber to light-brown resins result, soluble in aromatic hydrocarbons to 70% or 80% at syrupy viscosity.

After mounting in 75% ester gum in xylene, cover glasses become fairly immovable in an hour or two; but the resin remains rather soft, and slides will stick together if packed back to face, unless dried for some weeks.

Stains with basic aniline dyes and Romanovsky stains are well preserved. Prussian blue is reduced and decolorized. Cobalt sulfide is well preserved. Fuchsin and acid fuchsin tend to decolorize.
Those now in use include styrene polymers (Monsanto's Lustron L 2020 and Lustrex L-15, Zirkle's L-15, Gurr's Depex, Distrene, and others); Groat's styrene + isobutyl methacrylate copolymer; synthetic terpene resins, especially β-pinene polymers (Harleco synthetic resin HSR, the new Fisher Permount, and the Will Corporation's Bioloid); a coumarone + indene copolymer (Technicon resin), a coumarone mixture with other resins (Gurr's medium), a maleic polymer with plasticizer (Gurr's Xam), the naphthalene or cyclopentadiene polymers (Clarke and Clarke's introduced by Groat and now unavailable, and the old Fisher Permount, now replaced by a new β-pinene polymer sold under the same name); and some entirely secret proprietary preparations such as Rhenohistol of the Rheinpreussen Chemical Works, Homberg-Niederrhein, Germany; and Mahady's Micromount. According to the Farbenfabriken Bayer, Leverkusen-Bayerwerk, Germany (letters, 1951) Caedax is a mixture of a chlorinated aromatic hydrocarbon and a cyclohexanone dissolved in xylene.

Brief mention is made also of a few media of very high refractive index, such as Hyrax, nD 1.82248 (Hanna, J. Roy. Micr. Soc. 50:424, 1930); Flemming's Naphrax, nD 1.76 to 1.80 (J. Roy. Micr. Soc. 63:34, 1943); and two media of the George T. Gurr Co.; Clearax, a diphenyl resin of 66° C. melting point and 1.666 index of refraction, furnished as a chloroform solution of nD 1.602; and Refrax, a naphthalene formaldehyde polymer, similar to Naphrax, of 60° C. melting point and 1.78 refractive index, furnished as a xylene solution containing plasticizer (Flemming used dibutylphthalate in Naphrax), nD of solution 1.598. These media are employed particularly by students of diatoms.

In general these synthetic resins are quite neutral, and basic aniline dyes are well preserved in them. The chlorinated aromatic hydrocarbon of the usually neutral Caedax may occasionally (in contact with water?) break down and become highly acid, and Mahady's Micromount appears to be acid from the start. Otherwise the resins differ importantly in refractive index (see p. 100 for discussion), in rate of setting and drying to a nonadhesive state, and in degree of residual unsaturation. The more unsaturated resins, and the ketonic resins including the natural resins and ester gums, the terpenes and β-pinene resins, the coumarones and coumarone-indene resins, though often quite satisfactory for stains where oxidation-reduction potential is not involved, appear to reduce Prussian blue to the greenish-white ferrous ferrocyanide, but preserve cobalt-sulfide preparations relatively well. Conversely, the more oxidized or saturated resins preserve Prussian blue well, but allow fading of cobalt sulfide preparations. There are also important differences in solubility in solvents, in viscosity of solutions, in rate of drying, and in tendency to form air bubbles in mounts. Plasticizers are added to combat this last tendency, but tend to retard drying to nonstickiness.
Some fading of the acid fuchsin component of Van Gieson stains is observed with the Bioloid resin, Euparal, Diaphane, and Canada balsam and to a less extent with the Micromount, Xam, and the ester gums. There is moderate fading of the azure component of azure-eosin stains with Diaphane and Xam; severe fading with Micromount, which bleaches even alum hematoxylin. The eosin component of this stain fades somewhat in Euparal and Bioloid, and occasionally in polystyrenes.

Polystyrene media are usually employed in aromatic hydrocarbon solvents. Their viscosity is too great to permit much over 20-25% concentration of resin. Consequently, they set rapidly but tend to form large air spaces under cover glasses. This tendency is combated either by use of a higher-boiling solvent, such as diethylbenzene, trimethylbenzene, p-cymenc, or a mixture of equal volumes of amyl benzene and xylene; or by addition of orthocresyl phosphate or dibutylphthalate as plasticizers. Excessive amounts of plasticizer must be avoided, as they retard setting and hardening. Addition of 5 cc. dibutylphthalate to 70 cc. xylene and 25 Gm. polystyrene seems adequate to prevent air aspiration, and does not greatly retard setting; 10 cc. dibutylphthalate and 65 cc. xylene did considerably delay setting of the mount.

I strongly recommend 20-25% solutions of Monsanto’s Lustron L-202D in diethylbenzene,* trimethylbenzene,* or a mixture of secondary amylbenzene and xylene in equal volumes. Some find the odor of the amylbenzene disagreeable. These fluids are easy to mount in: the cover glasses are immovable in an hour, and the slides may be packed tight together without sticking by the following morning.

Gurr’s Depex resin is quite satisfactory. Kirkpatrick and Lendrum’s DPX contained 20 Gm. polystyrene, 15 cc. tricresylphosphate and 80 cc. xylene. I have had excellent results also with solutions of 5 or 10 cc. dibutylphthalate, 70 or 65 cc. xylene and 25 Gm. polystyrene. The lower quantity of plasticizer permits faster setting and is usually adequate to prevent bubbles in thin sections. For thick sections Zirkle uses 20 Gm. Lustrex 15, 20 cc. dimethoxytetraethylene glycol and 60 cc. xylene. The medium sets slowly, but forms no bubbles even with 50 to 100 μ sections. Ollet (J. Path. & Bact. 63:166, 1951) also recommends Lustron L-2020, of which he dissolves 100 Gm. in 50 cc. dibutylphthalate and 300 cc. monochlorobenzene. Xylene is omitted because it “leads to fading of the Gram’s stain.”

Polystyrenes contain virtually no titratable acid and contain little or no residual unsaturated material. Consequently they are excellent for conservation of stains with basic aniline dyes, hematoxylin, Van Gieson’s stain, and Mallory anilin blue variants. The Prussian blue and Turnbull’s blue of Perl’s, Tirmann-Schmelzer’s, and Schmorl’s ferric ferricyanide reduction reactions are well preserved. Cobalt sulfide gradually disappears, presumably by oxida-

* Obtainable from the Eastman Chemical Company, Rochester, N. Y.
| RESIN                | CLASS                          | SOLVENT & % RESIN | REFRACTIVE INDEX
<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>solution</td>
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<tr>
<td>Canada balsam</td>
<td>dried natural resin</td>
<td>xylene 60%</td>
<td>1.5232</td>
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<tr>
<td>Oregon fir balsam</td>
<td>liquid natural resin</td>
<td>turpentine ca. 82%</td>
<td>1.5251</td>
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<tr>
<td>Gum dammar</td>
<td>dried natural resin</td>
<td>xylene 60%</td>
<td>1.5317</td>
</tr>
<tr>
<td>Cedar oil</td>
<td>liquid natural resin</td>
<td>turpentine ca. 48%</td>
<td>1.5030</td>
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<tr>
<td>Euparal</td>
<td>semisynthetic resin mixture</td>
<td>(see text)</td>
<td>1.4776</td>
</tr>
<tr>
<td>Diaphane</td>
<td>semisynthetic resin mixture</td>
<td>(see text)</td>
<td>1.4777</td>
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<tr>
<td>Ester gum</td>
<td>glycol resin</td>
<td>xylene 75%</td>
<td>1.5952</td>
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<td></td>
<td>acid esters</td>
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<td>1.5379</td>
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<td>Harleco HSR</td>
<td>β-pinene polymer</td>
<td>xylene 60%</td>
<td>1.5202</td>
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<tr>
<td>Fisher Permound</td>
<td>β-pinene polymer</td>
<td>toluene 60%</td>
<td>1.5144</td>
</tr>
<tr>
<td>Willco Bioiod</td>
<td>β-pinene polymer</td>
<td>xylene 60%</td>
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<td>Technicon Resin</td>
<td>coumarone indene polymer</td>
<td>benzene + xylene 60%</td>
<td>1.5649</td>
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<tr>
<td>Gurr's Medium</td>
<td>coumarone resin mixture</td>
<td>cineole ca. 77%</td>
<td>1.5310</td>
</tr>
<tr>
<td>Gurr's Pale Medium</td>
<td></td>
<td>cineole ca. 77%</td>
<td>1.5082</td>
</tr>
<tr>
<td>Product</td>
<td>Description</td>
<td>Reference</td>
<td>Specific Gravity</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>Gurr's Xam</td>
<td>maleic polymer plasticized</td>
<td>xylene ca. 77%</td>
<td>1.5219 0.5401 0</td>
</tr>
<tr>
<td>Clarite</td>
<td>cycloparaffin polymer</td>
<td>toluene or xylene 60%</td>
<td>. .</td>
</tr>
<tr>
<td>Clarite X</td>
<td>cycloparaffin polymer</td>
<td>xylene 60%</td>
<td>1.5352 1.5647 c</td>
</tr>
<tr>
<td>Fisher's old Permount</td>
<td>cycloparaffin polymer</td>
<td>toluene 62.5%</td>
<td>1.5172 1.5376 c</td>
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<tr>
<td>Caedax</td>
<td>cyclohexanone &amp; chlorinated diphenyl resin</td>
<td>xylene 82%</td>
<td>1.6306 1.6724 c</td>
</tr>
<tr>
<td>Rhenohistol-</td>
<td>ketone + HCHO condensation</td>
<td>xylene 60%</td>
<td>1.520 1.533 c</td>
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<tr>
<td>Hyrax</td>
<td>naphthalene resin (secret)</td>
<td>aromatic hydrocarbon</td>
<td>1.8225 H</td>
</tr>
<tr>
<td>Naphrax</td>
<td>naphthalene HCHO polymer</td>
<td>xylene + dibutyl phthalate</td>
<td>1.76 to 1.80 F</td>
</tr>
<tr>
<td>Gurr's Clearax</td>
<td>diphenyl resin</td>
<td>chloroform</td>
<td>1.602 1.666 a</td>
</tr>
<tr>
<td>Gurr's Reffax</td>
<td>naphthalene HCHO polymer</td>
<td>xylene + plasticizer</td>
<td>1.598 1.780 a</td>
</tr>
<tr>
<td>Gurr's Depex</td>
<td>polystyrene</td>
<td>xylene + plasticizer</td>
<td>1.5228 1.6 a</td>
</tr>
<tr>
<td>Mahadey's Micromount</td>
<td>. . . . . .</td>
<td>xylene (?) ca. 43%</td>
<td>1.4918 1.4839 c</td>
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<tr>
<td>Groat's Copolymer</td>
<td>styrene + isobutyl methacrylate</td>
<td>toluene 45%</td>
<td>1.5193 1.5500 c</td>
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<tr>
<td>Lillie's polystyrene (Monsanto L-2020)</td>
<td>styrene polymer</td>
<td>diethylbenzene 20%</td>
<td>1.5150 1.6193 c</td>
</tr>
</tbody>
</table>

Footnotes: c calculated from solution data; o observed after concentration of commercial solution; a as advertised by manufacturer; R according to Romeis; H according to Hanna (see text); F according to Flemming (see text); G according to Groat (*Anat. Rec. 74:1, 1939); * Immersion oil; g determined by Greco.
tion, but may be restored by demounting and reimersion in ammonium sulfide.

Mineral Oil

This medium is unexcelled for preservation of azure eosin, Van Gieson, and similar stains. I used it for several years before the introduction of Clarite, for mounting azure eosin stains; and sections twenty years old still show excellent preservation of color. The oil does not dry, however, and preparations sealed with nitrocellulose cements often leaked badly. Nevertheless, stains are well preserved and preparations may be remounted after brief soaking in acetone to dissolve the cement. Refractive indices are low—1.460 to 1.483—the heavier, more viscous oils possessing the higher indices. Modified mineral oils, sold for immersion oil, may be obtained which possess refractive indices about 1.518 to 1.520, or nearly that of crown glass. I have used such oils only for temporary mounts; but it is to be noted that Giemsa stained blood films, put away without cleaning after use of such immersion oils, do not exhibit the fading in oil-wet areas that is usually seen in films where cedar oil has dried after use.

Aqueous Mounting Media

Permanent mounting media of this type fall perhaps into four general classes: simple syrups, gum arabic media, glycerol gelatins, and acid media of the lactophenol type. Of these the last is used principally in botany and insect histology. They do not in general conserve stains well, especially nuclear stains. Abopon,* recommended by Lieb (Am. J. Clin. Path. 17:413, 1947) for mounting crystal-violet stains of amyloid, is excellent for this purpose, but promptly bleaches alum hematoxylin stains. Gum arabic and glycerol gelatin media often cause diffusion of basic aniline dyes into the medium (“bleeding”). This bleeding occurs both in acid gum-arabic media and in the neutral glycerol gelatins. It is not prevented by addition of low quantities of potassium acetate that suffice to raise the pH to 6.5 or higher. It is prevented by high salt concentrations, even though the medium remains acid. It does not occur in strong sucrose, fructose or D-sorbitol syrups, though the pH of fructose may be as low as 4.0. Addition of large amounts of these sugars to glycerol gelatin, gelatin, or gum-arabic media prevents bleeding.

About 20% by weight of potassium acetate or about 60% of one of the sugars suffices to prevent bleeding of crystal-violet stains for amyloid.

Refractive indices of permanent aqueous mounting media are generally low (1.41 to 1.43). The highest levels, 1.49 to 1.50, are attained with media containing large proportions of sugars, notably fructose and D-sorbitol.

Gum-arabic media are generally acid (pH 3.5 to 4.2). The amount of potassium acetate needed to raise the pH to 6.5 or 7.0 is more than 1/2 and less than 3/4 the weight of gum arabic. Glycerol or sugar, and sometimes both

* Glyco Products Co., 148 Lafayette St., New York, N. Y.
are added to aqueous gum-arabic solutions to raise the refractive index or to retard overdrying.

Amann's Viscol was apparently a mixture of phenol, gum arabic, and glycerol, according to Dahl (Stain Technol. 26:97, 1951), who gave a substitute formula for Amann's secret preparation. Formulae for gum-arabic media are given below.

The commercial prepared media Clearcol* and Viscol† are quite acid (pH 1.5 and 2.9 respectively), and their refractive indices are low (1.4039 and 1.4167). They set promptly after mounting and are not sticky. Fat stains are well preserved, but crystal-violet stains of amyloid bleed badly. The Paragon‡ mountant is less strongly acid (pH 5.6), but affects stains similarly. Its refractive index is 1.4241. Alum hematoxylin counterstains faded in one month in Viscol, but were fairly well preserved in the other two.

Syrups often serve well as temporary mounting media, but they remain wet, sticky, and more or less fluid in moist climates, and they furnish excellent nourishment for molds. Sucrose, glucose, invert sugar, Karo and maltose syrups crystallize around and under cover glasses after a time. A fructose syrup available around 1900 (Ehrlich's Encyklopädie) did not crystallize and had a refractive index of 1.500. These properties are nearly duplicated by the modern crude sorbitol syrup Arlex. The commercial corn syrup Karo has had some vogue as a temporary mounting medium.

Addition of 10% gelatin to syrups is enough to render them solid at 20-25° C. I have used an Arlex gelatin thus prepared with some success, but it tends to be sticky in hot weather.

The glycerol gelatin media (formulae p. 110) require melting for use, but set firmly in a few days. Their refractive indices are low (1.41-1.42).

**Formulae**

*Farrant's glycerol gum arabic.* Dissolve 50 Gm. gum arabic (acacia) in 50 cc. warm distilled water. Add 1 Gm. arsenic trioxide and 50 cc. glycerol. The viscosity is rather high. The index of refraction is 1.43600 at 20° C. Addition of 1 Gm. potassium acetate is recommended if a relatively neutral medium is desired.

*Gum syrup,* modified from Apáthy by Lillie and Ashburn (Arch. Path. 36:432, 1943). Dissolve 50 Gm. acacia (gum arabic) and 50 Gm. cane sugar in 100 cc. distilled water by frequent shaking at 55°-60° C. Restore volume with distilled water. Add 15 mg. merthiolate (sodium ethylmercurithiosalicylate) or 100 mg. thymol as a preservative. Place in vacuum chamber for a few minutes while warm to remove air bubbles. Highman (Arch. Path. 41:559, 1946) adds 50 Gm. potassium acetate or 10 Gm. sodium chloride to this formula, to prevent bleeding of crystal-violet amyloid stains.

*Dahl's formula* to replace Amann's Viscol (Stain Technol. 26:97, 1951):

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* H. W. Clark Co., 33 South High St., Melrose, Mass. † Drogueries Réunies, Lausanne, Switzerland. ‡ Paragon C. & C. Co., New York 58, N. Y.
Dissolve 80 Gm. gum arabic in 40 Gm. (32 cc.) glycerol and 90 Gm. water; then add 20 Gm. phenol.

Various sugars may be added in varying amounts to gum-arabic media. Their effect is to raise the refractive index and to diminish the setting quality of the mountant.

Kaiser's Glycerol Gelatin as Modified by Mallory. Soak 40 Gm. gelatin two hours in 210 cc. distilled water. Add 250 cc. glycerol and 5 cc. melted phenol. Heat gently, stirring constantly for 10-15 minutes until the mixture is smooth. Store in refrigerator and melt as needed. Mallory notes a deleterious effect of the phenol on alum hematoxylin stains. The substitution of 50 mg. merthiolate (sodium ethylmercurithiosalicylate) for the phenol is suggested.

Glychrogel (Zwemer, Anat. Rec. 57:41, 1933). Dissolve 0.2 Gm. chrome alum (KCr(SO₄)₂·12H₂O) in 30 cc. distilled water by heating. Dissolve 3 Gm. granulated gelatin in 50 cc. distilled water by heating. Add 20 cc. glycerol to the still warm gelatin solution and mix thoroughly; then add the warm chrome alum solution, mix thoroughly, and filter in a 37° C. incubator. Add a crystal of thymol or camphor as a preservative, or 10 mg. merthiolate (sodium ethylmercurithiosalicylate). According to Wotton and Zwemer (Stain Technol. 10:21, 1935), this medium possesses a quite high index of refraction (1.75) after drying a week. This compares with 1.46 for glycerol, 1.47 for glycerin jelly, and 1.54 for Canada balsam. On occasion we have had great difficulty with the filtration of this medium, and see no great advantage in it.

Fructose (Levulose) Syrup. Dissolve 30 Gm. fructose (levulose) in 20 cc. distilled water by gentle heat (Mallory). This sugar formerly occurred in commerce only as a syrup of about 1.500 refractive index (Ehrlich's Encyclopedia), which fact probably accounts for its introduction as a mounting medium.

In my experience this concentration is too low. A 70-75% solution has a more suitable viscosity and a higher refractive index (60% fructose 1.43892, 70% 1.46011, 75% 1.4762, 80% 1.4906 at 20° C.). Fructose syrups do not crystallize in the mounts and when sealed (p. 99) can well serve as permanent mounting media. In dry climates sealing may be unnecessary, since the preparations become quite hard. Fructose is much higher in cost.

Most sugars crystallize badly after a time, spoiling the mounts. A 70%-sucrose syrup has suitable viscosity for mounting and a refractive index of 1.46468. It serves well for a temporary mountant, but crystallizes in a month or so. The refractive index of 70% glucose is 1.4614, of 80% maltose 1.4512, of 75% fructose 1.4762, of white Karo 1.4799, and of commercial Arlex d-sorbitol syrup 1.4660. Glucose, invert sugar, and maltose syrups crystallize in a month after mounting. Karo is somewhat acid (pH 5.8) and crystallizes after a time with or without addition of 1% potassium acetate. The Karo and maltose syrups set hard, but both crystallize. Arlex d-sorbitol syrup did
## Table 13
### Composition and Properties of Water-miscible Mounting Media

<table>
<thead>
<tr>
<th></th>
<th>Gum Arabic Gm.</th>
<th>Gelatin Gm.</th>
<th>Glycerol Gm.</th>
<th>Sugar or Syrup Gm.</th>
<th>Potassium Acetate Gm.</th>
<th>Merthiolate mg.</th>
<th>Other Ingredients Gm.</th>
<th>Water Gm.</th>
<th>Refractive Index</th>
<th>pH</th>
<th>Bleeding Methyl Violet</th>
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<tbody>
<tr>
<td>Apáthy gum syrup (LA)</td>
<td>50</td>
<td>sucr. 50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>1.4170</td>
<td>4.1</td>
<td>+</td>
</tr>
<tr>
<td>Apáthy gum syrup (H-a)</td>
<td>50</td>
<td>sucr. 50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>1.4266</td>
<td>6.8</td>
<td>—</td>
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<tr>
<td>Apáthy gum syrup (H-b)</td>
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<td></td>
<td>50</td>
<td>1.4252</td>
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<td>20</td>
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<td></td>
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<td>33</td>
<td>1.4519</td>
<td>6.6</td>
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<tr>
<td>Arlex gelatin</td>
<td>10</td>
<td>Arlex 89</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td>1.4936</td>
<td>6.0</td>
<td>—</td>
</tr>
<tr>
<td>Zwemer glychrologel</td>
<td>3</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>chrome alum 0.2</td>
<td>80</td>
<td>1.75 Z</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LA: Lillie & Ashburn; H-a & H-b: Highman; L: Lillie; KAc: potassium acetate; M: Mallory; F: Friedenwald; * water included in the Arlex sorbitol syrup; Z: according to Zwemer.
not crystallize and had the highest refractive index, and it was found that addition of 10% gelatin produced a medium which set well and preserved fat and amyloid stains well.

Lillie and Greco's Arlex gelatin. Heat 69 Gm. Arlex D-sorbitol syrup in a boiling-water bath, add 10 Gm. gelatin and stir until dissolved. Add 1 Gm. potassium acetate and 10 mg. merthiolate. The pH is 6.0 or higher, the refractive index 1.4936. Preparations set promptly, but remain sticky for some time (see p. 99).

Much of the foregoing material is derived from the reports of the Committee on Mounting Media of the Biological Stain Commission (Stain Technol. 25:1,11, 1950; 28:57, 1953).

Table 14

<table>
<thead>
<tr>
<th>FORMULAE OF INDIFFERENT DILUTING FLUIDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Physiologic saline</td>
</tr>
<tr>
<td>Sodium chloride 8.5 Gm., usually 9.0</td>
</tr>
<tr>
<td>Distilled water 1,000 cc.</td>
</tr>
<tr>
<td>Sterilize in autoclave</td>
</tr>
</tbody>
</table>

| 2. Ringer’s solution and Locke variant |
| Sodium chloride 8.5 Gm. 8.5 Gm.        |
| Potassium chloride 250 mg. 420 mg.     |
| Calcium chloride 300 mg. 250 mg.       |
| Sodium bicarbonate (200 mg.)* 200 mg.  |
| Distilled water 1,000 cc. 1,000 cc.    |
| Sterilize by filtration with Berkefeld.|
| Add calcium chloride last. Make fresh. |

| 3. Locke-Lewis solution                |
| Sodium chloride 8.5 Gm.               |
| Potassium chloride 420 mg.            |
| Sodium bicarbonate* 200 mg.           |
| Glucose 100 to 250 mg.                |
| Calcium chloride 250 mg.              |
| Distilled water 1,000 cc.             |
| Add calcium chloride last. Sterilize by Berkefeld filtration. Make fresh as needed. |

| 4. Tyrode solution                     |
| pH 7.5 to 7.8                           |
| Sodium chloride 8.0 Gm.                |
| Potassium chloride 200 mg.             |
| Calcium chloride 200 mg.               |
| Magnesium chloride 100 mg.             |
| Sodium acid phosphate 50 mg.           |
| Sodium bicarbonate 1 Gm.               |
| Glucose 1 Gm.                          |
| Distilled water 1,000 cc.              |
| Add salts to water in order given.     |
| Sterilize by Berkefeld filtration.     |

| 5. Pannett and Compton’s buffered saline† | pH 7.5 |
| Sodium chloride 6.4 Gm.                  |
| Potassium chloride 366 mg.               |
| Calcium chloride 160 mg.                 |
| Distilled water 960 cc.                  |
| Boil or autoclave, cool and add 40 cc. of the following: |
| Monosodium phosphate monohydrate 12.5 mg.|
| Disodium phosphate, anhydrous 67.6 mg.   |
| Distilled water 40 cc. which has been similarly sterilized. |
| The last is approximately 5.7 cc. of stock M/10 phosphate buffer pH 7.5 diluted with 34.3 cc. water (p. 451). |

* Sodium bicarbonate is often omitted from Ringer’s solution. It may be added to the Ringer-Locke solution and to the Locke-Lewis after the remaining constituents have been boiled to sterilize. The bicarbonate decomposes at 80° C in solution; hence it should not be added until solutions are below that temperature.

† In the Pannett-Compton solution, the chlorides and phosphates must be autoclaved separately and mixed after cooling.

The formulas are quoted as follows: No. 1, traditional at National Institute of Health; most authors give 0.9%. No. 2, both formulas are as in Lee, Romeis, and Cowdry. No. 3, as in Romeis and Cowdry. No. 4, as in Cowdry, Lee, Mallory, and Romeis. No. 5, emended from Lee.
Indifferent Examination Media

Many procedures exist for the examination of tissues, blood, or their constituent cells or products in the fresh state or in aqueous media. For the study of surviving cells, protozoa, and bacteria, a drop of tissue—perhaps diluted in serum or some indifferent fluid (see p. 112 for formulae) such as physiologic ("normal") saline, Ringer's fluid, or Locke's fluid—is placed on a clean slide, and at once covered with a cover glass. The edges of the cover glass may then be covered with petrolatum to prevent evaporation. Various reagents may be introduced by placing a few drops on the slide at one margin of the cover glass and drawing it into the observed space by applying filter paper to the opposite side of the cover glass.

Some procedures for the observation of living cells demand the use of a warm stage. Warm stages may be procured in various designs from the instrument makers. One worker found the most practical procedure was to enclose his microscope within a box with hand holes and holes for the eyepieces, and to keep the whole chamber warmed by using the heat of the microscope lamp or other heat source to maintain the desired temperature, which he controlled with a thermometer. For extended investigations and prolonged observation, thermostatic control is necessary.
Chapter 7

General Oversight Methods

Hematoxylin and Eosin

For permanency with reasonable differentiation of nuclei and cytoplasm, the preferred method is still alum hematoxylin (C. I. No. 1246) and eosin Y (C. I. No. 768). A large number of variants of this method have been proposed. Some use acid hematoxylin, others differentiate with acid after staining with a relatively neutral or even alkalinized hematoxylin. Some use strong eosin solutions before the hematoxylin; some mix the hematoxylin and eosin solutions; others use aqueous or alcoholic solutions of eosin after the hematoxylin; and some insist on the color acid of eosin Y dissolved in alcohol. Others prefer other dyes to eosin Y, such as eosin B (C. I. No. 771), erythrosin B (C. I. No. 773), or phloxin B (C. I. No. 778). Eosin B is a slightly bluish pink, erythrosin B is quite similar, phloxin B is a deeper red, while eosin Y is a yellowish pink. The technic follows.

I. The Acid Hematoxylin Variant.

1. Bring sections to water.
2. Stain 2–5 or more minutes in an acid hematoxylin (p. 76). Zenker-fixed material may need as much as an hour. Sections do not overstain.
3. Wash thoroughly in tap water until sections are blue. Some add a few drops of ammonium hydroxide to 500 cc. water. Some use a weak (1%) sodium acetate, or sodium bicarbonate or disodium phosphate solution.
4. Stain one minute in 0.5% aqueous eosin.
5. Rinse in water.
6. Dehydrate in two changes each of 95% and 100% alcohol.
7. Pass through 100% alcohol + xylene (50:50) mixture into two changes of xylene. Mount in xylene clarite (or balsam). Nuclei are
blue; cartilage and calcium deposits, dark blue; cytoplasm, muscle, and other structures, varying shades of pink; mucin, often light blue.

II. The Neutral Hematoxylin Variant.

1. Brings sections to water.
2. Stain sections five minutes or more in an unacidified hematoxylin (p. 76). The sections are overstained.
3. Differentiate in acid alcohol until red. (Concentrated hydrochloric acid 3 cc., 70% alcohol 97 cc.).
4. Wash thoroughly in tap water or 1% sodium acetate or bicarbonate or disodium phosphate until blue.
5. Counterstain one minute in 0.5% eosin.
6. Rinse in water.
7. Dehydrate with two changes each of 95% and 100% alcohol.
8. 100% alcohol + xylene, then two changes of xylene.

Results differ only slightly from the preceding method. Myelin and similar substances may retain some blue if hematoxylin staining has been prolonged and formalin or a chromate fixation has been used. 

Substitution of certain acid azo dyes for the eosins may give pleasing effects. Orange G (C. I. No. 27) gives orange to yellow tones, chromotrope 2R (C. I. No. 29) pink and orange-red tones, Bordeaux red (C. I. No. 88) somewhat redder tone, Biebrich scarlet (C. I. No. 280) varying pink to scarlet tones. Lee recommended this last in 1% solution as the best plasma stain he had tried.

Neumeyer (Zentralbl. f. allg. Path. u. path. Anat. 84:109, 1948) recommends as a substitute for alum hematoxylin a 0.1% solution of anthracene blue WR extra of Badisch Anilin (C. I. No. 1062, 1, 2, 4, 5, 6, 8-hexahydroxyanthraquinone) in 5% aqueous aluminum sulfate solution. Boil the fresh solution eight minutes, filter while hot, and add 1 cc. 40% formaldehyde per liter. The solution is quite stable.

Neumeyer's Anthracene Blue and Eosin Technic.

1. Hydrate paraffin sections (10 μ) of formalin-fixed tissue as usual.
2. Stain 10 to 30 minutes in the anthracene blue solution.
3. Differentiate 30 seconds to 2 minutes in 2% HCl alcohol. Nuclei resist longer differentiation quite well.
4. Wash 15 minutes in running water.
5. Counterstain in 0.5% eosin ½ to 2 minutes; or other suitable method.
6. Alcohols, xylene alcohol, xylenes, synthetic resin.

Results: Nuclei, blue black; muscle, violet; connective tissue, bright red; cytoplasm of glycogen laden cells, blue violet.
Azure–Eosin Methods

Excellent as the hematoxylin-eosin stains are for permanence, I now use them only infrequently, preferring for routine use procedures of the azure-eosin or hematoxylin-azure-eosin type. The latter give definitely good staining of bacteria and rickettsiae in tissues, and demonstrate tissue mast cells, which are not evident with hematoxylin. They act as sensitive indicators of early necrobiotic changes in cells, in that the normal light-blue staining of cytoplasm is replaced abruptly by a brilliant pink. Regenerating liver cells are blue, in contrast with the normal, more or less violet tone. With the buffer procedure now in use it is possible to stain as many as 100 slides at a time by a routine technic without the troublesome individual differentiation formerly required, and to attain a great uniformity of results even on miscellaneous formalin material sent in by mail from various places.

These azure-eosin procedures comprise sequence stains such as Mallory’s eosin or phloxine “methylene blue,” and neutral stains employing azures and eosin in extempore mixtures or in preformed azure eosinates in glycerol and methanol stock solutions. In the neutral stain group, such methods as Maximow’s and Wolbach’s were carried out at approximate neutrality and the excess of azure subsequently differentiated out by means of alcohol, dilute acid, colophonium alcohol, or sunlight. I have preferred to regulate the red-blue balance of the final stain by variation of the pH of the stain mixture by means of buffers. This last procedure gives very constant results on material fixed in formalin, chromate, and mercury fixatives, with some adjustment of pH for the fixative employed. It is less suitable for picric acid and absolute alcohol-acetic fixations. With this method, more acid pH levels give sharper and more selective chromatin staining, denser eosin staining of muscle and red cells, and less cytoplasmic basophilia. Less acid, higher pH levels give denser nuclei, pink to blue-gray muscle, orange erythrocytes, and increased cytoplasmic basophilia. Results are more or less similar for the following methods, and are given in detail on pp. 119–120 only to avoid duplication.

For Mallory’s eosin– or phloxin–methylene blue method, Zenker fixation was prescribed. Sections were deparaffinized and hydrated in the usual manner, treated with 0.5% iodine in alcohol for five to ten minutes and with 0.5% sodium thiosulfate for five minutes (5% removes iodine at once) and washed in water.

1. Stain one hour or longer at 52°–55° C. in 2.5% eosin Y or, preferably, phloxin B (C. I. No. 778).
2. Cool, decant, and rinse carefully in water.
3. Stain 5–20 minutes in a mixture of equal volumes of 1% azure II in distilled water and of 1% methylene blue in 1% borax solution. This is filtered just before use and poured onto and off the slides several times to insure even staining.
4. Then place slides in the water and decolorize individually in 0.2–0.5% colophonium alcohol. Mallory recommended adding 2–5 cc. of a stock 10% solution in 100% to 100 cc. of 95% alcohol. With formalin-fixed material 3–10% colophonium alcohol is recommended for differentiation. Slides are kept in constant motion during differentiation. When the general background becomes pink and nuclei are still blue (microscopic control),

5. Dehydrate in three changes of 100% alcohol.

6. Pass through one change of equal parts 100% alcohol and xylene and two changes of xylene. Mount in xylene clarite.

Mallory preferred phloxine B (C. I. No. 778) to eosin Y (C. I. No. 768) in this technic because of the deeper reds achieved. Borax methylene blue is essentially an alkaline methylene blue which gradually alters to azures and methylene violets, faster when kept warm than when cold. Azure II was a mixture of equal parts of methylene blue and of azure I, which was originally azure B or trimethylthionin. However, the usual azure furnished in the United States from 1925 to 1940 was azure A, or asymmetrical dimethylthionin, a more violet dye than azure B, and it seems probable that this dye was meant by Mallory. The borax methylene blue varies in composition with age, and may contain all three azures—A, B, and C—as well as methylene blue and methylene violets.

Maximow’s Hematoxylin–Azure II–Eosin (Ztschr. wiss. Mikr., 26:177, 1909; Mallory). Fixation in Spuler’s (Maximow’s) variant of Zenker formalin (p. 39) was prescribed. Originally Maximow removed mercury precipitates with iodine alcohol before imbedding and insisted on celloidin (nitrocellulose) imbedding. He attached the sections to slides with ether vapor before staining. I have successfully used paraffin sections and the usual iodine and thiosulfate treatment after sectioning, and have found that the method works quite well on Orth- and formalin-fixed material.

1. Stain sections with alum hematoxylin as usual. I use a five-minute stain in an acid, iodate ripened solution of full (0.5%) strength. Mallory recommended 24 hours in very dilute Delafield’s hematoxylin (1 or 2 drops per 100 cc. water). Wash in water.

2. Stain 18–24 hours in azure II–eosin: Dilute 5 cc. 1:1,000 eosin Y with 40 cc. distilled water and add 5 cc. 1:1,000 azure II. Mallory recommended water buffered to pH 6.8–7.0 with Sörensen’s phosphate mixture, but see under Lillie’s methods (pp. 118–119).

3. Differentiate sections individually in 95% alcohol until gross blue clouds cease to come out into the alcohol, and red cells and collagen are pink.

4. Two changes of 100% alcohol, 100% alcohol and xylene, two changes of xylene, mount in xylene clarite.
Nuclei stain blue; basophil leukocyte and mast cell granules, purple to violet; cartilage, purple; red corpuscles, pink; cytoplasm, blue to pink; secretion granules and eosinophil granules, pink. (See also pp. 119-120.)

**Giemsa Stain.** In place of the extempro mixture of azure II and eosin, a 1:50 dilution of *Giemsa's blood stain* (pp. 387-388) may be used with identical results. Wolbach (Mallory; personal communications) recommended addition of 2 to 4 drops (0.1-0.25 cc.) of 0.5% sodium bicarbonate to a 1:40 dilution of Giemsa’s stain. After staining he differentiated in weak colophonium alcohol, dehydrated in 100% alcohol, cleared in xylene, and mounted in cedar oil. Exposure to diffuse daylight or even sunlight after mounting produced a further differentiation which was valuable for rickettsiae. Wolbach now prescribes fixation in Möller’s (Regaud’s) fluid rather than Zenker.

**Lillie and Pasternack’s azure eosinate method** was derived from a staining accident with the Maximow method, in which the use of acid distilled water obviated the necessity for the usual differentiation. Any commercial azure eosinate may be used, or eosinates may be prepared with the yellowish eosin Y or the deeper red eosin B (C. I. No. 771) from methylene blue, azure B, toluidin blue, azure A, azure C, thionin, or new methylene blue. Nuclei vary in color from pure blue at the methylene-blue end of the series to purple at the new methylene blue end; mast-cell granules, mucin, and cartilage matrix, from blue violet to purplish red (the metachromatic colors). Contrast is greatest between the normal and metachromatic colors in the middle of the series, hence I usually prefer azure A or the often nearly identical azure C. It makes little difference which. Commercial samples labeled with either name usually contain more or less of the other, so that on spectroscopic examination some samples of azure A have been found to be more nearly azure C than some other samples labeled azure C, and vice versa. Other zinc-free thiazin dyes may be substituted in the same amounts, and the zinc double salts may be used as well, if appropriate allowance is made for their lower dye content. The phloxins, rose Bengal, and the erythrosins do not yield satisfactory thiazin salts for use in this technic. The eosinates are made up as 1% stock solutions in equal volumes of glycerol and C.P. methyl or 100% ethyl alcohol.

Latterly we have found it preferable to use in place of the prepared eosinates aqueous solutions of the eosin and the thiazin dye preferred, mixed at time of using (*J. Tech. Methods* 24:43, 1944). The same dyes may be used. The following technic may be carried out in Coplin jars or in the Technicon staining racks.

**Lillie’s Azure A–Eosin B:**

1. Bring paraffin sections to water as usual, using the 0.5% iodine, 5% sodium thiosulfate sequence for material fixed with mercuric chloride.
2. Stain 3–5 minutes in alum hematoxylin, blue and wash in water. This
step increases the density of the stain, but impairs brilliancy and I prefer to omit it. It has been noted that exposure to a pH 4.0 buffer for one hour almost completely extracts the hematoxylin stain.

3. Stain one hour in

<table>
<thead>
<tr>
<th></th>
<th><em>Coplin jar</em></th>
<th><em>Technicon</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Azure A</td>
<td>4 cc. 0.1%</td>
<td>6 cc. 1%</td>
</tr>
<tr>
<td>Eosin B</td>
<td>4 cc. 0.1%</td>
<td>6 cc. 1%</td>
</tr>
<tr>
<td>M/10 citric acid</td>
<td>1.2 cc.</td>
<td>21 cc.</td>
</tr>
<tr>
<td>M/5 disodium phosphate</td>
<td>0.8 cc.</td>
<td>14 cc.</td>
</tr>
<tr>
<td>C.P. acetone</td>
<td>5 cc.</td>
<td>90 cc.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>25 cc.</td>
<td>585 cc.</td>
</tr>
</tbody>
</table>

The larger quantities prescribed for the Technicon schedule are allowed to stand one hour after mixing, and then filtered. The mixture is used repeatedly, discarding at the end of each week. The smaller, Coplin-jar quantities are used at once and discarded after use.

4. Dehydrate in 2–3 changes of acetone, clear in a 50:50 acetone xylene mixture and two changes of xylene. Mount in synthetic resin. Clarite, Permount, HSR, polystyrene, Groat’s copolymer, and ester gum are satisfactory.

The buffer mixture may be altered as desired. Lower pH levels give redder effects, higher levels more blue. Zenker mixtures require pH 4.5 to 5.0, formalin fixation gives satisfactory results at pH 3.75 to 4.3. (See Buffer Tables p. 450.) In the mixture one may substitute 0.5 cc. 1% azure eosinate in glycerol and methanol for the separate aqueous solutions of azure and eosin (10 cc. for the Technicon schedule).

Nitrocellulose sections and collodionized sections require 2 to 2½ hours staining, or 2–3 times as much dye. If it is necessary to preserve the collodion coat, substitute isopropanol for acetone in the dehydration and clearing schedule.

**Results with Azure-Eosin Methods**

The following results apply generally to the foregoing azure-eosin technics (pp. 116–119): Blue nuclei, tigroid, bacteria, and rickettsiae; blue violet mast-cell and basophil leukocyte granules; reddish violet cartilage matrix; dark blue calcium deposits; light blue to violet or lavender cytoplasm of surviving cells; bright pink cytoplasm of necrosing and necrotic cells, and muscle fibers (Zenker’s degeneration or necrosis); pink secretion granules in pancreatic and salivary gland acini and Paneth cells; pink cytoplasm of gastric gland parietal cells, and blue cytoplasm of chief cells; yellowish green to green chromaffin after chromate fixation only; pink eosinophil and pseudoeosinophil granules; pink keratin, amyloid, fibrin, muscle cytoplasm, thyroid col-
loid, nuclear and cytoplasmic oxyphil inclusion bodies, and bone matrix; orange-pink erythrocytes and hemoglobin. Various hyaline degeneration products in liver cells, in Hassall corpuscles of the thymus, in the follicles of the spleen, and elsewhere also stain pink. The mucins vary in color from unstained through pale greenish blues to fairly deep blue violet. Variation of buffer level toward the acid side increases precision of nuclear staining and decreases cytoplasmic basophilia. Conversely, variation to the alkaline side increases the amount of blue in various elements. At perhaps a 1:1 ratio of citric acid and phosphate (pH 5.0) Orth- and formalin-fixed material will present pale gray-blue smooth muscle; and at higher pH levels perhaps only eosinophil granules and erythrocytes remain pink. Conversely, mast-cell granules, cartilage matrix, and lymphoid cell cytoplasm retain their blue or violet staining at lower pH levels than do most other elements. After certain alcoholic fixations the ellipsoids of retinal rods stain brilliantly in red.

The azure-eosin methods are also widely useful in the identification of the cells of inflammatory exudates in the tissues. Lymphocytes and plasma cells present the characteristic basophilic (blue) cytoplasm, broader and eccentric in the latter, and the round nucleus with relatively coarse, deeply stained chromatin granules, perhaps radially placed. The cytoplasm of monocytes, histiocytes, or macrophages is broader and less basophilic, and may enclose phagocytosed material. The nucleus of this cell type is characteristically larger than that of the lymphocyte, is round, oval, or indented in form, and is relatively pale in appearance (leptochromat), with quite finely divided chromatin. The nucleus of *Entamoeba histolytica* is much smaller, vesicular, as pale or paler, and contains a small nucleolus. Its cytoplasm is similar to that of the macrophage group, and may contain phagocytosed erythrocytes. The granules of the polymorphonuclear leukocyte are less well shown in section material than in Giemsa stained smears. They are much more conspicuous in some species, notably rabbits and guinea pigs, than in others.

Leishmania, and the leishmania forms of *Trypanosoma cruzi* in heart muscle, skeletal muscle, and skin are well shown, with deep-blue rod-shaped blepharoplasts and lighter-blue rounded trophonuclei in relatively lightly basophilic cytoplasm. Sarcosporidia, encephalitozoa, and toxoplasma all appear as fairly conspicuous bodies with blue-stained chromatin in the pink backgrounds of striated muscle and central nervous tissues. *Bartonella bacilliformis* in human liver is well shown by azure-eosin methods, notably Wolbach's Giemsa variant. I have not had opportunity to use the buffered azure-eosin techinics with this material. Plasmodia present deep-blue chromatin and lighter-blue cytoplasm. In human spleen they may sometimes be more easily discerned with an iron hematoxylin technic (pp. 80, 129).

Oxidation Schiff Technics

With the extensive application of the periodic acid leucofuchsin technics to a wide variety of tissue elements, this method, introduced by McManus
GENERAL OVERSIGHT METHODS

(Nature 158:202, 1946) for demonstration of mucins, and by me (J. Lab. & Clin. Med. 32:910, 1947; Bull. Internat. Assn. Med. Museums 27:23, 1947) for reticulum and for glycogen, has become a general method. The method is not specific for polysaccharides. It depends on the oxidation of 1,2-glycol groupings to aldehyde, according to the Malaprade reaction. In these groupings one of the hydroxyls may be replaced by a primary or secondary amine. It was first applied to polysaccharides by Jackson and Hudson (J. Am. Chem. Soc. 59:2049, 1937). Hotchkiss discovered the histochemical application of this test in 1945 but did not report it until 1948 (Arch. Biochem. 16:131, 1948).

In most instances a positive reaction is to be taken as denoting the presence of polysaccharides, mucopolysaccharides, glycoproteins, or glycolipoids. The positive reaction of chromaffin (p. 172) has not been fully explained chemically in relation to the Raper adrenochrome hypothesis and requires further study. The reaction is positive in many of the lipid pigments and in the Golgi substance, where it is usually accompanied by a positive peracetic acid Schiff reaction (p. 310). In these circumstances it seems to be assignable to the vicinal paired hydroxyls derived by hydrolysis from epoxidized unsaturated fatty acids:

\[
\text{CH}=-\text{CH}- + O \rightarrow \text{CH}=-\text{CH} + \text{H}_2\text{O} \rightarrow \text{CHOH}-\text{CHOH}=
\]

The question of protein reactions assignable to the amino acids serine, threonine, and hydroxylysine is still actively debated. No convincing experimental proof has been brought forward on either side at this writing (August, 1953). It is urged that serine and threonine should be nonreactive unless occurring as terminal groups with open NH₂ radicals, although the NH groups of peptide bonds still possess one available H atom, and possibly might comply with the requirement of a secondary amino group adjacent to a hydroxyl in these β-hydroxy-α-amino acids.

The Bauer (Ztschr. mikroskop. anat. Forsch. 33:143, 1933) chromic acid Schiff method and the Rossman-Casella permanganate Schiff method (Carnegie Contr. Embryol. 30:97, 1942; Anat. Anz. 93:289, 1942) apparently attack the same groupings to form aldehyde, but these two oxidants also oxidize aldehyde further, probably to carboxyl or to carbon dioxide. Consequently the reaction to Schiff's sulfite leucofuchsin reagent is generally less intense, and with substances having relatively few oxidizable groupings it may be entirely lacking. If exposure to chromic acid or to permanganate is prolonged to several hours (18 hours with 5% CrO₃, 6 hours with 0.5% KMnO₄) all of the reactive material is destroyed (Lillie, Stain Technol. 26:123, 1951). On the other hand, the oxidation with periodic acid does not destroy the aldehyde even when exposure is greatly prolonged (30 hours in
0.035 M), though aldehyde formation appears to be adequate even in five minutes.

The reagents used exhibit a considerable degree of uniformity, considering that the technics were developed quite independently in several laboratories.

Reagents. Concentrations, molarity, pH, and composition of the periodic acid baths and their duration are given in Table 15.

Table 15

<table>
<thead>
<tr>
<th>Author, Date</th>
<th>Source of HIO₃</th>
<th>Concentration</th>
<th>Molarity</th>
<th>pH</th>
<th>Solvent</th>
<th>Time in minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>McManus 1946</td>
<td>H₂IO₄</td>
<td>0.5%</td>
<td>0.022</td>
<td>2.1</td>
<td>Distilled water</td>
<td>5</td>
</tr>
<tr>
<td>Lillie 1947</td>
<td>Na₂IO₃</td>
<td>1.0%</td>
<td>0.036</td>
<td>1.6</td>
<td>0.5% Aq. HNO₃</td>
<td>10</td>
</tr>
<tr>
<td>Hotchkiss 1948</td>
<td>H₂IO₄</td>
<td>0.8%</td>
<td>0.035</td>
<td>2.5</td>
<td>0.02M Na Acet, Aq.</td>
<td>5</td>
</tr>
<tr>
<td>Hotchkiss 1948</td>
<td>H₂IO₄</td>
<td>0.8%</td>
<td>0.035</td>
<td>2.4</td>
<td>0.02M Na Acet, 70% Alcohol</td>
<td>5</td>
</tr>
<tr>
<td>Lillie 1949</td>
<td>KIO₄</td>
<td>0.8%*</td>
<td>0.035</td>
<td>1.9</td>
<td>0.3% Aq. HNO₃</td>
<td>10</td>
</tr>
<tr>
<td>Lillie 1950</td>
<td>KIO₄</td>
<td>0.69%</td>
<td>0.030</td>
<td>1.9</td>
<td>0.3% Aq. HNO₃</td>
<td>10</td>
</tr>
<tr>
<td>Mowry 1952</td>
<td>H₂IO₄</td>
<td>1.0%</td>
<td>0.044</td>
<td></td>
<td>90% Alcohol</td>
<td>120</td>
</tr>
</tbody>
</table>

* After using this solution for some months, it was found that it tended to deposit a few crystals at 20–25° C. temperatures, therefore the 1950 modification was made.

Periodic acid solutions apparently may be used for quite large numbers of sections and over several weeks without becoming exhausted. Nevertheless it is good practice to check a standard test section for adequacy of staining from time to time; for example, the completeness of staining of the fine reticular or sarcolemmal structure in human smooth muscle. Since 0.01 M periodic acid for ten minutes appears quite adequate, it is evident that there is a large margin of safety in the usual solutions.

Hotchkiss's recommendation of an alcoholic solution was based on the then current belief that glycogen in fixed tissues was readily soluble in water. Since 16 hours' exposure to boiled aqueous diastase solutions does not appreciably diminish the amount of demonstrable glycogen in liver tissue (Anat. Rec. 103:635, 1949) this precaution seems unnecessary for this purpose.

Hotchkiss's reducing rinse (KI 1 Gm., Na₂S₂O₅ • 5H₂O 1 Gm., water 20 cc., alcohol 30 cc., 2 N HCl 0.5 cc.) in McManus's experience as well as mine appears to partially block the coloration of collagen and reticulum, much as does a deliberately interposed bisulfite blockade step, and is not recommended as a routine procedure.

The Schiff reagent for routine use should be one of the stronger preparations (p. 155). McManus used a traditional Feulgen type containing 1 Gm.
The Periodic Acid, Schiff Sulfito-Leucofuchsin Reaction, short variant.

1. Deparaffinize and hydrate through xylene, alcohols, and distilled water as usual.
2. Oxidize ten minutes in 0.69% (0.03 M) KIO₄ in 0.3% HNO₃.
3. Wash five minutes in running water.
4. Immerse for ten minutes in Schiff reagent (p. 156).
5. Transfer quickly and directly to three successive baths: 1, 2 and 2 minutes in 0.5% sodium metabisulfite (Na₂S₂O₅). Replace sulfite rinses daily or more often.
6. Wash five minutes in running water.
7. Counterstain nuclei and cytoplasm as desired; e.g.,
   a. Stain two minutes in a 2% acetic hemalum solution of about 0.1% hematoxylin content (Mayer’s or a 1/6 dilution of Lillie’s, p. 76). Wash in water, and blue with a drop or two of 20% Na₂CO₃ in 200 cc, water.
   b. Stain two to four minutes in Weigert’s acid iron hematoxylin (p. 81); decolorize ten seconds in Pař’s bleach (p. 332) diluted 1/6 with distilled water. Wash four minutes in running water.
   c. Stain six minutes in Weigert’s acid iron hematoxylin, wash four min-
utes in running water, counterstain one minute in saturated aqueous picric acid solution. Other counterstains may be used.

8. Dehydrate (and differentiate) in two changes each of 95% and 100% alcohol. Clear in one change of alcohol xylene mixture (1:1) and two of xylene. Mount in suitable resin, such as polystyrene, HSR, permount, ester gum, clarite, Depex, or the like.

Results are presented on p. 126. It is to be noted that alum hematoxylin and freshly mixed Weigert's iron hematoxylin may stain nonnuclear structures excessively, sometimes partly converting the red-purple of Schiff reactions to gray-purple or violet. This may be overcome by keeping the staining period short and by allowing the Weigert mixture to age an hour or so before using. Acid differentiation is useful, and this is one of the functions of the picric acid counterstain. The dilute Pal's bleach renders the iron hematoxylin stain almost purely nuclear.

When diastase or other enzymatic digestion tests are employed, the sections are prone to become detached during the oxidation or aldehyde demonstration procedures. Hence it is recommended that collodionization be interposed immediately after the enzymatic digestion. Collodion films almost totally inhibit enzyme penetration; therefore the collodionization should not be inserted before enzyme digestion. Since anhydrous methyl alcohol dissolves nitrocellulose, of necessity collodionization if practiced at all must follow a methylation or methanol extraction procedure. Similarly, since pyridine dissolves both paraffin and collodion, collodionization must follow pyridine extractions and pyridine acetylation technics.

On the other hand saponification procedures (p. 161) and various other chemical blockade procedures which also tend to detach sections may more conveniently follow collodionization.

For the Bauer reaction, substitute at step 2 a one hour bath in 5% chromic anhydride (CrO₃); and for the Casella reaction, a 20 minute immersion in 1% potassium permanganate. These are the usual directions, though I have reduced the chromic acid treatment to 20 or 30 minutes at the same concentration, and the permanganate treatment to 0.5% for 10 minutes with essentially similar results.

The following long schedule has been prepared to indicate at what points and in what sequence various modifying procedures should be introduced.

The Periodic Acid Leucofuchsin Method in Relation to Various Modifying Procedures.

(Optional variables are set in italics; standard steps in roman.)

1. Deparaffinize in two changes of xylene, then two changes 100% alcohol.

2. Insert acetylation, benzoylation, or pyridine extraction: Immerse ten seconds in pyridine, then transfer to pure pyridine, to 40:60 acetic-
anhydride:pyridine, or to 2:38 benzoyl chloride:pyridine for prescribed period (pp. 160-161). Wash in 100% alcohol and collodionize if desired (Step 7).

3. Insert methylation, methanol extraction, or lipoid solvent extractions with alcohol ether, alcohol chloroform, petroleum ether, etc. Transfer from 100% alcohol to prescribed anhydrous reagent for prescribed time. Wash in 100% alcohol and collodionize, if required (Step 7).

4. After mercurial fixations, remove precipitates by iodizing in 0.5% iodine in 80% or 70% alcohol for five minutes; transfer directly to 5% sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) for one minute; wash five minutes in running water. This step precedes enzyme digestions, but may follow collodionization (Steps 5, 7).

5. Insert enzyme digestion procedures: Hydrate through 95% and 80% alcohols and water. Place in prescribed enzyme solution (pp. 150-152, 275, 291) and in solvent control at prescribed temperature for graded intervals or for a set time. Wash in water. If required, dehydrate and collodionize (Step 7).

6. Insert aldehyde blockade procedures: Hydrate through alcohols to water; immerse 30 minutes or more in 5% aqueous phenylhydrazine or molar aniline chloride; wash 5-10 minutes in water and proceed with oxidation (Step 10).

7. Collodionize, if required, by immersing from 100% alcohol in 1% collodion into equal parts of 100% alcohol and ether for 10 minutes. Drain on end for one minute and harden five minutes in 80% alcohol. This step must follow Steps 2, 3, and 5, but may precede Step 4 or 6 if desired. It should precede Step 8.

8. Deacetylation is inserted after collodionization: From 80% alcohol immerse for 24 hours in 10 cc. 28% ammonia, 10 cc. water, and 30 cc. 100% alcohol; or, according to McManus, for ten minutes in 0.1 N aqueous KOH. I have not tested this brief procedure for its effectiveness on the more stable acetylation complexes. Longer exposures (1–2 hours in 0.1 N KOH or NaOH, aqueous or in 80% alcohol) remove all sections, even with collodionization.

9. Bring sections to water, or to the appropriate concentration of alcohol.

10. Oxidize for prescribed period in the selected $\text{HIO}_4$ reagent (Table 15, p. 122).

11. Wash five minutes in running water, or in 70% alcohol in alcoholic variants.

12. Interpose Hotchkiss reducing rinse, five minutes, with preceding and following rinses in water or in 70% alcohol as desired.

13. Interpose aldehyde blockades: (a) Schiff solvent (p. 158) 1–4 hours as required. (b) Phenylhydrazine, semicarbazide or aniline chloride; use technics given on pp. 159–160. From the bisulfite transfer directly to Schiff reagent; from the other reagents wash five minutes in running water.
14. Immerse in Schiff reagent for ten minutes, or longer if specially required.
15. Transfer directly to 0.5% sodium (meta-) bisulfite; three changes, 1, 2, 2 minutes.
16. Wash ten minutes in running water.
17. Insert nuclear stain with acid iron hematoxylin (e.g. Weigert's) or alum hematoxylin or a blue or green basic aniline dye.
18. Wash in water, blue alum hematoxylin with 0.01% sodium carbonate solution; dehydrate with alcohol or acetone as indicated by dye used.
19. After hematoxylin nuclear stains, further counterstains may be added: A one-minute plasma stain in 1.25% aqueous picric acid or 2% orange G or other; a picro-methyl blue connective tissue stain (p. 357); acid-fast or fat stains.
20. Dehydrate and differentiate in two changes each of 95% and 100% alcohol. Clear in one change of alcohol xylene mixture and two changes of xylene. Mount in synthetic resin or ester gum.

One may also combine such procedures as the ammine silver reticulum methods or the ferric mannitol or dialyzed iron mucin methods with the periodic acid Schiff by interposing them before the periodic acid oxidation, before Step 9. See p. 290.

Results. Oxidation Schiff reactions have been recorded as positive from a quite extensive list of substances (Lillie, Stain Technol. 26:123, 1951). The substances reacting more strongly with the periodic-acid method usually give positive Bauer and Casella reactions as well. These include the polysaccharides glycogen, starch, cellulose, and the rather slowly digestible polysaccharide of Sarcocystis. Similar Bauer-positive polysaccharides are present in Eimeria stiedae, in Klossiella muris, in the egg cytoplasm of Capillaria hepatica, and in Toxoplasma. sp., for which the periodic and permanganate reactions have not been recorded. Among the epithelial mucins those of the gastric-surface epithelium, peptic-gland neck cells, cardial and pyloric glands, uterine cervical glands, and rodent vaginal epithelium give all three reactions, as do also the prostatic-gland secretion and corpora amylacea, the seminal-vesicle contents and renal hyaline casts. The ocular-lens capsule and the membrane of Descemet and the colloids of the thyroid and anterior hypophysis also give strong periodic acid and positive Bauer and Casella reactions. Rossman noted the latter two reactions for ovarian pigment; it often reacts strongly to periodic acid.

With a second group of substances the Casella reaction is generally weak or negative; the Bauer, moderately strong; and the periodic Schiff, positive to strongly positive. This group includes the mucins of the salivary glands, of the conjunctiva, of the oropharyngeal glands of most of the species studied, of Brunner's duodenal glands, of intestinal goblet cells, of tracheobronchial glands and goblet cells, and of ovarian follicles and cysts. The
cuticular borders of the epithelium of the villi of the small intestine and of
the renal convoluted tubules react similarly, as does the zona pellucida of
rodent ova. The Casella reaction of cartilage matrix is weak or negative; the
Bauer and periodic acid Schiff reactions are positive, with or without ante­
cedent diastase digestion. Agar used as an imbedding matrix behaves
similarly. Hypophyseal cyanophil or β cells react similarly, as do cerebral
corpora amylacea. Amyloid gives a rather light red coloration with the
periodic acid Schiff, and weakly positive Bauer and Casella reactions. Posi­
tive Bauer and HIO₄ Schiff reactions, with negative Casella reaction, are
given by the chitins of various yeasts and molds, by the filamentous material
in the granules of actinomycosis and botryomycosis, and by certain diph­
theroid organisms. The coagulum in the vitreous humor and corneal collagen
usually stain well with the HIO₄ Schiff and weakly with the Bauer and
Casella methods.

With a third group of substances the periodic acid Schiff reaction is posi­
tive and the Bauer and Casella reactions vary from weakly positive to nega­
tive. This category includes the acromere lipoid of the retinal rods, the
ceroid pigment of the choline deficiency cirrhosis of rats, the pharyngeal-
gland mucin of the rabbit, the mucins in the bases of the colonic glands,
chordoma mucin, Russell bodies, and the keratin of Gaucher's disease.

Collagen from areolar connective tissue and sclera give positive HIO₄
Schiff reactions, negative Bauer, and weak or negative Casella tests. Mega-
karyocyte granules color pink with the HIO₄ Schiff, and vary according to
species with Bauer and Casella reactions. Chromaffin in chromate fixed
adrenal gives a gray red after HIO₄ only.

Vascular and certain epithelial basement membranes, and reticulum give
good positive HIO₄ Schiff reactions, a negative Bauer, and a dubious or
negative Casella reaction. The colonic melanosis pigment of man; a similar
pigment in the guinea pig intestine; adrenal lipofuscin pigment of man,
guinea pigs, and mice; fibrin; often plasma and serum; salivary zymogen
granules of man and various rodents; pancreatic zymogen granules of man,
rabbits and some mice, but not rats or guinea pigs; Paneth cell granules of
rats, guinea pigs, and rabbits, but not regularly those of men and mice;
granules of many mast cells in man, few in mice, none in rats; and the bac­
terial chitin of some organisms (including anthrax, streptococcus, and vari­
ous intestinal bacteria) give fairly good positive HIO₄ Schiff reactions and
negative Bauer and Casella tests.

A relatively weak periodic acid Schiff reaction and negative Bauer and
Casella are recorded for bone matrix; myxoma mucin, the mucinous coagula
in the lymphatic spaces of the umbilical cord matrix; nucleus pulposus
mucin; collacin, or collagen in the state of basophil degeneration; and the
lipofuscin of heart muscle.

Finally, positive HIO₄ Schiff reactions (without information as to Bauer
and Casella tests) are recorded for eosinophil leucocytes in the monkey but
not in man (see p. 393); for the vitamin E deficiency ceroid pigment of the rat uterus; for the ceroid pigment of the mouse testis and adrenal; for Kurloff bodies in guinea pigs; for goblet cells in the pancreatic ducts; for certain nuclear inclusion bodies in the human vas deferens; for acrosomes and head caps of intratesticular spermatozoa; for egg albumen and gelatin; for cytoplasmic granules in the rat lacrimal gland; and for a nonlipoid component of the Golgi apparatus. Bauer and periodic Schiff-positive materials have been recorded in schistosome egg shells and in opercula of the eggs of Capillaria (Hepaticola) hepatica.

The reactions of blood cells are presented on pp. 431-432.

Keratin and keratohyalin,—of pharynx and esophagus generally, of the proventriculus of muridae, and of epidermis and hair cortex—are not stained; but, curiously, hair cortex reacts strongly to the periodic acid Schiff procedure after bromination.

The positive reactions of arterial elastica and of elastic laminae and ligaments which are seen after periodic oxidation, especially in rodents, occur also when the oxidation step is omitted. They may be prevented by interposing a 30 minute bath in 5% aqueous phenylhydrazine hydrochloride before the periodic acid step. Wash 5-10 minutes in running water after the phenylhydrazine.
Chapter 8

Nuclei and Nucleic Acids

Nuclei are generally well stained by the general oversight methods (pp. 114–120) and by basic dyes generally. The use of acids or low pH buffers with basic dyes increases their selectivity for chromatin. Also, overstaining with following acid or similar differentiation gives quite selective pictures.

The acid alum and iron hematoxylin methods used as progressive stains are quite selective for chromatin, and again the practice of overstaining and decolorization to the desired point yields superior results. Particularly the sequence methods of staining with an iron alum mordant followed by overstaining in an aged or “ripened” (partly oxidized) hematoxylin solution and subsequent differentiation with iron alum or acids have been widely used for the study of nuclear structure, mitosis, centrosomes, spindles, and the like. Formulae are presented on pp. 76–81. The phosphotungstic acid hematoxylin of Mallory is also highly recommended (pp. 344–345).

These regressive iron hematoxylin methods are still much used for the identification of intestinal Entamoebae in smear preparations. For this purpose a preliminary 15 minutes’ fixation of fresh, still moist films in Schaudinn’s mercuric chloride alcohol (p. 38) is prescribed. Then treat with 0.5% iodine in 70% alcohol for three minutes; and decolorize with 95% alcohol, or for a minute or two in 5% sodium thiosulfate. Wash in water and stain as usual with Mallory’s, Heidenhain’s, or other technic (pp. 78–80).

I see no especially good reason why one of the premixed neutral or acid iron hematoxylin should not be used for this purpose. I have often used Weigert’s acid iron chloride hematoxylin with picro-fuchsin (p. 346) in the study and identification of Trypanosoma cruzi in heart muscle. The blepharoplasts appear as intensely black, rod shaped structures; the trophonuclei as rounded, gray bodies. Entamoeba histolytica is also well shown in sections by this method, and plasmodia and sarcosporidia are readily identified. How-
ever, such parasites are often more easily found when azure-eosin methods are employed (pp. 116–119).

**Basic Aniline Dyes.** Staining 5–30 minutes in 0.1–1% solutions of safranin O (C. I. No. 841), Janus green B (C. I. No. 133), thionin (C. I. No. 920), toluidin blue O (C. I. No. 925), azure A, methyl or ethyl green (C. I. Nos. 684, 685) in 0.5% to 1% acetic acid solution in water will give quite selective chromatin staining even without much further differentiation.

Even better is the practice of staining in solution of similar strength in distilled water (or 20% alcohol to prevent growth of molds) for 5–20 minutes, followed by one minute differentiation in 5% to five minutes in 1% acetic acid. Mitotic nuclei are rendered more conspicuous when these differentiations are carried to a point where resting nuclei remain only rather lightly stained. This corresponds to a point where the sections appear only lightly colored on gross inspection, and when the differentiating fluid ceases to extract color clouds.

Many writers use far stronger solutions for nuclear staining than the 0.1–1% aqueous solutions noted above, alleging superior results. Saturated alcoholic and aqueous solutions and mixtures of them are recommended in the case of safranin; and one widely used formula, that of Babes (Ztschr. wiss. Mikr. 4:470, 1887) recommends saturating 2% aniline water with (5–6%) safranin O (C. I. No. 841) by heating to 60°–80°C. This solution keeps only 1–2 months. It stains instantaneously. Differentiation in alcohol gives good staining of both resting and dividing nuclei; and use of 0.1–0.5% hydrochloric acid alcohol decolorizes resting nuclei more rapidly than chromosomes.

Material fixed with osmium tetroxide methods or subjected to prolonged chromation is more difficult to stain, and more prolonged exposures and stronger dye solutions are necessary.

**Acetic Orcein.** A very precise chromatin stain used on crush preparations is the acetic orcein method. This was shown me by A. J. Dalton of the National Cancer Institute.

1. **Tissue** either fresh or stored at 5°C is simultaneously fixed and stained by immersion for 48 hours in acetic orcein. Dissolve 1 Gm. natural or synthetic orcein in 45 cc. hot glacial acetic acid; cool, and add 55 cc. distilled water.
2. **After staining** take a small fragment and gently crush it between a slide and a cover glass to about a single cell layer. Slides should first be coated with Mayer’s glycerol albumen.
3. **Fix the preparations** as they are (with cover glass and slide in place) by standing on end for 48 hours in a closed vessel containing in its bottom absorbent cotton saturated with 95% alcohol.
4. **Then immerse** in 95% alcohol and take off the cover glass. The tissue may adhere to either the slide or the cover glass.
5. The film is then counterstained for a few seconds in 60 cc. 100% alcohol containing about 1 cc. 1% fast green FCF. The tissue should appear faintly greenish at this stage.

6. Mount directly from absolute alcohol in Diaphane or Euparal. Or if this clouds, rinse in two changes of 100% alcohol, one of equal volumes of 100% alcohol and xylene and two of xylene. Then mount in xylene balsam.

This is a very precise red chromatin stain. Chromosomes are very conspicuous. Cytoplasm is pale green, without appreciable difference in tone between apparently surviving and definitely necrotic cells. Herein the method seems to me inferior to the azure-eosin methods.

In vivo Staining of Nuclei. DeBruyn and co-workers (Exper. Cell Research 4:174, 1953) report that nuclei of living cells may be stained with fluorescent diaminoacridine dyes. The affinity appears to be specific for intranuclear nucleic acids. Proflavine hydrochloride is administered intravenously to mice in dosages of 25 mg./kg. Fresh frozen sections are observed under long-wave ultraviolet for fluorescence (pp. 11-15).

On fixed material these dyes show only the same specificity as other basic dyes.

Nucleic Acids

The Caspersson school have used extensively the ultraviolet absorption of the nucleic acids at 260 mp as a means of localizing these substances. Caspersson (J. Roy. Micr. Soc. 60:8, 1940) uses a rather complicated apparatus for photometric estimation of ultraviolet absorption in quite small field areas. The estimates obtained by photography are quantitatively cruder, though qualitatively more accurate. In its applicability to living cells the method has certain special values. Mellors (Science 111:627, 1950) found that living-tissue cultures would tolerate as many as 30 photographic exposures at 260 mp before being killed. The apparatus is expensive, whether for ultraviolet photography alone or for photometry as well, and seems adapted to highly specialized studies. For most purposes the histochemical procedures for the identification of nucleic acids are easier, more flexible, and more differential.

Desoxyribonucleic or Thymonucleic Acid

The Feulgen reaction is now generally considered specific for the desoxypentosenucleic acids. It depends on acid hydrolysis of the nucleic acid, which by liberating the purines and pyrimidines from the pentose phosphoric-acid complexes leaves a reactive aldehyde group free on the latter. While a similar liberation undoubtedly also occurs with ribonucleic acid, it is an observed fact that normal aldoses such as glucose, ribose, xylose, lyxose and the like do not form the deep red-purple color complex with Schiff's sulfite leucofuchsin reagent, except perhaps very slowly; and that 2-desoxy
aldoses, as well as other aldose sugars in which carbon 2 does not present a hydroxyl group, react promptly. Ketone also reacts, but more weakly and slowly. Hence it would appear that the application of Schiff reagent to sections should not be unduly prolonged. (See p. 313.)

Hydrochloric-acid hydrolysis of nucleic acids is not instantaneous. It is observed that longer hydrolysis may give a more intense reaction, and that further prolongation of the treatment beyond the optimum weakens and finally completely destroys it. This last is sometimes observed when bone is treated with mineral acids for an unduly long period. For routine tissues hydrolysis with normal hydrochloric acid for ten minutes gives a more intense reaction than it does in five minutes at 60° C. Usually, preceding and following one minute baths in normal hydrochloric acid at room temperature are prescribed. I fail to discern any advantage in these steps and have omitted them for some time as a matter of routine.

The optimum hydrolysis time for a maximal Feulgen reaction varies considerably with the object under study and the method of fixation employed. For the best results the time should be determined experimentally for each object studied.

The reaction of the hydrolyzed nuclear material with the Schiff reagent occurs quite promptly, and appears to be as strong in ten minutes as it is with the traditional two hour treatment. Use of the shorter period would appear to lessen the chance of confusing the slower reactions of ketones and normal aldoses.

The precise fuchsin content and method of manufacture of the Schiff reagent appear to have little influence. The same Schiff reagent as used for other purposes (p. 156) seems quite satisfactory for the Feulgen test.

The Feulgen Reaction. (modified from our 1948 text)

1. Bring paraffin sections through xylene and alcohols to water as usual, with the usual iodine thiosulfate sequence for removal of mercurial precipitates if required.
2. Place in normal hydrochloric acid (preheated) at 60° C. for ten minutes.
3. Immerse in Schiff reagent (p. 156) for ten minutes.
4. Wash 1, 2, and 2 minutes in three successive baths of M/20 sodium bisulfite (0.52% NaHSO₃; 0.475% Na₂S₂O₅; 0.55% K₂S₂O₆).
The sulfite baths should be discarded daily.
5. Wash five minutes in running water.
6. Counterstain a few seconds in 0.01% fast green FCF in 95% alcohol. The stain does not wash out in alcohol, but if it is too intense it may be removed promptly in water.
7. Complete the dehydration with 100% alcohol; clear through one change of alcohol and xylene (50:50) and two of xylene. Mount in
polystyrene, ester gum, permount, HSR or other synthetic resin, or in balsam.

**Results.** Nuclear chromatin is a deep red purple. The chromatin of plasmodia, sarcosporidia, toxoplasmatas, histoplasmatas, and some yeasts is Feulgen positive, though often paler red than that of host cells. Feulgen-positive globules are seen in *Torula histolytica* in some specimens and not in others. Similarly, small red bodies are seen in endosporulating coccidioides. No definite Feulgen-positive material was found in vegetative forms of this fungus or of *Haplosporangium parvum*. Mold mycelia, and Gram-positive and Gram-negative bacteria generally fail to stain. Typhus rickettsiae in yolk-sac material are Feulgen-negative; and *B. tularens* and psittacosis rickettsiae usually are Feulgen-negative, though I have seen occasional clusters of each stain red. Trophonuclei of *Trypanosoma cruzi* in its leishmania forms in heart muscle stain as deep purplish-red rings. In some clusters blepharoplasts are unstained; in others they appear as deeper red-purple rods. In vegetative trypanosomes in the blood (*T. brucei, T. equiperdum*) trophonuclei may stain with difficulty, and blepharoplasts may fail to stain. In encephalitozoa, chromatin varies from vague, poorly stained pink granules to fairly definite, oval and round, small red-pink vesicles. Many coccidia (*Eimeria*) lack Feulgen-positive material, but some young intraepithelial forms present more or less numerous small red rings apparently outlining the refractile granules seen in these organisms.


Although elastic fibers in arteries and elastic ligaments and laminae are often Schiff-positive after the Feulgen hydrochloric acid hydrolysis (especially in rodents), the same fibers tend to be more strongly Schiff-positive when the acid treatment is omitted; and the reaction of elastica in Feulgen stains may be prevented by interposing a 30 minute bath in 5% phenylhydrazine before the acid hydrolysis step.

*Methyl Green–Pyronin*

This method has had a revival of popularity in recent years. Some even claim that it differentiates specifically between desoxyribonucleic acid (green) and ribonucleic acid (red), and regard it as a definite histochemical reagent.

Its behavior is much influenced by prior fixation procedure. Best results have been obtained after neutral and acid alcoholic fixatives (including alcohol formalin and acetic alcohol formalin; p. 35) and after the aqueous bichromate formaldehyde fixatives. Aqueous formalin and Zenker's fluid give inferior results: sections stain either all red or all blue.

The proportions of methyl green (C. I. No. 684) or ethyl green (C. I. No. 685) and pyronin Y (C. I. No. 739) or pyronin B (C. I. No. 741) vary greatly in various formulae.
<table>
<thead>
<tr>
<th></th>
<th>mg. Methyl Green</th>
<th>mg. Pyronin</th>
<th>cc. Alcohol</th>
<th>cc. Glycerol</th>
<th>cc. Phenol</th>
<th>&quot;Carbolwater&quot;</th>
</tr>
</thead>
<tbody>
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<td>Water</td>
<td>%</td>
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<td>Saathoff, 1905</td>
<td>150</td>
<td>500</td>
<td>5</td>
<td>20</td>
<td>1.5</td>
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<tr>
<td>Saathoff–Conn</td>
<td>800</td>
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<td>4</td>
<td>16</td>
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<td>Unna</td>
<td>150</td>
<td>250</td>
<td>2.5</td>
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<td>0.39</td>
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<td>Ramón y Cajal</td>
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<td>2.5</td>
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<td>Weill</td>
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<td>Masson</td>
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<tr>
<td>Slides and Downey</td>
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<td>500</td>
<td>2.5</td>
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<td>Slides and Downey reduced to 100</td>
<td>408</td>
<td>408</td>
<td>2.04</td>
<td>16.3</td>
<td>0.41</td>
<td>81.3</td>
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<tr>
<td>Lillie (new data)</td>
<td>700</td>
<td>300</td>
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<td>20</td>
<td>0 to 2</td>
<td>80–78</td>
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<td>Trevan and Sharrock</td>
<td>36</td>
<td>157.6</td>
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<td>100</td>
</tr>
</tbody>
</table>

Pappenheim's original prescription was inexact (Romeis, Unna in Ehrlich's *Encyklopädie*, 1903) and is omitted.

7 Cowdry, E. V.: *Laboratory Technique in Biology and Medicine*. Baltimore, Williams & Wilkins, 1948.
The amount of phenol in the mixture should be small. More than 3% seems definitely deleterious. Some seems to be beneficial, though I have had good results without any.

The presence of glycerol is probably chiefly valuable in retarding evaporation when slides are stained flat with small volumes of solution. Alcohol seems unnecessary.

With fresh samples of methyl green and pyronin, it may be necessary to vary the proportions. Make 2% aqueous solutions of each dye, mix in the desired proportions, and add an equal volume of a mixture of 4 Gm. phenol, 56 cc. water, and 40 cc. glycerol. The phenol is 2% in the final mixture, and may be reduced to 2 Gm. or 1 Gm. if it is desired to try a 1% or 0.5% level.

The technic is simple.

1. Deparaffinize and hydrate paraffin sections as usual.
2. Stain 20 minutes in methyl green pyronin.
3. Wash in cold, recently boiled distilled water.
4. Dehydrate with acetone; clear through acetone + xylene (50:50) and two changes of xylene; mount in synthetic resin.
4b. Or: blot dry; differentiate a few seconds in equal volumes of triethyl phosphate and xylene; clear in xylene; mount in synthetic resin. (Rosa, Stain Technol. 25:165, 1950).

Results. Nuclei, blue green; bacteria and basophil cytoplasm, red.

Rosa’s procedure is said to preserve the methyl green better than the usual alcohol dehydration. I have used acetone for the same reason.

Some recent workers—notably Kurnick, and Trevan and Sharrock, among others—insist on the purification of methyl green by extraction of the aqueous solution with chloroform until no more methyl violets appear in the chloroform solution. Trevan and Sharrock use 2% aqueous methyl green, extracting it in a separatory funnel with chloroform, and state that the extracted solution remains free of further extractable violet for six months.

Kurnick noted that pyronin was considerably extracted from cytoplasm by dehydration and by the differentiating agents used for the removal of methyl green from background structures. Therefore he converted the method into a sequence procedure, and used the pyronin as a saturated or ½-saturated acetone solution after dehydration and differentiation of the methyl green, thus avoiding irregular extraction of the pyronin by the acetone.

Kurnick’s (Stain Technol. 27:233, 1952) Methyl Green–Pyronin Variant.

1. Fix in Carnoy, cold 80% alcohol, cold acetone, neutral formalin; imbed and section in paraffin as usual; or use frozen dried material imbedded in paraffin directly.
2. Deparaffinize and hydrate as usual.
3. Stain six minutes in repurified 0.2% methyl green in water or in pH 4.2 M/100 acetate buffer. (The solution should be extracted in a separatory funnel by shaking with successive changes of chloroform until no more color is extracted.)
4. Blot dry and dehydrate with two changes of n-butyl alcohol.
5. Stain 30–90 seconds in acetone freshly saturated with pyronin B. For more delicate staining dilute one part of the saturated solution with nine parts acetone and prolong the time somewhat.
6. Clear directly in cedar oil, wash in xylene, and mount in terpene resin (Permount, HSR).

Results. Blue green chromatin, red nucleoli, pink to red cytoplasm, green cartilage, purplish pink bone, brown erythrocytes.

Trevan and Sharrock used for formalin-fixed tissue a weaker formula than most others (Table 16) and added orange G as an acid cytoplasmic stain, selecting a slightly bluish pyronin which did not precipitate with orange G. Two stock solutions were kept: one of 360 mg. chloroform extracted methyl green and 1576 mg. pyronin in 500 cc. distilled water; the other of 650 mg. orange G in 500 cc. 0.2 M acetate buffer of pH 4.8. Working mixtures of equal volumes of the two stock solutions keep for about two weeks. They direct: Deparaffinize paraffin sections of 3–5 μ, hydrate; stain ten minutes to 24 hours; wash about seven seconds in flowing distilled water; blot; dehydrate with acetone; clear through acetone xylene and two changes of xylene; and mount in polystyrene. Nuclei and mucin, green to blue-green; tigroid and basophil cytoplasms, magenta; nucleoli, pink; oxyphil cytoplasmic components, orange; mast-cell granules, usually dark red brown.

I have no great faith in the specificity of this method. It is often capricious and difficult to control. Such methods as the acid iron hematoxylin safranin sequence stain (p. 285) seem to yield a similar differentiation with far greater certainty and less limitation as to fixing methods.

For assaying the effect of ribonuclease digestions, I find the azure A eosin B technic superior. The pink of the basic protein left after removal of ribonucleic acid contrasts much better with the blue than does the remaining pink of pyronin-staining with the red of undigested preparations.

It is possible that the hitherto little used Turchini method for nucleic acids may prove to be more reliable and more specific, if the reagent 9-methyl-2,3,7-trihydroxy-6-fluorone (also written 2,6,7-trihydroxy-3-fluorone) can be made readily available through the usual commercial sources.

Turchini's method depends on a color reaction for the sugars with this reagent. According to Turchini and co-workers (Trav. membres soc. chim. biol. 25:1329, 1943) neither phosphoric acid nor the purines and pyrimidines give important reactions. With the 9-phenyl derivative, which gives slightly deeper colors than the 9-methyl-2,3,7-trihydroxy-6-fluorone, desoxyribose gives a blue violet in spot tests; ribose, a yellowish rose; arabinose, purplish
violet; xylose, fructose, galactose, and sucrose, red; glucose and maltose, orange to red; and lactose, violet.


Backlar and Alexander’s Variant of the Turchini Method for Nucleic Acids.

Use frozen dried material, or tissues fixed in Bouin, Zenker, formalin, or formol saline.

1. Deparaffinize and hydrate as usual, including iodine thiosulfate sequence if mercury fixation was used.
2. Hydrolyze two minutes in normal hydrochloric acid at 60° C.
3. Pass directly to 80% alcohol for 15 seconds.
4. Stain 20 minutes in 1 Gm. 9-methyl-2,3,7-trihydroxy-6-fluorone in 1 cc. concentrated sulfuric acid and 100 cc. 95% alcohol. (The mixture is stable for several weeks.)
5. Wash two minutes in fresh 1% aqueous sodium carbonate.
6. Wash in fresh distilled water two minutes.
7. Dehydrate three minutes in 50% acetone, three minutes in pure acetone, two minutes in acetone and xylene (50:50).
8. Clear in xylene, two changes, for three minutes total. Mount in a synthetic terpene resin.

Results. Desoxyribonucleic acid, violet to blue-black; ribonucleic, red to yellow.

Desoxyribonuclease digestion. Kurnick (Stain Technol. 27:233, 1952) used a preparation DNase (obtained from the Worthington Laboratories, Freehold, N.J.) at a concentration of 2 mg./100 cc., in 0.01 M trihydroxymethylaminomethane buffer at pH 7.6 (Gomori’s, p. 453, diluted 1:5 with distilled water). On material fixed in 80% alcohol or Carnoy’s fluid, digestion periods of two hours at 37° C. or 24 hours at 21° C. were recommended. Controls treated with buffer alone for the same period and unextracted controls should be used. He used his variant of the methyl green pyronin technic as the demonstration method.

Wigglesworth reports some interesting results regarding iron uptake by fixed tissue sections, with particular reference to iron which apparently goes into a masked state and becomes nonreactive to ferrocyanides. This iron is unmasked by ammonium sulfide. It has been shown that nucleic acid solutions precipitate from saturated urea solution when a little ferric chloride is added, and that the iron thus precipitated is nonreactive to thiocyanate. He attributes iron uptake to mono- and di-esters of phosphoric acid, which form firm,
non-ionized unions, or to free carboxyl groups. The stability of the complexes formed is attributed to chelation. I cite his technics.

**Wigglesworth’s Iron Sulfide Technic** *(Quart. J. Micr. Sci. 93:105, 1952).*

1. Fix in Carnoy, Bouin, susa, Zenker or Helly. Prepare paraffin sections, deparaffinize, and hydrate as usual.
2. Immerse one minute in saturated iron alum solution.
3. Wash well in running water.
4. Immerse 15 to 20 seconds or more in dilute ammonium sulfide.
5. Rinse, dehydrate, and mount in balsam.

*Results.* Nuclei, blue black; chromosomes, intense blue black; cytoplasm, gray or brown; fibrous tissue, pale chocolate; basal layer of epidermis, blue; erythrocytes, pale blue gray.

Staining may be intensified by transferring after step 4 to potassium ferricyanide solution, which forms Turnbull’s blue. Then sections may be again put through Steps 2, 3, and 4. They acquire additional iron sulfide in the process without removal of the ferrous ferricyanide, and thus the intensity of the reaction is deepened.

Wigglesworth interprets the reaction as demonstrating free carboxyl and phosphoric-acid groups. He finds that iron uptake is greatly reduced by two or three days of methylation in 0.1 N HCl in methanol *(cf. p. 163).*

Maximum uptake of iron by nucleic acids occurs at pH 1.5; by proteins, at pH 4 or higher.

For further interesting details the original should be consulted.

**Ribonucleic Acid**

This substance constitutes the basophilic cytoplasmic material in a wide variety of cells, including the tigroid substance of nerve cells; yeast cells; the cytoplasm of plasma cells, lymphocytes, myeloblasts, osteoblasts, pancreatic acinar cells, salivary serous acinar cells, peptic gland chief cells, intestinal epithelial and gland cells and the basal layers of the various stratified squamous epithelia.

Various methods have been used for its demonstration; notably, variants of Unna’s methyl green pyronin, Giemsa’s stain, Mallory’s eosin methylene blue *(p. 116)*, our Nocht azure A–eosin B technic *(p. 118)*, and simple solutions of toluidin blue, pyronin, thionin, and the like.

Gersh and Bodian *(Biol. Symposia 10:161, 1943)* stained nerve cells overnight in filtered 1% toluidin blue, differentiated in alcohol, and mounted in balsam. I have found a 30-second stain in 0.1% aqueous thionin *(buffered to pH 4 with acetates)* followed by the acetone, xylene, clarite sequence quite effective for most of the tissues noted above, but generally prefer the azure A–eosin B technic *(p. 118)* because of the more definitive color change from blue to pink when the digestion tests are performed.
The definitive test for the histochemical identification of ribonucleic acid is its digestion by the enzyme ribonuclease. This is ordinarily prepared from pancreas. Brachet used a boiled acid extract of pancreas. Ribonuclease is thermostable. Most workers have preferred the crystalline substance isolated by the method of M. Kunitz (J. Gen. Physiol. 24:15, 1941) and now available commercially from Armour & Co. The commercial enzyme has proved active in my hands at a 1:100,000,000 dilution. It functions well in 0.8% saline solution buffered with phosphates to pH 6.0. I prefer this pH level to the 6.75–7.0 levels of other workers because of the decreased section losses at the more acid level. I have found that commercial malt diastase also contains a thermostable ribonuclease, which I have used extensively.

This digestion, at least in the case of the malt diastase ribonuclease, is largely inhibited by fixation in Koser’s, Orth’s, and Möller’s bichromate formalin fluids; less so by Zenker’s fluid fixation. With formalin fixation the digestibility is influenced both by the duration of fixation and by the diluent. Longer aqueous formalin fixations make tigroid, ribonucleic acids generally, cartilage, and nucleus pulposus more resistant. Brief alcoholic formalin fixations facilitate this ribonuclease activity of the diastase. Still better is Carnoy’s fluid. Bouin’s fluid fixation almost entirely prevents malt ribonuclease digestion of ribonucleic acids, but Gendre’s alcoholic picroformalin acetic fixation allows fairly prompt digestion. Pancreatic ribonuclease does not appear to affect the basophilia of cartilage matrix or nucleus pulposus, in contrast to the malt diastase (pp. 275, 291).

Technic of Ribonuclease and Malt Diastase Digestion of Tigroid and Cytoplasmic Ribonucleic Acid.

1. Fix in Carnoy’s fluid at 0° C. or in acetic alcohol formalin (p. 35) for 18 to 24 hours. Wash in 95% alcohol over calcium carbonate. Complete the dehydration, dealcoholization, and paraffin infiltration as usual.

2. Bring paraffin sections through xylene and alcohols to water. Do not collodionize. Collodion films prevent enzyme action almost completely.

3. Immerse for one or more hours at 37° C. (or 50° C. for more drastic action; the malt diastase is seriously weakened by heating an hour at 55° C. and is destroyed at 60° C.) in a buffered enzyme solution, using simultaneous digestion controls in the solvent without enzyme.

The solvent contains 8 Gm. sodium chloride, 0.28 Gm. anhydrous disodium phosphate and 1.97 Gm. sodium acid phosphate monohydrate per liter to give pH 6.0. This pH level has less detergent effect on sections than pH 7, and allows effective enzyme action. Commercial ribonuclease, obtained from Armour in 100 mg. lots, is quite effective on cytoplasmic ribonucleic acid and on tigroid at 1:1,000,000; but is ineffective on glycogen at 1:5000. A concentration of 1:100,000 seems appropriate for testing purposes. The ribonuclease activity of malt diastase is evident to about 1:3000.
dilution; its glycogenolytic action, to about 1:100,000. Hence a 1:1000 dilution is used for both purposes as a matter of routine.

4. Rinse in water and counterstain by the Nocht method, by thionin, or by the other procedures which effectively demonstrate the tissue elements under consideration in undigested controls.

For precise work, after determination of the fact of digestibility under the conditions of the test, it is desirable to determine the minimum enzyme concentration which is effective in a set period, such as one hour at 37° C. This figure may be used for rough comparison with other sets of conditions or other objects.

Both of these enzyme preparations may contain also small amounts of a desoxyribonuclease, whose action is manifest after 8 to 16 hour digestion periods. This activity (as well as the glycogenase and amylase activity) may be selectively destroyed by adding 0.57 cc. glacial acetic acid per 100 cc. solution in distilled water (0.1 M) and boiling for five minutes. Then cool and neutralize with 1 N sodium hydroxide to about pH 6.2, using one to two drops of 1% aurin (rosolic acid) or nitrazine as an indicator, or adjusting with the pH meter. Since the solution now contains approximately 0.1 M sodium acetate (0.82%), addition of sodium chloride is unnecessary and has been found deleterious under some circumstances.

Leuchtenberger and Lund (Exper. Cell. Res. 2:150, 1951) record that the keratohyalin granules of the dog epidermis are Feulgen-negative, and lose their basophilia to toluidin blue or azure A on digestion with ribonuclease.

As noted on p. 275, human saliva also exhibits ribonuclease activity. Its action on tigroid was noted by Szent-Györgyi in 1931 (Nature 128:761), who interpreted the action as indicating a polysaccharide nature of the tigroid.

Perchloric Acid Extraction


Koenig and Stahlecker (J. Nat. Canc. Inst. 12:225, 1951) report differential removal of ribonucleic acid from nerve cells by 10% aqueous perchloric acid in 15 minutes at 37° C., 2 hours at 25° C., 6 hours at 23° C., 12 hours at 20° C., and failure to extract in 4 days at 4° C. Liver cell ribonucleic acid was easier to remove, requiring 18 hours at 4° C. and only 1 hour at 23° C. As with ribonuclease, prolonged formalin fixation greatly retards solution of ribonucleic acid in perchloric acid.
Extraction in hot perchloric acid (90° C.) also removes the desoxyribonucleic acid. Most other proteins are dissolved only to a minor extent; so the method is fairly differential.

It may be noted that after brief alcohol fixation, cytoplasmic basophilia of blood lymphocytes (in smears) may be removed by extraction for an hour at 58° C. in distilled water or in 0.9% sodium chloride solution, and is impaired by even 10 minutes extraction at this temperature.

As noted before, other acids share this property of removing ribonucleic acid. Fisher (Stain Technol. 28:9, 1953) showed that 2 M dilutions of nitric, hydrochloric, or sulfuric acid at 0° C. to 5° C. would differentially remove cytoplasmic basophilia of pancreas, various epithelia, gastric chief cells, and Nissl substance of nerve cells in 16-24 hours. Extraction with stronger acid at the same temperature, or with the same concentrations at 25° or 37° C. was less differential; a good deal of nuclear basophilia was extracted as well.

These extractions induce sufficient hydrolysis of desoxyribonucleic acid to yield a Feulgen reaction with sulfuric acid leucofuchsin but under the recommended conditions do not appreciably impair nuclear staining with azure A, even when the extraction at 0-5° C. is prolonged to 32 hours.

The metachromasia of mast cells and of pyloric-gland mucin, as well as the periodic acid Schiff reaction, were unimpaired by these extractions. Atkinson (Science 116:303, 1952) showed the parallelism in action between normal perchloric and hydrochloric acids, and pointed out that both reagents also extracted mucus, even at 5° C. Only toluidin blue stains were mentioned. The basophilia of mast cells and of cartilage was almost as resistant to these extractions as was that of cell nuclei.

**Tigroid**

Tigroid or the Nissl substance occurs as angular and elongate particles of varying size in the cytoplasm of nerve cells. It stains deeply with thionin, azure A, methylene blue, toluidin blue, and the like; less conspicuously with safranin, fuchsin, or neutral red; more differentially from acid solutions or with acid differentiation; poorly or not at all with hematoxylin.

Generally, alcohol fixation is prescribed, formalin serves well, and quite good pictures may be obtained with the azure eosinate method on fresh material fixed in Orth’s fluid, or in formalin followed by chromate treatment before imbedding. A galloycyanin chrome alum method is highly recommended by Einarson (Am. J. Path. 8:295, 1932).

**Einarson’s Method.** Dissolve 10 Gm. chrome alum KCr(SO₄)₂•12H₂O in 200 cc. distilled water, add 300 mg. galloycyanin, and heat slowly to boiling. Boil with frequent shaking for 15-25 minutes, cool slowly, and filter. This solution has a pH of about 1.84. Sections of material fixed in alcohol, 20% formalin, 10% alcoholic formalin, sublimate alcohol, or in a solution of 10 cc. formalin, 40 cc. 95% alcohol, and 50 cc. 6% aqueous mercuric chloride solution, stain well in 24 hours; but acetic Zenker material requires longer.
Either paraffin or nitrocellulose sections may be used. These are brought to water in the usual way before staining, stained 24 to 48 hours, dehydrated as usual with alcohols, cleared with xylene, and mounted in balsam (or clarite). This stain is not extracted by alcohol.

Windle’s Thionin. Windle (Conn and Darrow) uses thionin in buffered solution for a so-called “end point” method of staining. He prescribes Carnoy or other alcoholic fixative or 10% formalin.

Paraffin or nitrocellulose sections are brought to water as usual. The water should be distilled and freshly boiled to expel carbon dioxide. Stain 20 minutes in 1 cc. 1% aqueous thionin solution in 40 cc. dilute acetate buffer of pH 3.0 to 4.0. Rinse in distilled water. Wash in two changes of 70% alcohol until no more color comes out (about five minutes). Dehydrate, clear, and mount. This yields blue violet tigroid, blue chromatin, blue glia cytoplasm, and a clear to faint blue background.

Windle also suggests a variant in which sections are stained at pH 4.0-5.0 and differentiated 2-5 minutes until no more color comes out in 0.2% aqueous acetic acid solution. This largely decolorizes glia cytoplasm as well.

Laskey’s Thionin Method for Frozen Sections.

Use old or freshly fixed formalin material. Cut frozen sections at 10-15 μm. Wash in distilled water, immerse five minutes in 70% alcohol, and wash in distilled water. Stain ten seconds in 0.1% thionin in 4 cc. M/5 acetic acid, 1 cc. M/5 sodium acetate, and 95 cc. distilled water. Wash in distilled water, dehydrate in 95% and 100% alcohol or two changes of acetone, and clear in 1:4 carbol xylene for a few seconds. Float onto slides, blot, and mount in HSR or polystyrene.

Results. Nissl bodies and glia nuclei, dark blue; background, nearly colorless.

Dempsey et al. (Anat. Rec. 98:417, 1947) have used the dye uptake at various pH levels for the characterization of the basophilic substances in tissues. They stained 24 hours at 25°C in M/2000 methylene blue (0.1595% of the pure dye, or 0.18% at 87.5% dye content; 0.2% should do about the same). They measured the dye uptake photometrically, obtaining quite characteristic “pH signatures” for each substance.

It should be pointed out, however, that these pH signatures, just as the isoelectric points which govern acidophilia and basophilia toward azure cosin stains (p. 119), are considerably altered by the fixative employed. With brief formaldehyde or Orth fixation, nuclei and tigroid still take up thionin at pH 2.0, and melanin as low as pH 1.2; after long storage in 10% formalin there is no staining of nuclei at pH 2.0 and only feeble blue coloration at pH 3.0. At the latter level melanin colors green, at pH 2 it remains almost pure brown.
The following data are based on material fixed 48 hours only in buffered 10% formalin or Orth's fluid. Dempsey used Zenker fixation of unstated duration.

Staining for 1–30 minutes in 0.05% thionin in .01 M acetate buffer of pH 3, or even in 0.01 M acetic acid (pH about 2.7) gives good staining of nuclei, tigroid, and strongly basophil cytoplasm; and metachromatic staining of mast cells and cartilage. Oxyphil material, muscle, and connective tissue remain unstained; and red corpuscles appear in light yellow. In a similar buffer of pH 4, muscle and connective tissues appear faintly greenish, and at pH 5 take a fair grade of light blue green. Phosphate buffers from pH 5 to pH 4 precipitate thionin almost quantitatively, but do not throw down azure A or toluidin blue.

At higher pH levels (6.0–7.5) and at higher dilutions (10 mg./liter = \(2.75 \times 10^{-6}\) M of 88% dye-content methylene blue, or similar concentrations of azure A, thionin, and toluidin blue) and overnight staining, connective tissue, keratin, muscle, and oxyphil materials in general assume a rather diffuse and light green; red corpuscles—a darker, somewhat olive green; basophil cytoplasms—blue violet; nuclei—fairly deep blue, greenish blue, or lighter bluish green; and cartilage matrix and mast cell granules (at least with azure A, thionin, and toluidin blue)—a brilliant purplish red. Epithelial mucins may fail to stain.

Azure Eosinate. I have found that azure eosinate stains of formalin or Orth material done at pH 4.0 show nuclei and tigroid well, with the advantage of a pink-stained background (pp. 118–120).
Chapter 9

Amino Acids, End Groups, etc.

Arginine

For the demonstration of arginine, Baker (Quart. J. Micr. Sci. 88(1): 115-121, 1947) gives a modification of the Sakaguchi reaction which appears to be specific for substances with the general formula

\[(2) - \text{NH-C-NH-C-(4)},\]

\[(1) - \text{N} (5)\]

where (1) and (2) are either hydrogen atoms or methyl groups, and (3), (4), and (5) may be various atoms or radicals. Dicyanamid, mono-, di- and tri-methyl-guanidines, glycocyamine, alacreatine, a-guanidino-n-butyric acid, galegine, agmatine, and arginine are listed as reacting to this test.


1. Fix blocks 3 mm. or less in thickness with Zenker, Bouin, Heidenhain's susa fluid, HgCl₂ acetic or formaldehyde saline, for 24 hours.
2. Wash, dehydrate, clear, and imbed in paraffin as usual.
3. Section at 16μ, attach sections with Mayer's albumen glycerol, dry, and pass through xylene and 100% alcohol.
4. Collodionize by immersion in 1% collodion in ether alcohol, drain, and wipe the back of the slide.
5. Pass through 90% and 70% alcohol, iodine, and thiosulfate if necessary, into water.
6. Mix 2 cc. 1% aqueous sodium hydroxide solution with 2 drops (0.05 cc.) 1% α-naphthol in 70% alcohol, and add 4 drops (0.2 cc.) 1% sodium hypochlorite solution. (Baker recommends a proprietary solution containing sodium chloride and other unspecified salts). Drain the
slide by shaking the water off (or perhaps blot with hard filter paper?) and immediately flood with the alkaline hypochlorite α-naphthol. Let stand 15 minutes for maximum color development.

7. Drain, blot with filter paper, and clear in a mixture of 3 parts pyridine and 1 part chloroform for three minutes.

8. Mount in pyridine chloroform, sealing with gold size. This is a turpentine-soluble cement used by opticians in mounting lenses.

Results. A pink or red color indicates presence of arginine or one of the other positively reacting guanidine derivatives. Sexually mature testis or intestine is a suitable test object. Nuclei of spermatozoa react very strongly; smooth muscle and intestinal epithelial cells, less so. The preparations fade after several hours or days. But see p. 146 for stability of the color in mineral oil when the Liebman-Thomas variant is used.

Thomas's (Stain Technol. 25:143, 1950) method for arginine is based on the Sakaguchi reaction. In this, α-naphthol and a hypochlorite or hypobromite produce an orange-red color in the presence of guanidine derivatives in which only one H atom of one or both of the NH₂ groups is replaced by an alkyl, fatty acid, or CN radical. Proteins containing arginine (NH₂C(NH) -NH(CH₃)₉CH(NH₂)COOH) react, and in fixed tissues the reaction is specific for protein arginine, since fixing and washing dissolve out free arginine and other guanidine derivatives. Guanidine, urea, creatine, creatinine, and amino acids other than arginine do not react.

Later, following Warren and McManus (Exp. Cell. Res. 2:703, 1951) a-naphthol was replaced by 8-hydroxyquinoline oxime. The orange color is said to be more stable than with the naphthol technic.

The Carnes, Brown and Thomas Hypochlorite, 8-Hydroxyquinoline Oxime Technic for Arginine (Stain Technol. 28:89, 1953, with notes from Liebmann and Thomas, Stain Technol. 26:261, 1951): Fix preferably in Bouin's fluid (p. 46). Fixation in Carnoy's fluid (p. 36) or in 10% formalin also gives successful preparations. Prepare paraffin sections as usual.

1. Take through xylenes and alcohols to 70% alcohol.
2. Immers 15 minutes at room temperature in a 0.3% solution of 8-hydroxyquinoline oxime in 30% alcohol. (Keep a stock 1% solution in 100% alcohol and dilute with water when needed.)
3. Transfer quickly, without draining, to alkaline hypochlorite at room temperature for 60 seconds. (Mix 9.4 cc. Clorox of 1.6 N chlorine content with 15 cc. 0.1 N potassium hydroxide and distilled water to make 100 cc.)
4. Transfer quickly, without draining, to alkaline butanol urea, two changes, ten seconds and two minutes respectively. (Dissolve 15 Gm. urea in 15 cc. 0.1 N potassium hydroxide, add 70 cc. n-butanol and distilled water to make 100 cc.)
5. Dehydrate in two changes of n-butanol, ten seconds and four minutes respectively.

6. Clear three minutes in aniline, rinse ten seconds in xylene, and mount in Permount containing 0.02% aniline (add 1 cc. 0.1% aniline in xylene to 4 cc. Permount).

The reagents in Steps 2, 3, and 4 are made fresh daily, and care is taken to keep anhydrous n-butanol in Step 5.

Liebmann and Thomas used in Step 2 a 15-minute bath at 5°C. in a fresh 5% aqueous dilution of a 1% solution of α-naphthol in 100% alcohol. In Step 3 they used a 90-second bath at 5°C. in 0.15 N sodium hypochlorite (11.17 Gm./liter) in 0.05 N potassium hydroxide solution (2.8 Gm./liter). In Step 4 the alkaline butanol urea contained 10 Gm. urea and 5 cc. N KOH per 100 cc. of 70% n-butanol, and two changes of 5 and 25 seconds at 5°C. were used. The butanol dehydration was followed directly by xylene, this was blotted off with hard filter paper and the sections were mounted in heavy white mineral oil. Cover glasses were cemented with rosin lanolin cement (p. 99). Persistence of the orange-red color for several months was reported.

The Ninhydrin Test

Serra (Stain Technol. 21:5, 1946) directs as follows: Deparaffinize and hydrate as usual, or use frozen sections. Flood sections with a mixture of equal volumes of pH 6.98 phosphate buffer (p. 451) and of a freshly prepared 0.4% aqueous solution of ninhydrin (triketo-hydrindene-hydrate). Place sections on a rack over boiling water and steam for 1–2 minutes. A blue or violet color develops. Drain, mount in pure glycerol, and cement with rosin lanolin cement (p. 99). Observe at once, since the color fades in a day or so.

Serra states that the reaction is given in adequate intensity by the amino acids except proline and hydroxyproline, and by peptides and proteins. Langeron notes ready diffusion of color.

The Alloxan Reaction

Langeron cites this for amino acids and proteins generally. Alloxan reacts with amine groups to form murexide, or purpurate of ammonium.

Flood sections with 1% alcoholic alloxan solution. Proteins color in red or rose. Serra (loc. cit.) recommends use of a neutral (pH 7) phosphate buffer as solvent and accelerates the reaction by heating over a boiling water bath. On fixed tissues the reaction is weak. The color is diffusible and is given also by nonprotein NH₂ groups and perhaps by SH groups.

The Ninhydrin and Alloxan Schiff Reactions

According to Yasuma and Ichikawa (J. Lab. & Clin. Med. 41:296, 1953) the ninhydrin reaction with α-amino acids may be used for the demonstra-
tion of protein with Schiff reagent. Briefly, ninhydrin decomposes amino acid residues to carbon dioxide, ammonia, and the next lower aldehyde, suffering reduction of one of its own hydroxyl groups in the process. This reduced ninhydrin then conjugates with a mole of ammonia and a mole of ninhydrin to form a blue-violet colored compound. This compound is quite diffusible, and its use for protein identification has been unsatisfactory.

However, the aldehyde formed on the oxidized amino-acid residues may be quite well demonstrated with Schiff reagent. Thus Dr. Longley in this laboratory has obtained quite good reactions with muscle protein, collagen, and thyroid colloid. It may be noted that deamination (p. 162) prevents their reaction.

Fixation with 10% formalin or with Zenker variants or with absolute alcohol is recommended. Paraffin sections at 5–10μ are deparaffinized and brought to 100% alcohol as usual. Then incubate 5–24 hours in 0.5% ninhydrin (or 1% alloxan) in 100% alcohol at 37° C. Wash for a few minutes in running water, immerse for 30 minutes in Schiff reagent (p. 156), wash 1, 2, and 2 minutes in three changes of 0.5% Na2S2O5, wash ten minutes in running water, dehydrate, and clear and mount through an alcohol, xylene, synthetic resin series. Counterstains have not been successful in our hands so far.

The Xanthoproteic Reaction

Serra (Stain Technol. 21:5, 1946) prefers “strong” fixations, but uses fresh material as well. Cover sections with concentrated nitric acid for several minutes until they become intensely yellow. Wash in distilled water. Expose to ammonia fumes or immerse in dilute ammonia solution. The color changes to orange. Mount in glycerol after rinsing in water.

The reaction is produced by tyrosine, phenylalanine, or tryptophane, as well as by phenols. Langeron (op. cit., 7th ed., p. 1264) notes in addition that purine bases give a violet to purple color by this method, the murexide reaction. Uric acid is distinguished from guanine by its relative solubility in piperazin hydrate and its relative insolubility in iron alum and mineral acids. A negative test means nothing.

The Millon Reaction

Cowdry, Romeis, and Glick each recommend the method of Bensley and Gersh for protein containing tyrosine.

Millon Reagent.

Saturate 500 cc. of 4% aqueous nitric acid with mercuric nitrate crystals. Filter, and to 40 cc. filtrate add 0.3 cc. 40% nitric acid and 0.14 Gm. sodium nitrite.

Prepare paraffin sections of frozen dried material, or material fixed in an
anhydrous fixative. Mount on slides by floating on 95% alcohol, or without flotation. Deparaffinize, dip briefly in 100% alcohol, blot and immerse directly in Millon reagent. Remove sections at intervals, inspecting for the presence of rose or red color. The action reaches its maximum in about three hours. Rinse in 1% nitric acid, dehydrate with 100% alcohol (dropping-bottle or several changes), clear in xylene, and mount in balsam.

Results. Tyrosine—rose red. Glick regards the reaction as specific.

Serra (Stain Technol. 21:5, 1946, emended) recommends the following modification of Millon's reaction as essentially specific for tyrosine.

Stock reagent. Dissolve 7.5 Gm. mercuric sulfate, 5.5 Gm. mercuric chloride and 7 Gm. anhydrous sodium sulfate in 85 cc. distilled water. Add 12.5 Gm. (6.8 cc.) concentrated sulfuric acid (sp. gr. 1.836) and when cool dilute to 100 cc.

Procedure. Sections—loose, on cover glasses, or on slides—are immersed in stock reagent for 30 minutes at 60° C. in covered vessel. Then cool to room temperature by immersion of container in cold water and let stand ten minutes. Add an equal volume of distilled water and a few drops of molar (6.9%) sodium nitrite solution. A red color develops, reaching a maximum in three minutes. Mount in glycerol and seal with rosin lanolin (p. 99).

Tryptophane

The Romieu reaction for proteins (according to Glick, Lison, and Cowdry) indicates the presence of tryptophane. Fix in alcohol, Bouin, or formalin. Prepare rather thick paraffin or nitrocellulose sections. Deparaffinize and hydrate as usual. Blot. Cover with a few drops syrupy phosphoric acid and heat for a few minutes at 56° C. (use a paraffin oven). Remove, apply cover glass, and examine at once. A red to violet color indicates the presence of tryptophane.

The Voisenet-Fürth reaction is recommended by Serra (Stain Technol. 21:5, 1946).

1. Fix in some formaldehyde-containing fixative, or mordant hydrated paraffin sections in 10% formalin for 1-5 hours.
2. Immerse in 12% sodium silicate solution (sp. gr. 1.10) for 3-5 seconds. If material is well hardened this step may be omitted. Drain on filter paper and
3. Transfer directly to fresh Voisenet reagent for 10 to 15 minutes. Voisenet reagent: to 10 cc. concentrated hydrochloric acid add one drop 2% aqueous formol and one drop 0.5% sodium nitrite (fresh aqueous solution). Mix well. Prepare fresh daily.
4. Mount directly in glycerol and examine at once (the color fades).

Results. Violet color indicates presence of tryptophane. The reaction is given by indolic compounds, and in proteins is specific for tryptophane.
Diazo Reactions

Danielli (Symp. Soc. Exp. Biol., 1:101–113, 1947) details a diazonium hydroxide test for histidine, tryptophane, tyrosine, and the purines and pyrimidines of the nucleic acids. Tetrazotized benzidine seems preferable to the commonly used sulfanilic acid, because of greater stability, deeper primary color, and the possibility of intensification by subsequent coupling of the second $N_2O$H groups with $\beta$-naphthol to yield a deep red, or with H acid (the monosodium salt of 1-amino, 8-naphthol, 3,6-disulfonic acid) to give a deep purple. The latter compound is relatively insoluble in alcohol or xylene, and sections treated by this variant stand the usual mounting in resinous media. The $\beta$-naphtholazobenzidine azo compounds are very soluble in xylene, and there is the hazard that tissue compounds of this nature would also be lost. I suggest trial of gum-arabic or gelatin media for mounting, as a control.

Tetrazotize 1.84 Gm. benzidine by suspending in 100 Gm. ice-cold distilled water $+\ 40$ Gm. ice and 4 cc. 33% hydrochloric acid, sp. gr. 1.17, (= 3.5 cc. 37.5%, sp. gr. 1.19) and adding 1.38 Gm. sodium nitrite dissolved in 2 cc. distilled water. Store in a refrigerator. For use, dilute 1 cc. stock tetrazotized benzidine with 30 cc. veronal buffer of pH 9.0 (p. 454) and use at once. This is about 0.05% of the tetrazonium compound.

Technic. The tissue fixation is not specified by Danielli. Paraffin sections are deparaffinized and hydrated as usual.

1. Stain 15 minutes at 0.4° C. in 0.05% tetrazotized benzidine in pH 9 veronal buffer, immediately after dilution of the acid benzidine with the alkaline buffer.
2. Wash 1–2 minutes in four changes of pH 9 veronal buffer, also at 0–4° C.
3. Destroy triazenes by washing in 0.01 N hydrochloric acid at 0–4° C.
4. Rinse in pH 9 veronal buffer to remove acid.
5. Immerse for 15 minutes at 0–4° C. in 0.05% H acid or $\beta$-naphthol, made up in pH 9 veronal buffer.
6. Wash thoroughly in distilled water.
7. Dehydrate in alcohols, clear in xylene, mount in balsam (or synthetic resin). If the $\beta$-naphtholazobenzidine color is visibly lost in this step, try blotting and mounting in Apáthy's gum-arabic syrup or a glycerol gelatin instead.

The H acid variant gives a deep purple, the $\beta$-naphthol variant, a deep red color to purines, pyrimidines, tyrosine, tryptophane, and histidine.

Pretreatment for 20 hours in a 10% solution of benzoyl chloride in anhydrous pyridine (deparaffinize sections, bring into alcohol, and wash off alcohol with few drops of pyridine) removes histidine, tryptophane, and tyrosine, with little evident effect on the nucleic acids. According to Mitchell (Brit.
J. Exp. Path. 23:296, 1942) benzyolation breaks the imidazole ring of histidine and histamine, while esterification of the phenol residue in tyrosine renders it incapable of coupling. Danielli states that treatment with ice-cold performic acid adjusted to pH 4.5–5.0 destroys tryptophane without affecting the nucleic acids, tyrosine, or histidine. Treatment for one hour with an ice-cold solution of iodine in methanol or in 5% aqueous potassium iodide inhibits the reaction of tyrosine with tetrazotized benzidine. Treatment with an ice-cold solution of dinitrofluorobenzene or with a hot solution of dinitrochlorobenzene, both in 90% alcohol saturated with sodium bicarbonate, also eliminates the reactivity of tyrosine to tetrazotized benzidine. When these compounds (C₆H₅Cl(NO₂)₂, C₆H₅F(NO₂)₂) are used, SH and NH₂ groups develop yellow colors. Pretreatment with hydrogen peroxide eliminates the SH reaction, and the NH₂ reaction can be destroyed by pretreatment with nitrous acid.

**Enzymatic Digestion Tests**

Before proceeding to individual tests it seems pertinent to offer the following commentary on this method of identification of tissue components.

1. Since even purified enzymes or crystalline enzymes are seldom free of all other enzymatic activities, digestion of a given tissue component by a given enzyme preparation does not necessarily identify that tissue component as consisting of the specific substrate of the enzyme employed.

2. When a given enzyme preparation is known to digest a given substrate under specified conditions of fixation and preparation, failure of that enzyme preparation to digest a tissue component under the same specified conditions may be taken to definitely exclude identification of that tissue component with the stated given substrate.

3. For even presumptive identification of a tissue component by reason of its digestion by a given enzyme, it must be shown that the tissue component in question is not destroyed by the enzyme solvent alone, or by the enzyme after inactivation by physical or chemical means, or in the presence of known specific inhibitors of the enzyme action in question.

**Tryptic Digestion**

I have used effectively a “1–300” trypsin of the Nutritional Biochemical Company at 0.1% concentration in 0.01 M phosphate buffer of pH 7.6 (p. 451), with or without 0.4% NaCl and 0.1% NaF. This trypsin had also a quite strong glycogenolytic activity. On digestion at 37° C., nuclear and cytoplasmic staining are destroyed in as little as one hour in material fixed in 80% alcohol, but persist much longer in material fixed in aqueous formalin for long periods or in chromate fixatives. Collagen and reticulum are quite resistant; basement membranes somewhat less so (Lab. Invest. 1:30, 1952). Sarcolemma swells and stains poorly, displaying prominently the fine connective-tissue fibrils traversing its outer surface.
Controls of the same buffer solvent without the enzyme should be employed. After alcohol fixation, distilled water removes ribonucleic acid at 60° C.

### Peptic Digestion

I have used Difco pepsin at 1:1000 concentration in 0.1 N hydrochloric acid, digesting for varying periods at 37° C. For controls use 0.1 N HCl without enzyme.

Cytoplasmic staining is abolished in 2–4 hours’ digestion after cold alcohol fixation, persisting longer in material fixed in aqueous formalin. Periodic acid Schiff-positive basement membranes are digested much more rapidly than the collagen or reticulum which is stained blue by the allochrome method (p. 357). Nuclei become Schiff positive in 4–6 hours and then swell and vacuolate and lose their sharp definition in 18 hours. Ocular-lens capsule and Descemet’s membrane are quite resistant to peptic digestion.

Chromation of tissue almost completely inhibits peptic digestion.

### Technics for Tryptic and Peptic Digestion.

1. Fix in 80% alcohol, Carnoy or the like. Alcoholic formalin for 18 hours (not more) may be used. Avoid use of prolonged fixation in aqueous formalin and especially in dichromate mixtures. Imbed and section in paraffin, not celloidin.

2. Sections may be digested without deparaffinizing according to the suggestion of Goetsch, Reynolds, and Bunting (Proc. Soc. Exp. Biol. Med. 80:71, 1952) regarding amylase digestion. I have not tested this. Generally, deparaffinize and hydrate as usual.

3. Immerse control sections in the same solvent as is used for the enzyme: a 0.01 M phosphate buffer of pH 7.6 containing 0.1% sodium fluoride and 0.4% sodium chloride for trypsin, and 0.1 N hydrochloric acid for pepsin. Leave controls in until the last test sections are taken out. Use 1:1000 solutions of trypsin and pepsin in the buffers described above. Digest for 30 minutes, 1, 2, 4, 6 or 7, and 16–18 hours, removing test slides at the various times.

4. Wash in water.

5. Counterstain to demonstrate the particular tissue elements under study.

6. Wash and mount in glycerol gelatin; or dehydrate, clear, and mount in polystyrene.

Compare digested preparations with each other, with undigested controls, and with solvent controls.

### Elastase

Recrystallized trypsin (Armour & Co.) has no effect on elastin, according to Lansing et al. (Anat. Rec. 114:555, 1952); but commercial trypsin does
digest elastin on overnight exposure at 37° C. This difference is due to
the presence of an elastase in the cruder product, which is extractable by
pH 6, 0.1 N phosphate buffer and is salted out by 0.4 saturated ammonium
sulfate. Lansing's group used digestion at pH 9 for one hour at room tem­
perature, or for less time at 37° C.

Baló and Banga (Biochem. J. 46:384, 1950) prepared an elastase concen­
trate from defatted and powdered pancreas: Suspend 1 Gm. pancreas pow­
der in 20 cc. 0.1 N phosphate buffer (pH 6.0) and shake for 30 minutes.
Centrifuge, decant, and save the supernatant. Resuspend the sediment in a
second portion of pH 6 0.1 N phosphate buffer, and again shake for 30 min­
utes. Centrifuge, decant, and combine supernatant with the previous portion.
To four volumes of the supernatant add one volume saturated ammonium
sulfate solution, and let stand at 2° C. for 12 hours. Centrifuge and discard
the precipitate. To three volumes of the supernatant add one volume satu­
rated ammonium sulfate, raising the (NH₄)₂SO₄ saturation level to 0.4.
Centrifuge, take up the precipitate in 15 cc. distilled water. Dialyze until
free of sulfate. Centrifuge. To the solution add NaCl to 1% concentration
(140 mg. in 14 cc. for example). Store at 0° C.

Baló and Banga recommended borate buffers for pH levels of 7-9.5, and
acetate + sodium carbonate buffers for pH levels of 9-11; and stated that
activity was maximal at pH 10.3.

Establishment of precise concentrations, pH levels, and salt content of
elastase solutions for optimal and selective action must wait on isolation of
the enzyme in at least relatively pure form. The activity of each lot prepared
should be determined on standard test sections. Use varying dilutions of the
stock solution for a standard interval at a set temperature.

Urea

Leschke's method: Fixation of tissue in a mixture of equal volumes of
saturated mercuric-nitrate solution and 2% nitric acid, followed by treat­
ment of sections with ammonium sulfide to convert the mercury urea com­
pound to black mercuric sulfide. This method is regarded by both Lison and
Glick as highly unspecific.

The xanthydrol reaction is quite specific; but the reaction is slow, urea is
highly diffusible, and the localization is therefore poor. Gomori recommends
Oliver's (J. Exper. Med. 33:177, 1921) method: Fix small pieces of tissue in
a filtered solution of 6 Gm. xanthydrol in 35 cc. alcohol and 65 cc. glacial
acetic acid for 6-12 hours. Alcohol dehydration, paraffin imbedding, and
sectioning follow. The xanthydrol urea crystals are demonstrated under
polarized light.

I have used an essentially identical fixing fluid by first freezing tissue in
solid carbon dioxide and 100% alcohol mixture and then fixing for 14 days
at -25° C. At this temperature the alcohol acetic mixture remains liquid,
and the tissue melts only as the fixative penetrates. The method needs further 
study to put it on a good routine basis.

**Uric Acid and Urates**

Sodium urate is the commonest of the urates occurring in gouty tophi. Its 
crystals are slightly soluble in cold water, and insoluble in alcohol and ether. Mallory directs fixation in 95% or absolute alcohol. Schmorl recommends Oestreicher's (*Virch. Arch. f. Path. Anat.* 257:614, 1925) preliminary treatment of six hours in 6% xanthydrol (9-hydroxy-xanthene) in glacial acetic acid, followed by 48 hours' fixation in 100% alcohol. Mallory preferred celloidin, Schmorl paraffin imbedding. Stain sections briefly—Schmorl says two minutes—in hemalum or other neutral alum hematoxylin. Wash in water, dehydrate and clear by an alcohol xylene sequence, and mount in balsam or clarite. According to Mallory's technic the crystals are colored deep blue; by Oestreicher's xanthydrol method, bright yellowish green.

Schultz (*Virchows Arch. f. Path. Anat.* 280:519, 1931) reported a rather complicated procedure which Mallory recommended. I have not tried it. Fix tissues in 100% alcohol. Pass through three changes of acetone of about 1½ hours each. Then place in equal volumes of acetone and benzene for 30 minutes, then two changes of benzene, 30 minutes each. Imbed in paraffin, and section.

1. Deparaffinize and bring to 100% alcohol.
2. Stain five minutes in carmine, keeping slide in motion. The carmine solution is essentially similar to Best's, and is prepared thus: Boil 1 Gm. carmine, 2 Gm. ammonium chloride, 0.5 Gm. lithium carbonate in 50 cc. distilled water. Cool and add 20 cc. 28% ammonia water. Mix 6 cc. of the filtered stock solution with 3 cc. 28% ammonia water and 5 cc. methyl alcohol for staining.
3. Wash in several changes of 100% alcohol.
4. Stain 30 seconds, keeping slide in motion, in a half-saturated (about 0.75 to 0.94%) solution of methylene blue in 100% alcohol.
5. Rinse in 100% alcohol.
6. Stain 15 seconds, keeping slide in motion in sodium sulfate-picric acid mixture: saturated aqueous picric-acid solution 9 cc., saturated aqueous sodium sulfate solution (nearly 50 Gm. of the anhydrous salt per 100 cc.) 1 cc. Filter.
7. Wash in several changes of 100% alcohol.
8. Clear in xylene and mount in balsam.

**Results.** Nuclei gray blue, cytoplasm yellowish, uric acid crystals deep blue green, sodium urate brilliant green.

Gerard and Cordier (*Arch. de Biol.* 43:367, 1932) used Carnoy fixation
and observation under polarized light for the demonstration of experimental uric-acid deposits.

Uric acid exists in tautomeric trihydroxyl (acid) and (predominant) tricarbonyl (ketonic) forms, and is readily oxidizable. It has been found to reduce silver nitrate, on which fact De Galantha's technic rests.


1. Fix immediately in two changes, 4–24 hours each, of 100% alcohol.
2. Clear with xylene and infiltrate with hard (56°) paraffin. Section at 8–10 μ.
3. Dry sections on slides for two hours at 37° C. I suggest flotation on alcohol rather than water.
4. Deparaffinize with xylene and wash in 100% alcohol.
5. Transfer directly to 20% silver nitrate solution and expose to sunlight or ultraviolet light for 1–4 hours. Urate crystals become bright rose colored.
6. Mix 10 cc. hot 3% gelatin solution, 3 cc. 20% silver nitrate and 2 cc. 2% hydroquinone and pour at once over slides. Leave until urates are black and connective tissues yellow.
7. Wash quickly in water preheated to 58–60° C.
8. Dehydrate with three or four changes of 100% alcohol, clear with carbol xylene, wash in two changes of xylene, and mount in Canada balsam.

Gomori recommends his methenamine silver solution (p. 278) for silvering of urates. Adjust to about pH 9.0, preheat the solution to 37° C., and incubate sections about 30 minutes or until urates are blackened. Rinse in distilled water. Tone if desired in 0.1% gold chloride for ten minutes. Rinse in distilled water. Remove excess silver in 5% aqueous sodium thiosulfate for two minutes. Wash in running water for five minutes. Counterstain as desired; e.g., 0.1% safranin O in 0.1% acetic acid for five minutes. Dehydrate with alcohol or acetone, clear with xylene, and mount in polystyrene or other synthetic resin.

I have amplified Gomori’s directions from the other methenamine silver technics.

**Aldehyde and Carbonyl Reactions**

*Schiff's Sulfurous Acid Leucofuchsin Reagent*

This reagent for carbonyl groups, especially aldehydes, has shown itself extremely well adapted to the demonstration of these groups in tissue sections. It is used in Feulgen’s nucleal reaction for the aldehyde group of desoxyribose when freed from purine and pyrimidine bases (nucleotide-base + sugar + acid) by acid hydrolysis (p. 132), for the aldehyde unmasked in acetal phosphatides by the action of mercuric chloride (the plasmal reaction, p. 316), for aldehydes arising from the peroxidation of unsaturated fats (p. 313), for the demonstration of aldehydes formed by Criegee cleavage reac-
ctions of 1,2-glycols and 1,2-amino-alcohols by periodic and chromic acids, potassium permanganate (p. 121), lead tetra-acetate and sodium bismuthate (p. 283), for the demonstration of aldehydes resulting from the peracetic and performic acid oxidation of carbon-carbon double bonds (p. 310), in the Oster amine oxidase method (p. 232), the Liang nerve-ending technic (p. 419), and other methods (p. 146).

Fuchsin in 0.5–1% solution may be reduced with 6–8% SO₂ water (sulfurous acid), with 0.5–2% sodium bisulfite, metabisulfite or the corresponding potassium salts (NaHSO₃, Na₂S₂O₅, KHSO₃, K₂S₂O₅), with 2.25% sodium thiosulfate (Na₂S₂O₅·5H₂O), with 0.5% sodium hydrosulfite (Na₂S₂O₄) (Alexander, McCarty, and Alexander-Jackson, Science 111:13, 1950) or with 1 cc. thionyl chloride (SOCl₂) per Gm. fuchsin (Barger and DeLamater, Science 103:121, 1948). The last three reducing agents have been little used in histology.

The directions of Feulgen called for dissolving the fuchsin in boiling hot water, filtering while cooling, adding the sulfite, and storing in the dark for a day or two before use. This practice has been followed by nearly all writers up to 1950, when we evolved a satisfactory, abbreviated cold procedure, which will be given here.

Coleman (Stain Technol. 13:123, 1938) introduced the procedure of completing the bleaching of the reagent with activated charcoal, which removed a variable amount of residual coloring matter not taken out by the sulfite. Barger and DeLamater state that the charcoal treatment may either precede or follow the sulfite bleaching. Amounts of fuchsin vary: the 1 Gm. in 220 cc. cited by Lee (9th ed. 1928), Coleman (loc. cit.) and others; the 0.25% of Barger and DeLamater (loc. cit.); the 1 Gm. in 120 cc. of Bauer (Ztschr. mikr. anat. Forsch 33:143, 1933), C. Bensley (Stain Technol. 14:47, 1939), Cowdry, and others; the 1 Gm. per 100 cc. of our method presented here; the 2 Gm. per 100 cc. which we have occasionally used.

Preparation of Schiff Reagent: Traditional Method.

1. Bring 100 cc. distilled water to boiling, remove from the flame and at once dissolve 1 Gm. basic fuchsin (pararosanilin, rosinilin or new fuchsin). When the solution cools to 60° C.:
2. Filter and add to the filtrate 2 Gm. sodium or potassium bisulfite or metabisulfite (NaHSO₃, KHSO₃, Na₂S₂O₅, K₂S₂O₅) and 20 cc. normal hydrochloric acid (p. 455).
3. Stopper and store solution in the dark at room temperature for 18 to 24 hours.
4. Add 300 mg. activated charcoal, shake vigorously for one minute and filter.
5. Store at 0–5° C. The solution should be a clear light yellow. Discard it when a pink color develops. We have kept this solution for several weeks.
If thionyl chloride is used in place of sulfite and acid, use 1 cc. per Gm. fuchsin. The pH of the final reagent is about 1.25 with this variant.

**Preparation of Reagent by “Cold Schiff” Procedure.**

1. Weigh out 1 Gm. fuchsin and 1.9 Gm. sodium metabisulfite (Na$_2$S$_2$O$_5$). Dissolve in 100 cc. 0.15 N hydrochloric acid.
2. Shake the solution at intervals or on a mechanical shaker for two hours. The solution is now clear and yellow to light brown in color.
3. Add 500 mg. fresh activated charcoal, shake one to two minutes.
4. Filter into graduated cylinder, washing the residue with a little distilled water to restore the original 100 cc. volume.

The solution should be water white. If it possesses a yellow color a fresh lot of activated charcoal should be obtained and the charcoal decolorization repeated. Store at 0-5°C. Such solutions have been so stored for two months, still remaining colorless. Stronger solutions may form a white precipitate on refrigeration. This precipitate is perhaps less apt to form at lower pH levels. It does not dissolve on warming gently. The pH of this fluid is about 2.2. For dilute Schiff reagent, add 1.9% (0.1 M) sodium metabisulfite in 0.15 N hydrochloric acid to desired dilution (p. 374).

The hydrosulfite solution of Alexander et al. (loc. cit.) is prepared by dissolving 1 Gm. fuchsin in 200 cc. boiling hot water, filtering while hot and adding at once 1 Gm. sodium hydrosulfite (Na$_2$S$_2$O$_4$) and 500 mg. activated charcoal. Shake thoroughly and filter. The finished product is of light amber color, and of unspecified pH.

**Schiff’s Base Reactions**

Aryl amines condense with aldehydes to form fairly stable, often colored, and often insoluble condensation products:

\[
\text{ArNH}_2 + \text{O=CHR} \rightarrow \text{ArN}=\text{CHR} + \text{H}_2\text{O}
\]

This reaction is probably responsible for the success of Arzac’s periodic acid, basic fuchsin method for the demonstration of glycogen, etc.

These bases are readily formed with the Feulgen HCl and the periodic acid engendered aldehydes by a variety of basic and acid dyes which present unsubstituted amino groups. Basic fuchsin and Bismarck brown react; methylene blue, crystal violet, methyl violet, iodine green, ethyl green, and Victoria blue 4R do not; and thionin, safranin O, and safranin 6B give weak reactions. The last three possess two, two, and one open amino group, fuchsin has three, Bismarck brown four; and in the others the amino groups are all completely or partly alkylated or arylated. For basic fuchsin, which appears to be the most successful, the following technic can be used:

**Schiff Base Reaction with Basic Fuchsin.** Hydrate paraffin sections as
usual and carry through the desired hydrolytic or oxidative procedure for the production of aldehyde. Wash thoroughly in water or in alcohol. Then place in 0.2% fuchsin in 0.2 N HCl in water or in alcohol for 2 hours. Decolorize with 3 successive 10-minute baths of 0.2 N HCl in alcohol (or water). Wash in alcohol, counterstain if required, dehydrate, clear, and mount in synthetic resin.

These Schiff bases resist extraction by 0.2 N HCl in water or alcohol for at least half an hour, but an exposure of one minute to 0.1 N acid suffices to decolorize acid-base type stains with alkylated amino dyes. Like Schiff's sulfite leucofuchsin reagent, this Schiff base reaction is also prevented by interposition of a phenylhydrazine blockade.

Pearse complains that the Schiff bases formed by the reaction of tissue aldehydes with rosanilin or pararosanilin will not resist alcohol dehydration. It would appear that Pearse must have used too brief a reaction time for the formation of Schiff bases. I have found staining periods of less than 45 minutes rather irregular in their results, and prefer to extend the time to two hours.

In any case this reaction appears to be less effective in the demonstration of periodic acid engendered aldehydes than is the Schiff reagent, and its greatest value would appear to lie in the fact that alcoholic reagents can be used throughout, as in Mowry's alcoholic periodic acid method (p. 281). Mowry has used the alcoholic fuchsin successfully in this method in place of Schiff reagent. It must be noted that cell nuclei often stain as well. This may, of course, be a Feulgen type of reaction.

Nuclear staining is avoided by the use of acid dyes with open amino groups. I have used anilin blue with some success, but the best dye which I have tried is acid fuchsin.

Schiff Base Reaction with Acid Fuchsin. After the usual periodic acid oxidation (5-10 minutes in 0.5-1% wash well in water and immerse in 1% acid fuchsin in 0.01 N HCl for 18 to 24 hours. (Shorter times may well suffice, but have not been tested in detail.) Wash 5 minutes in running water, decolorize 10 minutes (or more) in 1% borax (Na₂B₄O₇·10H₂O), which leaves control, unoxidized sections pale pink or colorless, except for rodent elastic fibers. Rinse in water, counterstain as desired, e.g., 10 seconds in 0.1% fast green FCF in 95% alcohol, then 100% alcohol, alcohol + xylene, xylene, synthetic resin.

A number of acid dyes with substituted amino groups failed to give this reaction: fast green FCF, wool green S, patent blue V, cyanol. The azo dye Biebrich scarlet also was unsuccessful.

Hydrazine Reactions

Bennett's plasmalogen reaction with dinitrophenylhydrazine is described on p. 316, and Seligman's method with naphthoic acid hydrazide followed by azo coupling on p. 315.
Silver Reactions

Though aldehydes quite readily reduce silver salts in weakly to moderately acid solutions, I know of very few instances in which it can be shown conclusively that tissue aldehydes are capable of reducing alkaline diammine or methenamine silver solutions. Most, but not all, of the aldehyde sites after periodic acid oxidation reduce methenamine silver about as well when the periodic acid step is omitted.

Noteworthy exceptions to the above, in which it clearly appears that aldehydes engendered by periodic acid oxidation are actually reducing methenamine silver, are the lens capsule and Descemet's membrane in the eye (but not glycogen, reticulum, or cornea), and the surface epithelial mucin of the stomach (but not that of its glands or of the intestine, nor the mucosal or muscular reticulum, nor the cuticular border of the villi). (J. Histochem. & Cytochem. 2:130, 1954.)

Blockade Methods

Blockade methods are chemical procedures which of themselves fail to give recognizable color reactions in tissue elements, but so alter the latter that they fail to give other color reactions. In this way some quite specific chemical procedures can be made to alter a general color reaction or stain so as to make the failure to react specific evidence of the presence of specific chemical groupings.

Thus deamination (p. 162) prevents certain tissue elements from staining with acid anilin dyes, and thereby gives evidence that the usual staining of those elements was due to the presence of amino groups.

Blockade of Aldehyde Groups

The use of sulfite, semicarbazide, and phenylhydrazine to combine with aldehydes and ketones, and thereby prevent color reactions with Schiff reagent or Seligman's carbonyl reagent, aids in the identification of aldehydes and ketones. The reaction of sulfite or sulfurous acid with most aldehydes is quite easily reversed by application of mild oxidants such as 3% hydrogen peroxide; 1% iodine or its equivalent as potassium hypoiodite; 1% sodium iodate or its equivalent in iodic acid; 1% potassium chlorate or its equivalent in chloric acid; or 1% ferric chloride. Intervals of 2–10 minutes in these reagents are sufficient to nullify a 2–4 hour blockade in 0.1 M NaHSO₃, and render the Schiff reaction of HI0₄⁻-generated aldehydes again fully positive. None of these oxidants by itself will produce aldehyde either from 1,2-glycols or from ethylenes.

Though a prolonged treatment with bisulfite after the Feulgen hydrochloric acid hydrolysis, the periodic acid glycol oxidation, or the performic or peracetic ethylene oxidation will completely prevent the positive reactions which normally occur in 5–10 minutes in Schiff reagent, a prolonged ex-
posure (2–3 hours) to the Schiff reagent completely overcomes the sulfite blockade; and during this prolonged bath the more strongly reacting tissue elements recover their positive reactions first.

Similarly a 30-minute exposure to 5% phenylhydrazine hydrochloride after periodic or performic acid oxidation completely prevents reaction to a ten-minute bath in Schiff reagent, but is overcome by a three-hour exposure. However, treatments with phenylhydrazine prolonged for several hours or overnight render some periodic-acid generated aldehydes permanently Schiff negative; others are more loosely combined and again become Schiff positive on three-hour exposure to Schiff reagent, or by interposition of a weak oxidizing agent. But cellulose, mucus, and cartilage remain reactive to a ten-minute Schiff bath after one hour in phenylhydrazine.

For aniline chloride in molar solution dissolve 9.3 Gm. (= 9.1 cc.) aniline in 8.1 cc. concentrated HCl (0.1 mole of each), shaking during addition; dilute to 100 cc. with distilled water. This solution effectively renders Schiff negative (with a ten-minute Schiff bath) the aldehydes formed in the Feulgen, peracetic-acid, and periodic-acid reactions. Even a 15-minute aniline-chloride bath is adequate to block the Feulgen reaction. A six-hour exposure to molar aniline chloride or 18 hours in 0.2 M prevents the aldehyde reaction of retinal-rod acromeres after peracetic acid oxidation. The periodic acid Schiff reaction of glycogen, retinal acromeres, and corneal and scleral collagen is inhibited by 2 hours in 1 M or 18 hours in 0.1 M aniline chloride. That of epithelial mucin requires 4 hours in 1 M or 18 hours in 0.4 M aniline chloride. That of the lens capsule and Descemet’s membrane is not blocked by 18 hours in molar aniline chloride; it requires a 2.5 M concentration for the same period.

Dimedone I have not used as a blocking agent for carbonyls. It is said to be specific for aldehydes, and some workers recommend it highly. Pearse writes that it is found to block extremely slowly in practice: “It is often difficult to distinguish the reduction in recolorization of leucofuchsin caused by dimedone from that observed in control sections treated with the solvent (acetic-alcohol) only.”

Nevertheless, when it does block, the reaction is to be regarded as specific for aldehyde. Failure to block does not exclude aldehyde. The same is true of phenylhydrazine, aniline chloride, and hydroxylamine.

Pearse uses a saturated solution of dimedone (5,5-dimethylcyclohexane-1,3-dione) in 5% acetic acid (in alcohol) for 1–16 hours at 60° C., or for 2–3 days at 22° C.

Pearse prefers hydroxylamine in aqueous sodium-acetate solution (10 Gm. hydroxylamine hydrochloride, 20 Gm. sodium acetate crystals, 40 cc. distilled water) and treats sections 1–3 hours at 22° C. He states that with this solution “condensation with tissue aldehydes of all varieties is rapid and apparently complete.”

I have noted some curious failures to block the Schiff reaction after peri-
odic acid with hydroxylamine, either as a 20% solution in 30% sodium acetate or as a 10% solution in pH 4.5 0.2 M acetate buffer. (The pH optimum for the hydroxylamine aldehyde condensation is said to be 4.7.) The Schiff reaction of gastric surface-epithelial mucin and gland mucin, of ocular-lens capsule and rod acromeres, of cellulose and lignin, of cartilage and nucleus pulposus, and sometimes of muscle glycogen remained positive with 2 and 24 hours of hydroxylamine blocking after periodic acid in one series of trials in which molar aniline chloride had been completely successful in 24 hours, and largely so in two hours. Only gastric gland mucin gave a slight Schiff coloration. Usually exposure to 5% phenylhydrazine hydrochloride for 24 hours completely prevents the Schiff reaction when interposed after periodic, peracetic, or hydrochloric acid; and two hours is often adequate; but on some objects this agent may also fail to block.

**Blockade of Hydroxyl and Amine Groups**

Benzoylation and acetylation are used to render NH₂ and OH groups non-reactive by esterifying them. In general esterification of amines is more quickly accomplished and at lower temperatures. Starch, glycogen, and cellulose are the most difficult objects to render nonreactive to the periodic acid Schiff reaction. With the three hexose polysaccharides, individual particles may remain strongly reactive after fairly prolonged treatment while most of the same material demonstrable in controls has disappeared. Cartilage matrix and thyroid colloid are also strongly resistant to acetylation and benzoylation. In cartilage the pericellular areas are more resistant than the intercellular. Thyroid colloid, in benzoylation, presents strongly reactive areas adjacent to negative areas.

Cartilage matrix is almost completely acetylated after 18–24 hours at 25° C. in 40% acetic anhydride pyridine mixture; but six hours at 58° C. in 2.5% benzoyl chloride pyridine was not quite adequate. While most of the glycogen is acetylated in similar periods, a few granules may remain. Benzoylation for six hours at 58° C. completely abolished the reactivity of glycogen. Two hours was nearly sufficient, but 24 hours at 25° C. was not quite adequate. Epithelial mucins are moderately resistant to acetylation, six hours at 25° C. being adequate for most of them, but are quite readily benzoylated, practically completely so in two hours at 25° C. in 10% benzoyl chloride pyridine. Connective tissues, mast-cell granules, cuticular borders, retinal-rod acromere lipoid, ocular membranes, amyloid, hyaline droplets, plasma, fibrin, and the like are acetylated and benzoylated quite promptly. Benzoylation for 20 hours in 10% benzoyl chloride pyridine mixture is used by Danielli to prevent the reaction of histidine, tryptophane, and tyrosine with diazonium salts (p. 149).

Stronger mixtures of benzoyl chloride with pyridine give rise to considerable amounts of crystalline precipitate, which redissolves when sections are brought into alcohol. These precipitates are suspected of causing localized areas of reaction failure. Weaker mixtures—2.5% or 5% benzoyl chloride
AMINO ACIDS, END GROUPS, ETC. 161

by volume in anhydrous pyridine—seem about as effective in blocking the periodic acid Schiff reaction as stronger, crystal-producing mixtures.

Benzoylation at 58° C. for six hours appears to destroy the capacity of nuclei to stain with iron hematoxylin. Hence, either a lower temperature (37° or 25° C.) for a longer period should be employed, or exposure to 58° C. should be limited to two or three hours. Similarly, acetylation in 40% acetic anhydride in pyridine for six hours at 58° C. is about as effective as a 24-hour treatment at 25° C. Neither can be relied upon to acetylate consistently all the glycogen, starch, and cellulose; but cartilage matrix appears to acetylate almost completely in six hours at 58° C. or in 18–24 hours at 25° C. Monné and Slautterback found hot acetylation more effective in blocking all amine groups than cold (Arkiv Zool. 1:455, 1950).

Hydrolysis of the acetyl esters thus formed has been employed to restore the periodic acid Schiff reaction by McManus and Cason (J. Exp. Med. 91: 651, 1950). I have found their 45-minute treatment with N/10 potassium hydroxide to be generally too drastic in removing sections, and prefer to use ammonia alcohol mixtures. In general, 70% alcohol, 20% ammonia is more effective than 80% alcohol. When the alcohol percentage is lowered to 60%, deacetylation is prompter, but section losses appear. Use of 37° C. temperature also expedites the process, but use of 58° C. gives rise to other alterations and is consequently to be avoided.

Technics used in our laboratory are as follows: Bring sections to 100% alcohol as usual. Dip in pyridine and transfer to the reagent solution.

**Acetylation.** 16 cc. acetic anhydride + 24 cc. anhydrous pyridine. Incubate 1–24 hours at 25° C., in accordance with desired effect; or ½–6 hours at 58° C. The longer intervals are necessary to approach complete acetylation of glycogen, starch, cellulose, and cartilage.

**Benzoylation.** 2 cc. benzoyl chloride + 38 cc. anhydrous pyridine. Incubate 1–24 hours at 25° C. or ½–6 hours at 58° C. Glycogen, starch, cellulose, thyroid colloid, and cartilage require the most drastic treatments. After either treatment wash with two changes each of 100%, 95%, and 80% alcohol.

**Deacetylation Procedure.** Incubate 24 hours in 70 cc. alcohol, 10 cc. water, and 20 cc. 28% ammonia water at 37° C. in closed container. Wash in 80% alcohol and in water, and proceed to a definitive demonstration technic.

**Results.** A two-hour acetylation blocks the periodic acid Schiff reaction of collagen, basement membranes, reticulum, adrenal lipofuscin, colonic melanin, and renal brush borders; but not that of glycogen, starch, cellulose, cornea, vitreous, lens capsule, Descemet’s membrane, gastrointestinal mucins, cartilage, nor (usually) adrenal chromaffin. With six hours of acetylation, starch, cellulose, glycogen, gastric mucin, cartilage, and sometimes chromaffin remain reactive. With 16 hours, cartilage matrix remains faintly reactive; but this is lost at 18–20 hours.

Deacetylation for shorter periods—for example, 4–6 hours at 37° C.—reveals as Schiff positive or partly so such tissue elements as lens capsule and
Descemet's membrane, cornea, vitreous humor, glycogen, gastric and intestinal mucins, cartilage, and chromaffin. Longer treatment intensifies these reactions. Overnight treatments are required to deacetylate most collagen, vascular basement membranes, cuticular and brush borders of epithelial cells, and some pigments.

The acromere substance of the retinal rods is one of the easiest to acetylate (40 minutes in acetic anhydride pyridine mixture) and probably the most difficult to deacetylate, requiring the full 24 hours in the 60% alcohol, 20% ammonia, water mixture. Even 4–8 hours in 80% alcohol 20% ammonia may be inadequate to deacetylate this material.

Deamination

Monné and Slatterback (Arkiv Zool. 1:455, 1950) consider that the acidophil components of nuclei, chromidia, and myoglobin, which stain preferentially with the plasma-stain component of procedures of the Mallory phosphomolybdic acid anilin blue type (pp. 350–355) and resist procedures which extract the nucleic acids, are probably histones, basic proteins, or protamines. This oxyphilia disappears on application of deamination or milder acetylation procedures.

Application of Van Slyke's nitrous acid reagent (10 cc. concentrated (6 Gm. in 10 cc.) sodium nitrite, 5 cc. glacial acetic acid, 25 cc. distilled water 1–12 hours at 20–25° C.) completely inhibits azocarmine and anilin-blue staining in the Heidenhain method (p. 351); this indicates complete destruction of amino groups. Use of 5% chloramine-T for 12 hours at 20–25° C. has the same effect. Treatment with 0.4% ninhydrin for 12 hours at 80° C. inhibited the anilin blue staining of yolk, but not the azocarmine staining of chromidia. At 4° C. exposure to pure acetic anhydride for one to three days did not inhibit the azocarmine staining of chromidia, but did prevent anilin blue staining of yolk, while acetylation for three hours at 80° C. prevented both staining reactions. The Van Slyke reagent and chloramine-T treatments do not render the H2O4 Schiff reaction of yolk negative; but acetylation does, since it esterifies hydroxyls as well as amines.

Application of Van Slyke's reagent to blood films promptly destroys the eosinophilia of erythrocytes, so that these stain yellowish green with Giemsa's stain after only ten minutes at 25° C. in the nitrous acid reagent. Eosinophil granules still stain red to pink with Giemsa after an hour in Van Slyke's reagent, though the intensity of the eosinophilia decreases with longer exposure so that only a pale pink is seen at 2–6 hours.

Van Slyke's reagent does not impair the periodic acid Schiff reaction of leucocytes or platelets, nor does it destroy the sudanophilia of neutrophil leucocytes.

Acetic acid mixtures with sodium nitrite are more rapid in their action on sections and smears than normal hydrochloric acid nitrite mixtures in spite of the higher pH levels.
Table 17

<table>
<thead>
<tr>
<th>Ph Levels of Nitrite Solutions</th>
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<tr>
<td>Van Slyke's reagent</td>
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<tr>
<td>1 N NaNO₂ in 1 N HCl</td>
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<tr>
<td>1 N NaNO₂ in 1 N Acetic</td>
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<td>1 N NaNO₂ in 1 N Acetic</td>
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Methylation

Methylation has been used for the blocking of open carboxyl and other acid groups. Previously basophilic substances are thereby rendered unstainable with basic dyes. The process is usually accomplished by prolonged exposure to hot methyl alcohol in the presence of a weak acid.

Cytoplasmic basophilia of pancreas, gastric chief cells, gastric proventricular and esophageal epithelia, tracheal epithelium, plasma cells, and liver cells disappears after about 2–3 days in 0.1 N HCl in methanol at 25°C, while nuclear staining with buffered eosin resists up to about 5–7 days. The metachromatic basophilia of cartilage and of rat mast-cell granules disappears in two days in this strength of methanol hydrochloric acid, and is severely impaired even in 24 hours, or in two days with half the amount of acid (0.05 N).

The azure staining of some bacteria is about as resistant to methylation as that of cell nuclei, while yeast cells lose their basophilia in about three days.

Methylation at 37°C in 1% dilution of concentrated HCl in methanol destroys nuclear and cytoplasmic staining with azure A, renders mucin non-metachromatic (but does not impair its HIO₄ Schiff reaction), and destroys the reactivity of argentaffin cells in the methenamine silver procedure.

I have found that a three-day methylation period at 58°C in methanol containing 0.1 N HCl is adequate to destroy the periodic acid Schiff reaction of glycogen, epithelial gland mucins of stomach and trachea, thyroid colloid, collagen, and reticulum. The same objects gave the usual positive reactions after seven-day treatments at 37° or 25°C.

When methylation is done at elevated temperatures the use of screw-capped Coplin jars is recommended and has been used successfully in this laboratory.

Demethylation with restoration of basophilia should also be possible, but is generally only partially successful. This is accomplished by hydrolysis in quite dilute aqueous acid solution. The incompleteness of the success of restoration of basophilia is probably due to coincident acid hydrolysis and solution of the ribose phosphoric acid moieties of ribonucleic acid. Hence the acid used for hydrolysis of the methyl esters should probably be weaker than 0.1 N.

Fisher (personal communication, 1953) finds that a 20-minute bath in
0.5% potassium permanganate followed by brief rinsing in 2% oxalic acid largely restores nuclear and cytoplasmic basophilia, but not the metachromatic staining of mast cells, mucins, or cartilage matrix. He employed a 24-hour methylation at 60° C. in 0.1 N HCl methanol, which completely abolishes nuclear and cytoplasmic basophilia as well as metachromasia. With 2 to 6 hour methylation periods, which are adequate to destroy metachromasia but not nucleic acid basophilia, it was found that permanganate restored metachromatic staining of cartilage, mast cells, and mucins.

**Phenols**

Wildi (Science 113:188, 1951) records reduction (in vitro) of neutral silver nitrate by various ortho- and para-dihydroxyaryl compounds: pomiferin, isopomiferin, dihydroisopomiferin, 3',4',7,8-tetrahydroxyflavanone, eriodictol, catechol, hydroquinone, tetrabromocatechol, and pyrogallol. Phenol, meta-dihydroxyaryl compounds, and compounds which formed insoluble silver salts failed to react. Wildi's reactions were performed in about 91% alcohol, with about 0.5% AgNO₃. Most reactive substances reacted in 15 minutes at 20–25° C. Some required heating to 60° C. for one minute.

**Enterochromaffin or Argentaffin Cells**

These cells occur in varying numbers in the epithelia of surface and glands of the stomach and intestines, characteristically appearing adjacent to the basement membrane between the basal portions of the adjacent epithelial or gland cells. They are characterized by the presence of a chromaffin substance which yields a yellowish brown color on fixation in formalin bichromate mixtures of pH 2.5–5.5 and, after formalin fixation, reduces ammoniacal silver solutions. Lison considered it to be a catechol derivative; Gomori, a resorcinol derivative, Erspamer and, recently, also Pearse, a 5-hydroxytryptamine. Masson (Am. J. Path. 4:181, 1928) considers these the type cells of the so-called carcinoid tumors and calls them argentaffin-cell tumors.

Masson reported a block method for demonstration of these cells, which was cited in the first edition of this book. I have abandoned it completely in favor of section methods which allow the comparison of a variety of other procedures on adjacent sections. I cite first Masson's section method.

**Masson's Section Method.** Frozen, paraffin, and celloidin sections of formalin-fixed material can be used. It is advisable to collodionize paraffin sections as for other alkaline silver methods (p. 94). Bring sections to distilled water.

1. Treat with diammine silver hydroxide (p. 335) for 12 to 48 hours in the dark in a covered vessel. Overexposure decreases specificity. Imbedded sections require longer than frozen sections.
2. Wash in distilled water.
3. Tone in 2 cc. 1% gold chloride, 3 Gm. ammonium thiocyanate, 3 Gm. sodium thiosulfate, 98 cc. distilled water for five minutes. This is our modification of Ramón y Cajal's toner.

4. Wash in distilled water.

5. In any case fix one minute in 5% sodium thiosulfate.

6. Wash 2-3 minutes in running water.

7. Counterstain one minute in 0.1% safranin, 2 to 3 minutes in distilled water.

8. Rinse and differentiate briefly in 1% acetic acid.

9. Wash briefly in water, dehydrate, and clear and mount with the usual alcohol, xylene, clarite sequence.

This method is modified by us from Masson (loc. cit.). Lison used a simple 0.1% gold chloride toner for 4-6 minutes, while Jacobson omitted the gold.

Lipoid Content. Some argentaffin or "carcinoid" tumors are made up of coherent masses of polygonal cells heavily laden with fine fat droplets. In some such, the fats reduce osmium tetroxide and no reduction of diammine silver is observed. Some tumors contain variable amounts of anisotropic fats in the form of fine needle-shaped crystals. Most of the fat present stains well with oil-soluble dyes. I have also seen argentaffin-positive tumors which contained no fats. Masson (Am. J. Path. 4:181, 1928) records tumors with both argentaffin cells and fat. Appropriate methods for study of the lipoids are found in Chapter 15.

Gomori (Arch. Path. 45:48, 1948) identified the argentaffin substance as a resorcinol derivative. In the same paper he detailed a modification of his methenamine silver method (p. 278) for this substance, which required 12 to 48 hours. Burtner and I have devised a much more rapid variant of this procedure which seems to be even more selective in practice.


Solutions. Stock methenamine silver solution: Dissolve 3 Gm. methenamine (hexamethylene tetramine) in 100 ml. distilled water. Add 5 cc. 5% aqueous silver nitrate. This solution can be stored in a cool, dark place for months.

Working solution: To 30 cc. stock methenamine silver solution add 8 cc. Holmes' pH 7.8 borate buffer (p. 453).

Glassware. The Coplin jars should be chemically clean. Previous silver mirror deposits should be removed with concentrated nitric acid.

Technic.

1. Treat deparaffinized sections with Weigert's iodine solution (I:KI: H₂O = 1:2:100), ten minutes.

2. Bleach with 5% sodium thiosulfate (Na₂S₂O₅·5H₂O), two minutes.

3. Wash in running water, ten minutes.

4. Rinse in two changes of distilled water.
5. Place sections in Coplin jars containing the buffered methenamine silver solution at room temperature and put in a 60° C. (paraffin) oven for 3-3½ hours. (Preheating to 60° reduces impregnation time by ½-1 hours.)
6. Rinse in distilled water.
7. Tone in 0.1% gold chloride (HAuCl₄), ten minutes.
8. Rinse in distilled water.
9. Fix in 5% aqueous sodium thiosulfate, two minutes.
10. Wash in running water, five minutes.
11. Counterstain with 0.1% safranin O in 0.1% acetic acid, five minutes.
12. Dehydrate with acetone, clear in xylene, and mount as usual.

**Results.** At 3 to 3½ hours impregnation is optimal. Argentaffin cells are well blackened and appear in numbers as great as with longer impregnations. Coarse connective tissue of the submucosa sometimes shows a variable amount of blackening at this time. In 2 to 2½ hours' incubation, partial blackening of argentaffin cells in reduced numbers is seen. At 1½ hours, only cellulose is black. At longer intervals than 3½ hours, besides the connective tissue, the granules of eosinophil leucocytes, nuclei, smooth muscle, and surface epithelium become blackened. By 4½ hours a silver mirror begins to appear on the sides of the slides and Coplin jars. Mast-cell granules remain brilliantly red after nuclei and reticulin are blackened.

Gomori (loc. cit.) used Gram's iodine in Step 1 (personal communication). In Step 2 he used either a bisulfite or a thiosulfate solution of unspecified strength. His borate buffer was slightly different: To 100 cc. 0.2 M boric acid add 8 or 12 cc. 0.2 M borax for pH 7.8 or 8.2 respectively. (0.2 M borax tends to crystallize out at 20-25° C.) His methenamine silver working solution contained 25 cc. of the above stock solution, 25 cc. distilled water, and 5 cc. borate buffer. He silvered at 37-45° C. for 12 to 24 or even 48 hours.

If preparations appeared oversilvered at step 6, Gomori differentiated in 0.5% sulfuric acid containing 0.1 to 0.2% iron alum until the background was almost clear. He then washed in 0.5-1% hydrochloric acid in 70% alcohol, and resumed the above procedure with Step 9.

Gomori's regressive differentiation of oversilvered preparations with weak acidified iron alum solution did not give particularly satisfactory results with our variant. With the shorter incubation periods used in this new technic, it is more practical to repeat the procedure with a briefer silvering time on duplicate sections.

If a ten-minute bath in 1% ferric chloride or a one-hour bath in 5% chromic acid is introduced at step 3, the silvering of enterochromaffin cells is inhibited and the method is converted to a more or less selective demonstration technic for collagen and mucin. The time in the methenamine silver is reduced to 2 or 2½ hours for this purpose.
Neither treatment prevents the silvering of the enterosiderin pigment of guinea pigs. Ferric chloride prevents the silvering of adrenal lipofuscin, but chromic acid does not.

Effective blackening of collagen, much reticulum, and the mucins of the colonic, pyloric, and duodenal glands and of intestinal goblet cells was obtained by both methods. Ferric chloride afforded partial blackening of gastric-surface epithelial mucin; chromic acid, a more complete silvering. The reaction of cellulose and lignin is enhanced and accelerated by both variants.

The chromic acid variant is of course essentially the Gomori silver method for mucin and glycogen (p. 278).

Gomori further records (Arch. Path. 45:48, 1948) the use of the azo reaction as specific for phenols; the pale-green staining of argentaffin cells by very dilute ferric chloride solution; the production of dark yellowish shades by nitric acid, bromine water, and iodates; Clara's reaction with very dilute hematoxylin (1:10,000 for 24–48 hours), brazilin, galloycyanin, gallamin blue, celestin blue, and alizarin cyanin in the absence of mordants as giving sharp, selective staining of argentaffin granules; a brilliant ruby to purplish or bluish red coloration with the Mallory-Heidenhain stain (p. 351); the application of Gibbs's reaction with 2,6-dichloroquinone chlorimine to produce blue indophenol dyes in the presence of phenols as giving an intense reaction with resorcinol, a distinct reaction with argentaffin granules, and faint or negative reactions with o- and p-dihydroxybenzenes. Diazotized nitroanisidine stains resorcinol, phloroglucinol, and argentaffin granules a fiery orange; tetrazotized p,p'-diaminodiphenylamine gives a deep maroon red; catechol and hydroquinone give pale yellows with both reagents.

Diazotized safranin O couples in alkaline solution with phenol to form the azo dye Janus black, with β-naphthol to yield blue dyes (C. I. Nos. 134 and 135). Lillie, Burtner and Henson employed this reaction to demonstrate enterochromaffin cells in black on a red background (J. Histochem. & Cytochem. 1:154, 1953).

Lillie, Burtner and Henson's Diazo-Safranin Method. Dissolve 360 mg. safranin O in 6 cc. distilled water and 3 cc. normal hydrochloric acid. Cool to 4° C. and add 69 mg. sodium nitrite dissolved in 1 cc. distilled water (1 cc. M/1 NaNO₂). Keep at 4° C. for 10–15 minutes. The solution turns blue and foams moderately. Bring paraffin sections of formalin-fixed intestine or stomach to water as usual. Place slides in an empty, ice-cold Coplin jar. Dilute 1 cc. of the diazotized safranin with 40 cc. of cold (4° C.) 0.1 M disodium phosphate (1.42%) and pour over slides at once. Let stand five minutes, decant, and wash with three changes of 0.1 N hydrochloric acid in 15 minutes. Wash, dehydrate, clear, and mount in synthetic resin.

The acid safranin solution is reasonably stable and may be kept for some time. It is made up at M/9 concentration, so that on addition of 1/9 of its volume of normal sodium nitrite solution the concentration of both reagents
becomes 0.1 M and the hydrochloric acid is reduced to 0.3 N. Since the normal sodium nitrite is also fairly stable and the preparation of the active diazo mixture requires only 10 to 15 minutes, use of the reaction is relatively easy. The acid diazo mixture is not very stable and should be used within two hours of mixing for best results. It is probable that internal acid coupling occurs on longer standing, since only one of the two amino groups of the safranin can be diazotized in acid aqueous solution, and therefore the dye residue remains capable of acid (amine) coupling with diazonium groups. On overnight storage at 3° C. the acid diazo mixture changes from blue to dark red in color and gives only feeble coloration of argentaffin cells on alkalinization. Addition of 1 cc. of acid diazo mixture to 40 cc. 0.1 M disodium phosphate solution brings the reaction to pH 7.7, which is sufficiently alkaline for phenol coupling, and not alkaline enough to remove the sections from the slides.

In vitro, alkaline diazo coupling reactions occasion no noteworthy color changes with catechol, anisole, phloroglucinol, o-aminophenol, amidol, tryptamine, tyramine, uracil, guanine, phenylalanine, thyroxin, tryptophane, or cysteine. Dark brown precipitates are produced with phenol, o-cresol and p-cresol; a very dark red brown precipitate with resorcinol; red-purple to dark red-purple or purple precipitates with hydroquinone, pyrogallol, and tyrosine; and dark blue-violet precipitates with α- and β-naphthols.

Model experiments on formalin-fixed, phenol-impregnated, gelatin-paper strips gave coupling colors with diazo-safranin as follows: Pink to colorless with the blank gelatin control, with phenol, catechol, hydroquinone, pyrogallol, phloroglucinol, o-aminophenol, 2,4-diaminophenol, tyrosine, tyramine, 3,4-dihydroxyphenylalanine, adrenaline, noradrenaline, indole, tryptophane, tryptamine, cysteine, guanine, m- and p-aminophenol. Pink to purple colors were obtained with o- and p-cresols and 5-hydroxytryptamine; Gomori has reported blue with the last, but I have obtained definite blues only with resorcinol and with o- and β-naphthols.

I have employed also the stabilized diazonium salts Black BS Salt and Red 3 GS Salt for this purpose. They yield respectively slightly orange-brown and brown colors which appear to be quite selective for enterochromaffin cells. Laskey and I have worked out the following (hitherto unpublished) technic.

**Coupling Reaction for Phenols with Stabilized Diazonium Salts.**

1. Fix in formalin, dehydrate, and imbed and section in paraffin.
2. Deparaffinize and hydrate as usual.
3. Make fresh 0.2% solution of stabilized diazonium salt. Mix with an equal volume of 0.2% disodium phosphate (Na₂HPO₄) solution and at once pour onto slides in a Coplin jar. Let stand two minutes, decant and
4. Replace with 0.1% acetic acid solution.
5. Counterstain nuclei with safranin (0.1% in 1% acetic, one minute) or acetic hemalum (1–2 minutes) if required. In critical studies one set of slides should be examined without counterstaining.
6. Wash in water, dehydrate, clear, and mount as usual by alcohol, xylene, synthetic resin sequence.

Most of the diazonium salts furnished us by the National Aniline Division of Allied Chemical and Dye Corp. yielded pH levels between 3.5 and 4.2 in 0.2% aqueous solution. On mixing with 0.2% Na₂HPO₄, all gave pH levels between 7.4 and 7.9.

For testing the reactions of various phenolic substances in comparison with argentaffin granules, Gomori followed the suggestion of Coujard and used the following technic.

"Substances to be tested are dissolved in a strength of 0.25 to 1 per cent in a mixture of equal parts of serum and 5 per cent gelatin. Marks are written with a clean pen dipped in a slightly warmed solution on a slide; more than twenty-five different substances can be tested on one slide. The slides are dried and subsequently exposed to fumes of a solution of formaldehyde U.S.P. in a closed jar for several hours; the excess solution of formaldehyde is driven off in the paraffin oven. The dry slides can be stored for weeks. For the test the slides are run through xylene and alcohols to distilled water and stained just as tissue sections would be."

Gomori also records the reduction of Schmorl's ferric-ferricyanide lipofuscin reagent which we utilized independently in Laskey's technic in extensive studies on the rodent gastrointestinal mucosa (p. 173).

**Chromaffin**

Chromaffin cells share the property of reducing diammine silver salts exhibited by argentaffin cells, and Masson considered the latter as belonging to the chromaffin system.


1. Treat fresh tissue for 1–2 hours in the dark in a 1% dilution of 28% ammonia water (1 cc. and 99 cc. water).
2. Place blocks in the Bielschowski-Maresch diammine silver solution (p. 334), diluted with an equal volume of distilled water, for 3–5 hours in the dark.
3. Transfer to several changes of 1:100 dilution of ammonia water, during 30 minutes, in the dark.
4. Fix one hour in 3% sodium thiosulfate in the dark.
5. Wash one hour in running water.
6. Fix 1–2 days in 10% formalin. Cut frozen sections, float onto slides, dehydrate, clear, and mount in balsam or clarite as usual.
Ogata and Ogata note also the reduction of osmium tetroxide by adrenal medulla cells, but this is overshadowed by the greater blackening of the cortical lipoids.

**Chromation.** Most other methods depend on the production of a brown basophilic substance by the action of chromates on adrenalin and perhaps on other reducing substances. According to Cowdry the same brown material is formed by iodates. Usually, chromate fixation is prescribed; but according to Lison, chromaffin substances can be rendered demonstrable after other "banal" fixations by treating sections for a few hours with 3% potassium bichromate or potassium iodate.

I have not been able to render chromaffin brown nor to obtain its characteristic yellowish green stain with azures in the azure eosin technic when primary formalin fixation is practiced, either by several days' mordanting in 2.5% potassium-bichromate solution before dehydration and paraffin embedding, or by overnight mordanting of paraffin sections in 5% potassium-bichromate solution. I have not been able to obtain brown chromaffin staining green with azures after fixation by Lison's iodate formalin procedure.

Even as little as one hour of preliminary treatment with formalin may prevent the chromaffin reaction, though the tissues are fixed thereafter for the usual length of time in Orth's fluid. The presence of acid in the chromate-containing fixatives may also prevent chromaffin from staining. Ophüls' acetic variant of Orth's fluid and Telyesniczky's acetic bichromate fluid (p. 44) and the acetic Zenker fluid may fail to demonstrate chromaffin in the same material as gave good chromaffin staining after primary Orth fixation. The Spuler-Maximow variant of Zenker's fluid (p. 39) demonstrates chromaffin better than the original acetic Zenker, but not as well as Orth's fluid.

After Orth, Kose, or Möller (Regaud) fixation, chromaffin is manifest, even in frozen sections stained with fat stains and in paraffin sections stained with alum hematoxylin, as a diffuse brown coloration of the cytoplasm of the chromaffin cells. This material is tinged orange by safranin O and green by toluidin blue O, thionin and azure A. The azure A–eosin B technic (p. 118) stains chromaffin yellowish green to bluish green. Schmorl used a 24-hour Giemsa stain in formalin-bichromate fixations. Wiesel's method (from Schmorl) required sequence staining in 1% toluidin blue or anilin blue and by safranin O. The two stains were used for 20 minutes each, with 5 minutes of washing between. Sections were differentiated in 95% alcohol until the blue color reappeared, then cleared in carbol xylene and xylene, and mounted in balsam. Chromaffin stained green; other cytoplasms, blue; nuclei, red.

The *Vulpian reaction*, according to Cowdry, indicates the presence of adrenalin. Immersion of fresh adrenal tissue in a dilute solution of ferric chloride produces a green coloration of the chromaffin cells of the medulla. According to Karrer, ferric chloride gives a green color with solutions of
pyrocatechol. Since adrenalin is a catechol derivative, this may be the explanation of the Vulpian reaction.

Ciaccio (Anat. Anz. 23:401, 1903) adapted the Vulpian reaction for histologic use. He fixed very thin slices in a 5% solution of ferric chloride in 100% alcohol for about 10 minutes, transferred them to a mixture of 1 part ammonia with 10 parts 100% alcohol, and followed this treatment by hardening in 100% alcohol. Penetration was quite poor, only to about 100 μ. The medulla cells and medullary veins contain granules of violet to brown material.

I have previously noted that fixation in aqueous formaldehyde ferric chloride mixtures does not give the Vulpian reaction nor preserve chromaffin. It would appear that Ciaccio’s procedure could well be applied to paraffin sections of frozen dried material.

Anilin Blue Orange G. Gomori recently (Am. J. Clin. Path., Tech. Sect. 10:115–117, 1946) reported on the use of the Mallory Heidenhain technic (p. 351) for demonstration of the chromaffin cells of the adrenal medulla. Aqueous formalin, Bouin’s fluid, and Heidenhain’s mercuric chloride formalin are the recommended fixatives. Bichromate fixatives were considered inferior, and alcoholic fixatives were unsuitable.

The azocarmine G of Step 2 is reduced to 0.05%, and staining is altered to 60–90 minutes at 55–60° C. only. The aniline alcohol differentiation is done under microscopic control until chromaffin cells stand out in deep pink with pale cortex cells. The phosphotungstic acid bath is reduced to 2% or 3% and to 20 minutes. Mallory’s anilin blue orange G mixture is used undiluted for 15–40 minutes only. Quinoline yellow (C. I. No. 801?) and tartrazine (C. I. No. 640) are recommended as substitutes for orange G in Mallory’s mixture and yield a greenish yellow cortex-cell stain in place of the dull orange of the original mixture, contrasting better with the purplish-red granules of the chromaffin cells.

Three quinoline yellows appear in the Colour Index. Nos. 800, 801, and 802. The first is insoluble in water; the other two are sulfonic acids. Probably C. I. No. 801 is meant.

Alpha cells of pancreatic islets, some cells of the anterior pituitary, neutrophil leukocytes and myelocytes, and enterochrome cells also possess granules staining deep purplish red to violet by this technic; hence it appears that this technic demonstrates other cell granules of chromaffin cells, and not chromaffin itself.

The Fast Green–Van Gieson Method (p. 349) is far simpler than the foregoing, and demonstrates adrenal medulla cells in pink to gray pink, and cortex cells in green when chromate fixation is not used. The presence of the chromaffin substance changes the color of the medulla cells to a dark gray-green which contrasts poorly with the green cortex cells.

Perhaps the best methods of demonstrating chromaffin substance in paraffin sections after chromate fixations are the Schmorl ferric ferricyanide re-
duction test (p. 175), which colors chromaffin a deep greenish blue; and the periodic acid Schiff reaction (p. 123), which colors medulla cells a dark grayish red to gray pink. Both tests should be employed in critical cases.

It has been shown in test-tube experiments that the potassium bichromate precipitation products formed with catechol, adrenalin, dopa, and the like promptly reduce ferric ferricyanide mixtures.

Adopting the quinhydrone theory of the chromate oxidation product of adrenalin, the side chain still possesses a secondary amine and a hydroxyl on adjacent carbon atoms to give the periodic acid Schiff reaction. But if the

\[
\text{Adrenalin} + \text{O}_2 \rightarrow \text{Quinone} + \text{adrenalin}
\]

Raper adrenochrome structure is adopted, and it seems to be the consensus that it should be, the amino group becomes tertiary, and the product should not give the Nicolet-Shinn reaction. However, if it is presumed that noradrenalin rather than adrenalin is being demonstrated, a Raper formulation for a noradrenochrome would furnish the necessary reactive group.

\[
\text{Noradrenalin} + \text{O}_2 \rightarrow \text{Quinone} + \text{O}_2
\]

\[
\text{Noradrenochrome} + \text{HIO}_4 \rightarrow \text{aldehyde products}
\]

The final periodic acid cleavage product is formulated with the presumption that the quinone ring is not further attacked. It has been shown that when periodic acid is reacted with an excess of catechol, no aldehyde can be detected in the reaction product.
The Ferric Ferricyanide Reduction Test

This test was introduced into histochemistry by Golodetz and Unna (Monatsh. f. prakt. Dermat. 48:149, 1909), for the demonstration of reduction sites in tissue. At first it was attributed to tyrosine, but all of Unna's later papers refer simply to "Reduktionsorte." Ferricyanide is reduced to ferrocyanide and in the presence of ferric ions in the solution is converted to insoluble Prussian blue.

The method was cited by Schmorl for the identification of lipofuscin pigments. I have found it applicable to the pigment in phagocytes and parenchyma cells of the adrenal cortex, to a granular pigment found in phagocytes in the ovary adjacent to involuting corpora lutea, to the so-called melanosis pigment of the human appendix and colon, and to a homologous pigment in the intestine of the guinea pig (Anat. Rec. 100:239, 1950).

The method also demonstrates chromaffin of the adrenal medulla in material appropriately fixed in chromate-containing fixatives (Lillie, loc. cit.).

Gomori (Arch. Path. 45:48, 1948) and I independently discovered that the enterochromaffin or argentaffin substance of the basal granular cells of the gastrointestinal mucosa gave this reaction. Laskey and Greco (Arch. Path. 46:83, 1948) compared it with the Masson silver method and found it nearly as efficient and much briefer.

Chèvremont and Frederic (Arch. Biol. 54:589, 1943), apparently unaware of the previous use of this reaction for lipofuscin, utilized it for the demonstration of fixed sulfhydryl groups. They recognized, however, that other unidentified substances might react, and required the use of specific sulfhydryl-blocking reagents for confirmation of the identification. They used mercuric chloride—Yao (Quart. J. Microscop. Sci. 90:401, 1949) prescribed one hour in 6% aqueous solution—or 1% alcoholic chloropicrin solution or 4% aqueous monoiodoacetic acid.

Fisher and I have recently noted that thyroid colloid colors quite deep blue by this method.

Cutaneous keratin, as noted by Golodetz and Unna (loc. cit.), and keratoxylin granules, as noted by Chèvremont and Frederic (loc. cit.), and the corresponding structures in the stratified epithelium of the forestomach of rats and mice color deep blue by this reaction. Mercurial fixations do not inhibit the reaction in the rodent forestomach, and incubation at 37° C. for 16 hours in 5% mercuric chloride does not stop it in skin sections. Chèvremont and Frederic disagree with us and with Golodetz and Unna and deny the reaction of cutaneous keratin. A 20-minute treatment with 2% CrO₃ destroys the reactivity, but 18-hour treatments with K₂Cr₂O₇ (0.1 M) and HI0₄ (0.03 M) do not.

In Vitro Tests with Ferric Ferricyanide. The histochemical reaction mixture is promptly reduced by ascorbic, oxalic, and uric acids, by phenols (phenol, resorcinol, hydroquinone, pyrogallol, o-cresol, α-naphthol, β-nap-
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thol, adrenalin, L-tyrosine, tyramine, thyroxin); by indols (indol, skatol, tryptophane, tryptamine, nitrosoindol, nitrosotryptophane); by aryl amines (aniline, α-naphthylamine, benzidine, diphenylamine); by thiols (ethyl mercaptan, mercaptoethanol, thiophenol, thio-2-naphthol, rubeanic acid, glutathione, cysteine, DL-methionine); by hydrazines (phenylhydrazine, semicarbazide, aminoxyanilidine); by stannous chloride, nascent hydrogen, carbon disulfide; by inorganic sulfides, sulfite, hydrosulfite, and thiosulfate; by glyoxal, benzaldehyde and benzyl alcohol; by nitrous acid; by hydrogen peroxide, lecithin, cod liver oil, and linseed oil.

Pyrocatechol gives a green-black precipitate with FeCl₃; p-dimethylaminoaniline and salicylic acid, a deep purple; the aminophenols (o-, 2,4-diaminophenol), red to brown; nitrosoresorcinol and 1-nitroso-2-naphthol, brown; p-phenylenediamine, dark brown; Na₂S, NaSH, (NH₄)₂S, and H₂S, greenish black; but premixed with potassium ferricyanide and allowed to stand for a minute or so, they all yield prompt blue or green colors with ferric chloride.

Other substances tested took an hour or more to produce blue or green colors, more often overnight; and probably need not be considered as demonstrable by the histochemical reaction when the reaction time is restricted to ten minutes. These included isopropanol, formaldehyde, acetaldehyde, acryl acid, tartaric acid, citric acid, cholesterol, and fructose, which gave blue or green colors in an hour but no precipitate; piperidine and pyridine, which gave brown precipitates with ferric chloride and whose premixed ferricyanide solutions which had stood for one hour or more gave Prussian blue reactions with ferric chloride; and a larger group giving no reaction at one hour: DL-serine, DL-lysine, histidine, histamine, DL-valine, L-glutamic acid, L-arginine, DL-aspartic acid, DL-threonine, L-proline, L-cystine, DL-alanine, DL-phenylalanine, methanol, ethanol, n-butanol, glycol, diethylene glycol, glycerol, 1,4-dioxane, allyl alcohol, acetone, formic acid, glucose, urca, guanine, caffeine, uracil, thioracil, inositol, chloral hydrate, HgCl₂, HCl, and distilled water.

Fixation of Material. For demonstration of the fuscin or lipofuscin pigments routine formalin fixation is satisfactory. I have had good results on the adrenal pigment after alcoholic formalin, acetic alcohol formalin, Carnoy, aqueous and alcoholic lead nitrate and mercuric chloride formalin mixtures, and a variety of other non-chromate-containing fixatives. Potassium-bichromate fixations generally weaken or abolish the reactivity of this pigment.

For the argentaffin or enterochromaffin substance, routine fixations of 2–15 days in aqueous 10% formalin are satisfactory. Acetic formalin fixation is unsatisfactory. Alcoholic fixations of all sorts remove the argentaffin substance. It is preserved poorly or not at all with aqueous fixatives lacking formaldehyde. Bichromate formaldehyde fixations weaken the reaction and give a green color in place of clear blue.

For chromaffin, fixation in potassium bichromate formaldehyde mixtures
without added acid is preferred. The Möller and Kose mixtures (p. 44) give good results.

For sulfhydryl Chèvremont and Frederic prescribe a 4–18 hour fixation in saline formalin or Bouin’s fluid, and avoid prolonged treatment with melted paraffin, or use frozen sections. They state that longer than 48 hour exposure to formaldehyde will reduce or abolish the reactivity of sulfhydryl groups.

While sulfhydryls form stable non-reactive mercaptals on reaction with aldehydes, strong HCl or ZnCl₂ is required as catalyst. Only thiol acids react directly without catalysts. Hence formalin fixation can be tolerated if not unduly prolonged.

I find Carnoy’s acetic chloroform alcohol mixture (p. 36) and a mixture of 5 Gm. trichloracetic acid, 75 cc. alcohol and 20 cc. water—both at 5° C. —superior to formalin for preservation of sulfhydryl in the basal layers of the epidermis and the hair-follicle cells. Use of sublimate formalin with sodium acetate (p. 41) inhibits the reaction of these structures, but not that of keratin. I use a 30-minute vacuum infiltration in paraffin for skin. It appears to be necessary to work up this material promptly after preparation. Paraffin sections which have been saved for some weeks after cutting have utterly failed to give the reaction at sulfhydryl sites, though those which were reacted at once from the same blocks gave good results.

For thyroid colloid the use of alcoholic fixatives seems preferable to that of aqueous formalin. The use of dichromate fixatives appears to be contraindicated, both because they tend to oxidize reducing groups and because they appear to engender a more or less diffuse, moderate blue-green coloration of collagen, epithelia, and the like.

The reagent. Schmorl prescribed a five-minute bath in a freshly prepared mixture of equal volumes of 1% ferric chloride and freshly prepared 1% potassium ferricyanide. The mixture should remain clear and greenish brown in color. Chèvremont and Frederic prescribed a mixture of one volume of fresh 0.1% potassium ferricyanide solution and three volumes of 1% ferric sulfate solution, noted that the mixture was acid (pH 2.4) and that it was stable for about two hours in daylight and longer in the dark. A 20–25 minute immersion in this was required for paraffin sections.

I have found this highly disproportionate mixture—about 50 moles of ferric salt to 1 of ferricyanide—advantageous in giving a much clearer background than the Schmorl mixture, which gives only two moles of ferric salt to one of ferricyanide, but the concentration of ferricyanide is so low that prolonged exposures are necessary; and even then the demonstration of enterochromaffin cells is uncertain, and the reactivity of some of the pigments is dubious.

After considerable experimentation we have settled on 30 cc. of 1% ferric chloride made by diluting 1 cc. of the official ferric chloride solution with 36.2 cc. water (p. 82) or 2.69 cc. to 100 cc. with water, 4 cc. fresh 1% potassium ferricyanide (K₃Fe(CN)₆) and 6 cc. distilled water. The final mixture
thus contains 0.75% FeCl₃ and 0.1% potassium ferricyanide, a molar proportion of about 15:1. A ten-minute bath in this solution appears adequate.

**Ferric Ferricyanide Reduction Technic (1951).**

1. Fix material in accord with requirements on p. 174, imbed in paraffin, section at 5 to 10 μ, and deparaffinize.
2. At the appropriate point in the hydration schedule, interpose blockade tests as indicated.
3. Wash in water and immerse in ferric ferricyanide reagent: 4 cc. fresh 1% potassium ferricyanide solution, 30 cc. 1% ferric chloride solution, and 6 cc. distilled water, freshly prepared (see p. 175) for ten minutes at 20–30° C.
4. Wash in 1% aqueous acetic acid solution.
5. Counterstain ten minutes in 1:5000 new fuchsin (C. I. No. 678) or fuchsin in 1% acetic acid.
6. Rinse in 1% acetic acid.
7. Dehydrate, clear, and mount through 95% and 100% alcohol, alcohol + xylene, xylenes, synthetic resin.

**Results.** Sites of reducing substances (p. 173) should be blue; background, pale pink or faint green; nuclei, pink to red.

Since the ferricyanide in the Schmorl mixture would react directly with ferrous salts in the sections to produce Turnbull’s blue, a series of controls should be used, substituting 2% potassium ferricyanide for the ferrocyanide in the Perl’s method (p. 243), to demonstrate ferrous salts as such.

For demonstration of disulfides in hair keratin, insert at Step 2 a ten minute bath at 25° C. in a 10% solution of sodium thioglycolate, adjusted with sodium hydroxide to pH 9.5, and wash thoroughly with distilled water.

Perhaps better for this purpose is the peracetic acid–azure A method on p. 197.

**Other Sulfhydryl Methods**

Glick cites only the nitroprusside technics, which so far are applicable only to frozen sections of fresh unfixed tissue. The following technic is cited from Giroud and Bulliard (*Protplasma* 19:381, 1933) with modifications from Glick.

**The Nitroprusside Methods for Sulphydryl.**

1. Cut frozen sections of fresh unfixed tissue. The use of the supercooled knife as in the Adamstone-Taylor method (p. 51) is recommended. Giroud and Bulliard suggest gelatin imbedding.
2. Immerse for 5–10 seconds in 5% zinc acetate.
3. Rinse in distilled water and take up on a slide.
4. Blot and apply 1–2 drops of 2% sodium nitroprusside. Allow color to develop.
5. Here Glick adds a small crystal of ammonium sulfate and a drop of ammonium hydroxide.
6. Giraud and Bulliard continue by dehydrating with alcohol, clearing in toluene, and mounting in balsam.

Results. According to Glick the method through Step 5 demonstrates free SH groups, as in glutathione. Giroud and Bulliard used the method for soluble SH compounds.

Glick states that a preliminary 5–10-minute immersion in 10% potassium cyanide solution makes the nitroprusside react with all sulfhydryls, but that the compounds formed by KCN are quite diffusible, and localization is consequently poor. A preliminary treatment for 15 minutes with 10% trichloroacetic acid makes protein-bound SH reactive, but these compounds are also diffusible and poorly localizable.

Bennett’s method (Anat. Rec. 110:231, 1951) uses a red, mercury-containing azo dye, p-chloromercuriphenyl-azo-β-naphthol, to form insoluble mercaptide linkages with sulfhydryl groups in tissue. The reaction appears to be specific. It does not occur with the corresponding mercury-free azo dyestuff. It is prevented by agents which oxidize: 25.3 mg./100 cc. iodine in propanol (0.001 M), molar hydrogen peroxide ferric chloride mixture in isopropanol or 0.001 M in water; or alkylate sulfhydryl: 1.85% iodoacetamide or 1.86% iodoacetic acid (0.1 M) in n-propanol. It is prevented also by organic mercurials which form mercaptides with sulfhydryl: 0.001 M n-propanol solutions of phenylmercuric chloride (31.3 mg./100 cc.), tolyl mercuric chloride (32.7 mg./100 cc.), or methyl mercuric iodide (34.5 mg./100 cc.). Tissues and sections are brought through appropriate solvents to pure n-propanol, then immersed for several hours in the propanol solutions of the blocking reagents, washed in several changes of propanol, and then transferred, along with control, unblocked, positive material of the same general character (i.e., sections with sections, smears with smears, teased tissue with teased tissue), to the sulfhydryl reagent.

Mescon and Flesh (J. Investigative Dermatol. 18:261, 1952) have further adapted Bennett’s method for frozen sections of unfixed and paraffin sections of formalin-fixed material.

Unfixed material is sectioned by the Adamstone-Taylor procedure (p. 51), mounted on slides, and at once immersed in the sulfhydryl reagent.

Routine paraffin sections of formalin-fixed tissue are deparaffinized and brought to 80% alcohol as usual. Then stain, dehydrate, clear, and mount as for the frozen sections.

Bennett fixed muscle tissue in 5% trichloracetic acid, washed in distilled water, dehydrated in graded alcohol, and transferred to propanol or butanol.
Alternatively, the tissue was quickly frozen in isopentane cooled with liquid nitrogen, and dehydrated while still frozen by several changes of n-propanol or n-butanol at $-20^\circ$ to $-25^\circ$ C. for 10-12 days. Muscle tissue was then teased out to isolate single fibers and small groups, which were stained whole. He also used paraffin and nitrocellulose sections, which were brought to n-propanol or n-butanol for reaction.

The reagent is $p$-chloromercuriphenyl-azo-$\beta$-naphthol. This is dissolved according to Bennett at $1.25 \times 10^{-6}$ M (6 mg./liter) in butanol or at $5.6 \times 10^{-6}$ M (3 mg./liter) in n-propanol; or according to Mescon and Flesch by dissolving 3 mg. in 100 cc. 100% ethyl alcohol at room temperature, and then adding 25 cc. distilled water to reduce the alcohol concentration to 80%. Mescon's reagent is stored at 4° C. and keeps fairly well.

Bennett's $p$-Chloromercuriphenyl-azo-$\beta$-naphthol Method for Sulfhydryl, with Modifications from Mescon and Flesch.

1. Deparaffinize paraffin sections and bring to 80% alcohol. Transfer Adamstone-Taylor frozen sections and nitrocellulose sections directly, and ordinary frozen sections after blotting on slides to
2. The Mescon-Flesch reagent for 1-3 hours.
3. Dehydrate, clear, and mount through 95% and 100% alcohol, alcohol $+$ xylene, two changes of xylene in a synthetic resin.
1a. Bring teased muscle preparations, tissue fragments to n-propanol; or nitrocellulose sections to isopropanol, which does not dissolve the nitrocellulose.
2a. Immerse several hours or overnight, according to size and thickness of material, in Bennett's propanol reagent solution.
3a. Wash two or three hours in n-propanol.
4a. Clear through propanol $+$ xylene in two changes of xylene and mount in synthetic resin.

Results. Sites of sulfhydryl are colored red. If phenyl-azo-$\beta$-naphthol is used instead of its mercury derivative there should be no reaction.

Interpose blockade reactions after Step 1. The reagents are described on p. 177. It is suggested that one of the mercaptide reagents be used as well as an alkylating or an oxidizing reagent; or better, one of each of the three types. The blockade reagent should be used for 4-18 hours, and should be followed by a 2-6 hour bath in the same solvent to remove excess blocking agent. Then transfer to 80% alcohol or to n-propanol and proceed with Step 2 or 2a.

The color is not extracted by toluene, benzene, 70% alcohol, 50% propanol, 0.1 N NaOH, 0.1 N CaCl$_2$ (about 0.55%), 0.1 N NH$_4$OH (about 0.68% dilution of 28% ammonia water), or molar NaCl (5.85%) on overnight exposure. The color is extracted by overnight immersion in 0.001 M
propanol solutions of mercaptans: \( \beta \)-mercaptoethanol, cysteine, 2,3-dimercaptopropanol (BAL), glutathione, and thioglycolic acid (M.W. = 78, 121, 124, 307, and 92 respectively).

Hence if extraction tests are required, interpose after Step 2 or 2a, hydrating as usual for the use of aqueous reagents, washing first with propanol for use of the propanol reagents, and dehydrating for use of toluene, benzene, or other similar solvent. After the prescribed exposure, wash out the extracting agent with the same solvent, dehydrate if necessary, clear, and mount as usual.

Barrnett and Seligman utilize 2,2'-dihydroxyl-6,6'-dinaphthyl disulfide as a reagent for both -SH and -SS- groups. Dr. Barrnett communicated this method to me after its presentation at the Histochemical Society in 1952.

Barrnett and Seligman's Dihydroxyldinaphthyl Disulfide Method for Sulfhydryl and Disulfide.

1. Fix 24 hours in 80% alcohol containing 1% trichloracetic acid.
2. Dehydrate, imbed in paraffin, and section at 5-10 \( \mu \). Use minimal amount of albumin fixative, or preferably none.
3. Deparaffinize, collodionize with 0.5% collodion, and hydrate through alcohols as usual.
4. To demonstrate -SS- groups as well as -SH groups, insert here a 2-4 hour bath at 50° C. in 0.2-0.5 M thioglycolic acid (1.8 to 4.6%), adjusted with sodium hydroxide to pH 8.0.
5. Dissolve 25 mg. 2,2'-dihydroxyl-6,6'-dinaphthyl disulfide* in 15 cc. 100% alcohol and add 35 cc. Michaelis's pH 8.5 veronal sodium HCl buffer (p. 454). Stain up to nine slides in a Coplin jar at 50° C. for one hour.
6. Remove the Coplin jar from the water bath and cool for ten minutes at room temperature.
7. Rinse briefly in distilled water.
8. Wash ten minutes in 0.01% acetic acid (pH 4.0-4.5).
9. Dehydrate with graded alcohols, pass through 100% alcohol + ether 50:50, and extract with ether for five minutes to remove excess reagent and reaction byproducts.
10. Rehydrate through graded alcohols to distilled water.
11. Stain two minutes in a fresh solution of 50 mg. tetrazotized diorthoansidine† in 50 cc. Sörensen's 0.1 M phosphate buffer of pH 7.4 (p. 451).
12. Wash two minutes in running water.
13. Mount in glycerol gelatin or dehydrate with acetone, clear in xylene.

† Available from Dajac Laboratories, or from E. I. DuPont de Nemours Company, Wilmington, Delaware.
and mount in synthetic resin (Clarite, Permount, HSR, polystyrene, etc.).

**Results.** Reaction sites show pink, red, and blue-red to blue with increasing intensity of the reaction.

Barnett writes me that interposition of an iodine treatment after Step 3 completely blocks the sulfhydryl reaction, thus: Immerse for four hours at 25°C in 0.0015 M iodine (378 mg./liter) containing a trace of KI at pH 3.2.

Blocking is also accomplished by a four-hour bath at 37°C in 0.1 M ethyl maleimide (1.25%), buffered with Sörensen's phosphates to pH 7.4; or by a 20-hour bath at 37°C in 0.1 M (Na) iodoacetate (ca. 2%) at pH 8.0.

Incubation in 0.03 M glutathione (0.92%) at pH 8.5 for three hours at 50°C between Step 10 and Step 11 completely prevents the development of color.

Barnett (*loc. cit.*) formerly used at Step 4 a 1½-hour bath at 50°C in 10% ammonium sulfide. Step 4 as given above represents his 1953 procedure (personal communication). I have found ammonium sulfide ineffective in rendering distal hair cortex reactive, and have had similar failures with sodium hydrosulfite, sulfite, and thiosulfate. Stannous chloride not only failed to render hair cortex reactive, but occasioned diffuse deposit of Prussian blue over most other structures in the sections.

Pearse notes that for opening disulfide groups, relatively brief treatments with alkaline potassium cyanide are preferable because of the destruction of sulfhydryl by alkali. I have had rather irregular and generally unsatisfactory results with KCN, and prefer 10% sodium thioglycolate adjusted with NaOH to pH 9.5. This reagent appears to render hair cortex strongly reactive to the ferric ferricyanide method on a ten-minute exposure at room temperature (25°C). Longer exposures at higher temperatures appear simply to increase section losses without any appreciable gain in reactivity. This reagent works well after Carnoy, hot methanol chloroform, formalin, and the like. After Spuler's "Zenker Formol," apparently only sulfhydryl groups become demonstrable, and prolonged chromate fixations decrease or abolish reactivity. This last finding suggests that the mercurial binding of sulfhydryl groups may operate to protect them against bichromate oxidation.

Unalkalinized 10% sodium thioglycolate solution (pH 5.5) may also be effective in opening disulfide bonds, but apparently requires heat (60°C) and longer exposures. Its use may be desirable when it is necessary to avoid alkali.

Kekule and Linnemann used iodine to convert mercaptans to disulfides in 1862 (Hickinbottom, p. 131), hence this method of blocking -SH groups prior to trying to localize -SS- groups would seem inappropriate, as the iodine-created -SS- groups might be difficult to distinguish from those occurring naturally.
Barrnett and Seligman (Science 116:323, 1952) noted in rat tissues strong reactions in hair cortex at the zone of keratinization, in pancreatic acinar cells, in intestinal smooth muscle, in Hassall's corpuscles of the thymus, in lens, and in Purkinje cells of the cerebellum; moderate reactions of hair cortex near the root, of epithelia of epidermis, hair sheaths, sebaceous glands, and intestinal villi, of Paneth cells and stroma cells of villi, and of striated and smooth muscle of the skin. Intense staining of vascular elastica occurred, but this was not prevented by iodine and hence is not a sulfhydryl reaction.

**Antibody Localization**

Coons, Leduc, and Kaplan (J. Exp. Med. 93:173, 1951) in their study of localization of fluorescent antibodies, first cut hard frozen sections (p. 23), then attached them to gelatin-coated slides in the cryostat, removed them from the cryostat, and melted the section by pressing a finger against the back of the slide under the section. Sections were then dried onto the slides in an air stream at room temperature for an hour, and stored at 4° C. overnight. They were then fixed to immobilize the egg albumin and gamma globulin by immersion for 30 minutes at 37° C. in preheated 95% alcohol. For bovine serum albumin, acetone was used at room temperature for 15 minutes. After fixation the slides were dried in a vertical position in the 37° C. incubator for 30 minutes.

Sections were then reacted with a drop of fluorescein-treated antibody for 30 minutes at 37° C., washed in buffered saline for ten minutes and mounted in buffered glycerol. They were then examined under the fluorescence microscope. After photographing, the cover glass is floated off; the section is fixed in 10% formalin for ten minutes, and counterstained with hematoxylin and eosin. The same area is then rephotographed.

For details of the synthesis of fluorescein isocyanate and the preparation of the fluorescein isocyanate protein conjugation product the reader is referred to Coons and Kaplan, J. Exper. Med. 91:1, 1950. Apparently the isocyanate must be freshly prepared from fluorescein amine with phosgene and immediately conjugated with the protein, and any exposure to water avoided up to the moment it is added to the aqueous protein solution.
Chapter 10

Cytoplasmic Granules

Mitochondria

**Supravital Technics**

These round, oval, rod-shaped, or filamentous structures are destroyed by such acid fixatives as Zenker's fluid. They may be stained specifically by supravital technics with Janus green B (C. I. No. 133), diethylsafranine, Janus blue, Janus black I, pinacyanol (C. I. No. 808), rhodamine B (C. I. No. 749), and methylene blue (C. I. No. 922). Cowdry identifies Janus black I as a mixture of a brown dye and Janus green B, on which latter its action depends. Janus blue is similar in its effectiveness to Janus green B. It is diethylsafranine-azo-β-naphthol (C. I. No. 135). Pinacyanol is a red basic dye said to be superior to Janus green B. Rhodamine B is a weakly basic red dye.

Supravital Staining of Blood. Cowdry directs: Mix on a clean slide a small drop of blood with a small drop of 1:10,000 Janus green B in 0.85% salt solution (1% aqueous solution 1 cc., distilled water 99 cc., sodium chloride 850 mg., or use 99 cc. of a 0.86% sodium-chloride solution). Drop on cover slip and let spread. Ring with petrolatum. In about 5-10 minutes mitochondria are colored deep bluish green. Similarly small fragments of fresh tissue may be crushed in the salt solution to a thin film.

The technics with methylene blue, Janus blue, Janus black, and rhodamine B are basically similar.

Hetherington (Stain Technol. 11:153, 1936) recommended pinacyanol (C. I. No. 808)* alone or in combination with neutral red. He used a 0.1% solution of pinacyanol in 100% alcohol, diluting 1:40 for use with 100% alcohol, and adding, when desired, between the same and double the amount of neutral red (C. I. No. 825). Schwind (Blood 5:597, 1950) prescribed stock solutions in 100% alcohol: 0.1% pinacyanol and 0.4% neutral red.

* Obtainable from Eastman Kodak Co., Research Laboratory, Rochester, N. Y.
9 drops of pinacyanol and 30 drops of neutral red in 5 cc. 100% alcohol. I suggest 0.3 cc., 1 cc., and 100% alcohol to make 10 cc.

Flame clean slides, flood with one of the dilute dye mixtures, drain in a vertical position, and let dry, using a gentle, warm air stream in hot humid weather. Place on a clean cover glass a small drop of blood (or marrow diluted with or crushed in a drop of serum), using only enough blood to give a film one cell-layer thick and avoid air bubbles. Cover with the stain-coated slide and seal edges with petrolatum (petroleum jelly). Schwind recommends application of soft petrolatum with a 5-cc. syringe and a short, 23-gauge hypodermic needle, and warns against moving the cover glass during the process, as this tends to rupture cells.

Mitochondria soon color a deep blue to violet in still living and motile cells. Hetherington’s neutral red mixture is reported to give a red color to nuclei, “neutral red granules and vacuoles.” Schwind recorded a purplish blue color for nuclei.

Staining occurs more rapidly at 37° C., but preparations do not keep as long. Motility ceases somewhat below this temperature. As the colors are feebler than those in fixed preparations, brilliant illumination and a darkened laboratory are recommended.

As pinacyanol is light sensitive, its solutions must be stored in the dark, and stained films must be kept in the dark. Preparations, even when stored in the cold, do not keep more than two weeks (Schwind). However, mitochondria remain deeply stained for many hours, contrasting with the fairly prompt fading in Janus green preparations.

**Fixed Tissue Methods**

Of more general application are methods using fixed tissue. Regaud’s method (*Arch. d’anat. micr.,* 11:291, 1910) requires fixation with Möller’s (Regaud’s) fluid (p. 45) for four days, followed by eight days (four changes of two days each according to Mallory) in 3% potassium bichromate. Wash in running water 24 hours. Dehydrate in alcohol, clear in benzene, and imbed in paraffin. Cut thin sections (2–5 μ.) Deparaffinize, hydrate, and mordant 8–10 days in 5–15% iron alum (NH₄Fe(SO₄)₂·12H₂O) solution. Cowdry reduces this iron alum step to 24 hours in a 5% solution. Wash in water for a few minutes. Stain 24 hours in aqueous 1% hematoxylin containing 10% each of alcohol and glycerol. Regaud specified 6 to 8 weeks, Cowdry required only three weeks’ aging. Differentiate in 5% iron alum solution with microscopic control. Wash 30 minutes in running water. Dehydrate by the 95%, 100% alcohol series. Clear in xylene and mount in balsam. Mitochondria are sharply stained black.

**Aniline-Acid Fuchsin-Methyl Green.** Cowdry especially recommends the aniline-acid fuchsin-methyl green method of Bensley (*Am. J. Anat. 12:297,* 1911), with the Möller-Regaud fixation in place of Bensley’s acetic acid, osmium tetroxide, potassium bichromate fixation:
1. Cowdry fixed thin sections in Möller's (Regaud's) fluid (p. 45) in an ice box for four days, changing the fluid daily; and mordanted in 3% potassium bichromate for eight days, changing every second day. Bensley fixed in 4% osmium tetroxide solution 2 cc., 2.5% potassium bichromate solution 8 cc., and glacial acetic acid 1 drop, for 24 hours. I suggest the following emendation of this formula: 4% osmium tetroxide 2 cc., 4% potassium bichromate 5 cc., 0.5% acetic acid 3 cc. This assumes a value of 0.015 cc. for 1 drop of glacial acetic acid.

2. With either fixation, wash several hours to overnight in running water.

3. Dehydrate (Cowdry) with graded alcohols, 70%, 80%, 95%, 100% giving 6-18 hour changes. Then clear through 100% alcohol and benzene, and two changes of benzene of 30-60 minutes each. Imbed in paraffin, and

4. Section at 3-5 μ. Deparaffinize and hydrate as usual through xylene and graded alcohols.

5. 1% potassium permanganate one minute.

6. Rinse in water.

7. 5% oxalic acid one minute.

8. Wash thoroughly in water.

9. Blot water from around the section and heat it to steaming in Altmann's aniline-acid fuchsin (filtered 5% anilin water 10 cc., acid fuchsin 1.5 Gm.; shake it at intervals for 24 hours before using), let it cool and continue staining about six minutes (Bensley and Mallory preheated to 60° C. and then stained five minutes as the solution cooled).

10. Wash one minute in distilled water.

11. Dip into 1% methyl green or drop it on the section (1-5 seconds). Bensley sometimes used 1% toluidin blue instead.

12. Wash in 95% alcohol (Cowdry).

13. Dehydrate in 100% alcohol, clear with one change of 100% alcohol and xylene (50:50) and two of xylene. Mount in clarite or neutral balsam.

**Results.** Mitochondria are crimson; nuclei, green (or blue); zymogen granules, red. If the alcohol takes out too much green, try acetone.

Cain (*Quart. J. Microscop. Sci.* 89:229, 1948) at Step 10 differentiates in dilute sodium carbonate solution (1/200 saturated: about 0.1%) until cytoplasm is pale pink or colorless. Stop the differentiation and brighten the color of the mitochondria by dipping briefly into 1% hydrochloric acid. Then wash in distilled water and counterstain in 0.5% aqueous methyl blue, rinse in water, dip for three seconds in 1% hydrochloric acid, wash with distilled water, dehydrate (at leisure) in alcohols, clear, and mount in balsam.

**Phosphotungstic Acid Hematoxylin.** Mallory recommended a technic with phosphotungstic acid hematoxylin. Fix 24 hours or more in 10% formalin, *Ehrlich* (*Encyklopädie*) and Bensley specified 2 Gm. acid fuchsin; Conn gives solubility of acid fuchsin in distilled water at 12-12.5%.
mordant 2–5 days in 3–4 changes of 5% aqueous ferric chloride solution, wash briefly in water, harden in 3–4 changes of 80% alcohol for 1–2 days, until the alcohol remains clear. Imbed in paraffin as usual, and bring paraffin sections to water.

1. 0.25% potassium permanganate 5–10 minutes.
2. Rinse.
3. 5% oxalic acid 3–5 minutes.
4. Wash thoroughly in tap water.
5. Stain in Mallory’s phosphotungstic acid hematoxylin (p. 344) for 1–2 days.
6. Rinse in tap water.
7. Differentiate in 95% alcohol.
8. Dehydrate with 100% alcohol, clear in xylene, and mount in balsam.

Results. Nuclei and mitochondria are stained deep blue; collagen and elastin, reddish; myoglia and neuroglia fibrils, blue.

Salivary Gland

The zymogen granules are only moderately oxyphil to neutral stain mixtures. They are dissolved by fixatives containing acetic acid, such as aqueous or alcoholic 5% acetic 10% formalin, Carnoy, Zenker, Telyesniczky’s acetic bichromate, Bouin, and Gendre. Their oxyphilia is better shown after fixation with such fluids as Kosc’s bichromate formalin or Spuler’s Zenker formalin than with neutral aqueous formalin. The azure A eosin B technic (p. 118) is suggested for this reaction. The granules resist digestion with 1:1000 malt diastase in pH 6.0 saline, which removes the basophilic ribonucleic acid from the basal cytoplasm.

The zymogen granules give a positive Schiff aldehyde reaction after oxidation with periodic acid (p. 123) but not after potassium-permanganate or chromic-acid oxidation. The periodic Schiff-positive material resists diastase digestion. These reactions have been recorded in man, rabbit, rat, and guinea pig. Positive (blue violet) Gram-Weigert staining is sometimes noted in rabbits and rats.

The mucin of mucous acini and of duct goblet cells is generally strongly metachromatic to acid safranin and thionin stains. Technics suggested are the buffered acid thionin stain (p. 286) and the iron hematoxylin fast-green safranin method (p. 285). This mucin is strongly Schiff positive after periodic acid or chromic acid oxidation, weakly positive or negative after potassium permanganate (p. 127). Chromate fixatives seem to strengthen the Casella KMnO₄ and Bauer CrO₃ Schiff reaction, as well as improving the metachromasia to safranin, thionin and the like.

Pancreas

Like those of the serous salivary-gland acini, pancreatic zymogen granules are destroyed by aqueous and alcoholic acetic formalins, and by Carnoy’s
and Bouin's fluids, and disappear from all but the most superficial acini on fixation with Telyesniccky's bichromate, Zenker's and Gendre's fluids. They are well preserved by buffered neutral formalin, by Orth's, Kose's, and Möller's bichromate formalin fluids and by formalin Zenker variants.

They stain conspicuously by the Nocht azure A–eosin B technic (p. 118) at pH 3.75 to 4.1. They become especially conspicuous if the cytoplasmic basophilia is first removed by digestion with ribonuclease or malt diastase. With the last, brief neutral formalin fixation is recommended, since this inhibits the ribonuclease activity less than the chromate fluids, and at the same time preserves the granules.

In contradistinction to salivary zymogen granules, those of the pancreas may stain or remain unstained by the periodic acid leucofuchsin technic. Both conditions have been seen in man and mice; the negative phase has been seen in rat and guinea pig pancreas; but in rabbits I have seen these granules HIO₄ Schiff-positive. They are Bauer and Casella (p. 127) negative, and are usually violet with the Gram-Weigert method (p. 372, Variant 4b).

Pancreatic Islets

Bensley's method with neutral stains was designed especially for differentiation of pancreatic-islet cells. The neutral stains were made by precipitating aqueous crystal violet with an approximate stoichiometric proportion of acid fuchsin or orange G, or safranin with an acid violet, which last procedure is presented in detail below. Prolonged staining with a 24 hour old 20% alcohol dilution of the stock 100% ethyl alcohol solution of the crystal violet salts was used. In all three, differentiation is carried out under microscopic control with clove oil and alcohol.

Safranin Acid Violet. Bensley's (Am. J. Anat. 12:297, 1911) safranin acid violet has been used with striking contrasts. This is a true neutral stain made by precipitating saturated aqueous safranine O (C. I. No. 841) by cautiously adding saturated aqueous acid violet. Bensley did not specify which acid violet, but fast acid violet 10B (C. I. No. 696) and eriocyanine A (C. I. No. 699)—two rather blue violets—and a reddish-violet formyl violet S4B (C. I. No. 698) readily form coarse granular precipitates with safranine; and all will probably serve. Eriocyanine A is the bluest of the three and resembles the color depicted in Maximow's Histology, 1st ed., p. 692. These are the three acid triphenylmethane violets now available in the United States. The violet is added until on splashing the mixture up the sides of the flask the color changes, rather abruptly, from red to violet. The precipitate is collected on a filter and dried, and dissolved in 100% alcohol as with Bensley's other neutral stains and the staining technic follows the same general procedure.

Bensley specified fixation with his acetic osmic bichromate mixture (p. 184) or with the Spuler-Maximow formalin Zenker fluid (p. 39). Sections should
be under 5 \( \mu \) in thickness. Dilute the stock alcoholic stain with an equal volume of distilled water, let stand 30 minutes, filter, and use at once. Stain 5–30 minutes (Bensley), 40–50 minutes (Maurer and Lewis: *J. Exp. Med.*, 36:141, 1922), or as long as two hours (Cowdry, McClung). Blot and dehydrate with acetone, clear in xylene or toluene. Maurer and Lewis passed rapidly through 95% alcohol to remove precipitates, dehydrated in 100% alcohol, and cleared in benzene. Then differentiate sections individually under microscopic control in a mixture of three volumes clove oil and one of 100% alcohol. Wash thoroughly with xylene (or toluene or benzene) and mount in clarite (balsam in originals).

Nuclei and granules of beta cells of pancreatic islets and of true basophil cells of the hypophysis stain red; granules of alpha cells of pancreatic islets (Bensley) and of true acidophil cells of the hypophysis and erythrocytes stain blue to violet. Hypophysial chromophobe cells show at most a faint violet stippling.

Maurer and Lewis identified the red-staining cells as alpha cells and the blue as beta, and commented on the basophilia of the so-called "acidophil" cells of the hypophysis produced by this method. They further stated that the various types of chromophil cells are impossible to differentiate cytologically, except by the specific granule stains. I find that the same cell areas in which nearly all the cells stain pink with azure-eosin, stain predominantly blue in the adjacent section with safranin eriocyanine A.

On trial of the foregoing method it is found that the high alcohol content (50%) of the final staining mixture inhibits staining in covered Coplin jars, just as it does with Wright's blood stain. However, only Maurer and Lewis spoke of staining on the slide as is customary with blood stains.

In order to test out various acid violets to ascertain which was the best for this method, we devised the following method. I cite this because it seems to work well in practice and entails far less trouble than the traditional Bensley method.

**Safranin O–Eriocyanine A Method.**

1. Bring thin paraffin sections through xylenes and alcohols to water as usual, treating mercury-fixed material five minutes with 0.5% iodine in 70% alcohol and one minute in 5% sodium thiosulfate.
2. Stain 30 minutes in

<table>
<thead>
<tr>
<th>Solution</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% safranin O (C. I. No. 841)</td>
<td>2 cc.</td>
</tr>
<tr>
<td>1% eriocyanine A (C. I. No. 699)</td>
<td>2 cc.</td>
</tr>
<tr>
<td>M/10 citric acid</td>
<td>1.3 cc.</td>
</tr>
<tr>
<td>M/5 disodium phosphate</td>
<td>0.7 cc.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>34 cc.</td>
</tr>
</tbody>
</table>

3. Rinse in water, dehydrate with acetone, clear in acetone and xylene and two changes of xylene, mount in Clarite.
Results. Nuclei, basophils of hypophysis—red; erythrocytes and acidophils—blue. Staining is already fairly good in 10 minutes, and is perhaps slightly better in an hour than in 30 minutes.

If desired the buffer may be omitted and Bensley’s 3:1 clove-oil + 100% alcohol mixture used for differentiation instead.

I have used this method on hypophyses fixed in neutral formalin as well as with Orth’s and Spuler’s fluids, with perhaps even more brilliant contrasts. Negri bodies and necrotic nerve cells appear conspicuous in light blue by this method.

Anilin Blue Orange G. Bensley also recommends a variant of Mallory’s anilin blue orange G stain for pancreatic islets, which I requote from Mallory with slight changes: Fix fresh tissue in thin slices with the Spuler-Maximow fluid (p. 39) for 4-24 hours:

1. Presumably after the usual iodine and sodium thiosulfate treatment,
2. Stain ten minutes in Altmann’s anilin water acid fuchsin (p. 184).
3. Wash rapidly in previously boiled and cooled distilled water.
4. Mordant ten minutes in 1% aqueous phosphomolybdic acid solution.
5. Drain and stain one hour or less in 0.5 Gm. anilin blue (C. I. No. 707), 2 Gm. orange G (C. I. No. 27), 100 cc. distilled water.
6. Drain and differentiate in 95% alcohol until gross color clouds cease coming off.
7. Dehydrate with 100% alcohol, clear in xylene, and mount in balsam.

Results. Alpha granules—orange red; beta granules—bluish; acinar cells—bluish violet, sometimes with orange zymogen granules; erythrocytes—red.

Bencosme (Arch. Path. 53:87, 1952) recommends a very elaborate schedule using a Masson trichrome procedure, for which the reader is referred to the journal.


1. Run sections through xylene and alcohols to water.
2. Refix in Bouin’s fluid for 12–24 hours.
3. Wash sections thoroughly in tap water to remove picric acid.
4. Treat sections for about one minute with a solution containing about 0.3% each of potassium permanganate and sulfuric acid.
5. Decolorize with a 2–5% solution of sodium bisulfite. Wash.
6. Stain in the following hematoxylin solution under microscopic control until the beta cells stand out deep blue (about 10–15 minutes): Mix equal parts of 1% aqueous hematoxylin and of 3% chrome alum. Add to
each 100 cc. of the mixture 2 cc. 5% potassium bichromate and 2 cc.
0.5 N sulfuric acid. The mixture is ripe after 48 hours and can be used as
long as a film with a metallic luster will continue to form on its surface
after one day's standing in a Coplin jar (about 4–8 weeks). Filter before
use.

7. Differentiate in 1% hydrochloric acid alcohol for about one minute.
8. Wash under the tap until the section is a clear blue.
9. Counterstain with 0.5% aqueous solution of phloxine (B?) for five min-
utes. Rinse:
10. Immerse in 5% phosphotungstic acid solution for one minute.
11. Wash under the tap for five minutes. The section should regain its red
color.
12. Differentiate in 95% alcohol. If the section is too red and the alpha cells
do not stand out clearly enough, rinse the section for about 15–20 sec-
onds in 80% alcohol.
13. Transfer to 100% alcohol, clear in xylene, and mount in balsam.

Results. Beta cells are blue; alpha cells—red; delta cells—from pink to red
and indistinguishable from alpha cells; acinar zymogen granules—red to un-
stained in pancreas. In hypophysis, alpha cells are pink; beta cells—gray
blue, and not readily distinguished from chromophobes; nuclei—red purple
to blue violet; erythrocytes—deep pink; smooth muscle—pink; collagen—un-
stained; goblet cell mucin—coarsely granular and dark, slightly greenish blue
in color.

Bell found that this method permitted ready distinction of \( \alpha \) and \( \beta \) gran-
ules in the pancreatic islets even on material fixed as much as 12 hours post
mortem.

In place of Steps 9–12 Bencosme (Arch. Path. 53:87, 1952) substitutes a
ponceau-acid fuchsin mixture as used for Masson stains (p. 351) and stains
15–45 minutes, rinses in 1% acetic acid, and differentiates in 1% phos-
phomolybdic acid until alpha and beta cells are clear (5–30 minutes). Rinse
in 1% acetic acid. Dehydrate with 100% alcohol.

Mitochondria of islet cells are pale red to orange red, alpha-cell granules
are deep red, beta-cell granules are blue gray to black, and cytoplasm of delta
cells is pale gray or gray orange.

Ferner (Virchows Arch. f. path. Anat. 319:390, 1951) used the Gros-
Schultze silver method for the specific silvering of islet \( \alpha \) cells and of certain
cells other than the enterochromaffin cells in the gastric mucosa (dog). The
\( \beta \) granules are not blackened, but Heidenhain-Kultschitzky cells do blacken
along with Ferner's "silver cells" in the gastric mucosa. Ferner's silver cells
do not react by the Masson argentaffin method (but see Bencosme, infra),
with methenamine silver (p. 165), nor with the ferric ferricyanide reduction
test (p. 175), which demonstrates the argentaffin cells of the duct epithelium.

The Gros-Schultze method was originally devised for demonstration of
axones and neurofibrils in frozen sections. Material fixed for ten days to several months in neutral formalin is used. Sections are first impregnated in silver nitrate, and then in an ammoniacal silver-nitrate solution containing a little excess of ammonia to inhibit silvering of nuclei and connective tissue. Exact prescriptions vary. The original prescription called for formaldehyde treatment between the silver nitrate and the ammoniacal silver steps. Landau's variant for paraffin sections used formaldehyde before the nitrate and after the diammine silver, but not between.

Bencosme (Arch. Path. 53:96, 1952) prescribes fixation in 10% formalin or in the trichloracetic acid variant of Bouin's fluid (saturated picric 75; 40% formaldehyde 25:2% trichloracetic 5). After paraffin imbedding he employed Masson's argentaffin cell stain (p. 164), the Laidlaw reticulum method (p. 336), or Roger's silver method according to Van Campenhout (Proc. Soc. Exper. Biol. & Med. 30:617, 1933).

Bensley's anilin acid fuchsin-methyl green method gives green acinar cells with deeper green nuclei; red zymogen granules, basal filaments and mitochondria, deep red alpha granules; green beta granules. For the method see pp. 183-184.

The foregoing methods may be used also for hypophysis, parathyroid, and other glands.

Hypophysis

The alpha or acidophil, beta or basophil or "cyanophil," and chromophobe cells of the anterior lobe of the hypophysis may be distinguished by use of stains of the hematoxylin eosin type, notably with phloxine according to Mallory, with which the granules of the acidophil cells stain pink or red, and those of the basophils blue. Alpha granules stain red by the eosin B-azure A technics (pp. 118-119).

Laqueur (personal communication) finds the Gomori aldehyde fuchsin method (p. 364) useful for anterior hypophysis. Using a Mallory-Heidenhain counterstain with fast green FCF instead of anilin blue, the alpha granules are orange red; beta granules, violet to purple; delta granules, green to greenish blue; chief cell cytoplasm, pale gray green; connective tissue, green; elastica, violet; nucleoli and erythrocytes, red.

By the Mallory-Heidenhain and Masson (p. 351) technics alpha granules stain red; beta granules, bright blue; delta granules, lighter blue. After mercuric chloride formalin fixation (p. 41) the Masson method gives dark violet alpha granules.

Laqueur considers the periodic acid Schiff method (p. 123) to be one of the most reliable for beta granules and colloid (both red purple). Delta granules are not distinguished from beta granules, though delta cells usually contain less Schiff-positive material.

One of the simplest and most uniformly satisfactory methods for differentiating eosinophil and cyanophil cells is the buffered Mann stain devised
in this laboratory. No differentiation after staining is required. Lower pH levels increase the number of components stained blue; higher levels increase those stained pink.

Buffered Mann-Lillie Eosin Methyl Blue. (Internat. Assoc. Med. Museums Bull. 29:1-53, esp. pp. 3-4, 1949). Fix in Spuler's, Holley's, or similar fluid (p. 39); or perhaps better, in sodium acetate mercuric chloride formalin mixture (p. 41, par. 8). Neutral formalin is less satisfactory, and acid and alcoholic fixatives are to be avoided.

1. Deparaffinize thin paraffin sections and pass through graded alcohols to 80% alcohol.
2. Treat mercury-fixed material five minutes in 0.5% iodine in 70% alcohol.
3. Dip for one minute in 5% sodium thiosulfate.
4. Wash thoroughly in water.
5. Stain five minutes in Weigert's acid iron chloride hematoxylin (p. 81).
6. Wash in water.
7. Stain one hour at 55-60° C. (paraffin oven) in
   1% aqueous solution of eosin Y (C. I. No. 768) 8 cc.
   1% aqueous solution of methyl blue* 8 cc.
   (C. I. No. 706)
   0.1 M citric acid solution 1.4 cc.
   0.2 M disodium phosphate 0.6 cc.
   Distilled water 22 cc.
   pH 3.6

This solution keeps five or six weeks and may be used repeatedly.
8. Wash quickly in distilled water, dehydrate with 3 changes of acetone, and clear with one change of 50:50 acetone xylene mixture and two of pure xylene. Mount in synthetic resin.

Results. With staining at pH 3.6 hypophyseal eosinophils, erythrocytes, keratinized epithelium, and muscle stain bright pink to red. Hypophyseal chief cells, and epithelial and gland cytoplasms stain purplish or grayish pink. Collagen, reticulin, and hypophyseal cyanophils stain blue; and some mucins tinge pale bluish. Nuclei stain black with iron hematoxylin, or red without it. Granules of eosinophil leucocytes stain red or blue or perhaps both in the same cell.

McLetchie's method for certain basophil granules in the pituitary is perhaps worthy of trial. It depends on pretreatment with iodine and with phosphotungstic acid, and utilizes an acid dye. For this method Lendrum (J. Path. & Bact. 57:267, 270, 1945) first prepares the "carbacid fuchsin" thus: Mix 1 Gm. acid fuchsin (C. I. No. 692) with 0.4 Gm. melted phenol crystals; cool and dissolve in 10 cc. 95% alcohol. Grind 0.5 Gm. starch fine. Add

* Note especially that the blue dye is methyl blue (cotton blue, water blue), an acid dye of the sulfonated triaminotriphenylmethane series, and not methylene blue, a basic thiazin dye. Water soluble anilin blue (C. I. No. 707) may be substituted for it.
0.5 Gm. dextrin, and grind to a fine powder. Suspend in 100 cc. water by grinding with gradual addition of the water. Heat to 80° C., cool, filter, and add it to the acid fuchsin phenol alcohol mixture to make a total of 100 cc.

For this technic Lendrum prescribes a formalin mercuric chloride fixation and an alcohol, chloroform, paraffin imbedding sequence. For the staining of basement membranes by this technic, Lendrum prescribes exposing the paraffin sections face down over a shallow dish of formalin at 55–60° C. for two hours, before removal of paraffin. (For cell-granule staining this step is not necessary.) Sections are then deparaffinized and brought to water as usual. The usual iodine thiosulfate sequence for treatment of material fixed with mercuric chloride is not mentioned by Lendrum, and would not be required for removal of mercury precipitates, since a treatment with iodine is contained in the technic at a later point. I have found that in Weigert and Gram technics no special iodine treatment is required after mercurial fixation. The Weigert and Gram iodine treatments suffice. The technic is given by Lendrum as follows:

1. Stain 3–5 minutes with alum hematoxylin.
2. Wash briefly in water.
3. Stain 1–2 minutes in 1% fast green FCF in 0.5% acetic acid, varying the time in accordance with the desired intensity of the green cytoplasmic staining.
4. Rinse in water.
5. Treat for two minutes with Lugol’s iodine solution (p. 92).
6. Wash off with 95% alcohol.
7. Immerse in 2% alcoholic solution of phosphotungstic acid for two minutes.
8. Rinse in water.
9. Stain 2–6 minutes in “carbacid fuchsin.” Longer intervals give more staining of collagen and basement membranes. Lendrum prescribes microscopic control, but gives no definite criteria at this point.
10. Rinse in water, dehydrate, clear, and mount.

The cell granules of apocrine cells and of pituitary basophils are stained a blackish red. Even without the formalin vapor treatment there is some staining of collagen and basement membranes; but with that treatment they are stained intensely red. The apocrine-cell granules are also iron positive with ferrocyanide, and the same cells also contain fat droplets.

Pearse’s (J. Path. & Bact. 61:195, 1949) combined periodic acid Schiff and granule stain for hypophysis: Fix in Helly, in Zenker, in half-saturated HgCl₂ in formalin saline, or in formalin saline. Paraffin sections.

1. Iodine and thiosulfate sequence to remove Hg precipitates.
2. Bring to 70% alcohol.
3. Treat 5 minutes in 0.8 Gm. H$_5$IO$_6$, 20 cc. distilled water, 10 cc. 0.2 M sodium acetate solution and 70 cc. ethyl alcohol.
4. Rinse in 70% alcohol, wash one minute in 1 Gm. KI, 1 Gm. Na$_2$S$_2$O$_5$·5H$_2$O, 20 cc. H$_2$O, 30 cc. ethyl alcohol and 0.5 cc. 2N HCl.
5. Rinse in 70% alcohol and treat 15–45 minutes in fuchsin sulfite solution (Schiff reagent).
7. Stain 30 seconds in 0.5% celestin blue in 5% iron alum and then 30 seconds or more in Mayer’s hemalum (no intervening wash).
8. Differentiate quickly in 2% acid alcohol and blue in water.
9. Optionally stain α granules in 2% orange G in 5% phosphotungstic acid for 5–10 seconds (orange II, C. I. No. 151, gives deeper color).
10. Wash in running water until a yellow tinge is just visible in acidophil areas (or use microscopic control).
11. Alcohols, alcohol + xylene, xylenes, polystyrene (DPX).

Results. Colloid of stalk and vesicles, magenta; cyanophil, “basiphil,” (or β) granules, dark red; acidophil (or α) granules, orange; erythrocytes, orange; nuclei, blue black.

Pearse also found that the cyanophil cells were Gram positive, and Foster and Wilson (Quart. J. Microscop. Sci. 93:142, 1952) use a variant of the Gram stain for differential coloration of cyanophil cells, thus:

Hydrate and stain 2–3 minutes in 1% aqueous crystal violet. Rinse in water and cover with Lugol’s (Gram’s) iodine for 2–3 minutes. Blot, and differentiate in clove oil. Rinse in xylene, and mount in Canada balsam. Beta granules are dark violet.

I find that 30 seconds in Weigert’s iodine is quite adequate and presume that the authors meant Gram’s solution (p. 92) rather than the strong pharmacopeial Lugol’s solution. As usual with oil-differentiated Gram stains, collagen tends to remain violet. Differentiation should be carried to the point where cell nuclei are fully decolorized.

Use of the usual aniline xylene mixture for differentiation, as in the routine Gram-Weigert method (p. 372) also gives excellent results. I have used a Feulgen nucleal reaction (p. 132) before a Gram-Weigert stain. The red-purple nuclei contrast beautifully with the violet granules of the cyanophil cells.

Another method for staining the eosinophil granules of anterior hypophysis cells as well as those of eosinophil leukocytes is a modified Weigert myelin stain, derived from my variant of the Weil method. The success of this procedure indicates the presence of a phospholipid component of these granules. Tissue should be fixed in an aqueous fixative containing formaldehyde. Chromation is not essential.
Lillie’s Eosinophil Myeloid Stain.

1. Bring paraffin sections to 80% alcohol as usual.
2. Stain 40 minutes at 58-60° C. in a fresh mixture of equal volumes of 4% aqueous iron alum solution and 1% fresh alcoholic hematoxylin.
3. Wash five minutes in running water.
4. Differentiate 15 minutes in 0.5% iron alum.
5. Wash 3–5 minutes in running water.
6. Differentiate ten minutes in half-strength Weigert’s borax ferricyanide mixture:
   - Potassium ferricyanide: 1.25 Gm.
   - Borax: 0.5 Gm.
   - Distilled water: 100 cc.
7. Wash 3–5 minutes in running water.
8. Stain five minutes in 0.1% safranin 0 in 0.1% acetic acid.
9. Dehydrate, and clear through the acetone xylene sequence and mount in synthetic resin.

Results. Hypophyseal alpha granules, black; cyanophil granules, gray; eosinophil leucocyte granules, black; erythrocytes, gray yellow, brown, or black; nuclei, red; and cytoplasm and muscle, pink to unstained.

Since decolorization is not always uniform, the best results are attained by watching microscopically during the borax ferricyanide step, and interrupting at a point where erythrocytes are still black or dark gray and nuclei are decolorized. Differentiation may be retarded by adding an equal volume of water after the first five minutes.

Testis

The interstitial cells of the testis contain lipoids, pigment, crystalloids, and sometimes glycogen. The fats include cholesterol esters at times. The pigment is a lipofuscin and gives a brown fluorescence in ultraviolet light. The crystalloids are rod-shaped bodies with rounded or pointed ends. They are singly refractile, dissolve in a pepsin hydrochloric acid mixture, swell in 10% potassium hydroxide, and are insoluble in 10% mineral acids and in fat solvents.

Under certain experimental conditions the lipofuscin pigment of the interstitial cells may be greatly increased in amount and assume the characteristics of the ceroid pigments: acid-fastness, staining with oil-soluble dyes after paraffin imbedding (p. 320), reactivity to the periodic acid (p. 123) and peracetic acid (p. 310) Schiff methods and to the ferric ferricyanide reduction test (p. 175). See also pp. 249, 253.

Peptic Gland Chief Cell Zymogen Granules

These may be demonstrated in the gastric mucosa of rats and rabbits after fixation with such chromate formalin mixtures as Spuler’s, Helly’s,
Orth’s, and Kose’s fluids by staining in 0.05 to 0.1% thionin buffered with 0.01 M acetate buffer to pH 4–5 for one or more minutes. The granules appear as discrete deep blue or greenish blue bodies when stained at pH 5, contrasting with the deep violet blue of the basal cytoplasm. At pH 3 they fail to stain.

The granules are destroyed by acetic acid fixatives and are poorly preserved by neutral formalin. Acid alcoholic fixatives destroy them.

The azure A–eosin B technic (p. 118) stains them faintly pink in rats and rabbits, and red in guinea pigs. In guinea pigs the same cells may contain concurrently present retiform periodic acid Schiff-positive material, but the granules themselves were recorded as periodic acid Schiff negative in the three species mentioned. The eosinophilic granules resist barley-malt ribonuclease digestion (p. 139), which destroys the basophilia of the basal cytoplasm.

**Paneth Cells**

These cells contain conspicuous eosinophilic granules of variable size. The granules may lie in clear vacuolar spaces in the cytoplasm between the nucleus and the gland lumen. Like the zymogen granules of the pancreas and the salivary glands, these eosinophilic granules are destroyed by acetic acid fixatives such as aqueous and alcoholic acetic formalin, and Bouin’s, Gendre’s, and Carnoy’s fluids. The acetic bichromate fluids, such as Tellyesniczky’s and Zenker’s, may preserve them entirely, in part, or not at all. They are well preserved by buffered neutral formalin or mercuric chloride formalin (p. 41), by Orth’s, Kose’s, and Möller’s bichromate formalin mixtures, and by Spuler’s formalin Zenker fluid.

They stain red by the Nocht azure A–eosin B method (p. 118) at pH 3.75–4.1. They resist malt diastase (ribonuclease) and pancreatic ribonuclease digestions, which render them more conspicuous by destroying the normal basophilia of the cytoplasm. They are sometimes Gram positive by the Gram-Weigert technic (variant 4b, p. 372), especially in man, sometimes in guinea pigs, usually not in rabbits or rats.

They often stain red purple by the periodic acid Schiff procedure (p. 123). Positive reactions have been observed in man, mouse, guinea pig, rat, and rabbit, as well as negative reactions in the first three. Application of a picric acid counterstain changes the color of Paneth granules to orange, thereby permitting their ready distinction from the still red-purple mucigen granules.

The mucigen granules and mucin stain also by the Bauer method (p. 124), as well as by safranin, thionin, and the like (pp. 284–286). The azure A–eosin B method leaves mucin unstained or light blue green.

**Hair, Keratin, and Keratohyalin**

Hair cortex is often fully or partly Gram positive, especially when Weigert technics (p. 372) are used. Keratohyalin also retains the violet; keratin less so. In Ziehl-Neelsen technics (p. 380) hair cortex is quite strongly acid-fast.
Hair cortex colors red purple with Schiff reagent on direct exposure of a few hours (p. 313). It becomes Schiff positive with ten-minute exposure after peracetic or performic acid oxidation, but not after periodic acid oxidation. Bromination prevents the peracetic acid Schiff reaction but renders the periodic acid Schiff reaction positive, so that even a ten-minute Schiff-reagent bath yields a positive reaction (p. 128).

Keratohyalin and keratin are periodic acid and peracetic acid Schiff negative.

Keratohyalin often colors intensely with progressive alum hematoxylin and iron hematoxylin stains, as well as moderately with basic anilin dyes. With iron hematoxylin myelin procedures such as our "eosinophil myelin" stain (p. 194), differentiated to the point where cell nuclei are decolorized but erythrocytes are still black, keratohyalin varies from black to red; keratin, from light to dark brown; and hair cortex, from black near the root to blue or light blue gray distally.

According to Unna eleidin may be differentially stained by a picric acid nigrosin sequence technic. Romeis directs as follows: Stain 5 minutes in saturated aqueous picric acid solution. (2) Rinse in water. (3) Stain 1 minute in 1% aqueous nigrosin (C. I. No. 865). (4) Wash in water, alcohol, oil, balsam. Results: Eleidin blue black, keratin bright yellow.

Unlike keratohyalin, trichohyalin does not stain with hematoxylin, but stains vividly with acid dyes. The azure A–eosin B technic (p. 118) displays these granules in bright red against the lightly to deeply basophilic cytoplasm of the hair follicle.

Near the root zone, hair cortex gives strong sulfhydryl reactions in appropriately fixed material. The ferric ferricyanide reduction test (p. 175) is positive after trichloracetic alcohol fixation, but negative after mercurial fixations or post-mordanting (–SH blockade). Cutaneous keratin and sometimes keratohyalin also color blue with this test, and in these structures the reaction is also positive in our hands after mercurial fixations and blockade treatments.

With the Barnett-Seligman dinaphthol disulfide method for sulfhydryl (p. 179), rat hair cortex colors blue at the zone of keratinization, red nearer the root and remains unstained distally. Epithelial cells in root sheaths, hair bulbs, and stratum germinativum of the epidermis color red; those of the stratum corneum, pink. The reaction of keratohyalin was not noted.

The sodium plumbite reaction for reactive sulfur in hair dates back to Salkowski's textbook of 1893, Physiologisches Praktikum, p. 94. Unna (Biochemie der Haut, Jena, Fischer, 1913) directs: Dissolve 0.5 Gm. lead acetate in 10 cc. water and add 10% NaOH until the lead hydroxide at first precipitated is redissolved as Na₂PbO₂. Treat celloidin sections of alcohol-fixed skin (plantar) with this reagent until the keratin is well blackened. This method demonstrates hard and soft keratin and sites chemically containing cystine, as well as the more easily reactive cysteine and glutathione.
Another group of methods for cystine is based on the fact that peracetic and performic acids convert cystine quantitatively to cysteic acid. Bromine in carbon tetrachloride solution (1 cc.:39 cc., one hour) and aqueous potassium permanganate solution (0.5%, 30 minutes followed by 5 minutes in 1% oxalic acid or bisulfite) are somewhat less effective; and periodic acid, which was recommended by Dempsey, Singer, and Wislocki (Stain Technol. 25:73, 1950), has been ineffective in our hands when elevated temperatures are not employed. In accordance with the strongly acid character of cysteic acid, keratin thus oxidized takes up thionin even from a solution in 0.1 N hydrochloric acid. In unoxidized skin sections only the mast cells stained with this solution.

The Peracetic Acid–Azure Eosin Method for Hair Keratin.

1. Paraffin sections of skin fixed in the usual fixatives are deparaffinized and hydrated through xylene and descending alcohols as usual.
2. Oxidize for 10–120 minutes in peracetic acid mixture prepared 1–10 days previously (p. 310).
3. Wash five minutes in running water.
4. Stain one hour in azure A–eosin B according to technic on p. 118; or for 30 minutes to overnight in thionin or methylene blue in 0.1 N HCl (pH 1.2) or in 0.1 M sodium citrate HCl buffer of pH 2, 3, or 4. The dye concentration should be 1/2000 for half-hour staining and may be as low as 1/100,000 for overnight.
5. Dehydrate with acetone, clear in acetone + xylene and two changes of xylene. Mount in synthetic resin.

Results. Hair cortex and cuticle—dark violet to black in dermal and free portions, grading to greenish blue toward the root in hypodermal portion; stratum corneum—moderate violet; melanin granules—dark green to dark violet; nuclei—blue violet; keratohyalin—red to purple; muscle—pink; connective tissue fibers—pale pink; cartilage matrix—violet to purple; mast-cell granules—dark purple or dark violet.

Methylation (p. 163) in 0.1 N HCl methanol at 60° C. for 24 hours, done after the peracetic acid step, abolishes all basophilia except that of the keratins and melanin. The basophilia of hair cortex, engendered by peracetic acid, is destroyed by methylation at 60° C. for about 3–6 days. Even 7-day methylation at 60° C. is without effect on the basophilia of hair cortex when applied before the peracetic acid oxidation. Since methylation acts to destroy basophilia by esterifying open acid groups, and no cysteic acid is present until after oxidation of the disulfide, this behavior is to be expected.

Russell Bodies

Russell bodies are colorless spheres, polyhedra, rods, or ovoids occurring singly and in clusters in the cytoplasm of cells of the plasma cell type, rang-
ing upward in size to spheres twice the diameter of surrounding plasma cells. In fixed tissue they are strongly oxyphil on staining with mixtures of the azure-eosin type (pp. 116–119) but, according to Kindred (Stain Technol. 10:7, 1935) they differ from hemoglobin in not staining with eosin at pH levels of 6.2–6.6 when in the unfixed state.

Russell (Ehrlich's Encyklopädie) prescribed Müller fixation, and stained 10–30 minutes in saturated fuchsin solution in 2% aqueous phenol, then washed 3–5 minutes in water and 30 seconds in 100% alcohol, counterstained in 1% iodine green (C. I. No. 686) in 2% aqueous phenol for five minutes, dehydrated with 100% alcohol, cleared in xylene, and mounted in balsam. Nuclei stained green; the fuchsin bodies, red. Klien (ibid.) prestained with alum hematoxylin, stained in warm carbol fuchsin, and differentiated in a strong fluorescein solution in alcohol. The usual alcohol, xylene, balsam sequence followed. Schmorl quotes Russell’s original method without change; Cowdry refers to Kindred’s paper (above); other recent authors make no reference to these bodies. Askanazy, writing in Aschoff’s Pathologische Anatomie (Jena, Fischer, 1936), notes that they are Gram positive. This is true with the Gram-Weigert method, but with acetone differentiation they are Gram negative. With hemalum and oil red O they do not take the fat stain in frozen sections. On application of the Weigert myelin technic they become deep gray while red corpuscles are still black; and retain some gray for a little while after the erythrocytes decolorize. The periodic acid leuco-fuchsin technic (p. 123) colors them red, with or without antecedent diastate digestion (p. 275); but after oxidation with chromic anhydride (p. 124) or with 1% potassium permanganate, only a pink or gray-pink color is produced by the leuco-fuchsin. They do not blacken with diammine silver (pp. 333–344) and are iron negative.
Chapter 11

**Enzymes**

The list of localizable enzymes has greatly expanded since the last edition of this book. Some of the procedures still depend on the use of frozen sections of unfixed tissue or on the use of the freezing-drying technic advocated by Gersh. An increasing number are demonstrable in paraffin sections of tissues “fixed” or perhaps better “dehydrated” by non-denaturant fluids such as acetone and in some instances alcohol.

The demonstration of enzymes *in situ* depends on their action on a specific substrate in the presence of other substances with which one of the decomposition products resulting from enzyme action will form an insoluble deposit at the site of enzyme action. If this deposit is not already colored, it is needful to render it visible by chemical manipulation.

Transfer of deposition to surfaces of sections facing others with active areas has been observed, even when the recipient section has been chemically or physically inactivated. This we avoid by not placing sections back to back in the slots of a Coplin jar. Instead, a jar is filled by first inserting five sections, all facing the operator, in the five slots of a Coplin jar, then inserting four more between them, going diagonally across the spaces between the first five sections, and parallel to each other. Thus, triangular spaces are left open alternately on each side, and each section faces the back of the slide in front of it.

The section-preparation technics fall naturally into five general classes.

1. The tetrazolium-formazan reaction appears to constitute a special class, in that it may be carried out in moist-chamber preparations of scrapings, aspirates, and the like, and on thin slices of tissue, but cannot be used on frozen sections. The failure with frozen sections may possibly be due to the leaching out of a soluble substrate such as systeine or succinic acid. It appears that tetrazolium does not serve as a substrate itself but as the reduction in-
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dicator of the desulfuration of cystein or the dehydrogenation of succinates and similar compounds.

2. Enzymes of the cytochrome c type tolerate freezing, and frozen sections of unfixed tissues can be prepared; but chemical fixations and the paraffin imbedding which follows the Altmann-Gersh freezing and in vacuo dessication procedure are poorly tolerated.

3. Esterases and lipases tolerate dehydration with cold acetone and brief paraffin infiltration.

4. Alkaline phosphatase is well preserved by fixation in 70–80% ethyl or isopropyl alcohol at about 20° C. Cold fixation with acetone or alcohol often gives very poor histologic detail, especially if brief. I have had atrocious results from 24–48-hour fixation in alcohol or acetone at −20° C.; but when storage at this temperature was prolonged to 10–14 days, histologic detail was much better, and alkaline phosphatase activity was apparently unimpaired.

5. Leukocyte peroxidase, M-nadi oxidase, dopamelanase, hemoglobin peroxidase, and a few other hardy enzymes will resist even 24-hour formalin fixation and routine paraffin imbedding.

Seligman, Chauncey, and Nachlas (Stain Technol. 26:19, 1951) record that fixation at 4° C. in phosphate buffered 10% formalin (p. 34) preserves some enzyme activities quite well, notably β-glucuronidase, sulfatase, esterase, and some acid and alkaline phosphatases. Paraffin imbedding is quite destructive of these activities; hence the use of frozen sections would appear to be indicated. Distribution patterns of the phosphatases with these methods are different from those seen after acetone paraffin technics.

Phosphatases

The question of multiplicity or identity of demonstrable alkaline and acid phosphatases is still moot. Gomori (Proc. Soc. Exper. Biol. & Med. 70:7, 1949) tried 19 substrates in the acid, neutral, and alkaline ranges on paraffin sections of a variety of tissues fixed with alcohol or acetone, and concluded that identical patterns were obtained with all substrates including ribonucleic and thymonucleic acids, except for p-chloranilidodiphosphate in the acid range. Friedenwald (Bull. Johns Hopkins Hosp. 84:568, 1949) tried a variety of substrates in the alkaline range on frozen sections of unfixed kidney and cornea. He concluded that nucleic acid phosphatases differed from glycerophosphatase not only in being diester phosphatases, but also in requiring the presence of .001% glucose and .0003 M potassium chloride (.0022%) for optimal action. Friedenwald agreed with Krugelis (Biol. Bull. 90:220, 1946) in stating that with depolymerized desoxyribonucleic acid as substrate, phosphate was deposited only in cell nuclei. De Nicola (Quart. J. Microscop. Sci. 90:391, 1949) reported that phosphate was deposited in both nuclei and cytoplasm from both ribonucleic acid and desoxyribonucleic acid. Moog (Science 103:144, 1946) considered that adenosinetriphosphatase was prob-
**Table 18**

**Preferred Fixation and Section Methods for Enzyme Localization Studies**

<table>
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<td>Phosphamidase</td>
<td>Dopa oxidase</td>
<td>Cystein desulfurase</td>
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<td>Urose</td>
<td>Esterase†</td>
<td>Cytochrome c oxidase</td>
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<td>Zymohexase</td>
<td>Glucuronidase</td>
<td>Esterase</td>
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<tr>
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HISTOPATHOLOGIC TECHNIC

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ably largely destroyed by cold acetone fixation, and the activity observed by Glick (Science 102:429, 1946; 103:599, 1946) is probably assignable to nonspecific phosphomonoesterase.

Friedenwald used 0.1% sodium glycerophosphate, adenosine di- and triphosphates, hexose diphosphate, ribonucleic acid, and a desoxyribonucleic acid depolymerized by boiling in acid or by desoxyribonuclease, dissolved in a 0.1 M barbiturate buffer (p. 454) of pH 8.5–9.5, containing .03 M CaCl₂ (0.333% of the anhydrous salt), incubated 3–18 hours and visualized by the usual cobalt, ammonium sulfide sequence.

**Alkaline Phosphatase**

The demonstration of alkaline phosphatase depends on the enzymatic decomposition of organic phosphates in the presence of calcium ions to form insoluble calcium phosphate *in situ*. This deposit was originally made evident by application of the von Kossa silver nitrate technic, but now more commonly by conversion to cobalt phosphate with a soluble cobaltous salt and demonstration of the cobalt as sulfide by application of ammonium sulfide. For demonstration of acid phosphatase a soluble lead salt is included in the substrate, and the insoluble lead phosphate which appears at sites of action is visualized by conversion to sulfide.

Cobalt sulfide deposits in sections gradually disappear when they are mounted in certain of the synthetic resins such as Clarite, old Permount, polystyrene, and the Euparal-Diaphane type mixtures. Preservation is best in natural Canada balsam and in β-pinene or piccolyte resins (Bioloid, Permount, HSR, etc.) and in ester gums. Preparations mounted in Apåthy’s gum syrup, in glycerol gelatin, Arlex gelatin, syrup, and the like fade rapidly. Faded preparations may be fully restored by demounting, hydrating, reimmersing in ammonium sulfide, and then remounting as before.

Yellow ammonium sulfide gradually deteriorates on the laboratory shelf. It is desirable to get a fresh bottle about once a year.

Sodium sulfide, in equivalent concentration, or hydrogen sulfide water may be used instead, and the pH adjusted to near neutrality. Sodium sulfide is deliquescent, and crystals should be drained and dried before weighing. Or use 2.5 cc. of saturated aqueous (about 20%) sodium sulfide and about 0.56 cc. glacial acetic acid and 97 cc. distilled water to give pH 6.8–7.5.

We currently use the following modification of the Gomori technic for alkaline phosphatase, both for soft tissues and for decalcified bone (p. 431). In it we have more definitely prescribed times and concentrations of reagents.

**The Alkaline Phosphatase Technic of Gomori, with Modifications.**

1. **Fix** 24 hours or less at 18–25° C. in 70–80% ethyl or isopropyl alcohol.
2. **Dehydrate** with 95% and 99–100% concentrations of the same alcohol.
3. Dealcoholize with gasoline or petroleum ether (B. P. 35–60° C.) and infiltrate 15 minutes in 56° paraffin at 58–60° C., under 10–15 mm. pressure in vacuo.

3a. (alternate) Or dealcoholize with isopentane (B. P. 28° C.), ethyl ether (B. P. 37° C.), or special petroleum ether (B. P. 20–40° C.), enclose in a perforated metal capsule or wire gauze to sink the blocks, and infiltrate 20–30 minutes in paraffin, without vacuum. Imbed and section at 5–10μ. Float sections onto slides, and dry without heating.

5. Deparaffinize and hydrate as usual. Place sections in Coplin jars so that no two sections face each other.

6. Preheat working substrate and buffer control solutions to 37° C. Incubate sections at 37° C. for one hour. Duplicate sections may be incubated for longer or shorter intervals in accordance with results. Thirty minutes is often adequate on fresh animal material.

**Buffered Solvent.**

- Sodium barbital: 6.1 Gm.
- Calcium chloride (CaCl₂): 1.2 Gm.
- Magnesium sulfate (MgSO₄·7H₂O): 0.5 Gm.
- Distilled water: 1000 cc.

Store in refrigerator.

**Stock substrate.** 1% mixed α and β or pure β Na glycerophosphate in distilled water. Other stock substrates may be substituted. These also are stored in the refrigerator.

**Working substrate mixture.** 30 cc. stock substrate + 50 cc. buffer solvent. Check pH and adjust if necessary with 0.1 N HCl or 0.1 M Na barbital to pH 9.0–9.5.

**Buffer control:** Buffer solvent 50 cc. + 30 cc. distilled water.

7. Wash five minutes in water.

8. Transfer to 2% cobalt acetate (or nitrate or chloride) for five minutes.

9. Wash in three to five changes of distilled water in six or eight minutes.

10. Transfer to a fresh 1% solution of yellow ammonium sulfide for 30 seconds (or 0.5% Na₂S buffered to pH 7, see p. 202).

11. Wash five minutes in running water.

12. Counterstain, if desired, for two minutes in 0.1% safranin in 0.1% acetic acid.

13. Rinse quickly in 2% acetic acid.

14. Dehydrate quickly with two changes each of 95% and 100% alcohol, 1% alcohol + xylene and clear in two changes of xylene. Mount preferably in Canada balsam, ester gum, or β-pinene resin (p. 104).

At Step 8 Gomori originally transferred to 5% silver nitrate and left in bright, diffuse daylight for 15–60 minutes, thereafter completing the von
Kóssa technic (p. 264) and demonstrating activity sites with reduced silver. At Step 11 various other counterstains can be used, provided oxidants which might bleach the sulfide deposits are avoided.

Molnar (Stain Technol. 27:221, 1952) suggests replacing the cobalt bath (Step 8) with a 2–5 minute bath in 2% lead nitrate solution; and the ammonium sulfide treatment (Step 10) with a 40-minute bath in 0.5% aqueous sodium or potassium rhodizonate solution. Dark brown lead rhodizonate deposits are formed, and hemosiderin does not react.

Gomori (letter, 1953) finds the rhodizonate color no better than the sulfide, and suggests instead the use of a very dilute solution of gallamin blue, which colors lead deposits selectively and intensely. Other counterstains may follow the gallamin blue.

The concentration of magnesium in the foregoing technic (0.3125% MgSO₄·7H₂O or 0.00126 M) compares with Lorch’s (0.01 M MgCl₂), Seligman’s (0.1% MgSO·7H₂O), Friedenwald’s (0.01 M MgSO₄·7H₂O to none), and Gomori’s varying amounts. Friedenwald stated specifically that the magnesium ion was unnecessary.

Goetsch, Reynolds, and Bunting (Proc. Soc. Exper. Biol. & Med. 80:71, 1952) report that nuclear staining in Gomori alkaline phosphatase technics with glycerophosphate substrates may be avoided by conducting the incubation on undeparaffinized sections. Sharper localization of enzyme activity is obtained.

Lorch encountered considerable difficulty with the Menten, Junge, and Green (J. Biol. Chem. 153:471, 1944) betanaphthol phosphatase method. Incubation had to be carried out at low temperatures to avoid disintegration of the diazotized alphanaphthylamine. This rendered the enzyme less active, and in any case the substrate had to be renewed in two hours or less. Sensitivity of the method also seemed to be low.

Since most other recent workers have also adhered to the Gomori method and variants using other organic phosphates, visualizing the phosphate liberated with silver or cobalt, I have omitted from the present edition detailed consideration of this method.

In brief, a substrate composed of calcium betanaphthol phosphate; freshly diazotized alphanaphthylamine and sodium hydroxide to give a pH level of 7.4–9.4. Betanaphthol was liberated by the enzyme and coupled with the diazotized alphanaphthylamine to yield the brilliant red naphthylamine Bordeaux.

Manheimer and Seligman (J. Nat. Cancer Inst. 9:181, 1948) have overcome part of the objections to this naphthylamine Bordeaux process by preparing a stabilized diazonium salt to use in place of the freshly diazotized α-naphthylamine, by adding to the last its molecular equivalent of 1,5-naphthalene disulfonic acid. This salt can be stored for months in the cold.

The substrate is prepared by dissolving in 84 cc. distilled water, 15 cc. Michaelis’s veronal buffer of pH 9.4 (p. 454), and 1 cc. 1% magnesium
sulfate (MgSO₄·7H₂O), 50 mg. each of calcium β-naphthol phosphate and α-naphthyl diazonium naphthalene 1,5-disulfonate.* The temperature is kept below 10°C and the sections are placed in the substrate at once, even before all of the calcium β-naphthol phosphate is dissolved. Deposition of dye may be evident in a minute and reaches a maximum in 20 minutes. Sections are then washed in water and counterstained lightly in dilute alum hematoxylin and mounted in glycerol gelatin or similar aqueous medium.

Thin slices of undecalcified bone can be treated en bloc, then fixed in formalin, decalcified, and sectioned on a freezing microtome. The sections are then lightly counterstained with alum hematoxylin and mounted as before in syrup or gelatin media.

Seligman writes me (March 1951) that the commercially available stable diazonium salt diazo blue B, a stabilized tetrazotized diorthoanisidine, may be substituted for the α-naphthyl diazonium naphthalene 1,5-disulfonate in the same amount. This gives a dark blue color.

**Nucleotidase**

Wachstein and Meisel (Science 115:652, 1952) used adenosine phosphoric acid as a substrate in a modified Gomori alkaline phosphatase procedure to demonstrate nucleotidase activity in cell nuclei. Fix 24 hours in acetone at 0-5°C, complete dehydration with one day in 100% alcohol or cedar oil, clear in two changes of xylene of one hour each, imbed in paraffin (preferably in vacuo, 15 minutes) and section at 5μ. Deparaffinize, hydrate, and incubate at 37°C in the following substrate:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
<th>Amount (mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine-5-phosphoric acid†</td>
<td>1.44 × 10⁻³ M</td>
<td>50 mg.</td>
</tr>
<tr>
<td>Calcium chloride, anhydrous</td>
<td>8.0 × 10⁻³ M</td>
<td>888 mg.</td>
</tr>
<tr>
<td>Magnesium sulfate (MgSO₄·7H₂O)</td>
<td>1 × 10⁻² M</td>
<td>246.5 mg.</td>
</tr>
<tr>
<td>2-methyl-2-amino-1,3-propanediol buffer</td>
<td>2 × 10⁻¹ M</td>
<td>100 cc. 0.2 M</td>
</tr>
</tbody>
</table>

pH 8

After varying incubation periods, 10-60 minutes for paraffin sections, 2-4 minutes for frozen sections of fresh unfixed material or of material fixed in cold acetone, visualize by treatment with 2% cobalt acetate (five minutes) and 1% ammonium sulfide (30 seconds) as usual (p. 203). Dehydrate and mount in Canada balsam.

Optimal incubation pH is about 8.0, and may be attained also with trihydroxymethylaminomethane + maleate buffer (p. 453) at the same 0.2 M concentration.

Pearse and Reis (Biochem. J. 50:534, 1952) use an essentially similar tech-

* Available from Dajac Laboratories, 3430 West Henderson St., Chicago 18, Ill.
† Obtainable from Sigma Chemical Co., St. Louis, Mo. and from Schwarz Laboratories, New York.
nic. They use one volume 0.04 M adenylic acid, one volume 12% calcium nitrate (M. W. 164), one volume 2% magnesium chloride (M. W. 95), and five volumes 0.1 M veronal sodium buffer of pH 7.5 (p. 454), incubate 3–18 hours, and visualize by the silver nitrate process (p. 203). The activity seems less than with the Wachstein method, in spite of the higher concentration of adenylic acid (173 mg./100 cc.) in the final mixture. Short vacuum infiltration in paraffin would probably help.

**Acid Phosphatase**

Gomori (Arch. Path. 32:189, 1941) fixed 2-mm. blocks in anhydrous acetone for 24 hours at 5° to −5° C., and dehydrated over a period of 24 hours in three further changes of acetone at room temperature. The alcohol hardening suggested in place of the second day in acetone by Wolf, Kabat, and Newman (Am. J. Path. 19:423, 1943) seems inadvisable in view of the quite pronounced inactivating effect of 100% alcohol recorded by McDonald (Quart. J. Microscop. Sci. 91:315, 1950). She found that 4% formaldehyde in 1% saline or 1% acetic acid apparently had as little inactivating effect as acetone with a 24-hour exposure, and consequently tried perfusion with a 1% acetic, 4% formaldehyde solution for fixation. In this material it was necessary to prolong the incubation in substrate from four hours to overnight to obtain the maximal reaction. Histologic preservation, however, was much improved. (See also p. 200.)

After acetone dehydration Gomori prescribed cedar-oil clearing followed by benzene, or direct benzene clearing. Wolf et al. used 24 hours in toluene. Gomori stated that paraffin infiltration should not exceed three hours, and by 1947 preferred a 15–30 minute infiltration in vacuo. The procedure of acetone dehydration followed by a 50:50 mixture of acetone and a petroleum ether—say 30 minutes—petroleum ether, two changes of 30 minutes each, and paraffin infiltration as in Step 3 of the alkaline phosphatase method (p. 203)—would seem applicable and probably advantageous.

Gomori (Arch. Path. 32:189, 1941) originally used a substrate of lead nitrate and sodium α-glycerophosphate buffered with acetates to pH levels from 4.7 to 5.3 at 0.05 M final dilution.

Gomori (Stain Technol. 25:81, 1950) now prefers to keep two stock solutions: a 0.1 M sodium glycerophosphate (about 3%) and a 0.05 M acetate buffer of pH 5 (p. 450) containing 1.2 Gm. lead nitrate (Pb(NO₃)₂) per liter. The stock solutions are stable at 0–5° C. For use, one part of glycerophosphate solution is mixed with ten parts of the buffered lead nitrate solution. The final concentration of lead is about 0.0035 M in this mixture, though Gomori states that 0.004 M is the optimal level, and this would require 1.457 Gm. lead nitrate per liter in the acetate buffer solution.

Before using, the mixture is warmed gently, and a precipitate of lead glycerophosphate settles out. The mixture is filtered and the clear filtrate used. McDonald (Quart. Jour. Microscop. Sci. 91:315, 1950) considered
0.001 M lead nitrate as about the optimal concentration for motor neurons.

Newman, Kabat, and Wolf (Am. J. Path. 26:489, 1950) have pointed out that a more or less widespread lead impregnation of the tissues occurs (even in the absence of the phosphate ester) at pH levels between 5.3 and 7.0; it is greatest at 5.6. They regard pH 4.7 as the optimum for acid phosphatase action, and report that the nonspecific lead impregnation is occasional and variable at this level. The specific enzyme action is inhibited by 0.01 M sodium fluoride, by heating sections in distilled water to 80° C. for 10 minutes, or by treatment with 5% trichloracetic acid for 10 minutes. The nonspecific lead impregnation is unchanged by sodium fluoride, but is destroyed by heating and by trichloracetic acid; while the deposition of lead salts on preformed calcium phosphate and carbonate depots is unaffected by fluoride or heat, but is prevented by trichloracetic acid. Gomori (J. Lab. & Clin. Med. 35:802, 1950) uses controls inactivated by 0.1 N mineral acid or by Gram’s iodine.

This nonspecific lead action accounts for the axone staining reported by Lassek (Stain Technol. 22:133, 1947) as occurring also after formalin fixation and other enzyme-destroying procedures. Alcohol fixation inactivates acid phosphatase, but permits the nonspecific lead effect.

Make two stock solutions, which may be stored in the refrigerator (4° C.):

Stock buffer: M/20 acetic acid–sodium acetate buffer of pH 5.0 (Gomori 1950) or 4.7 (McDonald 1950), 900 cc.
Dissolve in this lead nitrate 331 mg., 662 mg., 993 mg., or 1.324 gm. lead nitrate for 0.001, 0.002, 0.003 and 0.004 M concentration in the final mixture.

Stock substrate: Mixed α and β sodium glycerophosphate, 0.1 M (3.06%).
Working substrate. Mix 5 cc. of stock substrate and 45 cc. of buffer.
Buffer control. Add 5 cc. water to 45 cc. stock buffer.

Technic of Acid Phosphatase Method:

1. Bring 6–10 μ sections to water as usual.
2. Rinse in distilled water and
3. Incubate in the lead nitrate sodium glycerophosphate solution at 37° C. for 1½–24 hours. Prostate is adequately stained in 1½ hours; other tissues require at least 6; and Wachstein used 24 hours as a matter of routine, and 48 hours if needed.
4. Gomori then rinsed in distilled water.
5. Then in 2–3% acetic acid and
6. Again thoroughly in distilled water.
7. Then immerse one minute (Wolf, two minutes) in 1:40 or 1:50 dilution of yellow ammonium sulfide.
8. Wash thoroughly in tap water. Counterstain as desired. Wolf used hematoxylin and eosin; Wachstein, basic fuchsin.
Results. Sites of acid phosphatase activity are shown by dark brown deposits of lead sulfide.

At Step 7, one may adopt Molnar’s (Stain Technol. 27:221, 1952) replacement of the ammonium sulfide by a 40-minute bath in 0.5% aqueous sodium or potassium rhodizonate. A brown-black deposit of lead rhodizonate is formed.

Götsch and Reynolds (Stain Technol. 26:145, 1951) strongly recommend that sections should not be deparaffinized until after the reaction has been completed. Shifting of localization and over-all decrease of activity are prevented by deferring deparaffinization until after visualization of the deposits (and counterstaining; cf. p. 94).

Seligman and Manheimer (J. Nat. Cancer Inst. 9:427, 1949) reported a method for acid phosphatase which utilized α-naphthyl phosphate as substrate, and a stable anthraquinone-1-diazonium chloride as coupling agent for the liberated α-naphthol. A very insoluble brown-red pigment is formed at sites of acid phosphatase activity. Unfortunately, one commercial form of this diazonium salt, Naphthanil Diazo Red AL of E. I. DuPont de Nemours & Company, contains 5% zinc chloride and 12% aluminum sulfate, and inhibits nuclear phosphatase activity of liver and kidney. For laboratory preparation they direct as follows:

Dissolve 10 Gm. technical 1-aminoanthraquinone in 85% sulfuric acid. Pour into a large volume of water. Filter out the finely divided precipitate on a suction filter and wash free of acid. Add 20 cc. concentrated hydrochloric acid to the moist precipitate. Run in 50 cc. of 6.4% sodium nitrite beneath the surface, stirring the while. Temperature should be about 30–40° C. Pour reaction mixture into ten volumes of water previously heated to 75° C. Add 0.5 Gm. sodium nitrite. Filter rapidly and add sodium chloride to the filtrate to salt out the diazonium chloride, which separates as shiny yellow platelets. Collect crystals, rinse with cold water, alcohol, and ether in sequence. Dry and store in the dark. It has not deteriorated in 18 months at 0° C.

Seligman and Manheimer’s Alphanaphthyl Phosphate Method for Acid Phosphatase.

1. Paraffin sections of acetone-fixed tissues deparaffinized and hydrated as usual.
2. Make fresh substrate

<table>
<thead>
<tr>
<th>Substance</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium alpha-naphthyl phosphate</td>
<td>50 mg.</td>
</tr>
<tr>
<td>1% Aerosol OT 345 (American Cyanamid Co.)</td>
<td>1 cc.</td>
</tr>
<tr>
<td>Stock acetate buffer pH 5 (3 M)</td>
<td>10 cc.*</td>
</tr>
<tr>
<td>4 M sodium chloride (23.38 Gm. in 106 cc. solution)</td>
<td>90 cc.</td>
</tr>
</tbody>
</table>

* The acetate buffer contains 20 Gm. sodium acetate, 5 cc. glacial acetic acid and 100 cc. distilled water. This is about 3 M.
When dissolved, add 30 mg. anthraquinone-1-diazonium chloride, dissolve, warm to 37° C., and incubate sections. Human prostate requires one hour; most other tissues require 24 hours.

3. Wash five minutes in running water; mount in glycerol gelatin.

**Results.** Phosphatase sites, brownish red. The salt is added to prevent diffusion of acid phosphatase, which, though fairly insoluble in water and 3–5 M NaCl, is quite soluble in 1.1 M sodium chloride. Colloidization of the paraffin section before hydration should also help to prevent diffusion of enzyme. Addition of D-tartaric acid (maintaining the pH of 5) to the substrate tended to inhibit the acid phosphatase of prostatic epithelial and stroma-cell cytoplasm, but not so much that of prostatic nuclei, of skeletal muscle, and of spleen pulp. The greatest differential effect was observed perhaps at the 4% level.

Grogg and Pearse (J. Path. & Bact. 64:627, 1952) use frozen sections of tissue fixed for 10–16 hours in 15% formalin. Their substrate contains 10–20 mg. sodium a-naphthyl phosphate in 20 cc. of 0.1 M acetate or veronal HCl buffer of pH 5. At the time of using they add 10 mg. stabilized o-dianisidine tetrazotate (Fast Blue B Salt, I.C.I., Ltd.), shake and filter. Incubate about 30–60 seconds for dog prostate, 30–60 minutes for rat liver. Wash two minutes in running water, counterstain with hemalum, and mount in glycerol gelatin. Reaction sites are red; nuclei, blue.

**Glucose-6-phosphatase**

This phosphatase is distinguishable from ordinary non-specific acid phosphatase by its thermolability, its sensitivity to formaldehyde and to pH levels below 5.0. It is destroyed in material fixed in cold alcohol or acetone and imbedded in paraffin. Tissue may be quickly frozen and stored at —20° C. for several days without great loss of activity.

According to Chiquoine (J. Histochem. & Cytochem. 1:429, 1953) fresh unfixed tissue is frozen and sectioned by the Coons technic (p. 23) and dried briefly onto slides.

**Substrate.** Dissolve 250 mg. barium glucose-6-phosphate in 10 cc. distilled water, add 0.1 cc. 2 N HCl and 120 mg. K₂SO₄. Let stand 2 hours with frequent stirring. Centrifuge out BaSO₄ and test supernatant for absence of Ba by adding a grain or so more K₂SO₄. If clear, dilute to 30 cc. and adjust to pH 6.7 with normal KOH. For the working substrate dilute one volume of this solution with two volumes of 0.2% lead nitrate (0.00604 M). Slight turbidity results. Filter and place 0.5 cc. quantities in each of the required number of 3 cc. beakers. Coat edges of beakers with petrolatum (U.S.P.). Place sections face down over orifice of beaker, invert and incubate 5 to 15 minutes at 32° C. Rinse successively in distilled water, in 2% acetic acid and again thoroughly in distilled water. Immerse for 2 minutes in 1:50 yellow ammonium sulfide. Wash 2 to 5 minutes in running water. Stain 2 minutes
in 0.1% safranin in 1% acetic, or as desired, dehydrate, clear and mount in balsam. Activity sites in brown.

Immersion for 10 minutes in Weigert's iodine, or in boiling water, abolishes the activity, as does fixation with formaldehyde. Preparations should be compared with duplicates prepared by the glycerophosphate acid phosphatase method.

Phosphamidase

In the course of exploration of a variety of substrates in the acid and alkaline phosphatase methods, Gomori found that p-chloroanilidophosphonic acid as substrate gave a quite different distribution picture from that of other substrates when used in the acid range, though the distribution picture was typical of the other alkaline phosphatases when the alkaline to neutral ranges were employed. Gomori (Proc. Soc. Exper. Biol. & Med. 69:407, 1948) described the method of purification of the crude product made by Otto's synthesis (Ber. deutsch. chem. Ges. 28:617, 1895) (see also Bredereck and Geyer, Ztschr. f. physiol. Chem. 254:223, 1938).

Gomori's Phosphamidase Method.

Substrate. 0.1 M stock solution of p-chloroanilidophosphonic acid.* Dissolve 21 Gm. in an excess (say 150 cc.) of a 10% dilution of 28% ammonia water (the equivalent is about 28 cc.). Adjust to pH 8 with dilute (say 10%) acetic acid electrometrically (just colorless with phenolphthalein). Add distilled water to make 1000 cc. Store in refrigerator.

Working substrate is composed of substrate and following solvent:

\[
\begin{align*}
\text{MnCl}_2 & \quad 12.5\% \text{ (M) aqueous solution} \quad 0.4 \text{ cc. \ (.004 M)} \\
\text{Pb(NO}_3\text{)}_2 & \quad 3.31\% \text{ (0.1 M) aqueous solution} \quad 2.5 \text{ cc. \ (.0025 M)} \\
\text{Solvent} & \quad \text{Maleate buffer pH 5.6} \quad (5.8\% \text{ maleic acid} \ 10 \text{ cc.}, \ 0.1 \text{ N sodium hydroxide} \ 62 \text{ cc.}, \text{ distilled water} \ 28 \text{ cc.}) \quad 100 \text{ cc. \ (0.1 N)} \\
& \quad \text{Shake to dissolve initial precipitate.}
\end{align*}
\]

To make working substrate add 4 cc. stock 0.1 M p-chloroanilidophosphonic acid. Preheat in paraffin oven 30 minutes and filter out excess lead phosphate.

Technic. Fix tissues in acetone, 95% alcohol, or 100% alcohol at 0°C. (for 24 hours?), dehydrate with acetone or 100% alcohol accordingly. "Clear" with petroleum ether, infiltrate in paraffin at 58-60°C. in vacuo (10-15 mm. Hg) for 15 minutes. Imbed and section at 5-10 μ.

1. Deparaffinize and hydrate sections as usual. Inactivate a set of control sections by immersion for ten minutes in Gram's iodine solution (p. 92).

* Obtainable from Dajac Laboratories, 3430 West Henderson St., Chicago 18, Ill.
2. Immerse test sections and iodine-inactivated controls in preheated, filtered, working substrate; and a second set of untreated controls in the preheated, filtered solvent without substrate, for 10–24 hours at 37° C., arranging slides all facing in the same direction in Coplin jars so tilted that the slides lean about 30° out of perpendicular with faces obliquely downward.

3. Wipe precipitate off the backs of the slides and rinse in distilled water.

4. Wash slide in 0.1 M citrate buffer of pH 5, until the surface of the glass adjacent to the section appears clean.
   Caution: Undertreatment leaves precipitate on the section; overtreatment may remove part of the lead phosphate deposits at sites of activity.

5. Wash in running water for two to five minutes.

6. Treat in 1% dilution of yellow ammonium sulfide for 1–2 minutes (or 0.5% Na2S buffered to about pH 7, p. 202).

7. Wash in water, counterstain as desired. 0.1% safranin in 0.1% acetic for five minutes; or otherwise.

8. Dehydrate in alcohols, clear with xylene, and mount in balsam, HSR, Bioloid, Permount, or ester gum.

Results. Carcinoma cells and gray substance of brain and cord show much blackening; other tissues relatively little. Some benign tumor cells color quite heavily; others do not.

Glucuronidase

Friedenwald and Becker (J. Cell. & Comp. Physiol. 31:303, 1948) give two methods for the demonstration of glucuronidase in the cytoplasm of hepatic parenchyma cells, renal tubules, splenic follicles and pulp, bronchial epithelium, endometrium, bone marrow, cartilage, epididymis, seminal vesicle, corpus luteum, and intestine.

Glucuronidase Method with o-Hydroxyphenyl-azo-2-naphthol Glucuronide.

1. Cut frozen sections of unfixed fresh tissue at 35–40 μ.

2. Incubate at 37° C. for 1–5 hours in a filtered 1:20,000 (50 mg/liter) solution of o-hydroxyphenyl-azo-2-naphthol glucuronide in 0.1 M acetate buffer of pH 5.0.

3. Wash in distilled water; fix in 1% acetic, 10% formalin; stain in 0.5% methyl green in 0.5% acetic or in dilute alum hematoxylin (1:20 dilution of Delafield’s or 1:15 of Lillie’s or Harris’s for 3–5 minutes).

4. Mount in glycerol gelatin, glycerol, gum syrup or the like.

Results. Nuclei, green or blue according to counterstain; sites of glucuronidase activity, red; fats, pale orange or yellow.
Campbell (Brit. J. Exper. Path. 30:548, 1949) reduced the concentration of substrate to 40 mg./liter, dissolved warm, then cooled, and filtered. Incubate at 37° C. for 4-5 hours in small covered vessels with little air space over the fluid, and proceed as above, Step 3.

Friedenwald and Becker direct preparation of o-aminophenyl glucuronide according to Williams (Biochem. J. 37:329, 1903) recrystallization, diazotization at 5° C. and coupling in alkaline solution with β-naphthol, purification by dissolving in dilute alkali and precipitation with HCl, collection on filter and desiccation in desiccator over CaCl₂. The resulting O-hydroxyphenyl-azo-2-naphthol glucuronide is dissolved by heating in 0.1 N acetate buffer of pH 5 at 50 mg. per liter.

**Glucuronidase Method with 8-Hydroxyquinoline Glucuronide.**

**Substrate.**
- 8-hydroxyquinoline glucuronide 0.01 N (0.32%) in 0.1 N pH 5 acetate buffer 13 cc.
- 8-hydroxyquinoline 0.01 N (0.145%) in 0.1 N pH 5 acetate 13 cc.
- Ferric chloride 0.01 M in 0.1 N pH 5 acetate buffer 10 cc. (4.35 cc. official ferric chloride solution per liter of buffer)

**Control substrate:** Substitute 13 cc. 0.1 N pH 5 acetate buffer for the glucuronide in the mixture above.

**Procedure.**
1. Cut frozen sections of unfixed tissue at 35-40 μ.
2. Incubate 18 hours at 37° C. in filtered substrate or control.
3. Wash with distilled water, float onto slides coated with albumen glycerol, blot down, and heat gently to stick sections.
4. Flood slides with 0.5 N oxalate buffer of pH 4 (2.87 Gm. sodium oxalate, 0.47 Gm. oxalic acid, 100 cc. distilled water). Let stand 15 minutes.
5. Wash in distilled water.
6. Flood for 15 minutes with a mixture of equal volumes of 1% potassium ferrocyanide (K₄Fe(CN)₆) and normal hydrochloric acid.
7. Wash; fix in 1% acetic, 10% formalin; counterstain 15 minutes in 1:5000 basic fuchsin in 1% acetic (or in 0.1% safranin in 1% acetic for three minutes).
8. Wash in water (or in 1% acetic), dehydrate in alcohol, clear with xylene, and mount in synthetic resin (polystyrene) or balsam.

During incubation black ferric hydroxyquinoline is formed. This is insoluble in oxalate buffer at pH 4, which dissolves the other absorbed iron. Then, since the black iron salt is soluble in alcohol, it is converted to Prussian blue with ferrocyanide. The preparation then stands fixation, counter-
staining, dehydration, and mounting in resin. Sites of enzyme activity are demonstrated in blue.

Campbell noted that addition of one part of 0.1 M (2.48%) potassium acid saccharate to 100 parts of substrate inhibited the reaction. Becker and Friedenwald (Arch. Biochem. 22:101, 1949) reported 90% inhibition by ascorbic acid, but only about 50% inhibition by heparin, on the basis of test-tube experiments. Heparin showed a more pronounced inhibition histochemically on liver, kidney, submaxillary gland, seminal vesicle, and bronchial epithelium than on other tissues. Hyaluronic acid had a similar effect in vitro to that of heparin.

The preparation of 8-hydroxyquinoline glucuronide is according to Brahm (Ztschr. f. physiol. Chem. 28:439, 1899). This product is recrystallized from hot water.

β-D-Galactosidase


1. Prepare frozen sections 10 μ thick from fresh, unfixed tissue by the Coons method (p. 23).
2. Coat slides with egg albumen and affix sections.
3. Immerse successively in six two-minute baths of 1%, 2%, 3%, 4%, 5%, and 6% aqueous sodium chloride solution.
4. Incubate two hours at 37° C. in the following substrate:
   Dissolve 15 mg. 6-bromo-2-naphthyl-β-D-galactopyranoside in 10 cc. 100% methanol and 15 cc. distilled water at boiling point. Add 55 cc. distilled water containing 5.8 Gm. sodium chloride and 20 cc. McIlvaine phosphate citric acid buffer (p. 450) of pH 4.95.
5. Wash one minute in 1% sodium chloride.
6. Immerse for 1–2 minutes in tetrazotized diorthoanisidine: Dissolve 100 mg. of the “20%” commercial salt in 50 cc. distilled water at 3° C. and add 50 cc. 0.2% disodium phosphate (Na₂HPO₄), also at 3° C. Use immediately. This solution should be at approximately pH 7.5. (Cf. p. 91.)
7. Wash in water and mount in glycerol gelatin.

Results. Reaction sites, blue.

Zymohexase

Allen and Bourne’s (J. Exper. Biol. 20:61, 1943) method for zymohexase. Zymohexase is an aldolase which converts hexose diphosphate (fructofuranose 1,6-diphosphate) to dihydroxyacetone phosphate and phosphoglyceraldehyde, + an isomerase, which catalyzes equilibrium between the two products. Inclusion of iodoacetic acid stopped the decomposition at the triose stage.
The triose phosphates liberate phosphoric acid in alkaline solution, which is visualized in the usual manner.

Reagents.

A. Neutralize 1.86 Gm. iodoacetic acid (0.01 M) in 80 cc. water, with normal sodium hydroxide (about 10 cc.), using bromthymol blue as an indicator; and dilute to 100 cc. (0.1 M sodium iodoacetate).

B. 0.42% (0.1 M) sodium fluoride.

C. 2% cobaltous chloride (CoCl₂·6H₂O).

D. Dilute yellow ammonium sulfide 1 cc. in 49 cc. distilled water just before using.

E. Magnesia mixture: Dissolve 5.5 Gm. magnesium chloride (MgCl₂·6H₂O) (2.57 Gm. anhydrous MgCl₂) and 7.0 Gm. ammonium chloride in 35 cc. 5 M ammonia (see Table on p. 455). Filter after one hour and add 60 cc. 4 M ammonia to the filtrate.

F. Sodium hexose diphosphate.

Convert a 5% aqueous solution of calcium hexose diphosphate to the sodium salt by treatment with the equivalent amount of sodium oxalate. Filter out calcium oxalate and adjust volume of solution to contain 4% sodium hexose diphosphate.*

I. Mix 40 cc. of this solution with 20 cc. of magnesia mixture (E). Filter out the contaminant inorganic phosphate precipitate after 30 minutes.

II. Mix 20 cc. of 4% sodium hexose diphosphate with 20 cc. magnesia mixture and 20 cc. water. Filter after 30 minutes as before.

Substrate A: Mix 15 cc. of solution I with 2.5 cc. 0.1 M sodium iodoacetate (A) and 7.5 cc. distilled water. Proportion 6:1:3.

Substrate B (for inhibition of phosphatase): 15 cc. solution I, 2.5 cc. 0.1 M sodium iodoacetate, 2.5 cc. 0.1 M sodium fluoride (B) and 5.0 cc. distilled water. Proportion 6:1:1:2.

Substrate C: Mix 15 cc. solution II with 2.5 cc. sodium iodoacetate and 7.5 cc. distilled water. Proportion 6:1:3.

The final concentration of the hexose salt in substrates A and B is 1.6%, in substrate C, 0.8%.

Technic of Zymohexase Method:

1. Fix in 80% alcohol for 24 hours at 20–25° C.
2. Wash in water 10–15 minutes.
3. Freeze and section on freezing microtome at 10–15 μ.

* Dissolve 2.5 Gm. calcium hexose diphosphate in 50 cc. distilled water. Dissolve 1.61 Gm. sodium oxalate in 50 cc. distilled water. Mix and let stand half an hour to complete precipitation. Filter and concentrate filtrate to 60 cc.
4. Incubate sections 2–3 hours in each of the three substrates. Rinse in water. For controls omit this step.

5. Transfer to 2% cobaltous chloride (CoCl₂·6H₂O) for several hours to allow liberation and precipitation of phosphate from the trioses. Rinse in water.

6. Convert the cobalt phosphate to sulfide by ten minutes treatment in a 1/50 dilution of yellow ammonium sulfide. Wash thoroughly in water.

7. Counterstain as and if desired.

8. Dehydrate, clear and mount in balsam, ester gum, HSR, Bioloid, Permount, etc.

**Results.** Strong diffuse brown in striated and smooth muscle.

**Aryl Sulfatase**

Rutenburg, Cohen, and Seligman (*Science* 116:539, 1952) reported a method for aryl sulfatase depending on the decomposition of the sulfuric acid ester of 6-benzoyl-2-naphthol and the coupling of the liberated benzoyl naphthol with tetrazotized diortho-anisidine. Rat tissues may be fixed for some days or even months in cold neutral formalin. From these, frozen sections are prepared as usual. Human and monkey tissues are cut by the Coons-Linderström-Lang technic (p. 23), mounted on glass slides directly, and dried in air. With the unfixed tissues it is necessary to incubate in a substrate made up in hypertonic salt solution.

The substrate is prepared by dissolving 25 mg. potassium 6-benzoyl-2-naphthyl sulfate in 80 cc. hot 0.85% sodium chloride solution and adding 20 cc. 0.5 M acetate buffer of pH 6.1 (p. 450). For unfixed frozen sections the substrate is made hypertonic by the addition of 2.6 Gm. sodium chloride per 100 cc., thus raising the NaCl content to 3.28%. Unfixed frozen sections attached to slides are first immersed in three baths of NaCl solution of 0.85%, 1%, and 2% concentration, and then placed in hypertonic substrate. Fixed frozen sections are transferred first to a small portion of the normal substrate, and then to the regular incubation bath of 20 cc. Sections from each organ are to be incubated in separate containers to avoid transfer of activity. For organs with high activity incubate 2–3 hours at 37° C.; for other organs, 4–16 hours in hypertonic substrate.

After incubation, wash formalin-fixed tissues in water and unfixed tissues in descending grades of salt solution (2%, 1%, and 0.85%); and transfer to a freshly prepared cold (4° C.) 0.1% solution of diorthoanisidine tetrazo salt in M/20 phosphate buffer of pH 7.6. Gently agitate for five minutes. Then wash in three changes of cold 0.85% sodium chloride solution and mount in glycerol or glycerol gelatin.

Areas of high activity stain blue; of lower activity, purple to red. On storage even at 4° C. the blue gradually changes through purple to red, and some diffusion occurs. Activity is largely cytoplasmic.
Table 19
SULFATASE ACTIVITY IN ORGANS BY SPECIES

<table>
<thead>
<tr>
<th></th>
<th>Man</th>
<th>Monkey</th>
<th>Mouse</th>
<th>Rat</th>
<th>Rabbit</th>
<th>Guinea Pig</th>
<th>Hamster</th>
<th>Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>++</td>
<td>++</td>
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<td>++</td>
<td>±</td>
<td>±</td>
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<tr>
<td>Kidney</td>
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<td>++</td>
<td>++</td>
<td>±</td>
<td>±</td>
<td>++</td>
<td>±</td>
</tr>
<tr>
<td>Pancreas</td>
<td>++</td>
<td>++</td>
<td>±</td>
<td>++</td>
<td>±</td>
<td>±</td>
<td>++</td>
<td>±</td>
</tr>
<tr>
<td>Adrenal</td>
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<td>±</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>±</td>
</tr>
</tbody>
</table>

Esterases

Esterases which are histochemically localizable comprise nonspecific esterases which decompose glycercyl and other esters of short-chain aliphatic acids, lipases which attack esters of long-chain fatty acids and are found principally in pancreas, and cholinesterases which hydrolyze fatty acid esters of choline and acetylcholine and are found in motor end organs, neural synapses, nerve cells, and erythrocytes.

Nachlas and Seligman (J. Nat. Cancer Inst. 9:415, 1949) propose a nonspecific esterase method utilizing β-naphthyl acetate as substrate and visualizing by coupling with α-naphthyl diazonium naphthalene-1,5-disulphonate*, which gives a red color; or with the commercial stable tetrazotized diorthoanisidine Diazo Blue B, which gives a deep blue.

Nachlas and Seligman’s Method for Esterase.

1. Fix thin blocks in acetone at 0-5°C, using three changes in 24 hours.
2. Clear 18-24 hours in cedar oil.
3. Xylene two changes, 30 minutes each.
4. Infiltrate in three changes of paraffin (53°C) at 55°C in 2-3 hours.
   Imbed and section at 6 μ. Float on water, mount with Mayer’s glycerol albumen, and dry overnight at 37°C.
5. Deparaffinize with xylene, hydrate through graded acetone-water mixtures, NOT ALCOHOLS!
6. Incubate at room temperature in the following substrate mixtures for 20 minutes.

   Solvent 0.1 M barbiturate buffer of pH 7.8 (p. 454) 20 cc.
   Distilled water 29 cc.
   Just before using add α-naphthyl diazonium naphthalene-1,5-disulphonate 40 mg.
   or Diazo Blue B 40 mg.
   This is the solvent + diazonium salt, which fluid is used for control incubations. For specific substrate, add 1 cc. of 1% β-naphthyl acetate in acetone, stirring thoroughly.

* Available from Dajac Laboratories, 3430 West Henderson Street, Chicago 18, Ill.
7. Wash in tap water, counterstain lightly with alum hematoxylin or borax carmine, wash in water, and mount in glycerol gelatin.

**Results.** Sites of esterase activity, red or blue. In rat and dog the greatest activity was found in lung (especially bronchial epithelium and elastica of large vessels), in liver parenchyma cells generally (but least in periportal zones), in pancreatic acinar cells, and in the cortical tubule cells of the kidney. Leucocytes contain esterase. Esterase appears also in surface epithelium of the urogenital tract, including prostate; and some in glomerular and reticular zones of adrenal cortex.

More recently Barrnett and Seligman (*Science* 114:579, 1951) introduced indoxyl acetate and butyrate as substrates and described their preparation from sodium indoxyl.* These substrates yield indigo on hydrolysis in the presence of air. This pigment is quite insoluble in water and in fats. The method demonstrates nonspecific esterase, lipase, and cholinesterases, which are distinguished by use of the usual inhibitors (pp. 218–219).

Prepare frozen sections by the Coons method (p. 23) from fresh tissue quickly frozen and stored at −30° C. Dry on slides and incubate at 25° C. in the following substrate: 25 cc. 2 M sodium chloride (11.69%), 10 cc. of 0.1 M veronal sodium buffer of pH 8.5 (153 cc. 0.1 N HCl + 847 cc. 0.1 M veronal sodium), 250 mg. CaCl₂ in 14 cc. distilled water. Add 1 cc. 2% indoxyl acetate in acetone just before using and mix thoroughly. Hydrolysis is perceptible in two minutes as a blue coloration in the more active tissues; and in ten minutes a blue color is grossly evident in all tissues. At the end of ten minutes' incubation decant, wash five minutes in running water, and mount in glycerol gelatin.

Holt and Withers (*Nature* 170:1012, 1952) substitute 5-bromoindoxyl acetate for the above, and use ordinary frozen sections of tissues fixed 16 hours at 4° C. in neutral formol saline. Finer-grained deposits are produced, diffusion is decreased, and the consistency of performance is increased.

Their substrate contains 6.3 mg. 5-bromoindoxyl acetate, 165 mg. potassium ferricyanide (K₄Fe(CN)₆), 211 mg. potassium ferrocyanide (K₄Fe(CN)₆·3H₂O), 111 mg. calcium chloride (CaCl₂), and 20 cc. M/10 trihydroxymethylaminomethane maleate buffer (p. 453), with distilled water to make 100 cc.

**Lipases**

Following his work on the phosphatases, Gomori (*Proc. Soc. Exper. Biol. & Med.* 58:362, 1945) introduced a method for the demonstration of lipases. In this technic the water-soluble palmitic or stearic acid esters of certain polymer glycols or hexitans are hydrolyzed in the presence of a soluble calcium salt, and the calcium soaps formed in situ are converted into lead soaps.

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* Available from National Aniline Division, 40 Rector St., New York, N. Y.
by treatment with lead nitrate. The lead soaps are converted to black lead sulfide with ammonium sulfide.

**Technic.**

1. Fix thin slices of tissue in acetone at 0° C. for 12–24 hours. Complete dehydration with two further changes of acetone of 6–12 hours each, at room temperature. Clear in benzene, two changes, 30 minutes each. Infiltrate in paraffin at not more than 60° C. for not more than two hours. I suggest vacuum infiltration (p. 61) for 10–15 minutes only.

2. Cut 5μ sections. Deparaffinize with xylene and pass through 100% alcohol into 0.5% collodion in ether–100% alcohol mixture. After 5–10 minutes take out, drain one minute, and harden five minutes or more in 80% alcohol. Wash in distilled water.

3. Incubate at 37° C. for 6–12 hours in this mixture of a palmitic or stearic hexitan ester: Tween 40 and Tween 60 of the Atlas Powder Co. respectively, using

- 2% Tween 40 or Tween 60 in water
- *0.1 M buffer pH 7.3
- 30% glycerol
- 2% anhydrous calcium chloride

The sites of lipase activity become opaque.

4. Rinse in distilled water.

5. Treat with 2% lead nitrate solution for ten minutes.

6. Rinse repeatedly in distilled water.

7. Treat two minutes with a 1:100 dilution of yellow ammonium sulfide in distilled water. (Gomori said 10 drops (about 0.5 cc.) in a Coplin jar of water, or about 50 cc.)

8. Wash in running water, counterstain 3–5 minutes in alum hematoxylin, dehydrate, clear, and mount in Clarite. Gomori advises against xylene as a clearing agent and as solvent for the darites, alleging fading in xylene media. Instead use dichloroethylene or ligroin (cf. also Timm; p. 268), or mount in ester gum, balsam or an unsaturated resin (p. 104).

**Results.** Sites of lipase activity are evident as dark brown deposits of lead sulfide.

Gomori notes further that treatment for one minute with Lugol’s iodine solution or with 5% phenol, or boiling ten minutes in water destroys the enzyme. Addition of 0.2% sodium taurocholate intensifies the action of pancreatic lipase, but inhibits that of “all other organs.”

Nachlas and Seligman (J. Biol. Chem. 181:343, 1949) report that in the test-tube eserine at 10⁻³ × 3.5 M (962 mg./l.) had only a moderate inhibitory effect both on pancreatic hydrolysis of naphthyl laurate and stearate (lipase), and on hepatic and renal hydrolysis of naphthyl acetate (esterase).

* Trihydroxymethylaminomethane buffer with HCl or maleate.
especially in man. Sodium taurocholate at 0.1 M (0.54%) accelerated the lipase hydrolysis of naphthyl laurate and stearate, and depressed slightly the esterase decomposition of naphthyl acetate. Quinine hydrochloride at 0.05 M (1.894%) inhibited almost completely the pancreatic lipase hydrolysis of naphthyl laurate and stearate, while esterase showed a species-variable but lesser grade of inhibition or even (in the dog) acceleration. Atoxyl (sodium arsenilate) at 0.1 M (2.39%) and sodium fluoride (3 mg. per cc.) tended to inhibit the esterase activity of liver, kidney, and pancreas on naphthyl acetate, but were without effect on the pancreatic lipase hydrolysis of naphthyl laurate and stearate.

In their hands the hydrolysis in vitro of the polyglycol stearic acid esters, such as Gomori used, followed more the organ distribution pattern of esterase than that of true lipase. The activity against naphthyl laurate and stearate was similar in organ distribution to that against olive oil.

Unfortunately naphthyl stearate and laurate are too insoluble to use in the histochemical technics (Seligman et al., Ann. Surg. 130:333, 1949); but by including 0.1 M (2.39%) sodium arsenilate (atoxyl) in the naphthyl acetate substrate, they showed that the esterase activity of liver and kidney was inhibited, and considered the pronounced activity still evident in pancreas as due to lipase.

This finding suggests that the method may actually demonstrate lipase in pancreas and that the activity inhibited by taurocholate may be that of nonspecific esterase, since Seligman et al. (loc. cit.) showed that the brei esterases of other organs were relatively ineffective in hydrolyzing \( \beta \)-naphthyl palmitate and stearate, though pancreatic brei enzymes hydrolyzed this substrate readily. Seligman was not able to adapt this palmitate stearate substrate to histochemical use because of its great insolubility.

**Cholinesterase**

Gomori (Proc. Soc. Exper. Biol. & Med. 68:354, 1948) introduced a technic for localization of cholinesterase, for which he preferred myristoylcholine as substrate. He prescribed 12-24 hours fixation in acetone at 0° C., a 1-3-hour bath at 0° in equal parts of 100% alcohol and ether, a 12-hour infiltration in 4% collodion in alcohol ether mixture at 0° C., followed by two changes of chloroform of one hour each, and embedding in paraffin (preferably in vacuo) for 15 minutes, but not more than two hours. Section at 5-10 μ.

**Gomori's Technic for Cholinesterase.**

1. Hydrate sections as usual and incubate 2-16 hours in solvent (controls) or substrate.

   **Solvent:** 0.1 M cobaltous acetate, \( \text{Co} (\text{CO}_2\text{CH}_3)_2 \cdot 4\text{H}_2\text{O} \) (2.49%) 40 cc.
Histopathologic Technic

0.1 M maleate-trihydroxymethylaminomethane buffer pH 7.6 (p. 453) 60 cc.
Distilled water 200 cc.
Add 1 mg. each of CaCl₂, MgCl₂, and MnCl₂.

Substrate: To 50 cc. of solvent add 1 cc. of 0.02 M myristoylcholine (0.663%) in distilled water.
Store the stock solvent and the myristoyl chloride solutions separately in the refrigerator, adding a crystal of camphor to each.

2. Wash two minutes in running water.
3. Immerse 15 minutes in 0.5% dilution of yellow ammonium-sulfide solution or 0.5% sodium sulfide (p. 202). (The cobalt soaps react rather slowly to form the sulfide.)
4. Wash two minutes in running water.
5. Counterstain five minutes in 0.2% safranin in 1% acetic.
6. Dehydrate in alcohols, clear in xylene, and mount in Canada balsam, ester gum, or β-pinene resin.

Results. Sites of cholinesterase activity are shown as dark brown; nuclei, red; cytoplasm, etc., in various shades of pink. Addition of prostigmine bromide (10⁻⁶ M: 0.5 cc. of a 30 mg./100 cc. to a 50 cc. Coplin jar) specifically inhibits the hydrolysis of the substrate.

Nachlas and Seligman (J. Biol. Chem. 181:343, 1949) note that cholinesterase is inhibited by 10⁻⁵ M (2.75 mg./liter) of physostigmine (eserine) but lipase and esterase are only partly inhibited by 3.5 × 10⁻⁸ M (nearly 0.1%).

Gomori noted species differences in localization with myristoylcholine and the palmitic and lauric esters. Dog and mouse tissues reacted best with myristic and lauric esters; human and pigeon with palmitic. Hard, Peterson, and Fox (J. Neuropath. & Exper. Neurol. 10:48, 1951) preferred myristoylcholine for their experimental studies on dogs.

Koelle and Friedenwald (Proc. Soc. Exper. Biol. & Med. 70:617, 1949) substituted acetylthiocholine as substrate in the cholinesterase technic introduced by Gomori (loc. cit.) because the choline fatty acid esters originally employed were found to be only very slowly hydrolyzed by brain and purified cholinesterases from erythrocytes and electric organ, in comparison with acetylcholine. Acetylthiocholine was found to hydrolyze even more rapidly than acetylcholine. This substrate is hydrolyzed by both cholinesterase and by nonspecific esterase, and may be blocked by pretreatment of tissue with the irreversible cholinesterase inhibitor di-isopropyl fluorophosphate at 10⁻⁸ M (184 mg./liter).

Koelle and Friedenwald’s Technic for Cholinesterase:

Reagents. Buffer: 1 N glycine 50 cc., 1 N NaOH 18 cc. dist. water 32 cc. (pH 9.6). Solvent: Buffer 0.4 cc., 0.1 M copper sulfate (2.5% CuSO₄·

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5H₂O) 0.2 cc. Distilled water 8.6 cc. Add trace of copper thiocholine, dispersing thoroughly. Preheat to 37° C. for at least 15 minutes.

Specific substrate. Add 0.8 cc. acetylthiocholine solution to 9.2 cc. solvent, and filter and use at once.
Acetylthiocholine solution. Dissolve 14.5 mg. acetylthiocholine iodide in 0.75 cc. distilled water in centrifuge tube.
Add 0.25 cc. 2.5% copper sulfate (CuSO₄·5H₂O). Centrifuge out cupric iodide, and decant.

Copper thiocholine. Dissolve acetylthiocholine in copper glycinate solution, adjust to pH 12 with KOH. Let stand overnight, collect precipitate, and wash free of alkali with distilled water.

Technic.

1. Cut frozen sections of fresh unfixed tissue or make teased preparations of fresh muscle.
2. Place control preparations in .001 M (.0184%) di-isopropyl fluorophosphate in 0.85% sodium chloride solution and let stand for 30 minutes.
3. Wash in distilled water.
4. Place untreated preparations and blocked controls in specific substrate and incubate at 37° C. for 10–60 minutes.
5. Rinse in distilled water saturated with copper thiocholine.
6. Transfer to a 1% dilution of yellow ammonium sulfide solution (Koelle says (NH₄)₂S) (or 0.5% Na₂S buffered to pH 7, p. 202) to convert the deposited copper thiocholine to the dark brown amorphous copper sulfide.
7. Wash in water, float onto slides, blot down.
8. Counterstain as desired, say 0.2% safranin in 1% acetic for 3–5 minutes, rinse, dehydrate with alcohols or acetone, clear in xylene, and mount in balsam, ester gum or β-pinene resin.

Results. Sites of enzymatic activity are shown by dark brown deposits of copper sulfide.

In place of the di-isopropyl fluorophosphate inhibitor used by Koelle and Friedenwald, it should be possible to use the 10⁻⁶ M prostigmine bromide (M.W. 303) inhibitor of Gomori or the 10⁻⁶ M physostigmine (M.W. 275) solution of Nachlas and Seligman.

Urease

Sen (Indian J. M. Research 18:79, 1930) reported that urease would act in fairly strong alcoholic solution to liberate ammonia and carbon dioxide from urea, and made this the basis of a histochemical method. Of the Co, Ca, Ni, Cu, and Pb ions tested for localization of the carbonate formed, the first seemed best. His procedure follows:
Fix animal tissues one hour in 60% alcohol containing 1% cobalt nitrate. Add an equal volume of 60% alcohol containing 1% urea and let stand 48 hours at room temperature. Dehydrate with alcohols, imbed in paraffin or celloidin, section, hydrate sections, and immerse for some minutes in hydrogen sulfide water or dilute sodium sulfide solution. Wash and mount as usual.

Brief trials of this method have not been satisfactory, and I suggest following Glick's (J. Nat. Cancer Inst. 10:321, 1949) alternate section titrimetric procedure to give approximate localization of urease activity. A block of gastric mucosa is frozen quickly at -25°C. With a large cork-borer cut a cylindrical block from the surface down to muscularis. Cut serial frozen sections horizontally from the surface downward. Reserve alternate sections for staining and histologic evaluation in comparison with the results of titrimetric assay of intervening sections.

For the chemical details of this assay the reader is referred to the original paper.

Oxidases, Peroxidases, Dehydrogenases

For practical purposes these fall into three classes, the relatively stable, moderately formaldehyde-resistant hemoglobin peroxidase; the myeloperoxidase or verdoperoxidase—also relatively stable, but somewhat less resistant—of the granular leucocytes and their precursors; and the highly labile tissue oxidases exemplified by cytochrome c oxidase (or G-Nadi oxidase of the German writers), succinic dehydrogenase, cystein desulfurase, and probably other similar enzymes demonstrable by the tetrazolium formazan systems.

The methods for their detection may also be classed according to the substances used to yield colored products in the presence of hydrogen peroxide, or without it, and some of these methods may be used for both the stable and the highly labile enzymes.

I will cite two general methods for hemoglobin peroxidase. Dunn's modifications of the "Zinc leuco" sulfonated triphenylmethane dyes, and one of the nitroprusside benzidine methods.

Lison used first acid fuchsin, and later some patent blue reduced by nascent hydrogen to its leuco state. Dunn (Stain Technol. 21:65, 1946) identified the patent blue as patent blue V (C. I. No. 712) and later (Arch. Path. 41:676, 1946) substituted the related dye cyanol (C. I. No. 716), using identical amounts. The latter dye is slightly more violet but otherwise equivalent, and I prefer the patent blue V.

The Lison-Dunn leuco patent blue method.

Fix blocks 3–5 mm thick in buffered 10% formalin (p. 34) for 24 to not more than 48 hours. Prepare paraffin sections at 5–8 μ.

Make a stock 1% aqueous solution of patent blue V. To 100 cc. of this add 10 Gm. granulated metallic zinc and 2 cc. glacial acetic acid and boil until
completely decolorized. Stopper well and store. This leuco patent blue solution is stable.

For use filter out 10 cc., and add 2 cc. glacial acetic acid and 1 cc. 3% hydrogen peroxide. This must be freshly mixed.

1. Bring sections to water.
2. Stain 3–5 minutes in the leuco patent blue–peroxide reagent.
3. Rinse in water.
4. Counterstain 30–60 seconds in 0.1% safranin O in 1% acetic acid or in an aqueous carmine.
5. Dehydrate, and clear through a graded alcohol and xylene sequence, and mount in clarite.

**Results.** Nuclei, red; cytoplasm, light pink; hemoglobin, dark blue-green; oxidase granules, dark blue.

The Lepehne-Pickworth method for demonstration of cerebral capillary distribution is essentially a benzidine and nitroprusside oxidase method for hemoglobin. The technic follows (emended from Mallory):

1. Wash sections 30 minutes in distilled water.
2. Place sections for 30 minutes at 37° C. in benzidine and nitroprusside reagent: Dissolve 100 mg. benzidine in 0.5 cc. glacial acetic acid. Add 20 cc. distilled water. Dissolve 100 mg. sodium nitroprusside in 10 cc. distilled water. Mix the two solutions and add 70 cc. distilled water. Make this reagent fresh each time. While sections are in this mixture, agitate frequently.
3. Wash ten seconds in distilled water.
4. Place in 0.04–0.05% hydrogen peroxide at 37° C. for 30 minutes, shaking frequently. This dilution must be freshly prepared. Use 1 cc. 3% hydrogen peroxide in 70 cc. water.
5. Wash in distilled water.
6. Dehydrate in 70%, 95%, and 100% alcohol, allowing sections to stand in each until diffusion currents are no longer evident. Place in 100% alcohol and xylene, and then in two or more changes of xylene until clear. Mount in balsam.

**Results.** Blood cells in the capillaries are colored black; other structures remain pale gray.

Romeis cites the Doherty, Suh, and Alexander (Arch. Neurol. & Psych. 40:158, 1938) variant of the Pickworth method. Step 2 above is shortened to 10 minutes, Step 4 to 20 minutes at 20° C.; but if the capillary net is not yet black, incubate further at 37° until the background is bleached. The
benzidine solution is composed of 0.5 Gm. benzidine dissolved in 50 cc. 100% alcohol, mixed with 10 cc. 1% aqueous sodium nitroprusside solution (freshly made) and 40 cc. distilled water. Deteriorated nitroprusside gives a greenish color on mixing. The peroxide solution contains also 10 cc. fresh 1% aqueous sodium nitroprusside, 2 cc. glacial acetic acid, 0.5 cc. 30% hydrogen peroxide, 50 cc. 100% alcohol and distilled water to make 100 cc.

**Myeloperoxidase**

This enzyme has been isolated from leukocytes by Agner (*Act. Physiol. Scand.* 2: Suppl. 8, 1941). It appears to be the enzyme responsible for both the benzidine and naphthol peroxidase reactions and for the Winkler-Schultze M-Nadi oxidase reaction. Certainly there is a very considerable correspondence in reagents effective in inhibiting the Washburn benzidine nitroprusside peroxidase test and the Winkler-Schultze reaction. Apparently the necessary peroxide for the latter reaction must be added if the reagent is quite fresh, or is formed spontaneously in contact with air within the dimethyl-p-phenylenediamine and a-naphthol mixture in a fairly short time.

Gomori's recent statement that leukocyte oxidase is not an enzyme but is fat peroxide ignored Agner's work. It was apparently based on the demonstration by Lison (*Bull. Soc. chim. biol.* 18:185, 1936) that fatty peroxides in adrenal cortical substance were capable of completing the indophenol synthesis from dimethyl-p-phenylenediamine and a-naphthol without access of air or addition of peroxide, and the considerable correspondence shown by Sehrt (*München. med. Wchnschr.* 74:139, 1927) between the destruction by various reagents of the sudanophilia of leukocytes and of the Winkler-Schultze oxidase reaction.

Recent studies in this laboratory have shown that a number of reagents promptly destroy myeloperoxidase (and M-Nadi oxidase) while conserving sudanophilia, while certain other reagents conserve the enzyme reactions quite well and destroy sudanophilia. There also appears to be some doubt as to whether the sudanophilia of the granules in neutrophil leukocytes is due to a fat (*J. Histochem. & Cytochem.* 1:8, 1953).

Of all the various technics for leukocyte peroxidase, I generally prefer the Washburn (*J. Lab. & Clin. Med.* 14:246, 1928) method. For this I prefer fixation of films for ten minutes in 75% alcohol. This has less damaging effect on the enzyme than 100% or 95% alcohol or any of the formaldehyde methods.

**The Washburn Method (Lillie 1952) Modified from Washburn.**

1. Fix air-dried blood or marrow films ten minutes in 75% alcohol; or in formaldehyde vapor for 30 minutes as follows: Place a piece of glass rod diagonally in the bottom of a Coplin jar to raise slides a little off the bottom. Add 1–2 cc. 40% formaldehyde. Put in slides smear-
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end up. Cover jar. After fixing, wash slides in two changes of distilled water.

2. Mix 1 cc. 30% aqueous sodium nitroprusside with 99 cc. 0.3% benzidine in 95% alcohol. This mixture is said to keep quite well in the cold, though I prefer to keep the benzidine solution alone and add the freshly prepared nitroprusside solution at time of using.

To the alcoholic benzidine nitroprusside mixture add an equal volume of freshly diluted 0.04% hydrogen peroxide, diluting the 30% stock solution 0.1 cc. in 75 cc. of distilled water, and pipet at once onto slides and let stand five minutes. To exclude negative reactions, double the time.

3. Wash two minutes or more in running tap water.

4. Counterstain ten minutes in strong Giemsa solution (pp. 228, 388).

5. Rinse in water, dry smears, and examine in an immersion oil of the mineral-oil type. Dehydrate sections with acetone, clear with xylene, and mount in neutral synthetic resin.

Results. Peroxidase granules in neutrophils, greenish or bluish black; in eosinophils, somewhat greener or brownier in tone; nuclei, violet to red purple; erythrocytes, orange pink. When reagents which inhibit myeloperoxidase are interposed after Step 1, erythrocytes often take a brownier tone and may present much granular or crystalline black deposit or long needle-shaped crystals.

In the foregoing technic Washburn added 0.3 Gm. basic fuchsin to the alcoholic benzidine nitroprusside solution, and after washing the reaction mixture off, decolorized to faint pink with 95% alcohol, counterstained with Wright’s stain (p. 389), rinsed, dried, and examined.

The following technics include benzidine and hydrogen peroxide as reactive materials, but no nitroprusside.

The Benzidine Peroxidase Reaction. The following technics for the benzidine peroxidase reaction and statements regarding it are a composite derived from Graham (J. Med. Res. 39:15, 1918), Mallory, Romeis, and Schmorl’s texts and Endicott’s work in this laboratory. Graham fixed fresh smears in a fresh 10% formalin alcohol for 1–2 minutes and then washed in water. Loele used frozen sections of formalin fixed tissue which are collected from the microtome in water. The staining solution is a saturated aqueous or 40% alcohol solution of benzidine to which 0.2–0.67 cc. 3% hydrogen peroxide per 100 cc. is added. Loele considered the aqueous solution more stable and prescribed shaking up 0.5 Gm. benzidine per 100 cc. distilled water, filtering, and adding 2 cc. 1% hydrogen peroxide to each 100 cc. filtrate. The 1% peroxide is a 1:30 dilution of the concentrated 30% solution. The technic:

1. Stain smears 5–10 minutes, sections 3–5 minutes in the benzidine peroxide reagent.
2. Wash in water.
3. Counterstain briefly in 0.1–0.5% methylene blue.
4. Wash in water.
5a. Blot smears, dry, and mount in balsam (or clarite).
5b. Float sections onto slides, blot, dehydrate with 95% and 100% alcohol, and clear with 100% alcohol and xylene mixture and two changes of xylene. Blotting between changes is necessary to keep sections on slides. Mount in balsam or clarite.

Results. Peroxidase granules, yellow to brown; nuclei, blue.

In Sato’s technic (Tohoku J. Exper. Med. 3:7, 1926, cited in Sato: J. Lab. & Clin. Med. 13:1058, 1928) the same benzidine and hydrogen peroxide solution can be used, though usually only 0.07–0.13 cc. 3% peroxide is added to each 100 cc. of filtrate, and Sato’s benzidine solution was only 0.25%. The technic:

1. Mordant unfixed fresh air-dried smears in 0.5% aqueous copper sulfate (CuSO₄·5H₂O) solution for one minute. Sato used 0.3% copper sulfate in 0.1% acetic acid.
2. Rinse very quickly in water (Mallory dipped films thrice in water; Gradwohl simply drained the films).
3. Stain in the benzidine hydrogen peroxide for 2–8 minutes.
4. Wash in water.
5. Stain 20 seconds to 2 minutes in 1% aqueous safranin.
6. Wash in water, dry, and mount in balsam, cedar oil, or clarite, or examine directly in immersion oil.

Villamil and Mancini (Rev. Soc. argent. de biol. 23:215, 1947 and 24:337, 1948) described a benzidine peroxidase technic for the demonstration of labile oxidases in thyroid epithelial cells. Frozen sections of unfixed tissue are required, and as little as 10–15 minutes treatment with alcohol or formalin destroys the enzyme, though a similar exposure to physiologic saline solution is tolerated.

Villamil and Mancini’s Ammonium Molybdate–Benzidine Peroxidase Reaction.

1. Immediately on excision of human surgical or animal specimen cut frozen sections at 10–15μ.
2. Immerse sections for 3–6 minutes in 1% ammonium molybdate in 0.9% sodium chloride solution.
3. Transfer to a saturated benzidine solution in 0.9% sodium chloride solution, to which a few drops of hydrogen peroxide are added. In about three minutes the sections become an intense blue.
4. Transfer directly to saturated (9%) picric acid solution in 95% alcohol and fix for five minutes.
5. Wash in water, dehydrate, clear, and mount as usual.

Results. Oxidase granules, dark blue in yellow cytoplasm of gland cells, histiocytes, and adjacent endothelial cells. To distinguish the more stable leukocyte oxidase, some sections should be immersed in 10% formalin for 15 minutes before Step 2.

The Graham α-naphthol pyronin stain (J. Med. Res. 35:231, 1916) for oxidase granules prescribes 1–2 minute fixation of fresh air-dried smears in fresh 10% formalin alcohol (p. 34).

1. Then wash in water.
2. Stain 4–5 minutes in 1 Gm. α-naphthol dissolved in 100 cc. 40% alcohol to which 0.2 cc. 3% hydrogen peroxide is added shortly before using.
3. Wash 15 minutes in running water.
4. Stain two minutes in 0.1 Gm. pyronin (C. I. No. 739 = Y or 741 = B), 4 cc. aniline and 96 cc. 40% alcohol.
5. Wash in water.
6. Stain 30–60 seconds in 0.5% aqueous methylene blue.
7. Wash in water, blot, dry, and mount in balsam.

Results. Neutrophil granules giving the oxidase reaction are purplish red; eosinophil granules are larger, lighter red, and more refractile; basophil granules are deep purple; cell nuclei are blue; cytoplasm, pale blue; erythrocytes, greenish yellow to pink.

The above α-naphthol peroxide solution can be used for four or five days. The aniline pyronin is relatively stable.

Ritter and Olson (Arch. Path. 43:330, 1947) carried out the α-naphthol pyronin method on blocks of fixed tissue which were then imbedded in paraffin and sectioned. They first fix 24 hours in 10% alcoholic formalin (p. 34) containing 1 cc. of 0.1 N sodium hydroxide per 100 cc. After washing ten minutes in running water the blocks were immersed for 24 hours in 100 cc. fresh 1% α-naphthol in 40% alcohol, to which 0.2 cc. 30% hydrogen peroxide is added at the moment of using. Then, after washing ten minutes in running water they transferred to 0.1% pyronin, 4% aniline, 40% alcohol for 3–24 hours. After this tissues were dehydrated 1 hour in 80%, 2 hours in 95%, and 2 hours in two changes of xylene of 20 minutes each and imbedded in paraffin. Sections were cut at 4μ and mounted without counterstaining, or counterstained with alum hematoxylin, or with a rather bluish Romanowsky stain. Eosin stains, if used, should be kept light to avoid confusion with the red peroxidase granules.

They observed that treatment with 100% alcohol before performance of
the α-naphthol peroxide treatment weakens the reaction, and that xylene treatment abolishes it.

The Winkler-Schultze reaction and the Nadi reaction depend on the synthesis of indophenol, the indophenol blue of the German writers, from α-naphthol and dimethylparaphenylenediamine in the presence of air. Lison referred to the reaction as a phenolase reaction.

The Winkler-Schultze method depends on an alkaline solution of α-naphthol, the Nadi method dilutes a 10% alcoholic solution one hundredfold with water, and utilizes preferably somewhat alkaline buffers during the staining procedure.

The Colour Index prescribes a definite proportion of alkali for dissolving the α-naphthol for the indophenol synthesis. This I have adopted in place of the vague directions of the original. I also use the more stable hydrochloride of dimethylparaphenylenediamine.

The Winkler-Schultze reaction, modified from Romeis, Schmorl, and Mallory.

Burtner and I (J. Histochem. & Cytochem. 1:8, 1953) fix blood films by immersion for 10 minutes in 75% alcohol, by exposure to formaldehyde vapor for 30 minutes, or by immersion in 75% alcohol containing 10% formalin for 30 minutes.

1. Dissolve 576 mg. α-naphthol in 6 cc. 1 N sodium hydroxide, and dilute to 50 cc. Dissolve 691 mg. p-dimethylaminoaniline hydrochloride in 50 cc. distilled water. Mix equal volumes, filter, and at once pipet onto slide on a staining rack. Rock gently to cover smears, let stand three minutes, decant, and rinse quickly in distilled water.

2. Immerse in dilute Lugol’s solution (1:KI:H₂O = 1:2:500) for three minutes.

3. Blue for 30 minutes in 1/20:000 solution of lithium carbonate, rinse in distilled water, and counterstain by immersion for 10 minutes in a fresh mixture of Giemsa stain 4 cc., acetone 3 cc., pH 6.5 0.1 M phosphate buffer 2 cc., distilled water 31 cc. Wash in distilled water, blow dry with compressed air, and examine.

Results. The positive reaction varies from large confluent blue-black globules, which may protrude over the cell borders, to dispersed fine gray-green granules. The latter occurs when smears have been first subjected to deleterious agents.

Schmorl used 40% ammonium molybdate instead of iodine in Step 2, followed by a simple wash in water. Counterstaining was omitted.

The method may be applied to frozen sections of tissue fixed briefly in neutral 10% formalin.
Gräff's G-Nadi Reaction, Emended from Schmorl and Romeis.

Reagent A. Dissolve 100 mg. α-naphthol in 1 cc. alcohol and dilute with distilled water to 100 cc.

Reagent B. Dissolve 120 mg. dimethyl-p-phenylenediamine hydrochloride in 100 cc. distilled water. This solution should be colorless or faintly pink. Schmorl makes these solutions half as strong as above.

Store the two solutions in tightly stoppered brown glass bottles. For use, mix equal volumes just before using and to 30 cc. of the mixture add 6 cc. 0.1 M disodium phosphate solution or an appropriate glycine sodium hydroxide, mono- and dibasic phosphate, or acetic acid-sodium acetate buffer of similar molarity, to give the desired pH level. Romeis also recommends the use of a weaker Nadi mixture: 10 cc. of mixed Nadi reagent to 40 cc. buffer. For the greatest activity, pH levels of 7.5–8.5 are recommended, but pH levels as low as 3.0 (that of the unbuffered mixture) and as high as 12.0 have been used.

Technic.

1. Cut frozen sections of fresh unfixed tissue. Immerse in buffered Nadi mixture and incubate at 37° C. for 1 hour (or 50° C. for 30 minutes).
2. Float onto slides, drain, and draw off excess fluid by touching with filter paper.
3. Rinse in 0.9% sodium chloride solution and mount in saturated potassium acetate solution (about 3 Gm. to 1 cc. water).

To make permanent preparations Schmorl uses the same ammonium molybdate or iodine procedure as in the Winkler-Schultze method (p. 228).

Results. Dark blue indophenol granules (as with the Winkler-Schultze method) also in lymphocytes and other cells as well as in leukocyte granules, etc.

Pretreatment with 0.1% potassium cyanide solution for a few minutes abolishes the G-Nadi reaction, whereas the Winkler-Schultze reaction is not affected. The alkali α-naphthol solution of that method cannot be used for demonstration of the labile oxidase (even if buffered to the same pH level?). Ordinary formaldehyde fixation prevents the Nadi oxidase reaction, but not the Winkler-Schultze; though Gräff found tissues fixed in formalin and buffered with phosphates to pH 7.3–7.6 for an unstated period suitable for his Nadi oxidase reaction.

Dopamelanase

The "Dopa" reaction demonstrates a specific intracellular ferment, dopamelanase, which converts 3,4-dihydroxyphenylalanine (also called dioxyphenylalanin and dopa) into a dark brown pigment dopamelanin.
Bloch's Technic (Arch. f. Dermat. u. Syph. 124:129, 1917; Romeis, op. cit. §954). Place fresh skin pieces in lukewarm agar solution, cool and cut as thin sections as possible on the freezing microtome. Soak sections 24 hours in 0.1–0.2% aqueous dihydroxyphenylalanine solution. Wash in water and mount in glycerol; or dehydrate, clear, and mount in balsam. The reaction may be made more intense by incubation at 37° C. Romeis suggests a methyl green pyronin counterstain. I suggest counterstaining five minutes in 0.1% aqueous Janus green B, differentiating one minute in 5% acetic acid, washing in water, floating onto slides, and mounting in gum syrup (p. 109).

Cowdry employs 0.1% dihydroxyphenylalanine buffered to pH 7.4 with phosphates at M/15, 6 cc. to 25 of the dopa solution, and incubates at room temperature for three or four hours. He stated that sections could be fixed 2–3 hours in 5% formalin, but not longer. Hershberger (in this laboratory) has fixed tissue in acetone for 24 hours at 5° C., changed thrice, cleared with benzene, and imbedded in paraffin.

Miescher (Arch. mikroskop. Anat. 97:326–396, 1923) successfully employed brief 4–5% formalin fixation (3–4 hours) before infiltrating various embryo eyes with 2% agar for preparation of frozen sections, and stated that even several days' exposure to formaldehyde solutions would not destroy the dopaoxidase reaction. He observed reaction by leukocyte granules and erythrocytes as well as in the pigment epithelium of retina, ciliary, and iris, and in the choroidal, ciliary, and iris star cells. Tyrosine, adrenalin, and a polyphenol complex of the Winkler-Schultze reaction failed as substrates.

According to Miescher the native pigment of the eye blackens distinctly on a 24-hour immersion in 2% silver nitrate solution, while the pigment formed by the melanase from dihydroxyphenylalanine does not.

While postembryonic eyes do not give dopamelanase reactions, this reaction may be restored by X-ray radiation.

The Bloch-Peck Method. Mallory cited a method of Bloch and Peck adapting the dopa reaction to the demonstration of leukocyte oxidases. The stock 0.1% 3,4-dihydroxyphenylalanine is made in 0.85% sodium chloride solution and stored tightly stoppered in the refrigerator. When it turns red it is to be discarded.

1. Smears are fixed by steaming 20 minutes face down over boiling formaldehyde solution.
2. Immerse slides in 50 cc. dopa solution in a Coplin jar and add 1 cc. 0.1 N (0.4%) sodium hydroxide solution.
3. When leukocyte granules are stained brown (usually in 1–2 hours) wash in running water, dry, and examine; or (3b) wash in distilled water and
4. Silver for two hours in 2% silver nitrate solution.
5. Again wash in distilled water.
6. Treat ten minutes with sodium thiosulfate solution (50 Gm. crystals, distilled water to make 50 cc.).
7. Wash 3–4 minutes in running water.
8. Stain 15–20 minutes in an unacidified alum hematoxylin.
9. Wash in water, dry, and mount in balsam.

Results. Without silvering, leukocyte granules are yellow to brown; after silvering, yellowish brown to dark brown.

Though Bloch and his followers vigorously deny it, adrenalin and tyrosine have both been successfully substituted for dihydroxyphenylalanine in the skin melanase reaction. It appears that conditions of somewhat greater irritation are necessary to stimulate the enzymatic synthesis of melanin from adrenalin and still more from tyrosine. It must also be pointed out that some workers deny the whole enzymatic basis and insist that the whole reaction is purely autooxidative in nature.

Tyrosinase

Fitzpatrick, Becker, Lerner, and Montgomery (Science 112:223, 1950) demonstrated that tyrosine could replace its first oxidation product dihydroxyphenylalanine \((dopa)\) as a substrate in the production of a melanin-like pigment by dendritic melanoblasts in the human dermal-epidermal border, provided the skin had previously been exposed \((in vivo)\) to ultraviolet or X-ray irradiation. Dopa could be substituted for tyrosine in their substrate, with similar final results. Tyrosinase activity is abolished by heating the sections to 100° C. for ten minutes, or by a six hour exposure to 0.171% (.01 M) sodium diethyldithiocarbamate.

The Tyrosinase Technic.

1. Fix slices of skin one hour at 5° C. in 10% formalin.
2. Slice further into blocks 1–2 mm. thick.
3. Wash in distilled water to remove formaldehyde.
4. Incubate 24 hours at 5° C. in 0.1 M phosphate buffer of pH 6.8 (p. 451) containing 0.0906% (0.005 M) L-tyrosine.
5. Transfer to a fresh portion of the same substrate and incubate for another 24 hours, this time at 37° C.
6. Rinse and refix in Bouin's fluid for 24 hours. Dehydrate, clear, infiltrate, and imbed in paraffin as usual. Section at 15µ. Counterstain with borax carmine or by some other desired method.

Results. The dendritiform melanoblasts are outlined by dark brown granules which are absent in controls of unirradiated skin treated as above, and in control blocks of irradiated skin incubated in pH 6.8 phosphate buffer without tyrosine. Melanin pigment in the basal epidermal cells is seen in both controls as well as in the test slides.
Amine Oxidase and Decarboxylase

Oster and Schlossman (J. Cell. & Comp. Physiol. 20:373, 1942) described a method for the localization of amino-acid decarboxylase and amine oxidase in guinea-pig kidneys. The decarboxylase first converts the amino acid to the next lower amine, and the amine oxidase converts the amine to the corresponding aldehyde with liberation of ammonia. The aldehyde is then rendered visible by the application of Schiff reagent (p. 156). Because of the natural occurrence of plasmalogen which liberates aldehyde (slowly in the presence of acid, more rapidly with mercuric chloride) it was thought necessary to first block these aldehydes with sulfite before proceeding with the specific enzyme technic. In the latter, tyramine was used as a substrate. When the amino acids L-tyrosine or L-tryptophane were used as substrates instead, the same localization was observed, indicating that the active areas (in distal convoluted tubules) possessed both decarboxylase and amine oxidase activities.

Technic.

1. Incubate frozen sections of fresh kidney in 2% sodium bisulfite solution for 24 hours at 37° C.
2. Wash thoroughly in distilled water.
3. Plasmal controls: Take several sections at this point and immerse for five minutes in 1% mercuric chloride solution, wash in water and immerse in Schiff reagent for 15 minutes, wash in 2–3 changes of 0.05 M sodium bisulfite, wash in water for five minutes, counterstain 1–2 minutes in acetic hemalum (p. 76), and mount in gum syrup. There should be no red color.

Buffer control: incubate 24 hours in M/15 pH 7.2 phosphate buffer.

Enzyme inactivation control: First soak some sections in octyl alcohol for 24 hours after the bisulfite treatment, then as in Step 3 experimental.

Experimental: Incubate at 37° C. for 24 hours in 0.5% tyramine hydrochloride in pH 7.2 M/15 phosphate buffer.
4. Wash in distilled water.
5. Place in Schiff reagent until blue color appears, allowing 30 minutes for buffer and inactivation controls.
6. Pass through three changes 90 seconds each of 0.05 M sodium bisulfite.
7. Float out and mount in potassium acetate gum syrup. Sections may be counterstained with acetic safranin if desired.

Results. Sites of amine oxidase activity, blue. If L-tyrosine or L-tryptophane is substituted for tyramine in the substrate, the blue color indicates the presence of decarboxylase as well as of amine oxidase.

The above technic has been emended from Oster and Schlossman to supply omitted details, and has not been tested in this laboratory.
Succinic Dehydrogenase.

Semenoff (Ztschr. f. Zellforsch. u. mikroskop. Anat. 22:305, 1935) reports a method for demonstration of succinic dehydrogenase depending on the decoloration of methylene blue in the presence of sodium succinate. The quantity of the last is not critical, and increases in concentration accelerate the reaction. The technic follows:

1. Cut frozen sections of fresh unfixed tissue.
2. Mount on clean slides in a few drops of substrate, cover with coverglass, and seal with petrolatum. Observe periodically. The substrate is composed of
   - 0.05% methylene blue
   - 10% sodium succinate
   - M/15 phosphate buffer of pH 7.6–8.0 to a total of 10 cc.
3. The preparations are decolorized first in the central area of the coverglass; later peripherally. Sites of greatest dehydrogenase activity are the first to decolorize. In liver and muscle, decoloration is complete in 30–80 minutes.

Control sections, heated to 60° C. for ten minutes, fail to decolorize methylene blue. Treatment with potassium cyanide at $10^{-4}$ or $10^{-5}$ completely inhibits the reaction; at $10^{-6}$ the reaction is retarded, and at $10^{-7}$ no effect is observed. It is presumed that the cyanide is added to the substrate, rather than that pretreatment was practiced.

The Tetrazolium-Formazan Reaction

Colorless, soluble tetrazolium compounds are converted by the addition of hydrogen into water-insoluble deeply colored pigments known as formazans. In tissue, this is accomplished by highly labile enzyme systems which are destroyed by the cold alcohol and acetone fixation processes; and even freezing inhibits the action (McLane, Stain Technol. 26:65, 1951). Plant tissues have given the reaction adequately after direct infiltration with carbowax from water and sectioning by that procedure. Black, Opler, and Speer (Am. J. Path. 26:1097, 1950) carried out the reaction by immersing thin (2–3 mm.) slices of tissue in a 1% solution of 2,3,5-triphenyltetrazolium chloride in 0.01 M phosphate buffer of pH 7.2 and incubating 30 minutes at 37° C. They then fixed the blocks in 10% formalin. Similarly Rutenberg, Gotstein, and Seligman (Cancer Research 10:113, 1950) incubated small pieces of mouse organs in a 1:5000 solution of a ditetrazolium chloride in 0.1 M phosphate buffer of pH 7.4 for two hours at 37.5° C. Formation of the dark-blue diformazan extended only to a depth of 0.5 mm. They then fixed in 10% formalin, cut frozen sections, and mounted them in “glycerogel.”

MacKenzie and Fuller (J. Lab. & Clin. Med. 35:314, 1950) observed the
development of large red globules in many cells in tumor scrapings when these were incubated at 37° C. for 20 minutes in a 1% triphenyltetrazolium chloride solution in Ringer's fluid (p. 112). They were not able to preserve the color with various alcoholic fixatives and resinous mounting media. The obvious remedy would be to uncover the incubated smears, fix 20 or 30 minutes over formaldehyde vapor, and mount in an aqueous mounting medium.

The extent of development of the formazan in gross preparations and in vivo has been used for estimating the spread of carcinoma, since the enzyme activity is generally more pronounced in epithelial and carcinoma cells than in stroma.

The enzyme activity responsible for the hydrogenation of the tetrazoliums is destroyed by boiling, by treatment with 0.002 M sodium cyanide, apparently also by alcohol, acetone, and formaldehyde, by cold storage for two days at 5° or —5° C., and by freezing. In vitro tests by Rutenburg et al. (loc. cit.) indicated that the dehydrogenation of succinic acid by dehydrogenase would hydrogenate tetrazolium, and that the degradation of cysteine by desulfurase to pyruvic acid, ammonia, and hydrogen sulfide also colored the tetrazolium by reduction. In the case of malic and lactic dehydrogenases, the presence of coenzyme I was required.

Most used has been 2,3,5-triphenyltetrazolium chloride, which produces a red formazan pigment 2,3,5-triphenyl formazan. Ditetrazolium (3,3'-dianisole bis 4,4'(3,4-diphenyl) tetrazolium chloride) produces a dark blue diformazan pigment (3,3'-dianisole bis 4,4'(3,5-diphenyl) formazan). Ditetrazolium is about five times as toxic for mice as triphenyltetrazolium. Apparently in the low concentration used (1:5000) it does not penetrate tissue slices as well as the triphenyltetrazolium. The procedure of perfusion of excised organs before incubation would appear to be indicated to secure complete penetration.

It seems possible that the failure of frozen sections of fresh tissue to react may be due to loss by leaching of soluble substrate such as succinic acid or cysteine, rather than to inactivation of the enzymes. Mowry tells me that he has inactivated tissue slices by soaking them in water and then reactivated them by use of a succinate substrate containing the tetrazolium as an indicator.

It would seem that cysteine might be used in a similar manner. In this case, tetrazolium would serve as an indicator of desulfurase activity.

Seligman and Rutenburg (Science 113:317, 1951) incubated 15 or more 20-μ frozen sections of fresh unfixed tissue for two hours at 37° C. in 20-25 cc. of a mixture of equal volumes of 0.1% aqueous ditetrazolium chloride, 0.2 M (5.4%) sodium succinate and 0.1 M phosphate buffer of pH 7.6 (p. 451). Sections were then washed in 0.9% sodium chloride solution, hardened 30 minutes in 10% formalin (optional), and mounted in glycerol gelatin. Sites of succinic dehydrogenase activity were shown by deposits of the dark-blue diformazan pigment. Fat droplets sometimes assumed a purplish red color.
Thinner sections (under 10\(\mu\)) fail to stain; thicker ones (30–40\(\mu\)) stained intensely. Covered vessels should be used for incubation, and the solution should be deaerated before use, either in vacuo or by boiling.

The activity was not inhibited by 0.105% sodium fluoride, by 0.015% sodium iodoacetate, or (anaerobically) by 0.0125% sodium cyanide; but 0.37% sodium malonate caused complete inhibition. Fixation for four hours in cold acetone (0° C.) destroyed about 40%; cold 100% alcohol, 50%; cold 70% alcohol, 80%; cold methanol, 70%. Storage for four hours at 4° C. caused no appreciable loss of activity.

Shelton and Schneider (Anat. Rec. 112:61, 1952) record that although frozen sections lack endogenous capacity to reduce tetrazoliums to formazans, yet in the presence of sodium succinate as substrate 30-\(\mu\) fresh frozen sections will reduce them.

Substrates are composed of equal volumes of 0.25 M sodium succinate (6.75% of the hexahydrate), distilled water, 0.1 M phosphate buffer of pH 7.5 (p. 451), and 0.1% tetrazolium. With neotetrazolium at 37° C. coloration appeared within 1 minute, and 45 minutes incubation was considered optimal. Neotetrazolium gave the finest crystals and the best localization. Fat droplets stain diffusely with the tetrazoliums and are hence readily distinguished from the crystalline deposits which occur in nonfatty cytoplasm. The authors do not regard the coloration of fat droplets as truly indicative of the localization of succinic dehydrogenase.

McLane (Stain Technol. 26:63, 1951) records that endogenous dehydrogenase activity is preserved in meristematic tissue of plants after infiltration at 51–52° C. in Carbowax 4000 (M.P. 50° C.). He warns against cooling the Carbowax blocks below 0° C., fearing destruction of the enzymatic activity.

According to Rutenburg, Wolman, and Seligman (J. Histochem. & Cytochem. 1:66, 1953) succinic dehydrogenase occurs only in cytoplasm and is greatest in heart, kidney, and liver. In muscle the activity appears to be localized in the anisotropic bands along myofibrils in both cardiac and skeletal muscle. In kidney, activity occurred throughout the cytoplasm of Henle and convoluted tubules; more basally than toward the lumen. No activity was evident in glomeruli, collecting tubules, or pelvis epithelium. Liver parenchyma cells exhibited greater activity in the periportal areas. Peptic glands showed most activity in parietal cells, then in chief cells. Nerve cells showed activity, glia cells none. Myelin presented a nonspecific pink stain with blue tetrazolium. Hair shafts also color intensely; but in this case the same reaction is obtained when the sodium succinate is omitted, and the authors attribute the reaction to sulfhydryl.

Improvement in staining and intensification of the reaction was achieved by conducting the reaction in recently boiled water in covered vessels. Loose, fairly thick frozen sections reacted better than attached or thinner sections. Addition of CaCl\(_2\) and of NaHCO\(_3\) (0.0015 M and 0.03 M) considerably
enhanced the reaction. A 40-cc. volume of their substrate now contains 20 mg. ditetrazolium chloride (blue tetrazolium, 10 cc. 2 mg./cc.), 540 mg. sodium succinate (10 cc. M/5), 10 cc. 0.2 M pH 7.6 phosphate buffer, 7.3 mg. CaCl$_2$ (0.2 cc. 0.33 M), 0.12 mg. MgSO$_4$ (0.2 cc. 0.005 M), 100.8 mg. NaHCO$_3$ (2 cc. 0.6 M), and 1.07 mg. AlCl$_3$ (0.8 cc. of 0.01 M), plus 6.8 cc. distilled water to make the 40 cc. total.

Tellurite Reaction for Dehydrogenases (from Gomori). Preferably use frozen sections of fresh, unfixed tissue. Tissue may be stored at 4° C. for several hours without loss of activity. Fixation for four hours in cold acetone causes only 40% loss of activity. The substrate is a 0.1 M to 0.05 M phosphate buffer of pH 7.3–7.6 containing sodium succinate, lactate, etc., in 0.1% to 0.2 M concentration and 0.1% potassium tellurite.

Incubate at 37° C. for 20 minutes to 3 hours, inspecting at intervals. Activity sites are shown by deposits of brown to black elementary tellurium. Wash in pH 7.0–7.3 phosphate buffer. Counterstain as desired (avoiding strong acids or alkalies), dehydrate in alcohol, clear in xylene, and mount in balsam. (Tellurium is insoluble in water and in fat solvents, but soluble in strong acids, alkalies, and KCN.)

Leukocyte Protease

Leukocyte protease has long been recognized from its lytic action on fibrin of exudates (Wells, *Chemical Pathology*, 3rd ed. Philadelphia and London, Saunders, 1918, pp. 94–96). It resists formaldehyde for long periods.

Histochemically it may be made evident from the selective cytolysis and later karyolysis of the neutrophil leukocytes which occurs in blood films which are first fixed ten minutes in 75% alcohol and then exposed to distilled water at 60° C. The enzyme is destroyed by fixation in boiling xylene. Formaldehyde fixation renders cytoplasm and nuclei resistant to digestion, and also to trypsin. The autolytic digestion occurs readily after fixation in boiling acetone, benzene, or toluene (Lillie and Burtner, *J. Histochem. & Cytochem.* 1:8, 1953).
Chapter 12

Endogenous Pigments

The Hemoglobins

Hemoglobin is well stained in erythrocytes by many acid dyes such as the eosins, and often its own yellowish color sufficiently modifies the tinge of red given it by eosin to give it a distinctly different color from that of other cytoplasmic materials. Certain red azo dyes such as azofuchsin G (C. I. No. 153) and azofuchsin GN (C. I. No. 154) stain erythrocytes deep red and are rather poor plasma stains; while brilliant purpurin R (C. I. No. 454) gives good brown cytoplasm and muscle, but rather pale yellowish brown erythrocytes. A mixture of one of the azofuchsins at 1 part of 1% solution to 4 parts of 1% brilliant purpurin R gave red erythrocytes and pinkish brown cytoplasm and muscle. It is probable that with any individual dye samples, varying mixtures would have to be tried to find the most differential one (see p. 349).

Certain basic aniline dyes (notably toluidin blue O and thionin), when used for 30–60 seconds in 1:1,000 solution, stain erythrocytes a brilliant green or yellowish green, nuclei deep blue, cytoplasm light blue, cartilage purple, and mast-cell granules deep purplish violet.

Alum hematoxylin counterstains of frozen sections stained for fats with oil red O often present deep olive-green erythrocytes. When overstained with neutral iron hematoxylin (either in combined solution or sequence technics) hemoglobin is stained black and is among the more difficult substances to decolorize, losing its color only just before myelin in freshly fixed chromated formalin material.

Allied to these stains is the Dunn-Thompson (Arch. Path. 39:49, 1945) method. It apparently requires neutral formalin fixation, for I have had it behave somewhat erratically on mailed-in formalin material. However, on tissue fixed in neutral buffered formalin it gives quite consistent results. It may also be used on tissue smears provided these are fixed moist in 10%
neutral buffered formalin, or in 3% tannic acid in methyl alcohol (3–5 minutes), or in methyl alcohol and subsequently treated with 3% tannic acid methyl alcohol as above.

The Dunn-Thompson Hemoglobin Stain. Smears and sections are brought to water as usual for sections.

1. Stain 15 minutes in Mallory’s aqueous alum hematoxylin (p. 76).
   (Probably any other unacidified alum hematoxylin will serve.)
2. Wash in tap water.
3. Mordant one minute in 4% iron alum.
4. Rinse in tap water.
5. Stain 15 minutes in a picrofuchsin solution composed of 13 cc. 1% acid fuchsin and 87 cc. saturated aqueous picric acid solution.
6. Dehydrate and differentiate 3 minutes in one or more changes of 95% alcohol. Dehydrate with 100% alcohol (two changes), and clear in xylene. Mount in Clarite.

Results: Cytoplasm—brown to yellow; hemoglobin casts, phagocytosed particles, and erythrocytes—emerald green; collagen—red; nuclei—brown to purple to gray black.

The Okajima method (Anat. Rec. 11:295, 1916) is one of the older differential hemoglobin stains. The following is Dunn’s modification, worked out in this laboratory: Fix in buffered neutral 10% formalin, imbed, and section in paraffin. Bring to distilled water as usual.

1. Mordant one minute in 10% phosphomolybdic acid.
2. Wash in distilled water.
3. Stain one hour in 10% phosphomolybdic acid 9.0 cc., saturated aqueous (7.69%) alizarin red S (Cl. I. No. 1034) 30 cc. (Okajima: 20 minutes to 20 hours).
4. Wash in distilled water.
5. Counterstain if desired with an unacidified alum hematoxylin (3–5 minutes).
6. Wash in water, dehydrate, and clear through alcohols and xylene. Mount in Clarite.

Results: Hemoglobin is light to dark orange red; the background is light brown with hematoxylin; orange without.

A further identification of hemoglobin lies in the fact that with acid formaldehyde fixation (pH 3–3.5) or with acid treatment of sections for sufficient time after neutral formalin fixation, red corpuscles and hemoglobin may sometimes give the Prussian blue reaction of Perls.

For the unmasking of iron in hemoglobin, Gomori recommends a 30-minute treatment with a few drops of 30% hydrogen peroxide alkalized with
some dilute ammonia or sodium carbonate. Then wash and apply the ferrocyanide reaction (p. 243). He suggests a similar treatment for the unmasking of copper. Here the rubeanic acid method (p. 267) should be used for demonstration.

See also hemoglobin peroxidase methods, pp. 222–224.

**Altered Hemoglobins**

Methemoglobin is colored bright red by the addition of potassium hydroxide in fresh unfixed material. This reaction is less distinct or absent in fixed tissue. Methemoglobin is partly soluble in alcohol. It is colored by certain basic dyes and stains like hemoglobin with acid dyes. Also like hemoglobin it may be stained by myelin methods. It is bleached by hydrogen peroxide. Silver nitrate and osmium tetroxide do not alter its color. It is best identified by spectroscopic study of fresh material.

Sulfhemoglobin or sulfmethemoglobin is a greenish sulfur methemoglobin compound occasioning the greenish discoloration of the abdominal wall of cadavers. It is distinguishable from other hemoglobin derivatives by spectroscopy. (The foregoing statements concerning the altered hemoglobins are derived from Schmorl and Mallory.)

**The Blood Pigments**

Pigments derived from hemoglobin include the so-called formaldehyde pigment, the hemosiderins, a brown granular pigment aposiderin (of deeper color than hemosiderin and free of readily demonstrable iron) which apparently is derived from hemosiderin, and hematoidin. The origin of von Recklinghausen’s (Versamml. Gesell. Naturf. u. Ärzte 62:324, 1889) hemo-fuscin observed in cases of hemochromatosis, which Hueck classed with the lipofuscins, and which Gillman and Gillman (Arch. Path. 40:239, 1945) have recently renamed cytolipochrome, I consider nonhematogenous. To these blood pigments should be added the malaria pigment. For convenience other iron-positive endogenous pigments which may be assigned a nonhematogenous origin are considered with the hemosiderins.

The formaldehyde pigment or acid formaldehyde hematin is formed when acid aqueous solutions of formaldehyde act on blood-rich tissues. It is a dark-brown microcrystalline substance which rotates the plane of polarized light. Consequently the individual, minute, rhomboidal particles glow and darken alternately with each 90° rotation of the stage when examined with crossed Nicol prisms. The pigment withstands extraction with water, alcohol, acetone, glycols, glycerol, fat solvents, and dilute acids. Formalin pigment is bleached promptly or within an hour by concentrated nitric acid, and partially by 90% formic acid. It is bleached in 30 minutes by 3% hydrogen peroxide or by 5% chromic anhydride (CrO₃). Sequence treatment with 5% potassium permanganate (but not 0.5%) and 5% oxalic acid removes the pigment, though neither reagent alone appears to bleach it.
It withstands extraction by concentrated sulfuric, hydrochloric, phosphoric, and acetic acids. It does not give Prussian blue or Turnbull's blue reactions with ferrocyanides or ferricyanides. It is extracted by treatment with weak alcoholic, aqueous, hydroalcoholic, or water-acetone solutions of sodium, potassium, or ammonium hydroxide, and by ammonia solutions in glycerol and glycols. It is removed at once by saturated alcoholic picric acid solution. It occurs copiously within vascular spaces among apparently intact or laked erythrocytes and also apparently within phagocytes. It seems more probable that the particles within phagocytes are formed from previously phagocytosed erythrocytes or hemoglobin than that the pigment is ingested as such. Spectroscopically it is similar to but distinct from hydrochloric acid and acetic acid hematin.

Several methods have been suggested for its removal. Vcrocy immersed sections ten minutes in 0.01% potassium hydroxide in 80% alcohol, and then washed five minutes in two changes of water. Schmorl warns that this treatment impairs the alcohol-fastness of the Gram stain, and finds Kardaszewitsch's method harmless in this respect. This method employed a 5 minute to 4 hour extraction in a 1-5% dilution in 70% alcohol of 28% ammonia water. Subsequent washing with water or alcohol is necessary to remove the excess ammonia. The saturated alcoholic picric acid mordanting (p. 95) before Masson stains (p. 350) should remove formalin pigment, especially if prolonged to five minutes. A mixture of 50 cc. each of acetone and 3% hydrogen peroxide and 1 cc. 28% ammonia water removes the pigment in five minutes or less.

More important than methods of removal of formaldehyde pigment is the fact that it is not formed on fixation with formaldehyde buffered to pH levels above 6.0. An exception is sometimes noted in those portions of the spleen adjacent to the stomach, where one might expect formation of acid hematin from the action of the hydrochloric acid on the blood post mortem. Formalin at pH levels from 3.0 to 5.0 forms large quantities of the pigment, with or without obvious lysis of erythrocytes.

Similar pigment is often found on the surface of the gastric mucosa when hemorrhage has occurred. I have observed this principally in experimental toxicologic material fixed in formalin, and cannot say whether or not it would appear here with other fixations. However, similar pigment may be formed in spleen tissue fixed in 1% acetic or formic acid in 89% alcohol or acetone, without formaldehyde.


Malaria pigment occurs in the parasites (especially the quartan P. malariae), about brain capillaries, and in littoral phagocytes in spleen, liver, bone marrow, and lymph nodes. It is an amorphous, dark-brown, granular pigment which does not fluoresce in ultraviolet light but rotates the plane
of polarized light, resembling in the latter respect the otherwise quite similar, microcrystalline and doubly refractile acid formaldehyde hematin, the so-called formalin pigment. Kósa (Virchows Arch. f. path. Anat. 258:186, 1925) stated that that part of the malaria pigment which was free or in erythrocytes was doubly refractile, while that in phagocytes never was. Like formalin pigment it is soluble in dilute aqueous and alcoholic solutions of sodium, potassium, and ammonium hydroxides; is bleached within an hour by concentrated nitric acid, by hydrogen peroxide, and partially by 90% formic acid; and is insoluble in dilute (5%) aqueous solutions of mineral acids, and in concentrated acetic, hydrochloric, phosphoric, and sulfuric acids. On direct experiment it was found that both these pigments remained identifiable in slide preparations for two weeks if sections were first blotted dry, treated with the 96.5% sulfuric acid (sp. gr. 1.84), covered with a cover glass, and sealed with petrolatum. But tissue structure was destroyed (Hershberger, unpublished data).

Schmorl states that malaria pigment is soluble in 5% alcoholic solutions of sulfuric, nitric, and hydrochloric acids at 40–50° C. in one day or less. It is also soluble in aniline, in pyridine, and in a 4% solution of quinine in chloroform. It is insoluble in fat solvents and is not stained by fat or lipoid stains. It is not blackened by osmium tetroxide or silver nitrate. Malaria pigment gives no iron reaction on direct test, but according to M. Kósa (loc. cit.) it may give a Prussian blue reaction with potassium ferrocyanide when sections are first treated 10–12 hours with 2% oxalic acid or 1% hydrochloric acid to remove hemosiderin, washed with distilled water, treated for 5–10 minutes with 1% potassium hydroxide, and again washed with distilled water. In my experience the prescribed acid treatment may be inadequate to remove the ferric iron from hemosiderin in formalin-fixed material. Hence I consider the validity of Kósa’s statement questionable.

Brown (Exper. Med. 13:290, 1911) calls malaria pigment “hematin,” while Mallory refers by this name to a dark-brown amorphous pigment not further characterized which occurs in old extravasations of blood. Neither of these uses of the word should be confused with the usual meaning of an acid or alkaline, soluble hemoglobin derivative with a characteristic absorption spectrum.

**Hemosiderins**

The hemosiderins are by definition pigments which exhibit one or more of the reactions of ionic iron. Three principal reactions have been used: The formation of ferric ferrocyanide (Prussian blue) when the material is treated with acid solutions of ferrocyanides (Perls’ reaction); the formation of black iron sulfide (FeS) when material is treated with ammonium sulfide (Quincke’s reaction); and the formation of ferrous ferricyanide from the ferrous sulfide thus formed, by treatment with a ferricyanide and acid (the Tirmann-Schmelzer reaction). In addition, Mallory has proposed a reaction
with fresh unoxidized hematoxylin which gives a black color with iron and a clear blue with copper (p. 266).

It is often stated that the fixative of choice for demonstration of hemosiderin is alcohol. On comparative tests of the same material with various fixatives, I have found that positive Prussian-blue reactions are most often obtained when 10% formalin buffered to pH 7.0 is the fixing agent. This is definitely superior to alcohol, alcoholic formalin, Orth's fluid, and unbuffered 10% formalin. However, in more or less autolyzed human liver the iron positive pigment in liver cells may be better preserved with alcohol.

According to Schmorl, Hall has proposed fixation of fresh tissue in alcoholic solutions of ammonium sulfide containing 70 parts of 100% alcohol. For liver, spleen, and bone marrow he made up the remaining 30 parts with strong ammonium sulfide solution; for other tissues he used 5 parts of ammonium sulfide and 25 of distilled water. The fixation interval was 24 hours. This amounts to a Quincke reaction done in the block on tissue during fixation. Hall prescribed a ferrocyanide reaction to follow on the paraffin sections. A ferrocyanide test instead would seem more logical, and would make the method conform to the Tirmann-Schmelzer reaction.

The Quincke (Deut. Arch. klin. Med. 25:567, 1880) (omit Steps 4 and 5) and Tirmann-Schmelzer (Hueck; Schmorl; Mallory) reactions: Formalin or alcohol fixation and celloidin or paraffin sections may be used.

1. Bring sections to distilled water as usual.
2. Impregnate sections 1–2 hours, or as long as 1–2 days in strong, slightly yellow ammonium sulfide solution. Mallory prefers to dilute this with 3 volumes of 95% alcohol, to avoid loss of sections. Otherwise it would seem indicated to soak sections first in 1% collodion for 5–10 minutes after deparaffinizing, drain one minute, and harden 5–10 minutes in 80% alcohol before bringing to water. Highman (Internat. Assoc. Med. Museums Bull. 32:97, 1951) substitutes a 24-hour impregnation in saturated hydrogen sulfide water for hematite and other refractory iron-ore dusts which fail to react to the usual procedure.
3. Wash thoroughly in distilled water.
4. Soak sections 15 minutes in equal volumes of 1% hydrochloric acid and 20% potassium ferricyanide, freshly mixed. This step is omitted in the Quincke method.
5. Wash thoroughly with distilled water.
6. Counterstain with 0.5% basic fuchsin in 50% alcohol for 5–20 minutes, wash in water, differentiate in alcohol according to Mallory; or with alum carmine for 1–24 hours and wash in water according to Schmorl; or with 0.1% safranin for two minutes and wash with 2% acetic acid for two minutes.
7. Dehydrate with alcohols, clear in xylene, and mount in Clarite or poly styrene.
Results: Quincke's reaction gives a dark-brown to black color to the iron pigment; Tirmann and Schmelzer's, a dark blue.

The brown color of the sulfide is less readily distinguished from other brown-colored substances than the blues of the Prussian and Turnbull blue reactions. Silver, lead, and mercury also give dark-brown to black deposits with this method. Other brown and black insoluble sulfides are noted on p. 260. Unless special precautions are taken sections are often lost in the alkaline sulfide solution. The same objection applies also to the Tirmann-Schmelzer reaction. Further, if instead of potassium ferricyanide the ferrocyanide is applied to the sections treated with ammonium sulfide, a positive Prussian blue reaction is still obtainable. This indicates that only a portion of the ferric iron originally present was converted to ferrous sulfide. It might be objected that Perls's reaction was open to the same criticism; i.e., that it demonstrated only ferric iron. However, with numerous tests of hemosiderin-containing material, I have never obtained a direct positive Turnbull's blue reaction on an intrinsic hematogenous pigment with acidulated potassium ferricyanide, when that salt is substituted for potassium ferrocyanide in the technic given below. Bunting, however, reports positive diffuse ferricyanide staining as well as ferrocyanide staining in the mixed calcium iron deposits occurring in necrotic areas (J. Nat. Cancer Inst. 10:1368, 1950).

Other Ferricyanide Reactions. Other insoluble ferricyanides include cobaltic and cobaltous—which are brown and red; cupric and cuprous—greenish yellow and brownish red; lead—red and soluble in hot water; nickel—brown; silver—orange; and stannous—white (Lange).

Dinitrosoresorcinol* gives a dark-green color with iron salts. Humphrey (Arch. Path. 20:256, 1935) substituted this reagent for the ferricyanide of the Tirmann-Schmelzer procedure, directing as follows:

After a one-minute bath in 30% ammonium sulfide, rinse in water and stain 6–20 hours in dinitrosoresorcinol. Either a saturated aqueous solution is used or a 3% solution in 50% alcohol. Wash in the same solvent, dehydrate, clear, and mount in Canada balsam. The dark-green color is quite permanent in balsam.

The Ferrocyanide Reaction of M. Perls (Virchows Arch. f. path. Anat. 39:42, 1867). The following technic works well on the hemosiderins and most mineral iron. Hematite dust deposits may require more drastic treatment to obtain the reaction.

1. Fix 48 or more hours in 10% formalin buffered with phosphates to pH 7 (p. 34).
2. Dehydrate, clear, imbed in paraffin, and section as usual.
3. Make up fresh 2% solution of potassium ferrocyanide in distilled water and add an equal volume of 0.25 N (2%) hydrochloric acid (Bunting) or 5% acetic acid (Highman). Heat sections 30 minutes at 60° C. in

* Dinitrosoresorcinol (resorcin green, C. I. No. 1) was obtained from Eastman.
this mixture; or (Bunting) let stand an hour at room temperature. Gomori used a 30-minute bath at room temperature in a solution containing 2 Gm. potassium ferrocyanide, 36 cc. distilled water, and 4 cc. concentrated hydrochloric acid.

4. Rinse in distilled water.
5. Counterstain two minutes in 0.2% safranin O in 1% acetic acid.
6. Wash in 1% acetic acid.
7. Dehydrate with 95% and 100% alcohols, 100% alcohol + xylene; clear in two changes of xylene, and mount in polystyrene or other non-reducing resin (p. 105).

**Results:** Reaction sites, blue or green; nuclei, red; background, pink. Freshly formed deposits of iron pigment react well with the acetic variant and are less likely to be dissolved out. Older deposits may require the stronger acid for adequate reaction. See p. 261 for the demonstration of hematite and other highly insoluble iron compounds. Heating to 80° C. in the ferrocyanide, as in the Abbott variant, is apt to produce a finely granular, blue deposit throughout the section. The Abbott variant was quoted in the 8th edition of Mallory and Wright; the other references are Bunting, *Stain Technol.* 24:109, 1949; Highman, *Arch. Path.* 33:937, 1942; Gomori, *Am. J. Path.* 12:655, 1936.

**Other Ferrocyanide Reactions.** Uranium potassium ferrocyanide is dark brown; cupric and cuprous ferrocyanides, red brown; cobaltous, gray green; cobaltic, dark brownish red; mercuric, white; lead, yellowish white; nickel, greenish white; silver, yellow; barium, yellow; ferrous, bluish white; manganese, greenish white; and zinc, white. These are most of the insoluble ferrocyanides listed (Lange). Most of these reactions have not been used histologically.

**Characteristics of the Hemosiderins.** Among the iron-positive hematogenous pigments three subvarieties may be distinguished. The first, seen in relatively acute hemolytic intoxications, is manifested as a diffuse brownish staining of cytoplasm and reacts with a diffuse light-blue color when acid ferrocyanide is applied. The other two are seen as granules of variable size, yellow to brown in color, both colored deep-blue to green by ferrocyanide. The one stains green with azures, thionin, toluidin blue, and methylene blue; the other remains unstained by basic anilin dyes. These two sometimes appear to intergrade, and the diffuse and granular forms of iron-positive pigment may often be present in the same cell. The diffuse form is more easily lost on fixation in acid formalin. A similar diffuse Prussian blue reaction is sometimes seen in the contents of renal tubules in the presence of hemoglobinuria. This change is probably related to the positive Prussian blue reaction which may be induced in erythrocytes by fixation in 10% formalin buffered to pH 3.5 or 4.0, or by immersion of sections of material fixed by neutral formalin in solutions of mineral acids for varying periods.

In the unfixed state, hemosiderin is fairly readily soluble in acids. This
solubility is diminished by the simultaneous presence of formaldehyde in acid fixing fluids. Similarly, after fixation hemosiderin is more readily dissolved by acid when the previous fixing fluid contained no formaldehyde. Acid fixatives containing no formalin may either completely remove hemosiderin or convert it into a brown iron-free pigment. A similar conversion to brown iron-free pigment occurs with formalin buffered to pH 3–4, in material which presented only iron-positive pigment when fixed in formalin buffered to pH 7.0. The presence of formalin in the fixing fluid may protect the iron content of hemosiderin against acetic, formic, or hydrochloric acid in the fixing fluid, but not against sulfuric acid.

After formalin fixation, hemosiderin remains iron positive after perhaps 2–4 weeks’ exposure to 20% acetic acid, 3–7 days in a 2.5% dilution of concentrated hydrochloric acid in 70% alcohol, as long as 2 days in 1–10% aqueous dilutions of concentrated hydrochloric acid, perhaps several hours only in 20% formic acid (though it may resist 5% formic acid for 2–3 days during decalcification of cancellous bone), usually less than 2 hours in 5% oxalic acid, and less than 24 hours in 10% sulfuric acid (by volume = 18.4 Gm. per 100 cc.). Often when the iron reaction becomes negative after acid extraction (and especially if the extraction is done at 37° C. instead of at room temperature) a brown granular pigment is left (Am. J. Path. 15:225, 1939).

Hueck stated that hemosiderin is insoluble in alkali solutions, is unaltered by bleaching agents such as hydrogen peroxide, does not blacken with osmium tetroxide or silver nitrate, and does not stain with oil-soluble dyes. He apparently dissented from Schmidt’s view that there was also an iron-negative pigment originating from hemosiderin. He states further that hemosiderin does not stain with basic anilin dyes; but I find iron-positive pigments which do so stain as well as those which do not.

According to Popper (Arch. Path. 41:766, 1941) hemosiderin does not fluoresce in ultraviolet light.

Heart-Failure Cell Pigment. This usually iron-positive pigment, found in large phagocytic cells in pulmonary alveoli and in sputum in cases of chronic passive congestion of the lungs, is singly refractile, and brown in color. In some cases it is more successfully demonstrated by the hydrochloric acid ferrocyanide technic; in others this procedure produces blue halos about the granules. In some cases a positive periodic acid Schiff reaction is given, in others not. Ferric ferricyanide (p. 175) is not evidently reduced. The pigment is not acid-fast in the Ziehl-Neelsen technic. It does not retain iron hematoxylin in the myelin variant method of eosinophils (p. 194), nor fuchsin in the Mallory hemofuscin method. Some of the darker-brown examples of this pigment may be largely iron negative with even the hydrochloric acid ferrocyanide method. The iron reaction does not correlate with the periodic acid Schiff reaction. Usually the pigment granules are untinged by oil-soluble dyes.
Like some other iron-positive pigments, notably that of the involuting corpus hemorrhagicum of the ovary, this pigment may reduce methenamine silver while nuclei and red cells are still unblackened. This reaction is probably similar to that of mucins and collagen, which blacken selectively with methenamine silver after treatment with ferric chloride (p. 166). Diammine silver solutions such as are used for reticulum impregnation (p. 334) do not blacken the iron-positive pigments mentioned above.

Extraction of the iron by a 24-hour bath in 10% sulfuric acid does not remove much of the darker brown material, though the iron reaction is negative. The periodic acid Schiff reaction of the granules is not decreased by the acid extraction. The amount of material which is dark-brown to black after 3–3½ hours at 60° C. in methenamine silver (p. 165) is much reduced by the acid extraction. This supports the thesis that the methenamine silver reaction is due to the iron itself.

Cytosiderin. The iron positive pigment cytosiderin occurring in liver cells in hemochromatosis and derived from mitochondria by Gillman and Gillman (Arch. Path. 40:239, 1945) appears to have been closely associated with the so-called hemofuscin, lipofuscin, or cytolipochrome pigment, but is otherwise similar to granular hemosiderin in its behavior.

Other Iron-Positive Pigments. The iron-positive pigment in apocrine gland cells is of uncertain origin. That in renal epithelium I regard as granular hemosiderin.

The iron-positive pigment in cutaneous xanthomata often appears to be related to the lipoids.

Aposiderin. As noted above, hemosiderin fixed in formalin at pH 3–4 may be converted in part or entirely to a brown granular iron-negative pigment. This same loss of iron apparently occurs also in vivo, since in cases of renal epithelial pigmentation one finds in recent intoxications perhaps only iron-positive pigment granules in the epithelium of the convoluted tubules. In more chronic poisonings both iron-positive and iron-negative granules are seen, perhaps the one in one tubule, the other in an adjacent tubule, perhaps both in the cells of a single tubule or in the same cell. And with quite chronic intoxications perhaps only iron-negative darker-brown pigment granules are found in the renal epithelium (Pub. Health Rep. 58:304, 1943). This brown pigment is quite resistant to extraction (Miller, Pub. Health Rep. 56:1610, 1941), resisting concentrated sulfuric acid for 72 hours in one series of tests, and 28% ammonia for 6 hours in another. It is not appreciably bleached by 3% hydrogen peroxide in an hour. On overstaining with basic fuchsin it does not withstand alcohol differentiation. It thus differs from Mallory's hemofuscin. It is not doubly refractile. These granules gave negative hydrogen sulfide and bichromate reactions for lead.

It is suggested that the name hemosiderin be restricted to the yellow to brown, granular, iron positive pigment; that the diffuse brownish iron positive material be designated protosiderin (Gr. πρῶτος first, αἰθήρος iron); and
that the residual iron negative brown granular material be called *aposiderin* (deg. away from).

**Pseudomelanosis.** The pigment of the so-called pseudomelanosis of the intestine is apparently hemosiderin converted to ferrous sulfide by the post-mortem action of hydrogen sulfide originating in the intestinal contents. As usual with sulfide-treated hemosiderin it should give both Turnbull's blue and Prussian blue reactions.

In view of the frequently positive iron reaction of the enterosiderin pigment of guinea pigs, and the complete agreement in the other reactions of this pigment with those of the pigment of melanosis coli of man, it would seem that the relation between pseudomelanosis and melanosis pigments needs reexploration with modern histochemical technics.

**Hematoidin** occurs as reddish- or yellowish-brown crystals and amorphous granules in old hemorrhages and infarcts. On treatment with concentrated nitric or sulfuric acid it assumes a brownish- to purplish-red color, gradually changing through violet and blue to green (positive Gmelin reaction). It is also turned green by hydrogen peroxide, but not bleached. It gives no iron reactions. It is gradually decomposed by alkalis. It is poorly soluble in fat solvents, best in chloroform, slightly in alcohol, not in ether. It is not stained by basic dyes or by fat stains and is not blackened with silver nitrate or osmium tetroxide.

**Bilirubin** also gives the Gmelin reaction, which can be observed under the microscope by mounting the sections in water under a cover glass and finding the pigment. Then apply a few drops of nitric acid containing 1-2% nitrous acid to one edge of the cover glass, and a piece of dry filter or blotting paper to the other. The acid is drawn in, and reacts with the pigment to give the succession of green, red, and blue colors. It may be necessary to treat the section previously with a drop or two of very dilute sodium or potassium hydroxide solution. Oxidation with peroxides or bichromates converts bilirubin into green biliverdin. Bile pigments do not fluoresce in ultraviolet light (Popper, p. 14).

Stein's method for bilirubin (*Compt. rend. Soc. de biol. 120:1136, 1935*) depends on its conversion to a green substance, possibly biliverdin, by the action of iodine. Stein finds formalin-fixed material best, but material fixed in alcohol or in Helly's fluid can be used.

*Stein's Iodine Method for Bilirubin.*

1. Fasten paraffin sections to slides by floating on water with albumen fixative. Dry well.
2. Deparaffinize and hydrate as usual.
3. Immerse sections in a mixture of 2-3 parts Lugol's solution and 1 part tincture of iodine* for 6-12 hours.
4. Decolorize with sodium thiosulfate solution for 15-30 seconds.

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*Composition of solutions not further indicated.*
5. Counterstain 1–3 hours in alum carmine.
6. Wash several times in distilled water.
7. Dehydrate with acetone, clear with xylene, mount in balsam.

**Results:** Nuclei, red; bilirubin, green; other pigments, melanin, lipofuscins, hemosiderin, lipochrome, and lutein do not react.

**Hematoporphyrin** gives the same Gmelin reaction as bilirubin, and is insoluble in dilute acids and alkalis.

Mallory refers to a dark brown or bluish black pigment occurring in old extravasations of blood as hematin, but does not further characterize it. I infer that it is iron negative.

The foregoing statements regarding pseudomelanosis, hematoidin, bilirubin, and hematoporphyrin have been brought together from Mallory, Hueck, Schmorl, and Romeis.

Lison (1953) recommends use of fluorescence microscopy for the detection of porphyrins in the tissues. With long-wave ultraviolet (365 mÅ), orange to red fluorescence is observed. Harderian gland of rodents, shell gland of the hen's oviduct, rodent placenta, and regressing corpora lutea form good test objects.

**Ochronosis**

In ear cartilage Friderich and Nikolowski (*Arch. f. Dermat. u. Syph. 192:273, 1951*) described an irregularly distributed, yellowish brown pigment which gave a yellow to golden fluorescence with ultraviolet light. In hematoxylin eosin preparations the pigment was sepia brown; with Van Gieson, greenish and reddish overtones appeared in the basic yellow brown. It tinges red with Kernechtrot, purplish-red to gray-yellow by Mallory-Helendhain “Azan,” and blue-black with cresylecht violett. Elastic, silver, and iron reactions were negative.

Gomori regards the cresyl violet staining as its most characteristic property, and states that it is not argentaffin.

**The Lipofuscins**

The so-called brown atrophy, “waste,” Abnutzung, or lipofuscin pigments often found in heart-muscle cells, in liver cells, in adrenals, seminal vesicles, testes, epididymides, ganglion cells, and elsewhere probably constitute a heterogeneous group. They are generally yellowish-brown, iron-negative, granular pigments which resist the usual procedures in paraffin imbedding. They often stain with oil-soluble dyes, even after paraffin imbedding; but the preparations after staining must not be dehydrated or treated with fat solvents. The oil blue N technic (p. 303) gives a blue to green color which I find easier to discern than the alteration of the natural brown color to a brownish red with Sudan IV or oil red O. Mount in syrup or glycerol media (pp. 302–304, 109–112).
These pigments often blacken with osmium tetroxide. They blacken slowly with silver nitrate; better, according to Staemmler (cited from Schmorl), with diaminos silver hydroxide (Maresch's Bielschowski solution, p. 334, diluted with distilled water to 50 cc. instead of 25 cc.). Thus: Heat the dilute silver solution to boiling, filter through a doubled filter paper, and pour over sections which have previously been brought to water in the usual manner. Let stand three minutes, rinse in distilled water, fix 30 seconds in 5% sodium thiosulfate, wash, dehydrate, clear, and mount.

In fluorescence microscopy the lipofuscins of the liver, adrenal, testicular germinal epithelium, and heart muscle give a red brown fluorescence. Hepatic lipofuscin fluoresces brown before extraction with alcohol, and red brown after; and the extract gives the labile green fluorescence of vitamin A (cited from Popper: Arch. Path. 41:766, 1941).

Adrenal fuscin pigment is seen as variably numerous, usually fine brown granules in the parenchyma cells of the reticular zone of the cortex; and as coarser, usually darker brown granules staining similarly but more deeply and contained in scattered, small interstitial phagocytes in the reticular and inner fascicular zones of the cortex. This pigment is colored green to deep blue in the ferric ferricyanide reduction test (p. 175), blackens with methenamine silver in the argentaffin method (p. 165), and colors reddish brown to red purple with the periodic acid Schiff method (p. 123). The coarser globules, both in phagocytes and in parenchymal cells, are often peracetic acid Schiff (p. 310) positive, and while some granules react even after Carnoy fixation, there appears to be more reaction after bichromate fixations.

The capacity to reduce ferric ferricyanide is abolished by bromination (p. 311), and by oxidation for one hour in 5% chromic acid or for 20 minutes in 0.5% potassium permanganate; but not by the usual -SH blocking reagents, nor by periodic acid. Conversely, the periodic acid Schiff reaction is unaffected by bromination, weakened by chromic acid and permanganate oxidations, and abolished by acetylation. It may often be colored with oil-soluble dyes after paraffin imbedding. For this purpose the Sudan black method (p. 320) is recommended. Acid-fastness may be demonstrated in some cases. Here a night-blue or Victoria-blue technic may be more convincing than the usual fuchsin method because of the greater difficulty of distinguishing reddish brown from brown than blue or green from brown (p. 381).


The Leydig-cell lipofuscin is colored by Sudan black B and by oil red O in paraffin sections of formalin-fixed material. It reduces ferric ferricyanide (p. 175). Part of it is acid-fast by the Ziehl-Neelsen method, and more retains the basic fuchsin in Mallory's hemofuscin stain (p. 252). Methenamine silver is reduced in the argentaffin-cell technic (p. 165), but no definite reduction of diaminos silver hydroxide is seen in a reticulum method (pp. 343-344) in
which the Weigert bleach is employed (p. 337). A minority of the granules are Schiff-positive after peracetic acid (p. 310) and on 48-hour exposure to Schiff reagent directly (p. 313). The granules color red purple in the periodic acid Schiff method (pp. 123–124) and the reaction is not impaired by a diastase digestion which removes all glycogen from germinal epithelium. In azure eosin stains the pigment is brown to dark green, and the basophilia is destroyed by one-day methylation (p. 163) but not by methanol extraction for seven days. Ferrocyanide reactions for iron are negative, and iron hematoxylin is not retained in the eosinophil myelin technic.

A quite similar pigment occurs in the epithelial cells of some tubules of the epididymis. It is basophilic, readily methylated, and periodic acid Schiff positive with or without diastase digestion, as is the cuticular border of the epithelium. It is occasionally Schiff positive on direct 48-hour exposure, and after peracetic acid oxidation. It is partly acid-fast and positive by the Mallory hemofuscin technic. It reduces methenamine silver and ferric ferrocyanide. The ferrocyanide reaction for iron and the Weigert-Lillie myelin tests (p. 194) are negative.

Ovarian fuscin pigment occurs in phagocytes near involuting corpora lutea and perhaps in their walls. It colors light to dark green with thionin and with azure eosin. It stains greenish black and greenish blue respectively with Sudan black and spirit blue in paraffin sections (p. 320). The periodic acid Schiff (p. 123) reaction is moderately strongly positive. Part of the granules give the peracetic acid Schiff reaction as well (p. 310). The granules are only partially acid-fast with Ziehl-Neelsen and Victoria blue technics (p. 380). In myelin technics iron hematoxylin is retained longer than in nuclei, but not as well as in erythrocytes. The ferrocyanide test for ferric iron is negative (p. 243). Ferric ferricyanide is sometimes reduced (p. 175), the granules becoming green to deep blue; and the granules in part blacken with methenamine silver. With Mallory's hemofuscin method, retention of the dye is inconstant.

In corpora hemorrhagica, iron-positive pigment is also seen, associated with strongly eosinophilic iron-negative granular material which is perhaps only partly degraded hemoglobin. It is not clearly evident that the iron-positive pigment is necessarily unrelated to the fuscin.

In some instances both the iron-positive and the iron-negative pigments blacken with methenamine silver (p. 165). Sudan black B and oil red O clearly differentiate the two pigments, even when granules of both are present in the same cell. Likewise diaminine silver (p. 256) blackens the fuscin pigment and not the iron-bearing granules. This last is a true argentaffin reaction, since the blackening is observed with no following reduction bath after as little as two minutes' exposure to the silver solution.

Although the Winkler-Schultze indophenol synthesis is not usually effected by unaltered ovarian pigment, a preliminary two-hour oxidation by peracetic acid (p. 310) followed by thorough washing in water causes many ovarian pigment granules to stain a definite blue green with the Winkler-Schultze
procedure. This probably indicates the formation of (fatty acid?) peroxides from unsaturated compounds.

*Lutein.* Apparently the lutcin described by Schmorl was a mixture of lipofuscin and a carotenoid pigment (see p. 258).

*Cardiac lipofuscin* pigment often stains better than the other lipofuscin pigments with oil-soluble dyes after paraffin imbedding. Sudan black or oil blue technics are preferred to Sudan IV or oil red O, because a green discoloration of the brown pigment is easier to discern than an orange discoloration. Ferric ferricyanide reduction is observed in some but not all cases. Part of the pigment gives the periodic acid Schiff reaction, a minor proportion the peracetic Schiff. The pigment is not definitely acid-fast. It does not retain the myelin stain with iron hematoxylin (p. 194). With ferrocyanide it does not react for iron. A minor proportion of the granules reduce methenamine silver (p. 165). Diammine silver is not reduced, either with or without a sensitizing step in the reticulum technics (pp. 334, 343). When the permanganate oxidation is omitted then a minor proportion of the granules blacken. The pigment stains green with thionin at pH 3.

*Nerve Cell Fuscin.* According to Wolf and Pappenheimer (*J. Neuropath. & Exper. Neurol.* 4:402, 1945) the yellowish-brown lipofuscin pigment of nerve cells and glia cells is also acid-fast after formalin fixation. It is basophilic but less sudanophilic in paraffin sections than is ceroid. The melanin of the cells of the substantia nigra and locus caeruleus is not acid-fast.

Dixon and Herbertson (*J. Path. & Bact.* 62:335, 1950) describe a brown pigment of the lipofuscin group in nerve cells of the human olivary and other medullary nuclei and of the anterior horns of the spinal cord. This pigment gives a positive periodic acid Schiff reaction (p. 123), stains black with Sudan black after formalin fixation and paraffin imbedding (but only feebly with Sudan IV), and resists digestion with saliva. The intensity of the H\textsubscript{4}O\textsubscript{4} Schiff reaction is decreased by primary fixation in boiling alcohol ether (3:1) mixture in a reflux apparatus.

*Von Recklinghausen's hemofuscin* (*Versamml. Gesell. Natürf. u. Ärzte* 62:324, 1889) was described as a finely granular yellow pigment not reacting to the iron methods. It occurred along with hemosiderin in the liver and certain other tissues in cases of hemochromatosis. Hueck classified it as a lipofuscin, and recently Gillman and Gillman (*Arch. Path.* 40:239, 1945) have called it cytolipochrome. In frozen sections it may be stained with oil-soluble dyes, but according to Endicott and Lillie (*Am. J. Path.* 20:149, 1944) it does not so stain after paraffin imbedding and is not acid-fast. Endicott and Lillie's material had been stored for several years in formalin; and it is possible that, like myelin, hemofuscin may lose part of its resistance to decolorization on long storage in formalin. However, the hemofuscin in this material still resisted decolorization with alcohol after prolonged staining with basic fuchsin according to Mallory's technic for the demonstration of this pigment.
Mallory's Hemofuscin Method.

1. Fix in Zenker's fluid, alcohol, or 10% formalin. Make paraffin or celloidin sections and bring to water as usual, including an iodine sodium thiosulfate sequence in the case of Zenker fixation.
2. Stain 5–10 minutes in Mallory's alum hematoxylin (p. 76; other alum hematoxylins will serve).
3. Wash well in water.
4. Stain 5–20 minutes in 0.5% basic fuchsin solution in equal volumes of distilled water and 95% alcohol.
5. Wash in water.
6. Differentiate in 95% alcohol.
7. Dehydrate with 100% alcohol, clear in xylene, and mount in balsam.

Results: Nuclei, blue; hemofuscin, bright red; melanin and hemosiderin, unstained in their natural browns.

Ceroid is also stained by this procedure, and it is not impossible that various other myelin-like substances would be.

Post, Benton, and Breakstone (Arch. Path. 52:67, 1951) report a similar cytoplasmic pigment of normal, predominantly centrolobular human liver cells, which is insoluble in water, alcohols, aromatic and aliphatic solvents, acetone, acids, and alkalies. It is stained by the Mallory hemofuscin technic and resists alcohol decolorization, but is not acid fast. It gives negative reactions by the periodic acid Schiff method, by the Schmorl ferricyanide reduction test, by the Feulgen thymonucleic acid technic, by the acid ferrocyanide method for ferric iron, by Stern's methods for bile pigment, and by Gomori's alkaline phosphatase method. It resists digestion by ribonuclease, desoxyribonuclease, amylase, and trypsin. Unlike hemofuscin as characterized by Mallory, it is not dissolved by 5% hydrogen peroxide. It is considered to be otherwise similar to hemofuscin, and is thought to be a functional metabolic complex.

Melanosis Pigment

The pigment of colonic and appendiceal melanosis is coarsely granular, contained in phagocytes in the stroma between the mouths of the glands. Granules are round or oval, of uneven size, and light to dark brown in color. They are moderately basophilic to thionin and to azure eosin, and their basophilia is unimpaired by ribonuclease digestion. The natural color changes the blue of these dyes to green and the red of safranin to orange brown. They are Schiff-positive after HIO₄ and CrO₃, and slightly so after KMnO₄ oxidation. Diastase digestion does not alter the HIO₄ Schiff reaction. The granules blacken with methenamine silver in the argentaffin-cell technic (p. 165) and color deep greenish blue in the ferric ferricyanide reduction test (p.175). They show at most only slight retention of fuchsin in Ziehl-Neelsen acid-fast
(p. 380) and Mallory hemofuscin technics (p. 252). With a regressive iron hematoxylin method of the eosinophil myelin type (p. 194) they retain iron hematoxylin more than epithelial cytoplasm, but less than erythrocytes. Gmelin and direct Schiff reactions are negative. The pigment remains insoluble after formalin fixation on 24 hours exposure of sections to 10% acetic acid, 10% hydrochloric acid solution, and to 5% borax solution. While the pigment usually gives a negative reaction to Perls's ferrocyanide method, a few cells may be found with granules giving the Prussian-blue reaction for ferric iron, and the homologous pigment in the guinea-pig intestine is often iron positive.

This pigment is now often considered to be of exogenous or dietary origin. Piringer-Kuchinka (Virchows Arch. f. path. Anat. 322:433, 1952) showed experimentally that in guinea pigs deprivation of green fodder from birth prevented the formation of the pigment, but does not state whether the animals grew normally. Others have attempted to correlate the pigmentation with the use of cascara.

Ceroid


As characterized by Endicott and Lillie (Am. J. Path. 20:149, 1944) ceroid possesses a bronze-brown color in gross preparations, and occurs microscopically as yellow globules 1–20 μ in diameter, located sometimes in liver cells, most often in large phagocytes, and also as rims of acid-fast material surrounding large fat globules. Fluorescence microscopy of frozen sections shows a greenish-yellow fluorescence which fades to pale yellow. In paraffin sections the fluorescence is golden brown. It retains myelin stains, acid-fast stains, and Mallory's hemofuscin stain. It usually stains green in the azure-eosin technic (p. 118), and is stained by oil-soluble dyes, both in frozen and paraffin sections. The oil-soluble dyes are readily removed by acetone, alcohol, and the like; and preparations may be restained and decolorized repeatedly. With the usual brief hemalum and iron chloride hematoxylin stains it does not color. Though it is Gram negative, if crystal violet staining is prolonged or accelerated by heat, the violet is retained with or without iodine treatment.

Ceroid is insoluble in dilute acids and alkalies, but is saponified by boiling 10% NaOH (gross chemical procedure), and fatty acids are precipitated from the solution by acid. It is insoluble in alcohols, acetone, ether, aliphatic
and aromatic hydrocarbons, chloroform, carbon tetrachloride, pyridine, acetic anhydride, glycols, and glycerol. It is not bleached by permanganates, chromic acid, hydrogen peroxide, or bromine or chlorine water.

It is blackened by osmium tetroxide, blackens slowly with diammine silver carbonate (p. 336). Most of it fails to reduce ferric ferricyanide; but definite deposits of blue pigment are formed in isolated globules and foamy masses. The Prussian blue (Perls) reaction is usually negative; but iron-positive pigment is sometimes associated with it, and necrotic foci containing ceroid exhibit both iron and calcium deposition.

Much of the pigment is colored red purple in the periodic acid Schiff procedure (p. 123), but often clear, unstained, small globules are seen inclosed in foamy red-purple masses. The peracetic and performic acid Schiff reactions are also positive in small globules, in part of the foamy masses, and in some of the large globules. In parallel preparations it appears as if part of the material gave the HIO₄ reaction, part the CH₃CO₂H reaction and part both reactions.

Bromination prevents the peracetic Schiff reaction. Benzoylation or acetylation prevents the periodic Schiff reaction and retards but does not prevent the peracetic Schiff reaction. Ceroid is colored red by 2–3 day exposure to Schiff reagent, and yellow by similar long exposures to phenylhydrazine (see also pp. 310–313).

The basophilia of ceroid is abolished by benzoylation but not by bromination. Ferric ferricyanide reduction is prevented by benzoylation but not by bromination. Acid-fastness is unaffected by bromination, benzoylation, or strong halogen acids. Hydrochloric and hydriodic acids (16 hours, sp. gr. 1.19 and 1.70 respectively) reverse the peracetic and periodic acid Schiff reactions, but do not destroy acid-fastness or sudanophilia. Exposure to 5% phenylhydrazine or to molar aniline chloride for 24 hours prevents both the peracetic Schiff reaction, and the Schiff reaction which occurs directly in 2–3 days. The periodic acid Schiff reaction is unaffected by these blockades.

Melanin

Melanin is characteristically a dark-brown pigment occurring in the basal layer of the epidermis; locally in the conjunctival epithelium; in hairs and hair-follicle cells; in chromatophores in the pia mater, the iris, ciliary body, and choroid; in the pigment epithelial layer of the iris, ciliary body and retina; and in nerve cells of the substantia nigra and locus caeruleus. In most places it occurs in rounded and oval granules of variable size. In the pigment epithelial layer of the retina, it occurs characteristically also as tiny, rod-shaped masses or "crystals." It occurs also in certain ocular and cutaneous malignant tumors and in nevus cells.

Melanin is insoluble in water, alcohol, fat solvents, and dilute acids and alkalies. Though it is freed from the chorioidal and retinal epithelial cells and partly dispersed as granules and rods by peptic and tryptic digestions, the
individual granules appear to resist digestion long after cytoplasms and nuclei have disappeared. Likewise, digestion with ribonuclease, with malt diastase, and with chondromucinase is resisted. Whether the alkali removal mentioned by Mallory is actually a solution of the pigment or is a dispersal from digestion of the cytoplasmic matrix, I am still uncertain.

Melanin is slowly bleached by 10% hydrogen peroxide; it may require 1–2 days, and is not visibly affected by 3% peroxide in 24 hours. While slow bleaching with ferric chloride is reported, a 24-hour exposure to a 1% solution has no evident effect. Similarly, no evident bleaching occurs with 24-hour exposures to 1% sodium iodate in 0.3% nitric acid, to 3% potassium bichromate, to Weigert's iodine, to 0.5% sodium bisulfite, to 5% hydroquinone, to normal hydrochloric acid, to 5% formic acid, to 5% trichloracetic acid or to 5% acetic acid. Appreciable though often irregular and unpredictable bleaching occurs with 5% chromic acid (CrO₃) in 1–3 hours, and with 0.5% potassium permanganate in intervals of 20 minutes up to several hours. Chlorine water bleaches melanin, though treatment with 1 cc. bromine in 39 cc. carbon tetrachloride for an hour does not. The most pronounced bleaching occurs with performic and peracetic acids (p. 310), even on 1–2 hour exposures, and with 16–24 hour exposures ocular melanin may be completely removed. This effect may be due to the large amount of hydrogen peroxide present in these reagents. Even 30 hours' exposure to 0.03 M periodic acid does not bleach ocular melanin.

Schiff reactions of ocular melanins in paraffin-imbedded material are negative directly, and after warm hydrochloric acid hydrolysis (Feulgen nucleal), chromic acid (Bauer), potassium permanganate (Casella), periodic acid and performic acid (ethylenic reaction).

Melanins are commonly said to reduce osmium tetroxide and to blacken with silver salts. Miescher (Arch. mikroskop. Anat. 97:326, 1923) used a 24-hour immersion in 2% silver nitrate. With methenamine and diammine silver preparations, it is often difficult to discern any difference in the brownish-black color of the untreated preparations from that in preparations treated with these reagents. Melanin remains the same shade of dark brown after the ammonium sulfide treatment used in the alkaline phosphatase method (pp. 202–203).

Cutaneous melanins often tinge green with azures, thionin, toluidin blue, and the like. This is more difficult to discern in the darker-colored ocular melanins. The latter do not evidently tinge with oil-soluble dyes. They do not retain the iron hematoxylin of the Weigert-Smith-Dietrich type technics, nor the basic fuchsin of the Mallory hemofuscin or Ziehl-Neelsen acid-fast methods. Lack of acid-fastness is also specifically recorded for cutaneous melanin and for that of the nerve cells of the substantia nigra. Perl's Prussian blue reaction with ferrocyanides is negative.

Cutaneous melanin colors deep green or blue green in the ferric ferricyanide reduction test. This reaction is readily blocked by prior iodination or
Ocular melanins, perhaps because of their more nearly black color, do not evidently react by this method.

In embryos and after roentgen radiation, the ocular pigment cells in appropriately fixed material will give the dopamelenase reaction (p. 230); and cutaneous melanin-bearing cells—specifically the dendritic melanoblasts—will form a dark-brown pigment both from dihydroxyphenylalanine and from tyrosine itself after radiation. Tyrosinase activity is not evident in adult skin except after X-ray irradiation (p. 231).

The melanin of the locus caeruleus and of the substantia nigra stains green with azure, thionin, and the like. This basophilia is partially resistant to methylation in N/10 HCl in absolute methanol at 60° C. for 24 hours, which completely destroys the nuclear and cytoplasmic basophilia of non-chromate-fixed tissue. The pigment gives negative iron reactions with ferrocyanide (p. 243) and with the Tirmann-Schmelzer technic; but ferric ferricyanide is strongly reduced (p. 175). Schiff reactions are negative on direct brief or prolonged exposure, after warm normal hydrochloric acid hydrolysis (Feulgen procedure p. 132), and after oxidation with KMnO₄, Cr₂O₃, or H₂O₂ (Casella, Bauer, periodic acid procedures p. 123) or with peracetic acid (ethylene reaction (p. 310). However, peracetic acid oxidation for two hours causes the pigment to become oxidase positive by the Winkler-Schultze indophenol method (p. 228). This indicates probable formation of peroxides from unsaturated compounds. This melanin is not tinged by Sudan black B or oil red O. Its color is not altered in myelin stains differentiated to the point where nuclei are decolorized but red corpuscles are still black. Osmium tetroxide is not evidently reduced. The pigment does not retain fuchsin in the Mallory hemofuscin or Ziehl-Neelsen acid-fast stains.

The pigment is argentaffin, reducing both methenamine silver oxide solution (p. 165) and diammine silver oxide solution as on p. 335, with the addition of 10% silver nitrate solution (about 9-10 cc.) to 1 cc. 28% ammonia water until a faint permanent turbidity is formed. Dilute with an equal volume of distilled water.

1. Deparaffinize and hydrate sections as usual.
2. Rinse in distilled water.
   To prevent sulfhydryl reactions immerse for 5-10 minutes in 1% iodine (Weigert's solution, p. 92) and then in 5% sodium thiosulfate for 20-30 seconds. Wash well in tap and distilled water. With brain well fixed in formalin iodizing can be omitted.
3. Immerse in diammine silver solution for two minutes, or for graded longer intervals when fuscin pigments are being tested.
4. Wash in distilled water.
5. Tone five minutes in 0.2% acid gold chloride and wash in water.
6. Fix two minutes in 5% sodium thiosulfate. Wash five minutes in running water.
7. Counterstain two minutes in 0.2% safranin in 0.2% acetic, alcohol, xylene, polystyrene.

**Result:** Reducing pigments, dark brown to black.

**Bleaching.** According to Alfieri (cited from Romeis), melanin may be bleached by treatment first with 0.05% potassium permanganate until sections are thoroughly brown, and then decolorization with 0.33% oxalic acid, repeating the treatment if bleaching is inadequate. Schmorl cautions against loss of sections during this procedure, and states that it may be carried out with fewer losses by using sections from which the paraffin has not been removed. The collodion treatment (p. 94) would allow more rapid bleaching with as little section loss. Chlorine water or a mixture of potassium chlorate (KClO₃) crystals and hydrochloric acid may be used in the same way. With undeparaffinized sections this acid chlorate method may require one or two days to bleach the melanin completely. Similarly, 10% hydrogen peroxide may require one to two days to decolorize melanomata and ganglion-cell pigment.
Carotenes. Among the organic exogenous pigments the carotenes are perhaps most worthy of note. They are a series of oil-soluble pigments of orange-yellow color, originating in carrots, squash, and certain other vegetables. They are often deposited in the body fats, imparting to them a yellow color, and are then often designated as lipochrome pigment. They are readily extracted by fat solvents and must be studied in frozen sections or spread preparations such as mesentery.

In the fresh state Lison states that they are colored blue by concentrated sulfuric acid before being destroyed. Treatment with Lugol's iodine solution (p. 92) produces a brown, green, or black color. Lipochromes are readily bleached by sunlight, by hydrogen peroxide, and by ferric chloride. They do not reduce dianmine silver hydroxide (p. 256) (Lison, Mallory, Cowdry).

Lutein. According to Schmorl, the yellow pigment lutein is stained with Sudan III and IV not only in frozen sections but also after paraffin and celloidin imbedding. When sections of fresh tissue are treated with concentrated sulfuric acid, lutein is colored first blue green and then light blue. This reaction is given by vitamin A, and lutein may contain that vitamin. Treatment with Lugol's iodine (p. 92) produces a green color.

Schmorl also refers to other lipochromes which give a deep blue color on sulfuric acid treatment, and these also give a green color on addition of Lugol's solution.

These reactions with sulfuric acid and with iodine are those of the carotenoid pigments.

Vitamin A. For demonstration of vitamin A, Popper (Arch. Path. 31:766, 1941) recommends brief fixation in 10% formalin, preferably in the cold and not over 10-12 hours. Blocks are cut 3 mm. thick. Frozen sections are examined in water within three hours of cutting, with ultraviolet light at 365 mμ in a fluorescence microscope (p. 18). Vitamin A presents a brilliant green
fluorescence which fades quite promptly in 10–60 seconds or more, depending upon the amount present.

Vitamin A is soluble in fat solvents and occurs dissolved in body fats as well as in liver cells, Kupffer cells, lutein cells, adrenal cortex, and other places. It resists treatment with 0.1 N hydrochloric acid, 0.1 N ammonium hydroxide, and saturated sodium hydrosulfite (Na$_2$S$_2$O$_4$·2H$_2$O) solution. The fluorescence of vitamin B$_2$ is destroyed by the last. Hydrogen peroxide produces a blue fluorescence of fats, but does not destroy the green vitamin-A fluorescence.

Vitamin A, like the lipochromes, gives a green to blue color with sulfuric acid. It gives a blue color with antimony trichloride.

Riboflavin. Gomori quotes a method of Chèvremont and Comhaire's. After reduction of riboflavin to leucoflavin it reoxidizes in air to red rhodoflavin.

Place frozen sections of formalin-fixed tissue in 1–2% hydrochloric acid containing enough zinc dust to give a steady evolution of hydrogen, and stir about gently for 30 minutes. Then wash in water and expose to air in a shallow vessel for several hours. Mount in glycerol gelatin. Flavoproteins are stained red.

Gomori suggests replacing the hydrogen reduction with sodium hydrosulfite.

Vitamin C. For the demonstration of vitamin C, Deane and Morse (Anat. Rec. 100:127, 1948) recommended immediate fixation for 30 minutes in the acetic alcohol silver nitrate solution of Barnett and Bourne (J. Anat. 75:251, 1940), followed by transfer directly to an acid fixing solution for two hours, then overnight washing in running water.

Barnett and Bourne's fluid was ambiguously stated to be a “saturated solution of silver nitrate in ethyl alcohol (5 parts), water (4 parts) and glacial acetic acid (1 part)” and to give a concentration of slightly less than 10% silver nitrate. Since silver nitrate is only slightly soluble in pure alcohol, it seems that saturation in the mixture was meant. The following emended formula is suggested: Silver nitrate 10 Gm., distilled water 40 cc. Dissolve, and add glacial acetic acid 10 cc. and 100% alcohol 50 cc. This fluid does not keep.

The acid fixing solution contained sodium thiosulfate crystals (Na$_2$S$_2$O$_8$·5H$_2$O) 5 Gm., sodium bisulfite (NaHSO$_3$) 1 Gm., distilled water 100 cc.

After washing, the material is dehydrated, cleared, infiltrated and imbedded in paraffin as usual, and sectioned at 5 μ.

Deane and Morse deparaffinized and mounted for examination in the unstained state; or counterstained with paracarmine (p. 84) or hematoxylin and eosin (p. 114).

Barnett and Bourne varied the procedure by simply washing in distilled water after fixation, imbedding and sectioning, and then toning sections 4–10 minutes in “very dilute gold chloride solution,” fixing in sodium thiosul-
fate solution 4–10 minutes, followed by dehydration, clearing and mounting.

Cater (J. Path. & Bact. 63:269, 1951) claimed superior results by a three-hour fixation in aqueous 10% silver nitrate, 10% acetic acid solution, followed by three changes of distilled water of 30 minutes each and a 90-minute bath in 5% sodium thiosulfate, all in the dark. Washing in water is presumed. Dehydration, clearing, paraffin-imbedding and sectioning at 5 μ were followed by a light counterstain with neutral red.

I have not used these procedures.

Clara (Mikroskopie 7:387, 1952), though admitting the effectiveness of the acid silver nitrate method in demonstrating ascorbic acid, points out that a number of other substances also reduce silver under the prescribed conditions. He names melanin, phaeochrome substance, enterochromaffin substance, pancreatic alpha granules, and the neurosecretory granules of the supraoptic and paraventricular nuclei of the thalamus.

Langeron cites Massa’s (Soc. Pharm. Montpellier 5:14, 1945) use of the ferric ferricyanide reduction test (p. 175) for the demonstration of ascorbic acid in plant tissues. Massa made up his potassium ferricyanide at 0.2% and his ferric chloride at 3.24%, both in 15% acetic acid, and mixed equal volumes at the time of using. The reaction time should be restricted to 5–10 minutes. Because of the solubility and diffusibility of ascorbic acid, only fresh tissue can be used.

Ascorbic acid gave immediate blue coloration; phenols, tannoids, anthocyanins and flavones gave green colors or green-black precipitates. He noted the reaction of glutathione and cysteine (p. 174), and used the nitroprusside reaction as a control. The reaction of carotenoids, tocopherols and vitamin A was noted as much slower than that of ascorbic acid.

The method should be applicable to Adamstone-Taylor or Linderström-Lang frozen sections of fresh animal tissue, transferred directly into the freshly mixed reagent from the microtome.

Pneumonyssus Pigment. Sometimes difficult to distinguish from carbon is the pigment deposited in monkey lungs about cysts and remnants of the acarid parasite Pneumonyssus. This includes particles of a rather deep brown color which stain practically black with azures. However, examination of unstained preparations reveals angular black particles, brown granules, and doubly refractile needles.

For exogenous pigments Hueck lists the following, classified according to color. I have abbreviated, translated, and supplemented his list, chiefly from the mineral pigments in the Colour Index, and from the colored ores noted in Lange. Not all of these have been reported as pigments in tissues.

Black. Carbon as soot, coal, and graphite. Coal occurs as irregularly angular and jagged particles. Graphite crystallizes as hexagonal crystals.

Brownish, Greenish, and Grayish Black. Oxides and sulfides of various metals, not specified by Hueck, but including iron, cobalt, nickel, lead, silver, copper, antimony, chromium, gold, iridium, manganese, mercury, molyb-
denum (MoS$_2$), palladium, platinum, rhodium, tin, tantalum, thallium, thorium, tungsten, uranium, and vanadium.

**Brown.** Bismarck brown used in tattooing; manganite, umber, and cupric ferrocyanide.

**Brownish Red.** Iron and copper compounds, notably iron oxide.

**Red.** Cinnabar, carmine, and other dyes used in tattooing; alizarin and madder; to which I might add the native arsenic sulfides realgar and orpiment, the iron ore hematite (Fe$_2$O$_3$), and mercuric oxide.

**Blue.** Vivianite (Fe$_6$(PO$_4$)$_2$·8H$_2$O), ultramarine (a mixed silicate and sulfide of aluminum and sodium), steatite (a colored talc), azurite (Cu(OH)$_2$·2CuCO$_3$), azure blue (CoAl$_2$O$_4$), smalt (potassium cobaltous silicates), copper blue (CuS), blue dyes used in tattooing, etc.

**Green.** Casalis green (Cr$_2$O$_3$) and its hydrates; Kinmann’s green and related products resulting from the fusion of varying proportions of zinc and cobaltous oxides; turquoise green (similarly made from chromium, cobaltous and aluminum oxides); verdigris (basic copper acetates); Scheele’s green (copper arsenite); and ultramarine green (a sodium aluminum silicate and sulfide); also green dyestuffs used in tattooing.

**Yellow.** Chromates of lead, barium, and zinc; cadmium and stannic sulfides; Naples yellow (Pb$_6$(SbO$_4$)$_3$).

**Violet.** Chiefly dyestuffs used in tattooing.

**Gray.** Chiefly various silicates.

**White.** Various lead, zinc, and bismuth pigments, as well as barite (BaSO$_4$), and titanium oxide (TiO$_2$).

**Carbon** is one of the commonest extrinsic materials appearing as a pigment. It is commonly deposited in the lungs and mediastinal lymph nodes, but may appear in axillary nodes as well, and sometimes in the skin as a result of tattooing or sterilization of hypodermic needles in sooty flames. It is distinguished by its black color and resistance to all solvents and bleaching agents.

**Iron ores** may be black (magnetite, hematite), blue (vivianite), green (siderite), gray (siderite), red (hematite), and brown or yellow (siderite). Some react in acid solutions to ferrocyanides, others to ferricyanides, some to both (see p. 243). Iron-ore dusts may be removed by treatment with oxalic or dilute nitric acids. Generally, however, petrographic examination may be necessary to identify precisely the dusts in question. See also infra.

Highman (Inter. Assoc. Med. Museums Bull. 32:97, 1951) finds that some of the less soluble iron-ore dusts (notably hematite, Fe$_2$O$_3$) fail to react to the usual ferrocyanide test, pp. 243-244. He notes that by increasing the concentration of the hydrochloric acid to 4 N or to even higher concentration (11 N), and heating to 60-80° C., positive Prussian-blue reactions may be obtained even with the more refractory ores. For the Quincke and Tirmann-Schmelzer reactions a prolonged exposure (1-2 days) to saturated hydrogen sulfide water is substituted for the usual ammonium sulfide step, p. 242.
Aluminum has been demonstrated after aluminum-dust therapy of silicosis as globules and mulberry-like aggregates of dark red material lying in the center of the fibrous nodules, by the aurine technic of P. C. Irwin (personal communication 1948).

Fix in formalin, cut frozen or paraffin sections, and bring to water as usual.

1. Stain 5 minutes at 75° C. in 2% aurine tricarboxylic acid (NH₄ or Na salt) in a pH 5.2 buffer composed of 3.8 parts 5 M (267.5 Gm./liter) ammonium chloride, 3.8 parts 5 M (385.3 Gm./liter) ammonium acetate and 1 part 6 N (500 cc./liter) hydrochloric acid.
2. Rinse for a few seconds in cool distilled water.
3. Decolorize three seconds in pH 7.2 buffer composed of 3.6 parts of above buffer and 10 parts 1.6 M ammonium carbonate.
4. Rinse quickly in distilled water.
5. Counterstain briefly in saturated aqueous picric acid solution or in 1:10000 methylene blue.
6. Dehydrate, clear, and mount by alcohol, xylene, balsam sequence.

Asbestos occurs in tissues as fine, white, doubly refractile fibers and as the so-called asbestosis bodies. The latter occur as beaded rods with large rounded ends, as fusiform bodies with beaded centers, as rosettes, and in other similar forms. They are golden yellow in color and give the Prussian blue reaction for ferric iron. Also some of the fine asbestos fibers are colored blue by acidulated potassium ferrocyanide solution (pp. 243-244). The asbestosis bodies and the fine fibers giving the Prussian blue reaction are dark under crossed Nicol prisms. (J. W. Miller, Pub. Health Bull. No. 241, pp. 96-101, Washington, D.C., Government Printing Office, 1938.)

Beryllium. Denz (Quart. J. Microscop. Sci. 90:317, 1949) proposes a histochemical test for reactive beryllium compounds, utilizing a British dye (Clayton Aniline Co.) named naphthochrome green B and described as pheno-oxy-dinaphtho-fuchsondicarboxylate of sodium. The dye forms a green beryllium lake, optimally at pH 5. The dark blue-green ferric lake and the yellowish-green aluminum lakes are formed only at relatively high metal concentrations at this pH level.

Denz's Beryllium Method.

1. Fix tissues in formol saline or alcoholic formalin (p. 34). Dehydrate with alcohols, clear in xylene or cedar oil, imbed in paraffin, section, deparaffinize with xylene, and hydrate through descending alcohols.
2. Mix equal volumes of a phosphate buffer of pH 5 (sic) and of a freshly prepared 0.5% aqueous solution of naphthochrome green B. Place slides in this mixture in a Coplin jar and incubate 30 minutes at 37° C.
3. Wash in distilled water.
4. Differentiate 30 minutes in 100% alcohol.
5. Wash in distilled water.
6. Counterstain 5 minutes in 1% aqueous acridine red.
7. Wash in distilled water.
8. Differentiate rapidly in 100% alcohol.
9. Clear in xylene and mount in Canada balsam.

Results: Beryllium compounds, apple green; background, red.
The method demonstrates protein combinations of soluble beryllium salts, which apparently remain in place for fairly long periods. Beryllium oxide and silicate dusts in tissues fail to react.
The acridine red referred to may be acridine red 3B (C. I. No. 740), which is a basic dye of the pyronin class. Probably one of the latter could be substituted.

Barium and Strontium. Waterhouse (Nature 167:358, 1951) first fixed tissue in neutral 10% formalin in 70% alcohol. Then soak the blocks for 30–60 minutes in a freshly prepared 1–2% solution of sodium rhodizonate in distilled water or pH 7.0 phosphate buffer. Then wash out excess rhodizonate in 50% alcohol. Tissues can then be sectioned as usual, and permanent preparations obtained. Barium and strontium compounds both give intensely colored red compounds. Treatment with aqueous potassium chromate solution before the rhodizonate prevents formation of the color with barium salts, and chromate treatment after coloration removes the color from barium but not from strontium.

Calcium. The procedure of feeding or injecting alizarin compounds to mark newly deposited calcium salts during life is considered on p. 431.
Allied to this procedure are the alizarin red S and purpurin methods for demonstration of calcium deposits in tissues.

Langeron's Alizarin Red S. I have modified this technic as follows: Fixation apparently is not important, neutral 10% formalin is satisfactory, though Langeron prescribed 80 to 90% alcohol. Paraffin sections are brought to water as usual.

1. Stain one hour in 0.1% alizarin red S (C. I. No. 1034).
2. Rinse in water.
3. Counterstain one minute in 0.1% aqueous toluidin blue O, thionin, azure A, or methylene blue.
4. Rinse quickly in 1% aqueous acetic acid and then in tap water.
5. Dehydrate, clear, and mount in clarite by an acetone xylene sequence.

Results: Calcium deposits are scarlet; nuclei, violet to blue; cytoplasm and other structures, pale blue; cartilage, purple to violet.

Dahl (Proc. Soc. Exper. Biol. & Med. 80:474, 1952) modifies the foregoing procedure by increasing the concentration of alizarin red S to 1% and
including 10 cc. of a 1/100 dilution of 28% ammonia water in each 100 cc. of the solvent. He claims high specificity and quite complete demonstration of calcium salts. Dissolve the dye in the water (dark orange) and add the ammonia slowly with constant stirring. The resultant clear red solution gives a pH of 6.3–6.5 and is stable for at least a month. Several hundred slides may be stained with the same jarful if introduction of alcohol is scrupulously avoided.

He prescribes alcohol fixation (four changes of 95% alcohol in a 36-hour period, followed by 16 hours in 100% alcohol) and paraffin infiltration directly from the alcohol. Sections are floated onto clean glass slides without albumen fixative. I suggest thorough drying to prevent section losses. Deparaffinize and bring to 95% alcohol. Drain well and transfer directly to the stain for two minutes. Wash by pouring distilled water on, and then immersing in distilled water for 5–10 seconds to remove the excess stain. Dehydrate in alcohols, clear in xylene, and mount in cedar oil.

To further suppress background staining, a 15-second rinse with 0.001 N hydrochloric acid in 95% alcohol may be inserted as the first step in dehydration.

Alizarin red S forms a crimson lake with calcium, and scarlet lakes with aluminum and with barium. Magnesium gives a clear scarlet solution; mercury a clear dark red solution.

Grandis and Mainani's Purpurin. Schmorl recommended a similar method employing purpurin (C. I. No. 1037) or alizarin SX (anthrapurpurin) (C. I. No. 1040) in saturated alcoholic solution (they are respectively very sparingly soluble in water and insoluble in water). He prescribed fixation with strong alcohol or neutral 10% formalin. Paraffin sections are deparaffinized and brought through 100% alcohol into

1. The saturated alcoholic stain for 5–10 minutes. The alizarin SX solution should contain also 1% sodium chloride and a trace of ammonia water.
2. Wash in 0.75% aqueous sodium chloride solution for 3–5 minutes.
3. Wash thoroughly in 70% alcohol until no more color clouds come out of sections.
4. Dehydrate, clear, and mount as usual.

Results: Calcium deposits are stained red.

Von Kössa's Method. (Beitr. z. path. Anat. u. z. allg. Path., 29:163, 1901.) Widely used for demonstration of calcification is the von Kössa silver nitrate method, which actually demonstrates the presence of phosphates, soaps, and amorphous but not crystalline carbonates, rather than calcium itself (see also p. 430). Alcohol fixation is preferred for this method, but it works well after neutral 10% formalin.

Frozen or paraffin sections may be employed.
1. Wash in several changes of distilled water.
2. Immerse in 5% silver nitrate for 10–60 minutes (v. Kóssa: 5 minutes) and expose to bright daylight (Mallory, Romeis, Langeron), not direct sunlight. Schmorl used 1–5% silver nitrate for 30–60 minutes; Cowdry, 10% for 30 minutes or more.
3. Wash well in distilled water.
4. Treat for 2–3 minutes in 5% aqueous sodium thiosulfate solution.
5. Wash in water.
6. Counterstain 20–60 seconds in 0.5–0.1% aqueous safranin O (C. I. No. 841).
7. Differentiate and dehydrate in 95% and 100% alcohol.
8. Clear with 100% alcohol and xylene and two changes of xylene. Mount in clarite.

**Results:** Calcium deposits, black; nuclei, red; other tissues, pink.

In place of safranin other counterstains may be used. In case of hard calcium deposits, ragged sections may require the collodion treatment outlined on p. 94. With larger deposits it may be better to use the procedure of silvering before decalcification (p. 430).

The most widely used criterion for the detection of small calcareous deposits is their staining deep blue with alum hematoxylin or gray violet with iron hematoxylin. This is not specific but is very useful.

Some calcareous deposits are encrusted or admixed with ferric salts which may be detected by the usual ferrocyanide reaction (pp. 243–244).

**Cretin's Reaction.** Lison regarded this reaction as very sensitive and highly specific. Gomori agreed as to specificity but found the reaction so capricious as to be almost useless. I have always regarded it as too complicated for practical use and have not tried it; nevertheless, thus:

1. The sections are first deparaffinized with xylene and washed in chloroform and kept in the latter until the Cretin reagent is ready.
2. Grind together in a mortar 200 mg. gallic acid (3,4,5-trihydroxybenzoic acid) and 100 mg. metaformaldehyde (trioxymethylene).
3. Dissolve 250 mg. of the mixture in 5 cc. boiling distilled water.
4. Then add cautiously 0.5 cc. 14% ammonia water (18° B., or 9 cc. of the 28% diluted with 10 cc. water).
5. Agitate until reagent becomes a pale yellow and at once pour over the sections. If the reagent turns brown or rose-colored it is no longer usable.
6. After 10–15 seconds pour off the reagent and expose sections to air. A blue color develops at sites of calcification.
7. Rinse in filtered saturated aqueous calcium sulfate solution.
8. Counterstain in 1% eosin containing 5% of ammonia water.
9. Wash rapidly in 10% formalin.
10. Dehydrate with alcohols, clear with xylene, mount in balsam.
Results: Calcium, blue; background, pink. Barium and strontium give green lakes; silicon, yellow; iron, brownish violet; magnesium, rose.

The strongly alkaline reagent is apt to remove the sections. Collodionization could be tried, thus: First deparaffinize in xylene, then pass briefly through 100% alcohol into 1% collodion in 100% alcohol and ether for five minutes. Then drain one minute and wash in two changes of chloroform, leaving sections in the second until the Cretin reagent is ready.

The most specific chemical tests are those with sulfuric and oxalic acids. In the unstained state the calcium deposits are granular, opaque, and white. By mounting the sections in water and running in acetic acid under one edge of the cover glass while drawing water out with a piece of filter paper from the other, the deposits may be seen to dissolve; if carbonates, with the formation of gas bubbles; if phosphate only, without. If sulfuric acid is used in place of acetic, the deposits dissolve as before, but monoclinic gypsum crystals are formed; but if 5% or 10% oxalic acid is used, the characteristic cubic calcium-oxalate crystals appear.

Silver appears as brown to black granules which are turned black by soluble sulfides. Silver deposits are removed by treatment with a solution containing 1 Gm. potassium ferricyanide and 22.5 Gm. sodium thiosulfate (Na₃S₂O₅·5H₂O) per 100 cc., or by treatment for 1–2 hours with Lugol's iodine solution (p. 92) followed by rinsing and immersion in 5–10% sodium thiosulfate until white (Schmorl). According to Timm (Virchows Arch. f. path. Anat. 297:502, 1936) silver sulfide is also removed by potassium cyanide solution (p. 268). It is not dissolved by ammonium hydroxide or by sodium-sulfide solution.

Copper sulfide is also a dark brown to black material which is soluble in potassium cyanide solution (Timm, p. 268), but is not removed by sodium-sulfide or ammonium hydroxide. Copper ferrocyanide is red, and Lison quotes this as a specific reaction (p. 243). According to Mallory copper compounds give a light to clear dark-blue color with unoxidized fresh aqueous hematoxylin solution.

Mallory's Hematoxylin Stain for Iron and Copper. Fix in alcohol. After formalin fixation only yellow to brown colors are obtained with iron. Imbed in paraffin or celloidin. Hydrate sections as usual.

1. Dissolve 5–10 mg. hematoxylin in 0.5–1 cc. 100% alcohol. Add 10 cc. distilled water which has been boiled five minutes to drive off carbon dioxide. Stain sections one or more hours in the hematoxylin solution.
2. Wash one hour in several changes of tap water.
3. Dehydrate, clear, and mount with alcohol, xylene, and balsam.

Results: Nuclei, bluish gray; hemosiderin, black; copper, light to clear dark blue. I have had no experience with this method.

Okamoto and Utamura used rubesanic acid (dithio-oxamide) for the dem-
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onstration of copper. Romeis directs: Bring frozen, paraffin, or celloidin sections to water as usual. Add 2–5 cc. 0.1% alcoholic rubeanic acid to 100 cc. 10% aqueous sodium acetate solution. Incubate sections 12–24 hours at 37° C. in this solution in a tightly covered vessel. Wash in water, counterstain in alum carmine, alcohol, xylene, and balsam as usual.

Results: Copper rubeanate, greenish black; cobalt, yellowish brown; nickel, blue violet. Silver and lead yield black sulfides. According to Feigl (1949) copper rubeanate is formed also from weak acid solutions; for cobalt and nickel the sodium acetate is required.

Poulson and Bowen quote (Exper. Cell Res. Suppl. 2:161, 1952) Waterhouse's use of sodium diethyldithiocarbamate as a histochemical reagent for copper in blowflies. They applied equal volumes of an 0.2% solution of this reagent and of 2% HCl to freshly dissected Drosophila larvae. Copper gives a yellow brown color. Waterhouse (Bull. 191, Counc. Sci. & Indus. Res., 1945) used 0.1% in either acid or neutral solution, both on fresh tissue and on material coagulated by heat, alcohol, or formalin. Iron also forms a brown carbaminate compound, but the sensitivity to iron is much lower than to copper. The blowfly material failed to react to the rubeanic acid method.

Cobalt. Despite the wide use of its sulfide to demonstrate the precipitation of phosphate ions in calcium phosphate, little other histochemical information is available concerning this metal. Its phosphate reacts with solutions of 1-nitroso-2-naphthol to form a red brown deposit. Iron phosphate does not react. The more reactive iron salts form brown black with this reagent. Copper salts give a brown color, but may be rendered nonreactive by conversion to cuprous iodide by an iodide sulfite mixture. Cuprous iodide is soluble in potassium iodide solution.

Alcoholic thioglycolic acid anilide yields a red brown, acid, insoluble cobalt compound from ammoniacal solution. Black precipitates are yielded by 4-methyl- or 4-chloro-1,2-mercaptobenzene with copper, cobalt, and nickel; red with tin, bismuth, and molybdenum; yellow with lead, silver, and antimony; and pale yellow with cadmium, mercury, and arsenic.

The foregoing account is gleaned largely from Feigl (1949).

Nickel. According to Lison a fresh alcoholic hematoxylin solution stains nickel salts lilac, grading to blue in thicker sections. He prescribes fixation in formalin 30 cc., saline solution ("sérum physiologique") 100 cc., and ammonium sulfide 0.3 cc. Soak in ammonium phosphate solution to produce the insoluble double nickel ammonium phosphate. Decalcify, imbed, and section. Stain in fresh alcoholic hematoxylin.

Nickel sulfide (NiS) is black, soluble in nitric acid and aqua regia and partly in hydrochloric acid, and insoluble in ammonia water and in sodium sulfide solution. Its ferrocyanide is greenish white, its ferricyanide rusty brown (Lange). Since nickel phosphate is insoluble in water, the phosphate buffered neutral formalin (p. 34) should serve for preservation of relatively soluble nickel salts as well as the sulfide.
Lead. The Sulfide Method. For the detection of lead Timm (Virchows Arch. f. path. Anat. 297:502–508, 1936) fixed tissues, especially bone, in absolute alcohol saturated with hydrogen sulfide gas. He decalcified in 30% formic acid in water, also saturated with hydrogen sulfide. Acid was removed by several changes of 5% sodium sulfate and of water, all saturated with hydrogen sulfide. Frozen sections were cut and treated with potassium cyanide solution to remove copper and silver sulfides and with yellow ammonium sulfide to remove tin. Sections were washed in water, mounted on slides, dehydrated in alcohols, and cleared in bromobenzene. Mount in bromobenzene balsam. Lead remains as brown granules of sulfide. Lead sulfide is converted into the white sulfate by hydrogen peroxide.

The Chromate Method. Fairhall treated paraffin sections of formalin-fixed lung for several days in a solution of potassium chromate acidified with acetic acid. The lead salts are converted into yellow monoclinic lead chromate crystals (U. S. Public Health Service Pub. Health Bull. No. 253, pp. 22–24, Washington, D.C., Government Printing Office, 1940). Fixation in Orth’s or Møller’s (Regaud’s) fluid yields the same result according to Lison, who attributes the general method to Frankenberger and to Cretin.

The Hematoxylin Method. Mallory (1938, p. 143) prescribed fixation in alcohol or 10% formalin and imbedding in celloidin or paraffin.

1. Sections were stained 2–3 hours at 54° C, in fresh 0.05–0.1% solution of hematoxylin in water saturated previously with calcium carbonate.
2. Sections were then washed 10–20 minutes in running water (or several changes).
3. They were then dehydrated with 95% alcohol, cleared in terpineol, and mounted in terpineol balsam.

Results: Lead is stained bluish gray to black. Even slightly aged hematoxylin gives a brown color and is useless. Old xylene balsam also immediately turns the stain brown. I suggest the use of one of the nonreducing synthetic resins.

Rhodizonate Test for Lead. Following Molnar’s suggestion (Stain Technol. 27:221, 1952) for improving phosphatase technics by substituting a lead nitrate bath for the cobalt and a rhodizonate treatment for the ammonium sulfide, it would appear that a simple 40-minute bath in 0.5% sodium or potassium rhodizonate solution should demonstrate lead deposits in dark brown.

According to Feigl (1949) neutral rhodizonate metal salt mixtures yield precipitates as follows: lead, blue violet; zinc, brown violet; cadmium, bismuth, calcium, strontium and barium, red brown; uranyl salts, brown; and thallium, dark brown. Silver is reduced to a black (metallic?) state. Iron gives a soluble blue-green color, and hence does not confuse. At pH 2.8 only silver (black), tin (violet), thallium (brown black), cadmium and mercurous salts (brown red), barium (red brown), and lead (scarlet
red) yield precipitates. Prior treatment with sulfuric acid should render barium nonreactive, and should extract mercury, tin, cadmium, thallium, and silver. Under these conditions the test should be specific for lead.

**Arsenic.** Fix in 10% formalin containing 2.5% copper sulfate (CuSO₄•5H₂O) for five days. Wash 24 hours in running water. Imbed in paraffin. Deparaffinized sections present green granules of Scheele's green (CuH₂As O₆) which, though insoluble in water, is dissolved by acids and by ammonium hydroxide. By substituting cupric acetate for the sulfate, the green granular Paris green or cupric acetoarsenite is produced. Its solubilities are similar (Castel's method, *Bull. d'histol. appliq.* 13:106, 1936). I suggest a light safranin counterstain.

**Bismuth.** The Christeller- (Med. Klin. 22:619, 1926) Kornaya (Arch. Dermat. u. Syph. 149:277, 1925) reaction uses Leger's reagents which are a 4% aqueous solution of potassium iodide and a 2% solution of quinine sulfate (3 Gm. quinine sulfate, 1 cc. concentrated nitric acid (30 drops), 150 cc. distilled water). Mix 5 cc. of each, add 2 drops (0.067 cc.) nitric acid for use. Cut frozen sections of formalin-fixed material; prestain with carmine, fuchsin, or crystal violet; wash in water; apply the mixed Leger's reagent for one minute; rinse in water containing 2 drops nitric acid to each 10 cc. Mount, blot dry, treat with carbol xylene → alcohol, carbol xylene, xylene, and balsam in sequence. Christeller describes the bismuth compound as bright yellow; according to Schmorl the needle-shaped crystals are orange yellow. Cowdry refers to brown granules, and cites a modification of Castel's (*Arch. Soc. Sci. Med. Biol., Montpelier* 16:453, 1934-1935), which utilizes a few drops of sulfuric acid in place of the nitric acid to dissolve the quinine sulfate. This gives red quinine iodobismuthate.

Bismuth deposits are blackened by hydrogen sulfide water or with ammonium sulfide solution.

**Gold.** According to Elftman and Elftman (Stain Technol. 20:59, 1945) gold may be demonstrated by simple incubation of paraffin sections of formalin-fixed tissue in 3% hydrogen peroxide at 37° C. for 1-3 days. The hydrogen peroxide bleaches other interfering pigments; and gold appears as rose, purple, blue, and black deposits. For critical evaluation omit counterstains. An alum hematoxylin stain may be used to aid in topographic studies and interferes only slightly. Probably light green SF (C. I. No. 670) interferes least of the cytoplasmic stains.

**The Stannous Chloride Reaction.** The same authors also recommend the following modification of Christeller's (*Verhandl. d. deutsch. path. Gesellsch.* 22:173, 1927) stannous chloride reaction, which produces the purple of Cassius. Make a stock aqueous solution of 5% stannous chloride (SnCl₂•2H₂O) in which some pieces of metallic tin are placed to prevent oxidation to SnCl₄. For use, mix 40 cc. stannous chloride and 4 cc. concentrated hydrochloric acid. Incubate paraffin sections of formalin-fixed tissue at 56° C. for 24 hours in a covered Coplin jar. Wash repeatedly in distilled water, dehy-
drate, clear, and mount. Gold is evident as purple to brown particles, and the red, blue and black of colloidal gold may be present as well.

G. Brecher tells me that the Christeller reaction may also be applied to tissues fixed in Zenker's fluid, provided that the thiosulfate treatment is omitted after the customary iodizing to remove mercury precipitates.

**Mercury.** Almkvist (Schmorl) prescribes fixation in: 100 cc. saturated aqueous picric acid solution to which is added 3 Gm. 25% nitric acid. The mixture is allowed to stand one day, shaken, filtered, and saturated with hydrogen sulfide: Fix for 8–24 hours or up to three days for maximal effect. Imbed in paraffin. Mercury appears as fine yellow to brown granules of mercury sulfide. This is soluble in sodium sulfide solution but not in sodium thiosulfate. Other heavy-metal sulfides are also precipitated by this fixative solution, as iron, silver, cobalt, and others. The precipitate should be tested with acetylated potassium ferricyanide as in the Tirmann-Schmelzer reaction (pp. 242–243) to exclude iron (blue) and cobalt (red). Mercuric ferricyanide is soluble, and potassium ferricyanide solution does not dissolve mercuric sulfide.

Christeller's fixative (Schmorl) consisted of 15 cc. distilled water, 2 Gm. tin chloride, and 1 Gm. nitric acid, and produced a black granular precipitate.

Brandino's method (Lison, p. 102) tests sections of alcohol or formalin-fixed tissue with 1% 1,5-diphenylcarbohydrazide \( C_6H_4NH\cdot NH\cdot CO \) (in alcohol?: very slightly soluble in water). A violet precipitate is formed.

**Manganese.** From Lison's account, the suggested methods for manganese appear to be unsatisfactory. The lower oxides \( \text{MnO, Mn}_2\text{O}_3, \text{and MnO}_2 \) are insoluble and are respectively gray-green, brown to black, and black; and the two native sulfides \( \text{MnS} \) and \( \text{MnS}_2 \) are also insoluble and are respectively green or pink and black. There is also an insoluble pink native silicate.

**Potassium.** While Lison rejected Macallum's method as of little value, Gomori now considers it a usable reaction. He has modified it as follows: Dissolve 2 Gm. cobalt nitrate in 5 cc. 20% acetic acid. Dissolve 6 Gm. sodium nitrite in 10 cc. distilled water. Mix. Allow most of fumes to escape. Chill to 5° C. Immerse small fragments of fresh tissue (or Adamstone-Taylor frozen sections) in cold reagent for two minutes. Rinse in ice-cold distilled water and wash in 4–5 changes of ice-cold 50–70% alcohol. Convert cobalt to sulfide by immersion in 0.5–1% yellow ammonium sulfide for two minutes. Wash in water; counterstain with safranin; hemalum, carmine, or the like; dehydrate with alcohol, clear in xylene, spread out and mount in Canada balsam.

The specific precipitate of potassium cobaltinitrite is converted to dark brown cobalt sulfides, and these serve to localize potassium. It is uncertain whether this demonstrates ionic or bound potassium or both. The alleged nonspecific creatine reaction has been shown to be due to potassium contamination.
Thallium forms yellow crystals of iodide (TlI) on fixation of tissues in alcohol colored with Lugol's solution (Barbaglia, from Lison). Gomori suggests 2.5 Gm. iodine, 5 Gm. KI in 50–100 cc. 95% alcohol, tincture of iodine (U.S.P.), diluted with an equal quantity of alcohol, should serve. The yellow thallous iodide is insoluble in alcohol, acetone, potassium-iodide solution, or water; but is soluble in aqua regia.

Zinc. Lison cites a method of Mendel and Bradley (Am. J. Physiol. 14:311, 1905) for the demonstration of zinc. Treat paraffin sections 15 minutes at 50° C. in 10% sodium nitroprusside solution. Wash in gently flowing water for 15 minutes. Cover section with a cover glass and introduce at one side a drop of sodium or potassium sulfide solution. An intense purple color is produced. Lison regards the reaction as specific.

Mager, McNary and Lionetti (J. Histoch. & Cytoch. 1:486, 1953) propose the following variation of the dithizone method for zinc.

Reagents: Stock dithizone: Dissolve 10 mg. diphenylthiocarbazone in 100 cc. reagent grade anhydrous acetone. Store at 3°C. in brown glass bottle. Complexing buffer: Dissolve 55 Gm. sodium thiosulfate (Na2S2O3·10 H2O), 9 Gm. sodium acetate (NaCO3·CH3·3 H2O), and 1 Gm. potassium cyanide (KCN) in 100 cc. distilled water. Shake in separatory funnel with several successive portions of dithizone in carbon tetrachloride, until the CCl4 layer remains clear green, to remove traces of zinc. Normal acetic acid. Rochelle salt: a 20% aqueous solution of sodium potassium tartrate (NaK(CO3 CHOH)2·4 H2O).

Reaction mixture: Mix 24 cc. stock acetone dithizone with 18 cc. distilled water, adjust with normal acetic acid to pH 3.7, add 5.8 cc. complexing buffer and 0.2 cc. Rochelle salt solution. Use at once.

Procedure: 1. Fix thin tissue fragments one hour in two changes cold 100% ethanol.
2. Cut frozen sections at 15 μ, or clear in xylene, infiltrate 1.5 hours in paraffin, imbed and section at 6 μ, or prepare paraffin sections by the freezing-drying procedure (p. 48).
3. Dry frozen sections onto slides, deparaffinize paraffin sections in xylene and let dry.
4. Flood sections with dithizone reaction mixture and stain 5 to 10 minutes, drain.
5. Wash off excess dye by flooding with chloroform, drain.
6. Rinse quickly in distilled water and mount in Karo, fructose syrup, or Arlex gelatin.

Results: Zinc is demonstrated as red to purple granules or diffuse red. The complexing buffer serves to render nonreactive the other metals which normally react with dithizone: Mn, Fe, Co, Ni, Cu, Ag, Pd, Pt, Cd, Sn, In, Au, Hg, Tl, Pb, Bi. Yellow crystals of dithizone resulting from evaporation of acetone, are readily distinguished.

Uranium. The oxides of uranium are insoluble in water, as is uranyl phos-
phosphate (Lange). Hence, the phosphate buffered formalin (p. 34) should be adequate for fixation. Lison cites Schneider's fixation in 50 cc. saturated aqueous picric acid, 50 cc. 5% potassium ferrocyanide, and 10 cc. hydrochloric acid. This was followed by washing in 4% hydrochloric acid, then hydrochloric acid alcohol. Dehydration and imbedding as usual follow, and uranium salts appear as the deep brown double ferrocyanide of potassium and uranium. Gerard and Cordier (Arch. de Biol. 43:367, 1932) used the Prussian blue reaction as for iron, obtaining the same dark brown double ferrocyanide.

The alpha-particle autoradiographic technic (p. 439) might be used to study the distribution of uranium salts in tissues.

**Phosphates.** Bunting (Arch. Path. 52:458, 1951) used a variant of the molybdenum blue reaction adapted by him from Feigl and from Serra and Queiroz-Lopes for paraffin sections of formalin-fixed tissues.

**Bunting's Molybdenum Blue Technic for Phosphates.**

1. Deparaffinize and hydrate as usual.
2. Cover section with few drops of 5% ammonium molybdate and add an equal volume of 1% nitric acid. Let stand five minutes.
3. Wash thoroughly with water.
4. Cover with benzidine solution for one minute. (Dissolve 50 mg. benzidine base in 10 cc. glacial acetic acid, and dilute to 100 cc. with distilled water.)
5. Flood with 45% (saturated) sodium acetate solution. Apply cover glass and examine at once.

**Results:** Sites of phosphate ion, blue. The color fades and diffuses in a few hours.

Serra and Queiroz-Lopes (Portugaliae Acta Biol. 1:111, 1945) hydrolyzed small blocks of tissue for 2–3 weeks at 10–12° C. in a solution containing 1 Gm. ammonium molybdate in 100 cc. 2 N HCl and then continued the hydrolysis for 2–3 days in the same fluid at 20–25° C. They then added an equal (small) volume of the same benzidine solution as Bunting (Step 4), reacted for three minutes, and added two volumes of saturated sodium acetate. By mounting in glycerol and sealing with Romeis's rosin lanolin (p. 99) they were able to preserve the reaction for some months.

**Sulfates.** Gomori cites Macallum's (Abderhaldens Handbuch der biologischen Arbeitsmethoden, vol. 2: 1145–46, 1912) method: Treat frozen sections (Adamstone-Taylor or Linderström-Lang technics should be more successful) of fresh tissue with N/10 lead acetate for ten minutes or more. Wash thoroughly in water and then in N/10 nitric acid to remove lead phosphate, carbonate, and chloride. Wash in water and treat with equal volumes of glycerol and ammonium sulfide, converting the lead sulfate to the brown sulfide. Molnar's suggestion of substitution of a 40-minute bath in
0.5% sodium rhodizonate for the sulfide treatment might be tried. According to Feigl, neutral sodium rhodizonate reacts with lead sulfate or sulfide to give an insoluble deep violet, while rhodizonic acid, at pH 2.8, gives a red precipitate.

Macallum's ammonium sulfide is produced by saturating 16% ammonia (sp. gr. 0.96) with hydrogen sulfide gas.
Chapter 14

Various Cell Products

Glycogen

Glycogen is a polysaccharide derived from, and decomposing into, glucose. It is stored in liver cells, certain cells of the parathyroid, muscle, cartilage, and other locations. It is prone to hydrolyze post mortem, and consequently prompt fixation is especially required. However, prompt refrigeration, followed by adequate fixation, may avail. I have had good glycogen preservation in material refrigerated for 12–24 hours before fixation. Formerly, strong alcoholic fixatives were recommended as it was believed that glycogen itself was readily soluble in water, except in cartilage. Much work was done with 100% alcohol alone, which on a comparative basis now seems to be one of the poorest glycogen fixatives because of its poor penetration. Preservation seems adequate only in the relatively badly shrunken surface layers. Neutral aqueous form...n solutions often preserve glycogen quite well, especially in fresh animal...vers, but the action of certain acid alcoholic fluids seems prompter and more reliable.

I can recommend Carnoy's fluid (p. 36), alcohol formalin, and acetic alcohol formalin (p. 35). Picric acid variants of the alcohol formalins are often recommended (p. 47), but seem to offer no distinct advantage over the corresponding fluids without picric acid.

For simultaneous studies of glycogen and fats, aqueous fixations and frozen sections are required, and may be used also when the fat stains are not desired. Usually, however, nitrocellulose or paraffin sections are preferred, and the latter give better results if collodionized before applying the specific stain.

For digestion tests nitrocellulose sections are to be avoided, and on paraffin sections the test must be performed before collodionizing. I have seen glycogen resist digestion for 18 hours in collodionized sections, when the same enzyme solution removed it completely from uncollodionized control sections.
in 30 minutes. Apparently the larger enzyme molecules are unable to penetrate the collodion film.

Human saliva has been used as a digestant by many workers, but I have found malt diastase or ptyalin more convenient. The latter has been unavailable commercially for some time. Malt diastase in 1/1000 solution buffered to pH 6.0 with phosphates is an entirely adequate digesting fluid. Some samples are still active at 1/1,000,000. In addition to a diastase, human saliva, beef salivary gland ptyalin, and malt diastase contain a ribonuclease. In malt diastase this action is evident in 0.02–0.1% concentrations, and resists boiling in M/10 acetic acid. Consequently, if pure glycogenase action is desired it is necessary to use malt diastase solutions at 1/100,000 or higher dilution. At 1/1000 dilutions at pH levels of 5–8 both the diastatic and ribonuclease actions are destroyed at temperatures of 55–60° C. Hence digestion tests should be done at 35–45° C. for maximal action.


Diastase digestion test. Make a 0.1% solution of malt diastase in 0.02 M phosphate buffer of pH 6.0. Sodium chloride up to 0.8% may be added if desired, but larger quantities should be avoided as they may inhibit the enzyme action. Filter before using, to remove starch granules.

Technic. (1) Bring paraffin sections to water as usual; (2) digest 30–60 minutes at 45–35° C. in 1/1000 malt diastase; (3) wash in water; (4) dehydrate with alcohols or acetone; (5) soak ten minutes in 1% collodion in ether: alcohol (50:50); (6) drain one minute; (7) immerse in 80% alcohol for 5–10 minutes to harden the collodion and (8) proceed with the glycogen demonstration method chosen, using an undigested control section along with the digested one.

Goetsch, Reynolds, and Bunting (Proc. Soc. Exper. Biol. Med. 80:71, 1952) report successful digestion of glycogen from liver cells amylase, using undeparaffinized sections. This procedure could well void some of the section losses. In our hands diastase solutions required 6–12 hours to effect fairly complete digestion of liver glycogen, compared with about ten minutes for the usual deparaffinized ones. Heating of the sections to the melting point of the paraffin before digestion almost totally inhibits digestion, probably by forming a continuous paraffin film over the section.

McManus and Saunders (Science 111:204, 1950) reported on the use of pectinase, pectinol O, pectin esterase, polygalacturonase, and β-glucuronase, as microdissection agents for the study of tissues fixed in cold acetone (p. 36). The enzymes were used in 0.4% solution in an acetate buffer of pH 4.0 (p. 450) and unspecified molarity.

Pectinase and polygalacturonase remove periodic acid Schiff-positive materials: mucin, glycogen, splenic and lymphadenoid reticulum, cartilage matrix, hyalin, etc. Pectinol O gave qualitatively similar though less com-
plete removals. Pectin esterase and β-glucuronase did not attack the periodic acid Schiff-positive materials.

Incubation periods were 48 hours at 37° C.

The traditional methods for the demonstration of glycogen are the iodine method, which is applicable also to amyloid, corpora amylacea, starch, and other substances; and the alkaline carmine solution of Best. More recently we have added the aldehyde reaction with Schiff’s reagent after oxidation with chromic acid or with potassium permanganate (p. 124), with periodic acid or acid periodate solutions (pp. 123, 125, 281), or, lately, with lead tetra-acetate (p. 282) and sodium bismuthate (p. 284).

**Iodine Methods.** I omit the Langhans method cited in the first edition, since it is now demonstrated that ordinary aqueous formalin will preserve glycogen. I prefer to fix in my acetic alcohol formalin (p. 35), alcoholic formalin, or Carnoy’s fluid. Complete dehydration with ascending alcohols. Changes of one hour each suffice for the 100% alcohol; the lower grades should have 8–16 hours each. Clear in cedar oil and imbed in paraffin as usual (Schedule VI, p. 62). Carry sections through two changes each of xylene and of 100% alcohol into 1% collodion in equal volumes of ether and 100% alcohol. Soak 5–10 minutes, drain one minute, and harden in 80% alcohol for 5–10 minutes. Transfer to water and proceed.

1. **Gram’s iodine** for 5–10 minutes.
2. Dehydrate in two changes of 2% iodine in 100% alcohol.

**Best’s carmine solution** is prepared by gently boiling 2 Gm. carmine, 1 Gm. potassium carbonate, and 5 Gm. potassium chloride in 60 cc. distilled water for several minutes or until the color darkens. Cool and add 20 cc. 28% ammonia water. Ripen for 24 hours and store in icebox at 0–5° C. The carmine should be certified by the Biological Stain Commission. The stock solutions may be kept for several weeks only. The foregoing directions are derived from C. Bensley and from Mallory, and vary materially from Best’s as cited in Ehrlich’s *Encyklopaädie*.

The staining solution is composed of 8 cc. of the stock solution above, 12 cc. 28% ammonia water and 24 cc. methyl alcohol, total 44 cc. (Mallory used 10 cc., 15 cc., and 15 cc. respectively, total 40 cc., and directed filtration of the stock solution before mixing.) C. Bensley directed thorough mixing and warned strictly against filtration. This dilute solution is good for one or two days only, and it is preferable to use it only once.

The technic:

1. Mallory prescribes celloidin sections, Bensley paraffin, and I generally take the paraffin sections from 100% alcohol into 1% collodion for 5–10 minutes, drain one minute, harden five minutes in 80% alcohol, and transfer thence to water,
2. Stain five minutes in an acid alum hematoxylin (Ehrlich's, Lillie's, or other, p. 76).
3. Wash briefly in tap water and
4. Stain 20 minutes in the freshly diluted staining solution.
5a. Rinse paraffin sections with three changes of fresh methyl alcohol (preferably using a dropping bottle) then
6a. Dehydrate and remove collodion with two or three changes of acetone.
7a. Pass through acetone and xylene into two changes of xylene and mount in clarite.
5b. Wash celloidin sections in several changes of a mixture of water 10 cc., 100% ethyl alcohol 8 cc., and methyl alcohol 4 cc.
6b. Dehydrate in 80% and 95% alcohol.
7b. Clear in origanum oil and mount in balsam.

Results: Glycogen red, nuclei blue.

Cretin (Compt. rend. Soc. de biol. 135:355, 1941) fixed blocks of tissue in a fluid composed of 25 cc. 25% picric acid in dioxane and 30 cc. of a 5% trichloroacetic acid in 6% methanol for some hours up to two days, and then soaked them in 1% alcoholic solution of phenylhydrazine acidified with 1 cc. concentrated hydrochloric acid for an unstated period (three days?); then dehydrated, cleared, and imbedded in paraffin. Alternatively he fixed in 1% phenylhydrazine, 1% trichloracetic acid in 60% methanol for three days, and imbedded in paraffin as usual. It was also possible to utilize paraffin sections of material fixed in other ways, by mordanting 48 hours after deparaffinizing, with a 1% phenylhydrazine solution in methanol acidulated with 0.3% of concentrated HCl.

Sections after any one of the foregoing treatments are brought to water, immersed in Schiff reagent for an unstated period, washed in SO₂ water for several hours and then dehydrated and mounted as usual. Cretin specified mineral oil, but the synthetic resin should be satisfactory.

Cretin's procedure is prolonged and demands special fixation. I have no success with attempts to use it (in somewhat abbreviated form) on paraffin sections of material fixed by other methods.

The periodic acid Schiff procedure, with diastase digestion controls, is far simpler and appears to be quite uniformly successful.

The Bauer and Casella oxidation Schiff methods are discussed on p. 121 and their results are included in the detailed discussion on pp. 126–128. The preferred method for demonstration of glycogen is the periodic acid, Schiff leucofuchsin technic (p. 123). Variations and results are discussed in detail on pp. 120–128. A useful method, giving red-purple glycogen and other periodic Schiff-positive substances (but blue collagen and reticulum) is the allochrome procedure (p. 357).

It may be noted here that prolonged bromination (1 cc. bromine in 39 cc. carbon tetrachloride for 3–6 hours at 25° C.) completely prevents the re-
action of glycogen by the periodic acid Schiff method, without evidently affecting the reactivity of any other tissue substances. The reaction is probably analogous to the chlorination of starch, bromine replacing the hydrogen on carbons 1 and 4, and removing both hydrogens, leaving $\text{O}=$ on carbons 2 and 3 (Kerr, Chemistry and Industry of Starch, New York, Academic Press, 1950). This bromination effect is reversed by a three-day immersion in 5% silver nitrate.


The Methenamine Silver Method. For this purpose he found best a methenamine silver nitrate solution: Add 5 cc. 5% silver nitrate to 100 cc. 3% methenamine (hexamethylenetetramine, urotropin). A heavy white precipitate appears and easily redissolves on shaking. This stock solution may be kept for months in the icebox, or for about two weeks at room temperature.

Gomori prescribes an alcoholic fixative for glycogen. Mitchell and Wislocki used an alcoholic picroformalin. Gomori's technic follows:

1. Paraffin sections were deparaffinized and then collodionized in 0.5% collodion. For mucin this step was omitted.
2. Bring sections to water.
3. Treat 1–1½ hours in 5% chromic acid.
4. Wash ten minutes in running water.
5. Treat one minute in 1–2% sodium bisulfite ($\text{NaHSO}_3$) to remove traces of chromic acid.
6. Wash five minutes in running water.
7. Rinse in distilled water.
8. Silver at 37–45° C. in stock methenamine silver solution 25 cc., distilled water 25 cc., 5% borax 1 to 2 cc. Observe slides at intervals under the microscope until mucin or glycogen is dark brown while background remains yellow. This may take 1–3 hours.
9. Rinse in several changes of distilled water.
10. Tone five minutes in 0.1% gold chloride.
11. Rinse thoroughly in 2–3% sodium thiosulfate solution to remove excess silver.
12. Wash in running water.
13. Counterstain as desired, dehydrate, clear, and mount as usual. The collodion membrane sometimes stains rather intensely. It may be removed by acetone or by ether alcohol mixture during dehydration.

Results: Mucin, glycogen, and melanin color deep gray brown to black. Insoluble calcium salts may also blacken. In comparison with the Bauer
method this technic appears rather laborious, and on brief trial the visual contrasts appear inferior. I have had a number of failures with it on Bauer positive material. It shares with the Bauer reaction the staining of mucin which gives difficulties in the study of glycogen in muciparous cells.

McLean and Cook (p. 40) state that staining for 15–20 minutes in a saturated solution of chlorazol black E in 70% alcohol demonstrated nuclei in black; cytoplasm and inclusions, gray; chitin, green; and glycogen, red. If sections are overstained, differentiate with terpineol.

I have tried this with American chlorazol black E. Chitin of cestodes is fairly well shown in green, but much of the connective tissue is also gray green, and (to say the most for the method) it requires a certain amount of imagination to discern a reddish fluorescence where the glycogen ought to be. The carmine and periodic acid methods are much better for glycogen.

**Chitin**

Chitin, the material forming the exoskeleton of arthropods and occurring as well in other organisms, is insoluble in water, alcohol, ether, alkalies, dilute acids, and ammine copper hydroxide. It dissolves in hot concentrated hydrochloric acid or sulfuric acid.

When tested with the iodine and zinc chloride test it yields a violet color. Add 3–5 drops of concentrated iodine potassium iodide solution to 10 cc. 33% zinc chloride solution. Apply this to chitin which has previously been treated with potassium hydroxide and thoroughly washed. The chitin is colored brown on the surface, violet within.

A solution of iodine 50 mg., potassium iodide 500 mg., calcium chloride (probably CaCl₂·6H₂O is meant) 16 gm., and distilled water 4 cc., stains chitin red violet.

Zander directed as follows for sections: Mount (frozen or paraffin) section in water under a cover glass. Simultaneously draw water out from one side of the cover glass with filter paper and introduce Lugol's iodine solution (p. 92) on the other. Tissues are stained brown. Then draw out the iodine solution with filter paper and at the same time introduce (33%) zinc-chloride solution. The brown tissues are partly decolorized. In the same way replace the zinc chloride solution with distilled water. Chitin now assumes a violet color. The foregoing tests for chitin are derived from Ehrlich’s *Enzyklopädie*.

Insect chitin is an amino polysaccharide which is not dissolved by heating 15 minutes at 160° C. in saturated potassium hydroxide, but is changed by this treatment to chitosan, which is readily soluble in dilute acetic acid (Lee). It would be of interest to try the periodic acid Schiff method on this chitin. That of cestode parasites and of adult schistosomes in tissue is Bauer and periodic acid Schiff negative. The cuticle of schistosome eggs is strongly Bauer and periodic acid Schiff positive, but eggs of ascarids, trichurids, and the like do not react. (See p. 128.)
Cellulose and Starch

Cellulose colors violet when treated with a solution composed of 25 parts zinc chloride, 8 parts potassium iodide, and 8.5 parts distilled water, saturated with iodine crystals (Behrens, from Ehrlich's *Encyklopädie*).

If cellulose be treated with Lugol's solution and then with a mixture of 2 parts (by weight) of concentrated sulfuric acid and 1 part of water, it gives an intense blue color (*ibid.*).

Cellulose fibers and vegetable-tissue fragments stain red to purple by the permanganate, chromic acid and periodic acid oxidation, Schiff leucofuchsin technics. Some fibers are Schiff positive without oxidation, and more, but not all, react after peracetic acid treatment (p. 310). They still rotate the plane of polarized light after application of these methods. With other stains these fibers may give striking color alterations when rotated under crossed Nicols.

Cellulose resists ptyalin and malt diastase digestions; raw starch is digested quite slowly. When treated with Lugol's iodine solution starch granules give the familiar dark-blue color. This fades after a few days.

Starch granules remain almost unstained with most ordinary stains, but are doubly refractile, exhibiting the black cross of polarization separating four bright quadrants. On rotation the orientation of the black cross follows the position of the plane of polarization and not that of the starch granules. Also on rotation it is seen that in certain positions alternate quadrants are respectively yellowish and bluish white, and that on rotation these colors fade in some granules but are maintained in others.

On Gram-Weigert staining, starch granules stain black and transmit no polarized light. After potassium permanganate or chromic acid oxidation and sulfurous acid leucofuchsin staining the anisotropy is maintained, the quadrants appearing bright red purple on a dark background. On direct illumination with nonpolarized light these two methods give red-purple starch granules. After the periodic acid leucofuchsin technic the granules are dark purple and may remain dark under crossed Nicols, or give a dark red illumination. I assign the total extinction of polarized light noted above simply to the density of the stains, and not to any alteration in molecular structure.

Lignin, Inulin, Pectin

According to McLean and Cook (p. 77) a basic fuchsin solution decolorized by addition of strong ammonia water, drop by drop, is a test reagent for aldehyde, and may be used for the demonstration of lignin, which gives a purple color.

Plant cuticle and lignin because of their aldehyde content react also with Schiff's reagent.

Lignin according to McLean and Cook (p. 78) also gives Maule's reaction. Treat first with aqueous potassium permanganate (I suggest 0.5%
for ten minutes). Wash with dilute hydrochloric acid (10% HCl anhyd.), and then with dilute ammonia. A red color develops in lignified tissue only.

McLean and Cook describe the orcin test for inulin. Soak sections first in 0.5% orcin in 90% alcohol. Then transfer to strong hydrochloric acid and warm. An orange-red color is developed at sites of inulin deposit.

McLean and Cook (p. 77) state that ruthenium red (RuCl₃) colors pectin substances and pectic mucilage deep red.

Deparaffinize sections and soak 24 hours in 1 part hydrochloric acid and 3 parts alcohol. Then place in dilute ammonia for some hours. Then place in 1:5000 aqueous RuCl₃ in the dark until adequately colored.

**Dextran**

This highly water-soluble glucose polysaccharide may be demonstrated in the renal epithelium and in casts in the collecting tubules by using the periodic acid Schiff method. However, it is necessary to fix in strong alcoholic fixatives, to float paraffin sections on 95% alcohol, and to use alcoholic reagents at least through the periodic acid step, and preferably throughout.


1. Fix 48 hours at −5°C in 100% alcohol.
2. Transfer to fresh 100% alcohol for 18 hours.
3. Clear in petroleum ether or other paraffin solvent.
4. Infiltrate and imbed in paraffin. Section at 5μ.
5. Float sections on clean slides without albumen with warm 95% alcohol to flatten.
6. Dry sections in an incubator at 37°C for 24 hours and then transfer to a paraffin oven for 20 minutes.
7. Deparaffinize in xylene, transfer to two changes of 100% alcohol.
8. 1% periodic acid (H₅IO₆) in 90% alcohol for two hours (80 minutes is less adequate; three hours is no better than two). Keep alcoholic periodic acid in the dark. Discard when a brown color appears.
9. Wash in 95% alcohol for 5 minutes.
11. Immerse in Schiff reagent (p. 156) for 10 minutes.
12. Wash in three changes 0.5% sodium metabisulfite of 1, 2, and 2 minutes.
13. Wash 2–3 minutes in running water.
14. Counterstain in a 25% aqueous dilution of Weigert's iron hematoxylin (p. 81) or by the same iron hematoxylin picric acid sequence as for the aqueous method (p. 124) alcohol, alcohol xylene, xylene, polystyrene.
Results: Dextran, glycogen, and starch show dark red purple; other periodic acid Schiff-positive substances, much as usual (pp. 126–128). Glycerin albumen, if used on slides, may react quite intensely. The method may be useful for other highly water-soluble substances which contain HIO₄ reactive groupings.

Control sections stained by the routine aqueous periodic acid Schiff procedure (p. 123) should be compared. Dextran deposits are lost when the aqueous method is used.

Mucin, Cartilage, Mast Cells

Mucin is precipitated by dilute acetic acid and rendered insoluble in water. Pseudomucin and gastric mucin are not precipitated by acetic acid. When precipitated by alcohol mucin will redissolve in water. Dilute alkalies readily dissolve it.

Consequently alcoholic and acid fixatives are preferred. Formalin is quite serviceable if not alkaline, as it would become from magnesium carbonate neutralization. Telyesniczky's acetic formalin alcohol (p. 35) should be excellent. I have used most of the following methods on routine material fixed in formalin that was probably acid.

The most uniformly successful method for demonstration of epithelial mucins is the periodic acid Schiff method (p. 123). The mucin of the gastric-surface epithelium stains intensely by this method; then that of intestinal goblet cells. The mucins of intestinal glands, trachea and bronchi, minor salivary and buccal glands, female genital tract, and prostatic gland and seminal-vesicle contents are generally quite well stained. The mucins of the rabbit's pharyngeal glands, those of the basal portion of the colonic glands of some rodents, and connective-tissue mucins may stain quite poorly or not at all. Cartilage matrix, however, usually stains quite well, and more densely in the pericellular capsular areas than between them.

Bauer and Casella methods are generally successful on those mucins which color intensely with the periodic acid Schiff method, and fail where this method colors them lightly.

Technics for these procedures appear on pp. 123, 124. The allochrome procedure (p. 357) gives excellent contrasts for epithelial mucins—red purple, contrasting with gray-green or greenish-yellow epithelial cytoplasm and blue connective tissue.

Crippa (Boll. Soc. ital. biol. sper. 27:599, 1951) reports on the use of lead tetraacetate as an oxidant in place of chromic or periodic acid in a procedure utilizing the oxidation-induced aldehyde reaction for the demonstration of mucin and mucoid substances.

The reagent, which is stable for an indefinite period, is a 1% solution of recrystallized lead tetraacetate in glacial acetic acid. The solution is clear and colorless. The technic follows.
Crippa's Lead Tetraacetate Method for Mucins and Mucoids.

1. Fix as usual. Imbed in paraffin, section, and deparaffinize in xylene.
2. Wash in 50:50 xylene: glacial acetic acid and in glacial acetic acid.
3. Immerse for 30–60 minutes at room temperature 1% lead tetraacetate in glacial acetic acid.
4. Wash in three changes of glacial acetic acid to remove lead acetates.
5. Pass through xylene: glacial acetic acid 50:50 mixture, xylene, and descending alcohols to water.
7. Wash in three changes of 0.5% sodium bisulfite, 1, 2, and 2 minutes.
8. Wash in running water 10 minutes.
9. Counterstain if desired, as with the periodic acid Schiff method; or dehydrate, clear, and mount directly in balsam or synthetic resin.

Results: Aldehyde deposits engendered by lead tetraacetate oxidation show in red purple.

If desired, Steps 7 to 13 of Gomori's methenamine silver method for glycogen and mucins (p. 278) may be substituted for Steps 6 to 9 above. The mucins then appear in black.

McManus (Feb., 1952) writes me that lead tetraacetate renders Schiff positive essentially the same structures as does periodic acid. Jordan and McManus use a fresh mixture of 1 part of a strong to saturated solution of lead tetraacetate in glacial acetic acid with 3 parts of a mixture of 20 Gm. anhydrous sodium acetate, 25 cc. glacial acetic acid, and 50 cc. water. They use a shorter oxidation time, 30–120 seconds. Lhotka (Stain Technol. 27: 213, 1952) uses 5 Gm. potassium acetate dissolved in 100 cc. glacial acetic acid saturated with lead tetraacetate, and the same short oxidation times as McManus. Leblond and co-workers (Stain Technol. 27:277, 1952) observed that glycogen was not stained by room-temperature oxidation, but did react vigorously at higher temperatures. Our experience with the Lhotka reagent indicated prompt reaction of glycogen. Shimizu and Kumamoto (Stain Technol. 27:97, 1952) use a solution similar to McManus's:

1. Deparaffinize and hydrate as usual.
2. Molar sodium acetate solution five minutes (13.6% NaCO₃CH₃·3H₂O).
3. Oxidize ten minutes in lead tetraacetate 1 Gm. dissolved in 30 cc. glacial acetic acid + 70 cc. 46.5% (saturated) sodium acetate solution.
4. Wash five minutes in molar sodium acetate solution.
5. Wash ten minutes in running water.
6. Schiff reagent 15 minutes.
7. Wash in three changes of sulfite solution, two minutes each.
8. Wash ten minutes in running water.
9. Counterstain, dehydrate, clear, and mount as in periodic acid Schiff methods (p. 124).

Results: Vic-glycols in red purple.

While the authors prescribe the original Schiff reagent and sulfite rinse of Feulgen and Rossenbeck, undoubtedly the more recent variants will give fully satisfactory results.

It is to be noted that lead tetraacetate, in warm acetic acid solution, also reacts with olefines to form glycol diacetates and unsaturated acetoxy compounds (Hickinbottom, Reactions of Organic Compounds, New York, 1948, Longmans, Green & Co. p. 32).

Lhotka (Stain Technol. 27:259, 1952) recommends the use of sodium bismuthate in aqueous phosphoric acid solution as another reagent for the cleavage of vic-glycols and α-hydroxycarboxylic acids to carbonyl. Organic solvents can also be used. Overoxidation is said not to occur. The bismuthate solution in phosphoric acid is unstable and must be used at once.

Lhotka’s Sodium Bismuthate Schiff Procedure for Vic-glycols and α-Hydroxycarboxylic Acids.

1. Section formalin-fixed tissue in paraffin, deparaffinize, and hydrate sections as usual.
2. Suspend 1 Gm. sodium bismuthate in 100 cc. 20% phosphoric acid solution. The suspension is bright orange yellow and soon turns brown by precipitation of bismuth pentoxide.
Oxidize sections three minutes in the fresh suspension.
3. Wash in running water for one minute.
4. Rinse 15 seconds in normal hydrochloric acid to remove Bi₂O₅ from sections.
5. Rinse in distilled water.
6. Schiff reagent ten minutes.
7. 0.5% Na₂S₂O₅ or SO₂ water, three changes, two minutes each.
8. Wash in running water five minutes. (I prefer 10 minutes.)
9. Counterstain with alum hematoxylin or as desired.
10. Dehydrate, clear, and mount by alcohol, xylene, synthetic resin sequence.

Results: Pictures are similar to those obtained with periodic acid or lead tetraacetate. Sites of vic-glycols and α-hydroxycarboxylic acids in red purple.

Metachromatic Dyes. Perhaps the simplest stains are those using simple aqueous solutions of certain basic dyes which stain mucin and cartilage matrix metachromatically (pp. 84–87). Of these perhaps the best are thionin (C. I. No. 920), azure A, azure C (both C. I. No. 923), toluidin
blue O (C. I. No. 925), Bismarck brown Y (C. I. No. 331), and safranin O (C. I. No. 841). Highman recommends especially new methylene blue N (C. I. No. 927) in his buffer technic.

With these, one stains perhaps 30–60 seconds in a 1:1,000 aqueous solution, dehydrates with acetone, and clears in xylene. The azures, thionin, and toluidin blue give deep blue nuclei, light blue cytoplasm, green erythrocytes, red purple to violet mucus and cartilage matrix, and deep violet mast-cell granules. With the Bismarck brown Y, nuclei are brown; mucin and cartilage matrix, brownish yellow. With safranin O, mucin and cartilage are orange; nuclei, red; mast-cell granules, orange red. However, gastric mucus is said to stain with these dyes only when freshly formed (Mallory).

Iron Hematoxylin and Metachromatic Dyes. A useful variant of this simple procedure is made by combining an acid iron hematoxylin nuclear stain and a simple, contrasting plasma stain with it. Other basic dyes may be used which do not of themselves exhibit metachromasia.

The following technic of ours may be followed. Paraffin sections of formalin-fixed material are deparaffinized and brought to 80% alcohol as usual.

1. Weigert's acid iron chloride hematoxylin (p. 81) six minutes.
2. Wash in water.
3. 1:5,000 aqueous fast green FCF for three minutes.
4. Wash in 1% acetic acid.
5. Stain 4–6 minutes in 0.1% safranin O (C. I. No. 841) in water.
6. Dehydrate with 95% and two changes of 100% alcohol, clear with one change of 50:50 alcohol xylene mixture and two changes of xylene. Mount in synthetic resin.

Results: Nuclei, black; cytoplasm, gray green; mucus, cartilage, and mast-cell granules orange red. If cytoplasms, collagen, muscle, etc. do not stain green, reduce the time in Step 5.

By using 0.2% eosin Y in Step 3 and 0.1% crystal violet (C. I. No. 681) or malachite green (C. I. No. 657) in 1% acetic in Step 5, pink cytoplasm and violet or green mucus may be obtained. Other dye combinations also give good results, such as sequences of picric acid and basic fuchsin or new fuchsin or 1:5,000 anilin blue (C. I. No. 707) in place of the fast green FCF.

Safranin, Bismarck brown, and basic fuchsin variants with fast green FCF also do well on connective-tissue mucus and cartilage in mixed tumors, and often on the mucoid degeneration of the derma seen near some skin cancers. For mast cells I prefer the safranin variant. This variant is perhaps the most vivid of all methods for demonstration of nucleus pulposus mucin.

I have found brief staining with 1:2000 thionin (or German toluidin blue) buffered to pH 4 with 0.01 M acetate buffer very successful for connective-tissue mucins in connection with chondromucinase studies (Arch. Path. 52:363, 1951).
Buffered Thionin Stain.

1. Deparaffinize and hydrate sections as usual.
2. Stain 30 minutes in 0.05% thionin in 0.01 M acetate buffer.
3. Rinse, dehydrate in alcohol, clear with xylene, mount in polystyrene.

Results: Connective-tissue mucins, purplish red; mast-cell granules, red purple; nuclei, blue violet; red corpuscles, pale yellow; basophil cytoplasm, blue; muscle and connective tissue, faint greenish or unstained.

Using a pH 5 buffer demonstrates muscle and connective tissue in light blue-greens, while pH levels of 3 and 2 cause red corpuscles to remain unstained. Cytoplasm stains more poorly. Mucins appear to be only partly demonstrated at pH 3, and at pH 2 metachromatic staining seems restricted to cartilage and mast cells. Nuclear basophilia persists even in 0.1 N hydrochloric acid solution. (Note: Phosphates precipitate thionin.)

This method of staining with buffered thiazin dyes is based on that of Highman (Stain Technol. 20:85, 1945) and a basically similar procedure has been extensively used by Dempsey et al. (Anat. Rec. 98:417, 1947).

Mota (Blood 6:81, 1951) counts mast cells in rat bone marrow by making a 1:100 dilution of marrow in 3% acetic acid containing 1/50,000 toluidin blue. The mast cells are readily distinguished by their red-purple granules, and chamber counts of mast cells are readily compared with total nucleated cell counts.

Hematoxylin and Carmine Methods

Two of the traditional methods for mucus are Mayer’s mucihematein and mucicarmine stains. I have not used these to any great extent, but cite them because the first is said by Mallory to be excellent for connective-tissue mucin and the second for epithelial mucin. For both 100% ethyl alcohol fixation is prescribed. Paraffin and celloidin (nitrocellulose) sections may be used.

Mucihematein. For the mucihematein dissolve 0.2 Gm. hematein (not hematoxylin) and 0.1 Gm. aluminum chloride in 100 cc. of either 40% glycerol or 70% alcohol. To the alcoholic solution 1 or 2 drops of concentrated nitric acid are added. The latter is preferable when the mucus tends to swell in aqueous stains. With either solution stain sections 10-60 minutes or more, wash in several changes of distilled water for 5-10 minutes, dehydrate, clear, and mount with alcohols, xylene, and clarite. Mucus stains violet blue; other tissue elements remain unstained. Gastric mucin is not stained. Cowdry suggests prestaining with alum carmine to give red nuclei.

Laskey (Stain Technol. 25:33, 1950) uses hematoxylin and ripens with 40-100 mg. sodium iodate per Gm. Solutions are ready to use at once and remain useful for over six months. Her directions for preparation of the solution read: Dissolve 1 Gm. hematoxylin in 100 cc. 70% alcohol. Add 0.5 Gm.
aluminum chloride and 5 cc. 1% aqueous sodium iodate solution, and make up to 500 cc. with 70% alcohol.

*Laskey's Technic for Mucihematein Staining.*

1. Deparaffinize and hydrate as usual.
2. Lay slides face up on staining racks and flush off several times with distilled water.
3. Deposit 2 cc. of mucihematein on each slide and stain for 5-10 minutes.
4. Wash in three five-minute changes of distilled water.
5. Dehydrate with 95% and 100% alcohols, two changes each, and clear with alcohol + xylene and two changes of xylene. Mount in polystyrene or other resin.

*Results:* Epithelial mucins stain deep blue-violet; connective tissue mucins and cartilage matrix, lighter violets. Gastric surface epithelial mucus is unstained.

*Mucicarmine.* For mucicarmine heat a mixture of 1 Gm. carmine (alum lake), 0.5 Gm. anhydrous aluminum chloride, and 2 cc. distilled water over a small flame, agitating constantly, until the color darkens, about two minutes. Then add with constant stirring 100 cc. 50% alcohol. Let stand 24 hours and then filter. This stock solution is stable. According to Mallory, take 1 cc. stock mucicarmine and dilute with 10 cc. distilled water or 50-70% alcohol and stain 10-15 or more minutes, rinse in water, dehydrate with alcohols, clear with xylene, and mount in balsam. Cowdry first stained sections in alum hematoxylin, washed, and stained five minutes in undiluted mucicarmine. Only mucus, including gastric mucus, is stained red; other structures are unstained or stained with hematoxylin, according to the variant chosen.

Acid methyl blue and anilin blue stains in the various collagen technics (pp. 347-348, 350, 356) often stain epithelial mucus, including gastric mucus, selectively a light blue, contrasting well with counterstains and with the deep blue collagen. Mucin may remain deep violet by Gram-Weigert variants (p. 372).

*Alcian Blue.* Steedman (*Quart. J. Microscop. Sci.* 91:477, 1950) reported a technic for staining chondroitin and mucosulfuric acid mucins with the phthalocyanin dye Alcian blue 8GS.* This dye stains mucin a clear blue-green color. Mast cells are not stained. Prolonged staining will color almost all non-nuclear components of tissue. Treatment (after Step 3) for two hours or more with alkaline alcohol (pH 8 or higher) converts the dye into the insoluble pigment Monastral fast blue, which is highly resistant to decolorization, and thus permits the subsequent use of various histologic reagents.

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* Imperial Chemical Industries, Ltd.
**Steedman’s Alcian Blue 8GS for Mucins.**

Material fixed in Bouin, susa, Zenker is recommended. Formalin-fixed tissue is considered unsuitable.

1. Deparaffinize and hydrate as usual.
2. Stain 10-40 seconds in 1% aqueous Alcian blue 8GS.
3. Rinse in distilled water.
4. If required, treat two hours in 80% alcohol containing 0.5% borax.
5. Counterstain with alum hematoxylin and eosin, or otherwise as desired.
6. Dehydrate, clear in xylene, and mount in synthetic resin or balsam.

**Note:** The Alcian blue solution should contain thymol to prevent mold growth and should be filtered every week to ten days.

**Iron Methods**

The mucins, as well as collagen, reticulum, and various other tissue components, have the property of taking up in more or less selective fashion ferric ions from solutions of ferric salts. These combined ferric ions may then be demonstrated by the usual ferrocyanide reaction (p. 243) as Prussian blue. Alternatively, they may be subjected to a silvering procedure, such as the Gomori-Burtner methenamine silver method (p. 165) and the sites of iron uptake shown in black. A 10-minute bath in 1% aqueous ferric chloride is adequate for this purpose.

The number of reacting tissue elements is profoundly influenced by the pH of the iron solution. With alkaline solutions of the Lillie-Mowry type practically only gastric mucins react.


For pH 5 ferric mannitol dilute 2.18 cc. of U.S.P. Solution of Iron Chloride to 50 cc. with distilled water to make 0.1 M ferric chloride. Add 50 cc. 0.1 N acetic acid (p. 456) and 5 Gm. mannitol, and shake to dissolve. Then add 145 cc. 0.1 N sodium hydroxide with continuous agitation. Check pH. Cautiously add more 0.1 N NaOH, checking pH frequently, until pH 5 is reached. Avoid precipitation of ferric hydroxide. A total of about 220 cc. 0.1 N NaOH is required. Then add distilled water to make a total of 500 cc. Store in ice box.

For pH 8 ferric mannitol reagent dissolve 5 Gm. mannitol in 50 cc. of 0.1 M ferric chloride solution (2.18 cc. U.S.P. XI Solution of Iron Chloride + 47.82 cc. distilled water), add 125 cc. 0.1 N NaOH, shake thoroughly and then add more 0.1 N NaOH, 1-2 cc. at a time, until solution is almost clear. Then adjust with 1% acetic acid to pH 8. Store in the icebox.
The technic:

1. Deparaffinize and hydrate sections as usual. Wash in four changes of distilled water.
2. Incubate 30 minutes at 25° C. in ferric mannitol solution.
3. Wash in four changes of distilled water.
4. Mix 20 cc. 5% acetic acid and 20 cc. fresh 2% aqueous potassium ferrocyanide solution, preheat to 45° or 50° C., put sections in, and incubate 30 minutes in paraffin oven at 58° C.
5. Wash in 1% acetic acid.
6. Counterstain two minutes in 0.1% safranin O in 1% acetic acid.
7. Wash in 1% acetic acid, dehydrate with alcohols, clear with 100% alcohol + xylene and two changes of xylene, mount in polystyrene, Clarite, or other saturated synthetic resin.

Results: With the pH 8 ferric mannitol, gastric epithelial mucin and the mucigen granules of the necks of the peptic glands color blue. Esophageal keratohyalin is blue. The argentaffin cells of the rabbit gastric mucosa exhibit a fine blue cytoplasmic granulation after various alcoholic fixations only. Parietal and chief cells stain pink to faint gray-blue; collagen and reticulum remain unstained; intestinal gland and goblet-cell mucins stain metachromatically orange with the safranin; and nuclei stain red.

With the pH 5 ferric mannitol, all of the gastric and intestinal epithelial, goblet-cell, and gland mucins color an intense blue; the cuticular border of the villi is blue; reticulum, collagen, and connective-tissue mucin color a moderately deep blue; cartilage matrix stains partly deep blue; partly bright orange. Nuclei and the cytoplasm of pancreatic acini and chief cells are deep red; smooth muscle, parietal cells, and cytoplasm generally color pale blue-green.

With pH 3 ferric mannitol mucins are less intensely colored; collagen and reticulum are less conspicuous; cytoplasm and muscle are moderately gray-blue; and altogether contrasts are decreased.

The alkaline ferric mannitol technic does not demonstrate the enterochromaffin substance. In alcohol-fixed material in which this method succeeds, the Gomori-Burtner methenamine silver and Schmorl ferric ferrocyanide technics fail, and in aqueous formalin-fixed material, in which the two latter methods demonstrate numerous enterochromaffin cells, the alkaline ferric mannitol method fails completely.

The Hale Dialyzed Iron Method (Nature 157:802, 1946). Alcoholic fixations are preferred. Prepare paraffin sections. Prepare dialyzed iron reagent by mixing equal volumes of 2 M (12%: 11.5 cc. of glacial acetic acid + 88.5 cc. distilled water) acetic acid and of Merck's or other medicinal Dialyzed Iron (5% Fe₂O₃). Prepare 0.02 M potassium ferrocyanide in 0.14 N hydrochloric acid (approximately 86 cc. 1% ferrocyanide + 14 cc. normal acid).
The technic:

1. Deparaffinize and hydrate as usual.
2. Cover sections for ten minutes with dialyzed iron reagent.
3. Pour off and wash well in distilled water.
4. Immerse for ten minutes in the acid ferrocyanide (above).
5. Wash in water, counterstain in 1% safranin in 1% acetic, alcohols, alcohol + xylene, xylenes, polystyrene.

Results: In general the Hale method gives a less intense blue coloration of the same elements as the pH 5 Lillie-Mowry variant. Epithelial and connective-tissue mucins and nucleus pulposus are blue; connective tissue fibers, light blue; cartilage, part blue, part orange.

In the Ritter-Olson procedure (Am. J. Path. 26:639, 1950) one proceeds as above through Step 4, washes in water, and proceeds with the periodic acid leucofuchsin technic, starting with the HIO₄ oxidation step (p. 125). Ritter and Olson apparently used the Hotchkiss alcoholic periodic acid solution (p. 122) and the Hotchkiss reducing rinse.

Though very striking red-blue contrasts are obtained, the histochemical specificity of the procedure for the differentiation of the mucopolysaccharides seems open to some question.

Rinehart and Abul-Haj (Arch. Path. 52:189, 1951) have improved the Hale method by altering the colloidal iron preparation and adding the Van Gieson counterstain. Their iron solution is made thus: Dissolve 75 Gm. granular ferric chloride in 250 cc. distilled water, add 100 cc. glycerol and then add gradually 55 cc. 28% ammonia, stirring constantly to redissolve precipitate. Dialyze against distilled water for three days, changing water twice daily. Dialysis tubes should be large enough to hold at least 1000 cc. Longley finds this solution more selective than Hale's, and uses the following.

Longley's Variant of the Rinehart-Hale Method

1. Paraffin sections through xylene and alcohols to water.
2. Hydrolyze 10 minutes at 60° C. in N/1 HCl, wash and complete Feulgen reaction as usual (p. 132).
3. Wash in 3% acetic and immerse in Rinehart's iron solution for 10 minutes.
4. Wash in six changes of distilled water.
5. Immerse 10 minutes in 15 cc. 2% potassium ferrocyanide + 30 cc. 1% hydrochloric acid (N/8), freshly mixed.
6. Wash in distilled water.
7. Counterstain in Weigert's Van Gieson picrofuchsin (p. 346) for 6 minutes.
8. Alcohols, alcohol + xylene, xylenes, polystyrene.

Results: Nuclei red purple, cytoplasm, muscle and erythrocytes yellow, collagen and bone matrix red, mucins blue, except that of the gastric surface epithelium, cartilage matrix variably blue to colorless.
Chondromucinase and Hyaluronidase Digestions

The chondromucins or chondroitin sulfuric mucins are further identified by the fact that their metachromatic staining with thionin, toluidin blue, azure A, safranin O and the like may be prevented by chondromucinases or chondroitinases.

The chondromucinases so far characterized are enzymes which resist boiling for ten minutes in 0.1 M acetic acid, and may thus be separated from the hyaluronidase of bovine testes and from the amylase of barley malt, both of which are destroyed by this treatment.

Since the metachromatic staining of umbilical cord and nucleus pulposus mucins and of cartilage matrix are destroyed apparently equally by raw and by acid boiled testicular "hyaluronidase," it would appear that these metachromatically stained mucins are chondromucins and not hyaluronic acid. The Hale and ferric mannitol reactions of the same mucins are likewise equally impaired by raw and acid boiled hyaluronidase digestions. Hence it appears that these methods too demonstrate chondromucins rather than hyaluronic acid.

At this writing it is not clear whether we possess any method for the histochemical localization of hyaluronic acid as distinguished from the chondromucins.

Hotchkiss (Arch. Biochem. 16:131, 1948) listed hyaluronic acid among the substances giving a strongly positive spot test with his (alcoholic?) periodic acid Schiff method. Others have denied this reaction (using aqueous periodic acid?).

The vitreous humor, which contains a considerable amount of hyaluronic acid, exhibits a weblike coagulum, more copious after alcoholic fixations, which stains red with the periodic acid Schiff method (p. 123) and blue by the allochrome procedure (p. 357). It also colors blue with the Feulgen spirit blue method (p. 320), and this blue staining is retained after alcohol dehydration, clearing, and mounting in xylene polystyrene, as are those of ceroid and cartilage matrix.

Blue staining of this vitreous webwork is seen also with the Lillie-Mowry ferric mannitol method in the pH 5 variant (p. 288).

The periodic acid Schiff-positive material in the fibrillar stroma of the umbilical cord is not reduced in amount by 18 hours' digestion in a bovine hyaluronidase preparation which completely destroyed the metachromatically staining material in one hour.

Technic of Hyaluronidase and Chondromucinase Digestions.

1. Fix in alcohol, alcohol acetic formalin, Carnoy, lead or mercury salt mixtures, or the like. Dehydrate, imbed in paraffin, and section as usual. Bichromate fixatives impair the digestibility of cartilage matrix.
2. Deparaffinize and hydrate through alcohols, treating with iodine and thiosulfate to remove mercury precipitates if necessary. Do not colloidinize, for this greatly retards digestion. Fragile material may be digested without removal of paraffin, but the digestion may require days rather than hours.

3. Digest for graded intervals in hyaluronidase or malt diastase buffered to pH 5.5–6.5 (0.1 M) with phosphates or acetates. I have used testicular hyaluronidase concentrations of 25–2 mg. per 100 cc. Digestion times vary from 1 to 18 hours. Steps of 1, 2, 4, 6, and 18 hours are convenient. Malt diastase is used at 1:1000 dilution in pH 6 buffer (0.1 M acetate or phosphate). Digest at 37–45°C.

4. After digestion, wash in water and stain with toluidin blue, thionin (p. 286), the iron hematoxylin-fast green-safranin method (p. 285) or one of the iron methods (pp. 288–290), as usual. Dehydrate, clear, and mount by methods prescribed for the staining technic employed.

Mast Cells

Besides their ready staining with basic anilin dyes and the basic components of neutral stain mixtures, and their variable behavior under the periodic acid Schiff procedure (p. 127), it is specifically recorded that mast-cell granules are nonacid-fast (p. 381), Gram negative and Gram-Weigert negative, and iron negative with ferrocyanide and ferricyanide technics. They do not blacken selectively in the Masson argentaffin or the Gomori-Burtnner methenamine silver technics. They do not reduce ferric ferricyanide (p. 175). In ferric mannitol procedures at pH 5 and pH 8, the granules (in mice) do not take up iron selectively, but their staining with safranin is irregularly permitted or prevented.

The granules are not stained by iron or alum hematoxylin or by alum or borax carmine, by mucicarmine or mucihematin, or by Alcian blue.

Metachromatic staining has been attributed by Lison and others to the presence of sulfate esters of mucopolysaccharides, and the metachromasia of mast cells has been attributed to heparin (Jorpes, Heparin, New York, Oxford University Press, 1939; also Sylven, Acta. Path. et microbiol. Scandinav. 29:197, 1951). The metachromasia is readily abolished by even brief methylation (p. 163) and can be restored by demethylation with KMnO₄ only if methylation in 0.05 N HCl methanol did not exceed 6 hours at 60°C.

Amyloid

Amyloid is a chondroitin sulfuric acid-protein complex which is colored mahogany brown by iodine—potassium iodide solution in the fresh state. This reddish brown color is changed to violet or blue by sulfuric acid. Amyloid is insoluble in water, alcohol, ether, and dilute acids. Like mucin, it exhibits metachromatic staining with certain basic dyes, and stains light blue with
anilin blue and methyl blue methods for collagen fibers. It gives a dim blue fluorescence under ultraviolet light (p. 15).

Mallory's Iodine Reaction (emended).

1. Stain paraffin or frozen sections of formalin- or alcohol-fixed material in 1:10,000 iodine solution for 10–15 minutes. (Dilute 1 cc. Weigert's iodine solution (p. 92) with 99 cc. water or 3 cc. Gram's iodine solution with 97 cc. water.)
2. Wash in water, and examine in water or glycerol.

Results: Amyloid is mahogany brown. Post-mortem tissues should be rinsed in 1% acetic acid before applying the iodine.

According to Langhans (Ehrlich's Encyklopädie) permanent mounts may be made thus:

1. Prestain 10–15 minutes with carmine.
2. Stain 5–10 minutes in Gram's iodine solution (p. 92).
3. Dehydrate with 100% alcohol containing 1–2% of iodine crystals.
4. Clear and mount in origanum oil.

Bennhold's Congo Red Method (München. med. Wchnschr. 2:1537, 1922). Formalin or alcohol fixation; frozen, paraffin or celloidin sections.

1. Stain in 1% aqueous Congo red 10–30 minutes.
2. Immerse in saturated aqueous lithium carbonate 15 seconds.
3. Decolorize in 80% alcohol until stain clouds no longer come off.
4. Wash in running water 15 minutes.
5. Counterstain with alum hematoxylin.
6. Wash in water.
7. Dehydrate with alcohols and clear in xylene. Use isopropyl alcohol with celloidin sections. Mount in balsam (or Clarite).

Results: Amyloid red; nuclei blue.

In Highman's variant (Arch. Path. 41:559, 1946) the Congo red is 0.5% in 50% alcohol for 1–5 minutes. Steps 2 and 3 are replaced by 1–3 minutes' differentiation in 0.2% potassium hydroxide in 80% alcohol. (This reagent decolorizes amyloid more slowly and tissue more rapidly than does lithium carbonate.) Step 4 is reduced to a mere rinsing with water. The rest of the technic is unchanged.

The Crystal Violet Method. Mallory cites a crystal violet method which I have modified by adding methyl violet 2B to give a redder color. Formalin or alcohol fixation, and frozen or paraffin sections may be used. From water:

1. Stain sections 3–5 minutes in crystal violet (C. I. No. 681) 1 Gm., methyl violet 2B (C. I. No. 680) 0.5 Gm., alcohol 10 cc., distilled water 90 cc.
2. Wash in 1% aqueous acetic acid.
3. Wash thoroughly in tap water.
4. Examine directly in water or mount in glycerol, glycerol gelatin, fructose syrup, or Apáthy's gum-arabic syrup (p. 109).

**Results:** Nuclei and cytoplasm, varying shades of blue violet; amyloid and fibrinoid, red purple. The preparations fade after a time (Mallory 1938, Conn and Darrow 1943). Water mounts may be sealed temporarily with petrolatum.

*Highman's crystal violet method* (*Arch. Path.* 41:559, 1946) thus:

1. Stain five minutes in Weigert's acid iron hematoxylin.
2. Wash in water.
3. Stain 1–30 minutes in 0.5–0.1% crystal violet or methyl violet in 2.5% acetic acid (I suggest five minutes in 0.2% crystal violet).
4. Wash in water.
5. Mount in Highman's potassium acetate Apáthy gum syrup, glycerol gelatin, or Alex gelatin (pp. 109–112).

**Results:** Red purple amyloid, cartilage, and certain types of mucus, in a bluish background with blue black nuclei. The acetic acid in the crystal violet prevents overstaining of cytoplasm, and the salt added to the syrup prevents diffusion or “bleeding” of the violet. Omission of the iron hematoxylin (Step 1) renders the stain less dense.

Lieb (*Am. J. Clin. Path.* 17:413, 1947) used about 0.3% crystal violet (10 cc. saturated alcoholic solution in 1 cc. hydrochloric acid and 300 cc. water), stained 5 minutes to 24 hours, and mounted from water in a solution of 50 Gm. Abopon* in 25 cc. water.

According to Herzenberg (*Virchows Arch. f. path. Anat.* 253:656, 1924) amyloid may be stained intravitally by injection of 0.1–1 mg. Congo red in 1 cc. intravenously in mice.

The periodic acid Schiff procedure (p. 123) colors amyloid light red; and in its allochrome variant (p. 357) the color remains predominantly red, though with a bluish cast.

The so-called *paramyloid* is characterized by inconstancy and irregularity of staining with Congo red and with crystal violet. Commonly colors are displayed between that of typical amyloid and that of serum or plasma, and considerable variability is evident even in the same section. The Van Gieson stain gives a yellow color to paramyloid, whereas it stains typical amyloid more orange. Paramyloid bodies often present concentric lamination, like that of corpora amyloidea in prostatic acini, and may show central calcification. Its distribution is not that of typical amyloidosis. It occurs in bone, fat, fascia, etc., rather than in the liver, spleen, pancreas, kidney, adrenal, and heart (Bauer and Kuzma, *Am. J. Clin. Path.* 19:1097, 1949).

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*Glyco Products Co., 148 Lafayette St., New York, N. Y.*
VARIOUS CELL PRODUCTS

King (Am. J. Path. 24:1108, 1948) has applied a Del Rio-Hortega silver carbonate method for demonstration of both usual and atypical amyloid in frozen section of formalin-fixed tissues.

1. Cut thin frozen sections; wash in distilled water.
2. Add concentrated (28%) ammonia water drop by drop to 5 cc. 10% silver nitrate until the brown precipitate is just dissolved; then add 6–8 cc. of 3.5% sodium carbonate (Na₂CO₃), and dilute to 75 cc. with distilled water. Store surplus solution in a dark, cold place. To 10 cc. of the stock diammine silver carbonate solution in a small beaker add a few drops of pyridine. Introduce the sections with a glass rod and warm gently to about 45° C. with continuous gentle agitation until sections become a rather deep brown (“tobacco-brown”).
3. Transfer directly with a glass rod to (5%) sodium thiosulfate (Na₂S₂O₅•5H₂O) solution for 2–4 minutes.
4. Wash in several changes of tap water.
5. Float onto slides, smooth out, and blot down with hard filter paper.
6. Counterstain lightly if desired.
7. Dehydrate and clear by the usual alcohol xylene sequence, and mount in Canada balsam or an unsaturated synthetic resin.

Results: Amyloid, nuclei and lipochrome (lipofuscin?) pigment show dark brown to black. Some collagen may impregnate, and cytoplasm is often light brown, as are hyalin casts in the renal tubules.

For corpora amylacea the Langhans iodine method for glycogen may be used. Schmorl recommends a variant of Siegert’s (no reference) in which Müller or alcohol fixation is recommended:

1. Wash sections well in water, then
2. Stain deep brown with strong iodine-potassium iodide solution.
3. Decolorize with strong alcohol and then
4. Immerse in 20% hydrochloric acid until the corpora amylacea reappear as darkly colored points.
5. Wash out acid quickly with water.
6. Dehydrate in tincture of iodine diluted with four volumes of alcohol.
7. Mount in origanum oil.

Results: Corpora amylacea in prostate, brain, lung, and urinary tract are stained deep brown; other tissues are colorless.

Toluidin blue O stains the corpora amylacea of the central nervous system metachromatically reddish purple. They are red to purple with the periodic acid and chromic acid leucofuchsin methods (pp. 123–124) and resist diastase digestion (p. 275).

The periodic acid Schiff method (p. 123) colors prostatic secretion pink to
purplish red, and corpora amylacea appear in deeper purplish red. Diastase
digestion does not alter these reactions. When prostate sections are incubated
about 3½ hours in methenamine silver (p. 165) by the Gomori-Burtner tech­
nic, to the point where stroma reticulum and nuclei begin to blacken, pros­
tatic secretion still stains pink by the safranin counterstain and the corpora
amylacea are peripherally or (more often) completely blackened.

The Highman-Bennhold technic (p. 293) colors prostatic corpora amyla­
cea purplish to orange-red and the secretion gray-violet to pink. The crystal
violet amyloid technic (p. 294) colors them red-purple, while the secretion
remains violet.

Hyaline

Mallory's "alcoholic hyaline" is said to give reactions for phosphates and
to stain by Mallory's hematoxylin method for lead (p. 268). After Zenker
fixation it stains intensely red with Mallory's phloxin methylene blue method
(pp. 116–117) and blue with his phosphotungstic acid hematoxylin (p. 344). With alcohol or formalin fixation it is stained deep blue by Mallory's (re­
gressive sequence) iron chloride hematoxylin method (pp. 79–82).

**Mallory's Phloxin Method.** When overstained with phloxin and then
decolorized with lithium carbonate hyalin remains red: Fix in alcohol or
formalin, section in paraffin or celloidin.

1. Stain nuclei in Mallory's or other alum hematoxylin (p. 76).
2. Wash in water.
3. Stain 20–60 minutes in 0.5% phloxin B (C. I. No. 778) in 20% alcohol.
4. Wash in tap water.
5. Decolorize 30–60 seconds in 0.1% aqueous lithium carbonate.
6. Wash in tap water.
7. Dehydrate and clear through an alcohol xylene sequence and mount in
   balsam.

**Results:** Nuclei appear blue; fresh hyalin appears as red droplets and
threads; older hyalin, pink to colorless. Hyalin droplets in renal epithelium
are also well shown, and hyalin casts color purplish red more than pink.
Amyloid stains pale pink.

**Mallory's thionin method** uses similar material:

1. Stain 5–10 minutes in 0.5% thionin (C. I. No. 920) in 20% alcohol.
2. Differentiate several minutes in 80% alcohol and then
3. 95% alcohol and
4. Clear in terpineol and mount in terpineol balsam.

**Results:** Granules and networks of red to purple material; old hyalin,
blue; nuclei, blue.

1. Hydrate deparaffinized sections as usual, using the iodine thiosulfate sequence for Zenker-fixed tissue.
2. Stain five minutes in alum hematoxylin.
3. Wash in water.
4. Flood with 20% acid fuchsin in aniline water (shake 2 cc. aniline thoroughly with 100 cc. distilled water; filter) and heat with a small flame to fuming. Let stand five minutes.
5. Wash in water.
6. Differentiate in a mixture of 7 parts 20% alcohol and 1 part saturated alcoholic picric acid (about 1% picric acid in 30% alcohol) until only hyalin and red corpuscles remain red, and collagen is faint gray or unstained.
7. Wash thoroughly in water.
8. Mordant 4-18 hours in 1% phosphomolybdic acid solution.
9. Transfer directly to 1% light green SF in 1% acetic acid for one hour.
10. Wash in water.
11. Differentiate in 80% alcohol until collagen fibrils appear discrete. This occurs quickly, so care is needed.
12. 95% and 100% alcohol, xylene, synthetic resin.

**Results:** Mallory's alcoholic hyalin, brilliant red; cytoplasm, pale brown; bile pigment, green; collagen, green; hemosiderin and hemofuscin unstained or yellowish brown in their natural color.

Renal hyaline droplets and casts appear red purple with the periodic acid leucofuchsin technic (p. 123). Casts also react to the Casella and Bauer methods (p. 124) though less strongly. With the allochrome procedure (p. 357) the droplets and casts assume an orange color similar to that given by intravascular fibrin and that of pneumonic exudates. It contrasts well with the gray-green or gray-yellow of renal epithelial cytoplasm. Both droplets and casts often color red with Mallory's phloxin technic (p. 296) and may color violet with Weigert's fibrin method (pp. 372-373). They are eosinophilic with azure eosin methods.

The hyaline of intimal degeneration of small renal arteries gives a positive Sudan IV (p. 302) and a Liebermann-Burchard cholesterol reaction (p. 317) in frozen sections. It is often periodic acid Schiff positive (p. 123) (Baker, Am. J. Path. 27:680, 1931).

**Fibrin**

Fibrin is a fibrous protein occurring in various acute inflammatory processes, notably diphtheritic inflammation and lobar pneumonia, and in antemortem and post-mortem thrombi.
It is eosinophilic with hematoxylin eosin (p. 114) and azure eosin stains (p. 118). It retains the plasma stains in procedures of the Van Gieson and fast green–Van Gieson types (pp. 346, 349) and the Masson-Mallory methods (p. 351). It stains orange in the allochrome method (p. 357), and red to pink in the periodic acid Schiff method (p. 123) changing to orange if a picric acid counterstain is used. It retains the crystal violet iodine complex of the Gram stain when Weigert’s anilin xylene differentiation is used (p. 372), but not when alcohol or acetone is the differentiating agent.

Stains of the Masson type are more brilliant after Bouin, Zenker, or Spuler fixation. With material fixed in formalin, they may be improved by pre-mordanting in saturated alcoholic picric acid for two minutes at 25° C. or in saturated aqueous mercuric chloride for an hour at 58° C. Bouin fixation, however, interferes with the Weigert fibrin stain. The allochrome method is quite differential and does well on formalin-fixed tissue.

According to Glynn and Loewi (J. Path. & Bact. 64:329, 1952) fibrin is digested in three hours at 37° C. by 0.1% trypsin in pH 8 phosphate buffer, but fibrinoid and collagen are not.

**Fibrinoid**

Fibrinoid is a homogeneous, refractile, oxyphilic substance occurring in degenerating connective tissue, in term placentae, in rheumatoid nodules, in Aschoff bodies, and in pulmonary alveoli in some prolonged pneumonitides.

With toluidin blue it is often metachromatic, sometimes not; and in some instances this metachromasia is abolished by predigestion with hyaluronidase, in others not. With phosphomolybdic or phosphotungstic acid anilin blue stains of the Mallory type, it is described sometimes as staining red with the counterstain, sometimes as cyanophil. With phosphotungstic acid hematoxylin (p. 344) it colors partly blue, partly orange to yellow. Altshuler and Angevine record purple staining with crystal violet (p. 293). Its uptake of acid and of basic dyes at various pH levels are similar to those of fibrin. According to Altshuler and Angevine, fibrinoid contains considerable amounts of arginine (p. 144).

Regarding the Weigert fibrin stain, the literature is vague. Klinge (Virchows Arch. f. path. Anat. 278:486, 1930) spoke of most of the fibrinoid material as giving a positive fibrin reaction. The fibrin reaction in common use at that time was the Weigert fibrin stain (Schmorl 1928, Romeis 1932).

Fibrinoid is conceived of as a precipitation of acid mucopolysaccharide with a basic protein (Altshuler and Angevine, Am. J. Path. 25:1061, 1949).

Like Altshuler and Angevine, Glynn and Loewi (J. Path. & Bact. 64:329, 1952) report a strongly positive periodic acid Schiff reaction (p. 123). This reaction is abolished by pectinase digestion and resists tryptic digestion, but is weakened by tryptic digestion after denaturation for 18 hours in 36% (6 M) urea.

They also report blackening and demonstration of a fibrillar structure by
Gomori's reticulum technic (p. 335). Fibrin did not react by this method. This reaction of fibrinoid was also abolished by pectinase digestion.

For tryptic digestion they used a 0.1% solution of Armour's crystalline trypsin in pH 8 phosphate buffer for three hours at 37° C.

In the pectinase digestion a crude preparation from onions was used at 0.4% in pH 4 acetate buffer, for three hours at 37° C.
Chapter 15

Fats and Lipoids

Neutral Fats

The most ancient method for demonstration of fatty substances in tissues is the reduction of osmium tetroxide (commonly called osmic acid). This reagent is reduced to a black substance by unsaturated fats and fatty acids and by a variety of other reducing agents, such as eleidin and tannin; and if osmium tetroxide is followed by 60–70% alcohol, stearin and palmitin are also blackened. Myelin is also blackened by osmium tetroxide; but if previously treated with chromate solutions, only degenerating myelin is so blackened.

Generally speaking, the oil-soluble dye methods are much less troublesome and more satisfactory for the demonstration of fatty substances. The first of these was Sudan III, introduced by Daddi in 1896 (Arch. ital. biol. 26:143, 1896). Michaelis (Virchows Arch. f. path. Anat. 164:263, 1901) later introduced the use of Sudan IV or Scharlach R which has been the most popular of the oil-soluble dyes.

Following the long use of these two Sudans, III and IV, the property of staining with oil-soluble dyes generally has come to be referred to as sudanophilia, even though the oil-soluble dye used may not have the word Sudan included in its name.

Osmium Tetroxide Method. However, since the osmium tetroxide method may have some value in assisting in the identification of fat-like substances, the following is suggested.

Cut frozen sections of formalin-fixed material. Soak part of the sections in 2.5% potassium bichromate for two days, then a day or two further in a mixture of 6 cc. 2.5% potassium bichromate and 3 cc. 1% osmium tetroxide. Wash in water and mount in gum syrup. Degenerating myelin and neutral fats are blackened.
Treat a second group of sections 24 hours in 1% osmium tetroxide, wash in water 6-12 hours, soak several hours in absolute alcohol to obtain the secondary staining of fats. Wash in water and mount in gum syrup. This procedure is supposed to demonstrate saturated neutral fats as well as unsaturated.

Material treated in the block with osmium tetroxide, alone or with chromates, can be imbedded in paraffin or nitrocellulose. For paraffin imbedding, clearing with cedar oil or briefly with chloroform is preferred, and chloroform balsam is said to be better for mounting.

The Marchi Method. A method which I formerly used for degenerating myelin was slightly modified from Schmorl:

1. Fix two days in Orth or 10% formalin.
2. Mordant seven days in 2.5% potassium bichromate, changing on the third and fifth days.
3. Osmicate 14 days in the dark in two changes of seven days each of a mixture of 2 volumes 2.5% potassium bichromate and 1 volume 1% osmium tetroxide.
4. Wash 24 hours in running water.
5. Dehydrate with four changes of acetone, 30-45 minutes each.
6. Clear in petroleum ether, two changes of 30 minutes each.
7. Infiltrate with paraffin, three changes, 30 minutes each or one change of 30 minutes in vacuo.
8. Imbed and section.
9. Deparaffinize with chloroform and mount in chloroform balsam.

Results: Degenerating myelin, black; background, brownish yellow.

Recently I have preferred a frozen-section method with a modified Weigert myelin stain combined with Sudan II (C. I. No. 73) for the degenerating myelin (p. 305).

Fat Stains with Oil-Soluble Dyes

Staining with oil-soluble dyes is based on the greater solubility of the dye in lipid substances than in the usual hydroalcoholic (and other) dye solvents. Michaelis so described it in Ehrlich's *Encyklopädie*, Lison so characterized it, and Cain and Harrison (*J. Anat.* 84:196, 1950) still state that these substances operate "not as a dye, but only as an oil soluble colorant."

This staining has long been regarded as quite specific for lipoids. Cain and Harrison make the important exception that fats of high melting point do not color with Sudan dyes unless heated to near or above their melting points. Thus cholesterol and its esters, carotenoids, tristearin, and high-melting paraffins may be stained when melted, but not at room temperature.

There is an important corollary to this theory of staining by oil-soluble dyes. The dye should be again extracted by an excess of a suitable dye sol-
vent which does not dissolve the lipoid, and the lipoid should then be again restainable by the original technic. Most of the lipoids which survive paraffin imbedding can thus be completely decolorized in a matter of a few seconds to one or two minutes with acetone, and stained again with oil-soluble dyes. The decolorization and restaining can be reperformed repeatedly. Similarly, frozen sections dried onto slides and stained by Sudan black or oil red O can be decolorized by three or four hours extraction in diethylene glycol, restained, and decolorized repeatedly. It is found, however, that some substances such as the sudanophil granules of leucocytes cannot be decolorized by prolonged exposure to acetone, xylene, chloroform, and the like, even at elevated temperature. Erythrocytes stain intensely on prolonged heating with Sudan black B, but not with oil red O or Sudan IV, and the stain is not extracted by acetone or xylene.

It is evident that the Sudans are not inert chemically (Lillie and Burtner, J. Histochem. & Cytochem. 1:8, 1953) as had been supposed, but are capable of forming firm unions with certain tissue elements some of which, at least, are probably not lipoids. These combinations occur less promptly than with ordinary fat stains, and may require elevation of temperature for their accomplishment. Hence any Sudan or other oil-soluble dye staining is suspect in which one dye is allegedly much better as an oil-soluble dye than another or in which resistance to immersion oil or to xylene or absolute alcohol has been noted; and the lipoid nature of the stained substance needs confirmation by the extraction and restaining tests mentioned above.

It is perhaps noteworthy that acetylated and benzoylated Sudan dyes still stain ordinary fats quite effectively, but fail to color leukocyte granules. It is possible that these ester dyes may be useful in discriminating true fats from chemically sudanophilic substances.

The original method of Daddi’s (1896) called for a saturated solution of Sudan III in 70% alcohol. This takes a half hour or more to stain, gives a rather light orange color, and dissolves out an appreciable amount of fat, in some instances all of the demonstrable fat that was present.

Herxheimer’s technic (Centralbl. allg. Path u. path. Anat. 14:891, 1903), employing a saturated solution of Sudan IV (Scarlet or Scharlach R) in a mixture of equal volumes of acetone and of 70% alcohol, gave deep orange-red staining in a few minutes, but also removed a considerable amount of fat, and in some cases all of the fat present.

Herxheimer’s alkaline Sudan IV (Deut. med. Wchnschr. 27:607, 1901) has been much used by neurohistologists. This saturated dye solution contained 1% (Conn) or 2% (Mallory) sodium hydroxide in 70% alcohol. The solution is said to be unstable and must be discarded after two or three days. Globus (Practical Neuroanatomy, Baltimore, Wm. Wood & Co., 1937, p. 256) used 3.33% sodium hydroxide in 50% alcohol, again saturated with the dye.

The technic:
1. Place sections in a covered watch glass and heat until condensation droplets appear on the lid, and then allow to stand 15 minutes.
2. Wash five minutes in distilled water.
3. Counterstain in dilute Ehrlich's hematoxylin (p. 76).
4. Wash in distilled water.
5. Mount in glycerol.

Results: Fats bright red.
This variant of Globus's method preserves some fats which are least in the original Herxheimer solution, probably because of its lower (50%) alcohol content, as compared with the 70% of the original.
Romeis used a solution of Sudan IV in 40% alcohol and avoided fat losses, but took some 18–24 hours to stain.
Gross's (Ztschr. wiss. Mikr. 47:64, 1930) technic, which used a saturated solution of Sudan IV in 50% diacetin, stained fats deep red promptly, and did not dissolve out lipoids. However, the solvent gradually decomposed and impaired the stain.
Lillie and Ashburn (Arch. Path. 36:432, 1943) developed the principle of using fresh aqueous dilutions of a saturated 99% isopropyl alcohol (isopropanol) stock solution to 60% or 50% strength. These solutions were supersaturated with dye and stained vigorously and promptly in the first few hours after dilution, later becoming slow and relatively inefficient stains. Sudan IV, first used in this procedure, was soon replaced by dyes giving more stable supersaturated solutions and better color effects.

Oil red O, which is xylene-azo-xylene-azo-β-naphthol, a disazo dye introduced by French (Stain Technol. 1:79, 1926) in the Herxheimer technic, is found to be one of the best fat stains available in this supersaturated isopropanol method (Stain Technol. 19:55, 1944). It gives a deep scarlet color to fats. Oil red 4B, xylene-azo-toluene-azo-β-naphthol, is similar.
Sudan II (C. I. No. 73) gives a bright orange yellow in this technic which contrasts well with a myelin stain. Sudan brown (C. I. No. 81) gives a deep brownish red.

Coccinell red (Stain Technol. 20:73, 1945) stains successfully from as low as 30% isopropanol, and also gives a deep scarlet color. This dye is 1,5-bisamyl-amino-anthraquinone, and its 1,4 isomer oil blue N (Stain Technol. 20:7, 1945) gives deep blue fats from 40% isopropanol solution.

The Supersaturated Isopropanol Method. The following technic may be used with Sudan brown, oil red 4B, or oil red O, any of which are better than Sudan IV or Sudan III.
Prepare a stock saturated solution in 99% isopropanol (isopropyl alcohol), using 250 to 500 mg. of dye per 100 cc.
1. Dilute 6 cc. of stock solution with 4 cc. water.
2. Let stand 5–10 minutes and then filter. The filtrate can be used for several hours.
3. Stain thin frozen sections for ten minutes.
4. Wash in water.
5. Stain five minutes in an acid alum hematoxylin of about 0.1% hematoxylin content (undiluted Mayer, 1 part of Lillie's to 4 of 2% acetic, or 1 of Ehrlich's to 5 of 2% acetic, see p. 76).
6. Blue in 1% disodium phosphate or in tap water.
7. Float out in water, mount on slides, and
8. Drain and mount in gum syrup (pp. 109-110).

Rinehart (Arch. Path. 51:666, 1951) uses oil red O, Sudan IV, Sudan black B, and coccinel red as supersaturated 60% ethanol solutions made in the same way. His staining interval is five minutes.

The amylamino-anthraquinone dyes oil blue N, caccinel red, and carycinel red were quite satisfactory in the supersaturated isopropanol technics. The first stained well from a final 40% isopropanol concentration; the other two, from as low as 30% isopropanol. Deep blue, scarlet, and deep crimson colors respectively were imparted to fats (Stain Technol. 20:7, 73, 1945).

Chiffelle and Putt (Stain Technol. 26:51, 1951) strongly recommend propylene or ethylene glycol as a solvent for fat stains. These solvents virtually do not attack aliphatic fatty acid esters, but are said to have some solvent capacity for aromatic compounds, such as cholesterols and ketosteroids. The dyes recommended are Sudan IV and Sudan black B. These are soluble to about 0.5% in propylene glycol.

Chiffelle and Putt's Propylene Glycol Method. Dissolve 0.7 Gm. of Sudan IV or Sudan black B in 100 cc. propylene glycol at 100-110° C. Do not exceed 110° C. Filter hot through Whatman No. 2 filter paper. Cool and refilter with vacuum through a medium-porosity frilled glass filter.

Technic:
1. Cut frozen sections, wash in water 2-5 minutes to remove formaldehyde.
2. Dehydrate 3-5 minutes in pure propylene glycol, moving sections at intervals.
3. Transfer to the dye solution for 5-7 minutes. Agitate occasionally.
4. Differentiate in 85% propylene glycol for 2-3 minutes.
5. Wash in distilled water 3-5 minutes.
6. Counterstain if desired.
7. Float onto slides, drain, and mount in glycerol gelatin.

Results: Neutral fats, myelin, mitochondria and other lipids—orange red or greenish black; cytoplasm—unstained.

Staining of Lipoids with Esterified Sudan Dyes

Benzoylated oil red O was made for me by the National Aniline Division, Allied Chemical & Dyestuffs Corp. Acetylated Sudan IV and Sudan black B can be made quite simply by dissolving 2-2.5 Gm. of dye in 60 cc. pyridine,
then adding 40 cc. acetic anhydride and let stand overnight. Then pour into three or four liters of distilled water, let stand a few hours, and filter out the precipitated dyestuff on a Buchner funnel with vacuum. Dry the precipitate, the funnel and the glassware used in the precipitation. Dissolve the precipitate from glass, funnel and filter paper with acetone and evaporate to constant weight at 60° C. Yields range from 75% up to nearly theoretical. While acetylated oil red O may also be prepared, the resultant ester is a tarry red mass at room temperature.

Acetylated Sudan black B appears to give less background staining and just as intense lipoid staining as the untreated dye. Acetylated and benzoylated oil red O and acetyl Sudan IV fail to stain human neutrophil leukocytes in 16 hours at 37° C., but at 60° C. there is apparently some hydrolysis of the esters, and some coloration results on prolonged staining.

Observing the precaution of staining at 37° C. or lower, and using a control human-blood film fixed either with formaldehyde gas or with 75% alcohol for ten minutes, it is believed that these esterified dyes can be used to aid in discriminating lipoid staining from the stable sudanophilia.

The technics used are those usual for fat stains. We have employed stock solutions at 550 to 600 mg. per 100 cc. in 100% ethanol, diluting at time of use to 60% or 50% alcohol content with distilled water. Filtration of the freshly diluted mixture reduces precipitation on sections, and washing after staining in 50-60% alcohol is helpful.

Fluorescence Microscopy. For the demonstration of fats in fluorescence microscopy, Popper (Arch. Path. 31:766, 1941) used either a ten-second stain in 1% aqueous methylene blue (C. I. No. 922) or a three-minute stain in 0.1% aqueous phosphine (C. I. No. 793). The first gave a blue fluorescence which suppressed green fluorescences other than that of vitamin A. The second produced a silvery-white fluorescence, and demonstrated more fats than traditional strong alcoholic Sudan methods.

As with stains with Sudan dyes, the fluorescence color should be extractable by a dye solvent which does not dissolve the lipoid, and the fatty substance should be restainable, if the fluorescence demonstration is to be regarded as evidence of lipoid nature of the stained substance.

Myelin Stain with Sudan II and Iron Hematoxylin. Use frozen sections at 10-15 µ of material fixed in formalin and chromated in 2.5% potassium bichromate for 2-4 days, then washed in water.

1. Make fresh mixture of 5 cc. of fresh 1% aqueous hematoxylin and 5 cc. of 4% iron alum in covered dish. Stain sections 45 minutes at 55-60° C., agitating dish gently from time to time to insure even staining.
2. Wash sections in water. Save out some sections in water.
3. Decolorize in 0.5% iron alum for 1 hour, agitating from time to time.
4. Wash in water.
5. Treat with 1% borax, 2.5% potassium ferricyanide solution 10 minutes,
agitating several times. (This is Lillie's variant of Weigert's borax ferri-
cyanide, formula 15, p. 332.) At start of this step dilute the Sudan II for
Step 7.
6. Wash in water.
7. Take 6 cc. of a stock solution of Sudan II (C. I. No. 73) saturated in
99% isopropanol, and dilute with 4 cc. water, let stand 7 to 8 minutes,
filter. Stain sections 10 minutes in the fresh filtrate.
8. Wash in water.
9. While a nuclear stain with a red, green, or brown dye might be useful
at this point, I have had little success in attempting to insert one.
10. Float onto slides, drain, and mount in gum syrup.

*Results:* Normal myelin blue black, nerve cells gray, nuclei deeper gray,
red corpuscles yellow to black, fats orange yellow.

The persistence of black in nerve cells indicates underdifferentiation with
the iron alum, loss of color in myelin overdifferentiation. If sections are not
satisfactory, those saved at Step 2 may be differentiated for a shorter time in
0.5% iron alum if the myelin was too pale, or an hour in 1% iron alum if
other structures were too dark. With freshly fixed formalin or Orth material
chromated not over four days, this should not be necessary.

Feyrter developed a staining technic which is said to be specific for the
so-called "onkocytes," Hamperl (Arch. Path. 49:563, 1950) performs it as
follows:
1. Fix in 10% formalin and cut frozen sections at 10-15 μ.
2. Float from distilled water onto a clean slide.
3. Cover with 1% thionin or 1% cresyl violet in 0.5% tartaric acid solution.
   (Let stand a few days before using. Do not filter.)
4. Place cover glass on the stain drop, and seal with lanolin rosin (p. 99).

*Results:* Nuclei, blue; myelin, red purple; other lipids, onkocytes, and
mucus, pink to red. Cardiolipids show blue metachromasia with cresyl violet.
The onkocytes owe their metachromasia to poorly sudanophilic lipoids in
their cytoplasm. The full color of the staining develops only after some hours.
Hamperl states that the preparations are stable for years. Romeis comments
on their limited life.

Consideration of the special requirement of Feyrter (Romeis) that the
thionin of only one German firm is suitable for his metachromatic staining
of myelin, and of the fact that some materials staining metachromatically by
Feyrter's method are themselves removed from frozen sections by brief (two-
hour) alcohol extraction (Thorén, Acta Soc. Med. Upsal. 55:125, 1950) sug-
gests that the dye concerned may contain a good deal of a relatively fat-solu-
ble dye, such as Bernthsen's methylene violet. The solutions of this dye in
chloroform are red. It would appear that the procedure may be closely re-
lated to the Ciaccio type methods in so far as it applies to myelin.
Paraffin

Paraffin may occur in sections as a result of incomplete deparaffinization, as birefringent, often intranuclear crystals. The birefringence disappears at the melting point of the paraffin. In the cold these crystals do not stain appreciably with oil-soluble dyes; but if the staining solution is heated to above the melting point of paraffin, staining occurs (Nedzel, Quart. J. Microscop. Sci. 92:343, 1951). Xylene, alcohol, water, alcohol, xylene sequence treatments are more effective in removal of these birefringent crystals than a similar exposure to xylene alone, both at room temperature.

Fatty Acids

Fatty acids are readily soluble in ether and alcohol. They form calcium soaps when fixed in a 10% formalin which has been saturated with calcium salicylate (about 1.5%). These calcium soaps are insoluble in ether and 100% alcohol mixture and in dilute hydrochloric acid when tested with these reagents separately, but dissolve in a hydrochloric acid solution in ether and alcohol. After mordanting with copper acetate, fatty acids and their calcium soaps form a black hematoxylin lake which is very resistant to decolorization with Weigert's borax ferricyanide mixture. This forms the basis of Fischler's (Centralbl. f. allg. Path. u. path. Anat. 15:913, 1904) method for fatty acids.

1. Fix in 10% formalin and cut frozen sections.
2. To differentiate fatty acids from calcium soaps, extract some of the sections with two or three changes of 100% alcohol ether mixture (50:50), first dehydrating through 95% and 100% alcohol, and afterwards rehydrating through 100%, 95%, and 80% alcohol. Only the soaps will remain in these.
3. Treat both extracted and unextracted sections with saturated (perhaps 10%?) aqueous cupric acetate solution for 2-24 hours at 20-30° C.
4. Wash in distilled water.
5. Stain 20 minutes in Weigert's lithium carbonate hematoxylin (p. 332).
6. Differentiate in Weigert's borax ferricyanide mixture (p. 332) "greatly diluted" with distilled water, until red corpuscles are decolorized.
7. Wash thoroughly in distilled water and mount in glycerol, glycerol gelatin, or gum syrup. If desired, one may add a counterstain with a yellow or red oil-soluble dye before mounting (pp. 302-306).

Mallory notes that iron, hemoglobin, and calcium may stain as well. If differentiation be carried to the point where red corpuscles are thoroughly decolorized, hemoglobin should give no difficulty. Pretreatment of a few sections with dilute hydrochloric acid in place of Step 2 should serve to eliminate calcium as a source of error. Similarly, overnight extraction in 5%
oxalic acid solution should serve to remove iron. Or a control section may be subjected to the ferrocyanide test (pp. 243–244).

Since Wigglesworth's iron sulfide method (p. 138) is said to demonstrate carboxylic acids as well as phosphoric acid complexes, this method might also be applicable to the demonstration of fatty acids.

Tandler's lead sulfide methods are said to demonstrate fatty acids (Cien. e invest. 8:44, 1932) and phosphoric acids (Arch. histol. norm. y pat. 4:275, 1951). He employs a primary 12-hour fixation in 10% neutral lead acetate, with or without about 0.2% picric acid included, or in 5% lead acetate with a few drops up to 1% acetic acid. After this, tissues are washed thoroughly in water.

At this point tissues intended for demonstration of phosphoric esters are dehydrated, cleared, imbedded in paraffin, sectioned, deparaffinized and brought either into 70% alcohol containing 2% yellow ammonium sulfide or into saturated hydrogen sulfide water.

Tissues intended for demonstration of fatty acid esters are treated with very dilute (0.7–0.5%) ammonium sulfide without prior alcohol treatment and washed and mounted in glycerol.

Lead phosphate esters are insoluble in alcohol, chloroform, xylene, and alcohol ether, but soluble in dilute acetic acid. Even 0.5% will remove them in a short time. Unsaturated fatty acid lead soaps are soluble in alcohol (70–80%), while saturated fatty acid lead salts resist alcohol, but are dissolved by xylene, chloroform, etc. Both types of lead soaps resist extraction in 40% acetic acid.

Substitution of Molnar's rhodizonate method (p. 268) should give a darker color in both technics, and permit final dehydration and mounting in a resinous medium.

I question whether the differential solubilities of the lead soaps are sufficiently great to permit their histochemical differentiation.

Meyer-Brunot (Ztschr. wiss. Mikroskop. 60:476, 1952) adapts the Gomori lead sulfide lipase technic for the differentiation of fatty acid and neutral fats.

1. Fix smears by heating on an albumen-glycerol smeared slide over a small flame.
2. Immense in 0.5% calcium chloride in 0.5% veronal sodium solution (pH 9.4) for ten minutes, thereby converting free fatty acids to calcium soaps.
3. Wash thoroughly and immerse for ten minutes in 2% lead nitrate solution, converting the calcium soaps to lead soaps.
4. Wash thoroughly in water and immerse for 30 seconds in dilute ammonium sulfide— I suggest 1 to 50 dilution—thus converting lead soaps to sulfide; or in 0.5% potassium bichromate, forming the yellow lead chromate (p. 268).
5. For neutral fats, counterstain with Sudan III as usual (p 302).
Undoubtedly the method can be applied to frozen sections of formalin-fixed material. Treatment with 1% acetic acid after Step 3 to dissolve lead phosphate would tend, according to Tandler (p. 308), to eliminate lead phosphate derived from cell nuclei. Use of rhodizonate (p. 268) in place of ammonium sulfide could give a darker color to the lead deposits.

Cain (J. Anat. 84:196, 1950) again stresses the view (with which I thoroughly agree) that Nile blue sulfate solutions containing the red oxazone which Lison calls Nile red behave simply as mixtures of a (rather inefficient) oil-soluble dye and a blue basic dye which colors nucleic acids and other acid-bearing tissue elements as well as the open carboxyl groups of free fatty acids. Staining with the red oxazone component does not indicate unsaturation, but appears to be governed by the staining temperature and the melting point of the specific fats and fatty acids concerned. Solid fats do not readily stain with oil-soluble dyes at temperatures far below their melting points.

The melting points of the fatty acids are: oleic 14° C., palmitic 63° C., and stearic 70° C.; of their glyceryl esters, respectively —4° C., 65° C., and 71° C.

The technics are simple. Fix in formalin, cut frozen sections, stain 20-30 minutes in 0.1-0.15% aqueous solution of Nile blue sulfate, differentiate 1-20 minutes in 1% acetic acid, wash well in tap water, and mount in lukewarm glycerol gelatin, or in a buffered neutral gum syrup (p. 109). (Modified from Romeis.) To include the high-melting fats in the stained substances, raise the staining temperature to 70° C. I suggest buffering the Nile blue to pH 2.5 or 3.0 with acetates to eliminate if possible the necessity for regressive differentiation. Staining for 20 minutes in 0.1% Nile blue sulfate in 1% acetic, followed by washing in water and mounting in glycerol gelatin, has given sharply selective staining of certain lipofuscin pigments.

Ethlenic Linkages, Unsaturated Fats

Certain substances, such as ceroid, the lipoid of the retinal rod acromeres, and some of the fuscin or lipofuscin pigments may be oxidized with performic or peracetic acid to yield aldehyde, which may then be demonstrated with Schiff reagent. Prior bromination (one hour at 25° C. in 1 cc. bromine to 39 cc. carbon tetrachloride) completely blocks the reaction, whereas acetylation does not. After the oxidation step, interposition of sulfite blockade, phenylhydrazine, or semicarbazide treatments, prevents the lipoids from coloring with Schiff reagent when the usual ten-minute exposure is used. Long exposures (2–3 hours) overcome sulfite blockades and brief (30 minutes) phenylhydrazine blockades; and the aldehydes react with the usual red purple color. Prolonged phenylhydrazine and anilin hydrochloride blockades (4–72 hours) appear to be permanently effective.

The action of performic acid and of peracetic acid is quite comparable, and both reagents are readily prepared. According to Greenspan (J. Am. Chem. Soc. 68:907, 1946) performic acid is formed in adequate concentra-
tion in 30–60 minutes, but deteriorates practically to inactivity by the next
day. Peracetic acid takes 2–3 days to reach maximum concentration, and
may be kept several weeks. I have used the same jar repeatedly. Hence the
performic acid is preferable for an occasional test and the peracetic for fre­
quent routine use. The following directions for manufacture of the reagents
are derived from Greenspan.

Performic Acid Reagent: To 8 cc. of 90% formic acid add 31 cc. of 30%
hydrogen peroxide and 0.22 cc. concentrated sulfuric acid. Keep at or below
25° C. About 4.7% performic acid (HCO₂H) is formed within two hours,
and the solution deteriorates after a few more hours. Make fresh daily.

Peracetic Acid Reagent. To 95.6 cc. glacial acetic acid add 259 cc. 30%
hydrogen peroxide and 2.2 cc. concentrated sulfuric acid. Let stand 1–3
days. Add 40 mg. disodium phosphate as stabilizer. Store at 0–5° C. I have
kept such solutions in the icebox for months. A single Coplin jar of this re­
agent may be used for 8–10 groups of nine slides before discarding; but a
positive control should be included, at least in the later groups.

Lillie's Performic or Peracetic Acid Schiff Reaction.

1. Fix and section in accordance with the solubility requirements of the
lipoid under study. For ceroid, any fixation and paraffin or frozen sec­
tions; for fuscin pigments, routine formalin and paraffin sections; for
retina and myelin, aqueous formaldehyde or, better, formaldehyde bi­
chromate sequence fixations and paraffin sections.
2. Bring sections to water as usual.
3. Oxidize 90 minutes in performic acid reagent or two hours in peracetic
acid reagent.
4. Wash ten minutes in running water.
5. Immerse in Schiff reagent for ten minutes.
6. Wash in three changes of 0.5% sodium bisulfite or metabisulfite, 1, 2,
and 2 minutes.
7. Wash ten minutes in running water.
8. If desired, counterstain 1–2 minutes in Weigert's acid iron hematoxylin
(p. 81), wash four minutes in running water, and stain one minute in
saturated aqueous picric acid solution.
9. Dehydrate, clear, and mount by the alcohol, alcohol + xylene, xylene,
polystyrene sequence. Or, if required because of the solubility of the
lipoids concerned, wash in water and mount in Arlex gelatin.

Results: Ceroid, retinal-rod acromere lipoid, some adrenal and ovarian
fuscin pigments react with a red-purple color. Myelin sometimes reacts quite
well, especially in frozen sections or in well-chromated paraffin material.
If no counterstain is employed, nuclei are also colored red by the concur­
rent Feulgen reaction. The latter may be induced by corresponding mixtures
of formic or acetic acid with a little sulfuric acid and distilled water replacing the hydrogen peroxide solution. The red color of nuclei is almost completely suppressed by iron or alum hematoxylin counterstains.

Hair cortex also colors red-purple, a reaction which Pearse (Quart. J. Microscop. Sci. 92:393, 1951) attributes to cystine, although he was not able to repeat the reaction with cystine in vitro. Like that of ceroid, this reaction is prevented by prior bromination and is not reversed by even prolonged extraction in fat solvents, hot or cold. Like ceroid, hair cortex is also acid-fast by Ziehl-Neelsen technics; but unlike ceroid, it is not sudanophilic in paraffin sections.

Pearse makes peracetic acid by mixing 5 cc. 30% hydrogen peroxide with 20 cc. acetic anhydride and notes that it is less active than his performic acid mixture. Apparently he used it immediately, without allowing the 24-48 hour interval which Greenspan recommends for reaction to form peracetic acid. His performic acid reagent is made by adding 4 cc. 30% hydrogen peroxide to 40 cc. 98% formic acid and reacting for one hour before using.

Greenspan used about 1.57 moles of $H_2O_2$ per mole of acetic acid, a proportion designed to give a maximum yield of peracid. Pearse's prescription calls for only 0.10 mole of $H_2O_2$ per half mole of acetic anhydride. Thus the latter contains much less water and much more acetic acid in proportion than does Greenspan's mixture.

With performic acid, Greenspan's directions call for about 1.55 moles of peroxide per mole of formic acid; Pearse's, for only 0.038 mole; again providing less water and a far greater excess of formic over performic acid.

This reaction is controlled by omission of the hydrogen peroxide from the performic or peracetic acid reagent, substituting the same amount of water; or is prevented by bromination (infra) or by treatment of duplicate blocks by concentrated aqueous halogen acid solutions (Karrer).

I have treated blocks of formalin-fixed cirrhotic rat liver containing ceroid for 16 hours with hydrochloric (sp. gr. 1.19) and hydriodic (sp. gr. 1.70) acids, subsequently washing, dehydrating, imbedding in paraffin, and sectioning as usual.

After these treatments the acid-fastness and sudanophilia of ceroid persist, but the periodic acid and peracetic acid Schiff reactions are abolished.

Bromination is done as follows:

Bromination Technic for Paraffin Sections.

1. Deparaffinize in xylene as usual.
2. Wash in two changes of carbon tetrachloride, five seconds each.
3. Brominate one hour in 1 cc. bromine (3.1 Gm.) and 39 cc. carbon tetrachloride (63 Gm.). Expose control sections to pure carbon tetrachloride for 1-6 hours, to eliminate solubility in that solvent as a confusing factor.
4. Wash in two changes of carbon tetrachloride, five seconds each.
5. Descending alcohols etc., as usual to demonstration technic.

Results: Carbon–carbon double bonds are blocked and no longer react with organic peracids or potassium permanganate to form aldehyde. Glycogen is partly brominated and may become periodic acid Schiff negative, especially with longer bromination (3–6 hours).

Bromination Technic for Readily Soluble Fats.

1. Carbowax, gelatin, or frozen sections are attached to slides by floating on, blotting lightly, and allowing to dry for 10 or 15 minutes.
2. Immerse in freshly mixed bromine water (1 cc. to 39 cc.) for one or more hours.
3. Wash in water.
4. Dip into 0.5% sodium metabisulfite for 1–2 minutes, to remove free bromine.
5. Wash 2–4 minutes in running water and proceed with usual demonstration technic.

I have also used potassium permanganate for the oxidation of ethylenes to aldehyde, but since this reagent also attacks vic-glycols, converting them also to aldehyde (Stain Technol. 27:37, 1952) and further, destroys the aldehydes which it produces (Stain Technol. 26:123, 1951), its use is scarcely convenient for general technical purposes. By blocking ethylene by bromination and vic-glycol by acetylation, one may obtain a completely negative permanganate Schiff reaction of ceroid. Omission of acetylation allows vic-glycols to react, omission of bromination allows ethylene to react. Reagent concentrations and times are as in the Casella reaction (pp. 121, 124), and the usual acetylation (p. 161) and bromination (p. 311) were employed.

Cain (Quart. J. Microscop. Sci. 90:411, 1949) and Chu (Anat. Rec. 108:723, 1950) have drawn attention to the fact that certain unsaturated compounds (Chu names oleic, linoleic, and linolenic acids and certain lecithins) may react directly with Schiff reagent to form red to purple complexes. The lecithin Schiff complex is alcohol soluble, but may be made alcohol resistant by posttreatment with bromine or by pre- or posttreatment with picric acid. Prior bromination prevents the reaction. Cain reported blocking of this Schiff reaction by pretreatment with bisulfite or with phenylhydrazine. Chu denied the blocking with aldehyde reagents.

The foregoing statements are apparently based largely on test-tube experiments and do not accord well with the failure of ceroid, retinal acromere lipoid, and certain lipofuscin pigments to give direct Schiff reactions from paraffin sections, though they do give positive Schiff reactions after oxidation with performic or peracetic acid and this reaction is blocked by prior bromination.
Freshly distilled allyl alcohol (CH₂=CH—CH₂OH) gives an immediate positive reaction with Schiff reagent when mixed in equal-volume mixture at a final concentration of 10–50% allyl alcohol. About 1/2000 of this amount of formaldehyde (50 µg. per cc.) suffices to give a reaction of the same intensity in the same time. Hence the ethylene reaction with Schiff reagent is far too faint for detection under histochemical conditions when normal short exposures are used. But when allyl alcohol–Schiff reagent mixtures are allowed to stand in air for 24 hours, strong red colors develop at considerably higher dilutions and these are not decolorized by passing SO₂ through the mixtures.

Consistently with these findings, the peracetic Schiff-positive substances hair cortex, ceroid, retinal-rod acromere lipid, and ovarian lipofuscin assume definite red-purple colors on 72-hour immersion in Schiff reagent. With hair cortex a fair red color is evident even in two hours. The next two take six hours to develop an appreciable pink, and ovarian pigment requires 18 hours. By 30 hours the last three have developed a light purplish red; the ten-minute reactions are negative. All these reactions are blocked by bromination: that of hair only partially and irregularly but at times completely; of the rest, completely. They are much weakened by 24-hour exposure to 5% phenylhydrazine hydrochloride or molar anilin chloride.

The reaction of allyl alcohol with Schiff reagent appears to be retarded somewhat by sulfite treatment; more, by semicarbazide, and still more by phenylhydrazine; but after an hour or so the color becomes about the same as in untreated allyl alcohol samples of the same dilution.

Cain (J. Anat. 84:196, 1950) states that dinitrophenylhydrazine may react with ethylene groups as well as with carbonyl groups; although almost in the same breath he notes the ease of oxidation of unsaturated lipoids in air to “hydroperoxides.” It seems probable that the hydrazine reactions observed are due to the aldehydes resulting from rearrangement (and cleavage) of the peroxides rather than to the ethylenic linkages per se.

However, if allyl alcohol is mixed with phenylhydrazine in stoichiometric proportions and allowed to stand for 24 hours, the usual bromine consumption of the unsaturated alcohol is reduced by about 95 to 100%.

It must be noted, however, that if care is taken to prepare ceroid liver sections from previously unsectioned paraffin blocks, and to use them immediately for the 72-hour Schiff reaction, only a faint to quite light coloration is developed, and that this is completely preventable by even two-hour immersion in 5% phenylhydrazine solution. Paraffin sections which have been kept for some time after sectioning develop a much stronger Schiff reaction on prolonged exposure. They will also give a positive Winkler-Schultze reaction (p. 228) under relatively anaerobic conditions, as Sehrt and Lison have described for oxidized unsaturated fats in vitro. It may be noted that this positive Winkler-Schultze indophenol synthesis is considerably enhanced, or even engendered anew, in ovarian lipoids by prior two-
hour peracetic acid oxidation. This tends to confirm the theory that peracetic oxidation of ethylenes to aldehydes goes through a peroxide stage rather than through epoxide or 1,2-glycol.

Prolonged phenylhydrazine treatment (three days, 5%) prevents the 72-hour Schiff reaction, but not always the peracetic acid Schiff reaction. Bromination does not always prevent the 72-hour Schiff, but does prevent the peracetic Schiff. It would appear that if peroxides are already present (perhaps by atmospheric oxidation of sections still in paraffin) the bromination does not interfere with the subsequent Schiff reaction; but if a considerable proportion of the lipoid pigment remains unperoxidized, phenylhydrazine does not and bromination does prevent its peracetic acid Schiff reaction.

**Carbonyl Lipoids**

This class probably includes ketosteroids, as well as plasmalogens and various ill-defined readily soluble lipoids which give aldehyde and ketone reactions. Verne derives them from ethylenic oxidation products. Since ethylenic linkages readily oxidize in air to epoxides and peroxides which hydrolyze respectively to 1,2-glycols and to aldehydes, this derivation is not improbable.

Cain (Quart. J. Microscop. Sci. 90:411, 1949) considers this latter group distinct from true plasmalogens, which become Schiff positive promptly on treatment with mercuric chloride solutions, while ethylenic derivatives oxidize slowly to aldehydes, and the ethylenic linkages themselves react slowly with Schiff reagent.

The lipoids demonstrated by Liang (p. 419) in axis cylinders of nerves are believed by Chu (Anat. Rec. 108:723, 1950) to be unsaturated fatty acid lecithin esters, which he says react directly with Schiff reagent. In view of the slow reaction of ethylenic groups observed by Cain and by us, it seems more probable that Chu was dealing with intermediate peroxidation products of the unsaturated fat.

Seligman considers that his hydroxynaphthoic acid hydrazide method demonstrates ketones as well as aldehydes, and gives two supplemental tests for the distinction of the latter.

The following carbonyl method of Seligman and Ashbel (Cancer 4:579, 1951) has been altered slightly to accord with the report of Herman and Dempsey (Stain Technol. 26:185, 1951) with certain variations of our own, and with Seligman and Ashbel (Endocrinol. 50:338, 1952).

**The Seligman-Ashbel Method for Active Carbonyl Groups.**

1. Fix in 10% formalin, cut frozen sections at 10-20 μ, float onto slides, and dry for ten minutes to make sections adhere.
2. Wash in water for two hours to remove formaldehyde.
3. Stain controls by oil red O as usual (p. 303). Perform solvent tests on experimen
tal material and bring sections back to water. Omit this step in plain demonstration technic.

4. Immerse extracted and unextracted sections for two hours at 25° C. in a fresh 0.1% solution of 2-hydroxy-3-naphthoic acid hydrazide.* (Dissolve 40 mg. in 2 cc. hot glacial acetic acid, add 38 cc. freshly prepared 50% alcohol.)

5. Wash in four changes each of 5% acetic in 50% alcohol, of 50% alcohol, and of distilled water, of two minutes each (total 12 washes, 24 minutes). Or (Seligman and Ashbel, 1952) six washes of 50% alcohol, 20 minutes each; and one of 30 minutes in 0.5 N hydrochloric acid; followed by rinses in water and in 1% sodium bicarbonate.

6. Mix 25 cc. of M/15 pH 7.4 phosphate buffer with 25 cc. of 100% alcohol. Introduce slides, add 50 mg. dry tetrazotized di-o-anisidine, and let stand two minutes. Or dissolve 50 mg. Black BS Salt or Blue BNS Salt (NAC) in 50 cc. 0.1% disodium phosphate (Na₂HPO₄), pour at once over slides in a Coplin jar, and let stand two minutes. The last mixtures have pH levels of 7.5-7.8.

7. Wash in 0.1% acetic acid and in water.

8. Counterstain in 0.1% safranin in 0.1% acetic acid for 1–2 minutes, if desired. Wash in water.

9. Mount in glycerol gelatin, Arloc gelatin, or the like; or dehydrate quickly with alcohol, clear with xylene, and mount in polystyrene or other synthetic resin.

Results: Active carbonyl in dark blue or greenish blue. Myelin and ceroid from frozen sections are stained.

Plasmalogens

Acetal phosphatides or plasmalogens are composed of the colamine ester of glycerophosphoric acid and the aldehyde of a fatty acid which is combined with the two free hydroxyl groups of the glyceryl moiety of the ester. This combination may be broken liberating the aldehyde from the colamine glycerophosphate by hydrolysis with mercuric chloride (Karrer). This aldehyde may then be demonstrated with Schiff reagent as in Feulgen’s plasmal reaction, or with a phenylhydrazine as in Bennett’s reaction (Am. J. Anat. 67:151, 1940).

These plasmalogens occur in adrenal cortex, corpus luteum, mammary and preputial glands, myelin sheaths, and fat cells. They also are found in some tissues showing no sudanophilic substances, such as muscle, kidney, liver, thyroid epithelium, prostatic and seminal vesicular epithelium, and elastic fibrils generally. They are readily soluble in alcohol and other fat

* Obtainable from Dajac Laboratories, 3430 West Henderson St., Chicago 18, Ill., thus labelled; Seligman and Ashbel say “3-hydroxy-2-naphthoic.”
solvents. Nuclei react neither to the Feulgen plasmal method nor to the dinitrophenylhydrazine method.

The technics generally used employ Schiff reagent or a hydrazine to couple with the open carbonyl group. The first group includes the technic of Feulgen—the use of frozen sections of unfixed tissue oxidized or hydrolyzed with mercuric chloride to release plasmal from acetal phosphatides (plasmalogens); the technic of Verne—the use of tissue fixed in mercuric chloride or platinum chloride without controls; and the technic of Gerard—in which frozen sections of formaldehyde-fixed tissue are carefully washed in water and then treated with mercuric chloride. Untreated controls are used with the Feulgen and Gerard methods.

The second group includes the methods of Bennett, employing phenylhydrazine or dinitrophenylhydrazine, which form yellow aldehyde complexes; and those of Seligman and Ashbel, in which 3-hydroxy-2-naphthoic acid hydrazide is first reacted with tissue carbonyls and then coupled with a stabilized diazonium salt to yield a colored compound.

For the dinitrophenylhydrazine reaction Albert and Leblond prescribe 48-hour fixation in 10% formalin (neutralized with magnesium carbonate), 24 hours' washing in running water, and frozen sections at 10-15 μ. (1) Extract frozen sections for four hours in 17% alcohol. (2) Soak overnight in saturated 30% alcohol solution of 2,4-dinitrophenylhydrazine 17 cc. plus 0.2 N (1.64%) aqueous sodium acetate 13 cc. (emended; authors stated simply "enough to raise the pH to neutrality," noting a decrease of alcohol content to 17%). (3) Wash 20 minutes in 17% alcohol to remove excess stain. (4) Wash in distilled water and mount in glycerol gelatin. The positive reaction is a yellow color.

For the Feulgen plasmal reaction Albert and Leblond fix, wash, and section as above.

Hayes (Stain Technol. 24:19, 1949) has redefined the plasmal reaction to make it more specific for acetal lipids.

The Plasmal Reaction of Feulgen and Voit, Hayes' 1949 Modification.

1. Cut frozen sections of tissues at 15 μ either unfixed, or fixed in neutral 10% formalin for less than six hours. Or use unfixed smears or impression preparations. (Immerse unfixed tissue in [15%] aqueous gumarabic solution for 5-10 minutes and freeze in a drop of the same to facilitate sectioning; or use Hack's polyethylene glycol method, p. 54.)

2. Wash in several changes of 0.9% sodium chloride solution. (Use distilled water if sections are formalin-fixed.)

3. Place sections in 1% mercuric chloride solution for 2-10 minutes to allow complete penetration. For controls omit this step. Rinse glass section lifters before going into mercury solution.

* Cf. Walpole's buffers, p. 450.
4. Transfer mercury treated and control sections directly to separate small, closed dishes of Schiff reagent and let stand 5–15 minutes. Controls should remain negative. (Discard used Schiff reagent daily.)

5. Wash in three changes of 0.5% NaHSO₃ in 0.05 N HCl, of two minutes each.

6. Wash in several changes of water, float onto slides, and blot down.

7. Counterstain briefly in 0.5% methyl green in 0.5% acetic or in acetic hemalum (0.1% hematoxylin 2% acetic) for two minutes. Wash in water. Blot.

8. Treat successively with 95% alcohol, 100% alcohol, 100% alcohol + xylene (1:1), and two changes of xylene. Blot between changes if necessary. Mount in synthetic resin: polystyrene, Permount, HSR, etc.

Lipoids

Polarized Light. When examined in the dark field produced by crossing the Nicol prisms, neutral fats ordinarily remain dark. Substances forming Lehmann’s “liquid crystals,” such as cholesteryl esters, phosphatides, and cerebrosides, may exhibit the black cross of polarization with luminous quadrants between the arms of the cross filling out a circle. This phenomenon is suppressed if the temperature is above that at which the liquid crystals in question can exist and the globules remain dark. Any fatty substance in solid crystalline form may be luminous under polarized light. Sections showing such crystals should be compared with paraffin sections of the same tissue, where the luminous crystals should be absent if they are fatty in nature.

The use of polarized light has been suggested by Prickett and Stevens (Am. J. Path. 15:241, 1939) as a means of differentiating between normal birefringent myelin and degenerating singly refractile myelin. In practice I have not found this method particularly helpful.

In cutaneous xanthomata, this means of examination reveals more or less numerous, fine, needle-shaped, doubly refractile crystals associated with more plentiful isotropic sudanophilic fat droplets. These tumors often contain iron-positive pigment as well, and the fat may be cholesterol positive.

Cholesterol

Cholesterol is often manifest in necrotic tissue and in the granulomatous tissue replacing it as long rhomboidal crystals which glow under polarized light and are extinguished and light up alternately once in each 90° of rotation of the stage.

The Schultz Method. Cholesterol may be more definitely identified by Schultz’s (Centralbl. allg. Path. u. path. Anat. 35:314, 1924) adaptation to histology of the Liebermann-Burchardt sterol reaction. The technic as modified by Mallory follows:
1. Cut thin (10–15 μ) frozen sections of formalin-fixed tissue.
2. Mordant sections in a closely stoppered bottle for three days (Schmorl: 2–4 days) at 37°C in 2.5% iron alum solution. (Hershberger, in my laboratory, finds that 1–3 minutes in 3% hydrogen peroxide or in 1% sodium iodate will suffice in place of the iron alum.)
3. Rinse in distilled water, float onto slides, and blot dry.
4. Treat with a few drops of acetic sulfuric mixture made as follows: Place 2–5 cc. glacial acetic acid in a small test tube and immerse in ice water. Then add gradually the same volume of concentrated sulfuric acid while the tube is still in the ice water.
5. Cover with a cover glass and examine at once. The preparations may be kept for a few days if sealed with petrolatum.

Results: A blue-green color appears in a few seconds, becoming stronger in the first few minutes and often turning to brown in half an hour. Or the blue-green color may persist as long as 24 hours. Positive controls such as sections of previously tested adrenal cortex should always be used. At least two and better three sections of the material under test should be treated with the acetic sulfuric mixture and examined, before considering the test negative. The presence of glycerol inhibits the test; but if stearic acid is present as well, the inhibition is destroyed. Both cholesterol and cholesteryl esters react.

Windaus's digitonin reaction (Ztschr. physiol. Chem. 65:110, 1910) is a reaction for free sterols. All writers caution about absolute cleanliness of slides and cover slips, since cholesterol is present in sweat, and hence fingerprints give positive reactions. This is particularly important when the reaction is carried out on the slide under a cover glass on fresh or formalin-fixed material. Cowdry recommends a variant of Lison's which seems to avoid this difficulty, thus: Fix in formalin, and cut frozen sections. Immerse sections in a 0.5% solution of digitonin in 50% alcohol in a small covered dish for several hours. Rinse in 50% alcohol. Counterstain part of the sections only, by the usual hematoxylin Sudan IV or oil red O technic. Mount all sections as usual in Apáthy's gum syrup or a glycerol gelatin.

Examine the uncounterstained sections under polarized light with crossed nicols. Needles or rosettes of complex cholesteryl digitonids are formed. In the counterstained preparations the cholesterol compound remains doubly refractile and does not stain, but the cholesteryl ester compound colors with the oil-soluble dye and loses its birefringence.

The digitonin cholesterid crystals are insoluble in cold 95% alcohol, acetone, ether, or water; slightly soluble in hot ethyl alcohol and in methyl alcohol; readily soluble in glacial acetic acid; and very soluble in pyridine and in chloral hydrate.

The Golodetz reaction (Schmorl, Romeis), a brown-red color on treatment of frozen sections for one to two minutes with 2 parts 30% formalin
and 5 parts concentrated sulfuric acid, may be confusing, since a blackish-brown color is given also by various phenols, fats, and oils (Romeis).

Cholesterol is not blackened by osmium tetroxide. It is soluble in acetone, ether, benzene, xylene, and strong alcohol. There is some loss of doubly refractile material from adrenal cortex after treatment with 70% alcohol, more loss with 80%, and complete loss with 95-100%.

Other Lipoid Substances

Fatty substances which resist extraction with alcohol, acetone, benzene, and similar fat solvents and consequently may be demonstrated in paraffin sections with oil-soluble dyes include such substances as ceroid, some lipofuscin pigments, lutein, Ciaccio's lipoids, the Golgi substance, unsaturated fats, and myelins. Some of these require previous chromate treatment to render them resistant to fat solvents. With some, formaldehyde is adequate; and some are resistant to fixation with alcohol chloroform mixtures without previous treatment. For myelin, formaldehyde treatment is essential, and subsequent or simultaneous chromation definitely improves the resistance to fat solvents.

For the cerebroside phrenosin, I find no specific methods. It is soluble in benzene, in pyridine, and in hot alcohol; almost insoluble in ether; and insoluble in water. It is included in Lison's general class of lipins, some of which give the liquid crystal maltese cross under polarized light and of which some are Smith-Dietrich- and Ciaccio-positive. Specific statements are lacking except that Baker finds that it stains gray by his variant of the Smith-Dietrich procedure (pp. 321-322), whereas the phosphatides are black.

Kerasin is composed of lignoceric acid, sphingosine, and a hexose (Morrison and Hack, Am. J. Path. 25:597, 1949). It is soluble in warm methyl and ethyl alcohols, chloroform, and pyridine; and insoluble in cold alcohols, acetone, ether, petroleum ether, and xylene. These authors did not find the spectroscopic absorption peak at 267.5-270 μμ reported by Dworaak and Pesta (Wien. klin. Wchnschr. 52:332, 1939) when purified kerasin was used. Both in frozen-dried preparations and after formalin fixation the Gaucher keraisin gives the periodic acid leucofuchsin reaction (pp. 123-124). Morrison and Hack reported (loc. cit.) this reaction as present in the foam cells of Gaucher's disease, but not in those of the Niemann-Pick syndrome. Unlike experimentally introduced purified keraisin, the Gaucher periodic acid Schiff-positive compound was not extracted by warm methyl alcohol and chloroform, and Uzman (Arch. Path. 51:329, 1951) considers that it is bound as a lipoprotein. In material furnished me by Dr. Morrison this lipoid gave the red-purple Schiff coloration after periodic acid but failed to give a positive Schiff reaction after permanganate or chromic acid oxidation, and did not retain iron hematoxylin in the myelin eosinophil stain (p. 194).

Sphingomyelin—the Niemann-Pick lipoid—contains no hexose and is negative to the periodic acid leucofuchsin reaction.
Ciaccio's method (Anat. Anz. 35:17, 1909) depends on simultaneous formaldehyde and chromate treatment of fresh or formalin fixed tissue, followed by prolonged chromation, dehydration, clearing, and paraffin imbedding. Sections are stained in a supersaturated solution of Sudan III or Sudan IV, counterstained in hemalum, and mounted in gum syrup. This method stains myelin red; Ciaccio positive lipoids orange; and nuclei blue. By introducing an osmium tetroxide step just after chromation, Ciaccio stained neutral fats black.

Simple chromation for 2–4 days after two days' or more fixation in 10% formalin seems as effective as Ciaccio's rather elaborate schedule, and nuclear staining is less impaired with the shorter chromation.

Though this and other variants of the classical Ciaccio procedure using Sudan III and Sudan IV are often quite satisfactory for uncolored insoluble lipoids, in lipid pigments possessing natural brown colors, the orange tingeing of the native color is often difficult to discern with any degree of certainty. With such lipoids it is better to use a blue or dark-green oil-soluble dye. Blue to green colorations are easily distinguished from unmodified yellows and brown.

The following technics, devised by Lillie and Laskey (Bull. Internat. Assn. Med. Museums 32:77, 1951) for the demonstration of the rod acromeres in the retina, work very well on cardiac, ovarian, and other similar lipofuscins and ceroid pigments.

Lillie and Laskey's Modified Ciaccio Procedure with Sudan Black B or Spirit Blue (C. I. No. 689).

1. Fix in calcium acetate formalin or calcium-chloride formalin (pp. 33–34). For those lipoids which require chromate oxidation, soak in 3% potassium bichromate for 4–8 days, changing every two days. Wash out chromate overnight in running water. For many lipoids chromation is unnecessary; and with some, it renders the material useless for other histochemical procedures by oxidizing their reactive groups. Prepare paraffin sections as usual. Deparaffinize and hydrate paraffin sections as usual. Frozen sections may be used for comparison or for special multiple-solubility tests.

2. Hydrolyze 15 minutes at 60° C. in preheated normal hydrochloric acid.

3. Transfer directly to Schiff reagent (p. 156) for ten minutes.

4. Wash for about five minutes in 0.5% sodium metabisulfite, three changes of ½1, 2, and 2 minutes.

5. Wash in running water or in several changes of water.

6a. Sudan black variant. Dilute 20 cc. of a 1% solution of Sudan black B in 99% isopropanol with 20 cc. of a 1% aqueous solution of borax, NaB₄O₇·10H₂O (.026 M). Let stand ten minutes. Filter into a Coplin jar. Stain sections five minutes.
6b. Spirit blue variant. Dilute 20 cc. of a 1% spirit blue (C. I. No. 689, alcohol soluble anilin blue, Lyons blue) in 100% alcohol with 20 cc. 1% aqueous acetic acid solution. Let stand ten minutes. Filter into a Coplin jar. Stain sections five minutes.

7. After either stain, wash five minutes in running water.

8. Mount in Arlex gelatin, glycerol gelatin, or other aqueous mountant.

Results: Nuclei, red purple; lipoids, dark gray-green with the Sudan black variant, blue with the spirit blue variant; brown pigments, definitely altered in color toward green or respectively greenish-black or blue according to the dye used, if lipoid positive; backgrounds, respectively light greenish-gray and pale greenish-blue.

Several batches of slides may be stained successively in the same Coplin jar within the first hour or so after the dilution and filtration of either dye. Fresh dilutions must be made each day, as further precipitation occurs on longer standing.

Longer staining in Sudan black B increases the density of background staining and decreases the contrast. The 50% alcohol solution turns brown in a few days and stains cytoplasm and various other structures gray-green, but fails to stain fats.

Oil red O, similarly supersaturated in 50% alcohol, can be used for a red lipoid stain in the above technic, but requires 1–2 hours for adequate staining. With this a following dilute alum hematoxylin counterstain is used in place of the preceding Feulgen procedure, Steps 1–5.

If the alcoholic Sudan black solution and the aqueous diluent are preheated to 60° C., and then mixed and filtered in the paraffin oven as above, a five-minute stain at 60° C. will color erythrocytes and the granules of eosinophil and neutrophil leukocytes and myelocytes dark gray-green, even after formalin fixation and routine paraffin imbedding.

Thomas (Quart. J. Microscop. Sci. 89:333, 1948) gives a technic basically similar to the Lillie-Laskey, using three-day chromation in saturated aqueous potassium bichromate (12% at 20° C.) before imbedding and a 7–10 minute stain in a saturated solution of Sudan black B in 70% alcohol, a quick rinse in 50% alcohol, a 3–5 minute counterstain in alum carmine, and mounting in Farrant’s glycerol gum arabic (p. 109).

With the Smith-Dietrich procedure (Verhandl. d. deutsch. path. Gesellsch. 14:263, 1910) there is a sequence formalin chromate treatment and an overstaining with ripened acetic hematoxylin followed by differentiation with Weigert’s borax potassium ferricyanide and mounting in fructose syrup. In this procedure frozen sections are made after the formalin fixation, and actually no test of solubility in fat solvents is made. Dietrich states that sections may be carried through alcohol and xylene into balsam, with loss of part of the lipoids. Mallory notes that the procedure may also stain iron, hemoglobin, and blood pigments. Baker (Quart. J. Microscop. Sci. 85:1,
1944) used it as a reagent for demonstration of the Golgi substance, reducing the borax ferricyanide time from 15 hours to 8 hours, as follows:

1. Add 1% calcium chloride to 10% formalin and neutralize it with suspended calcium carbonate. Fix tissue in this for three days.
2. Imbed tissue in 25% gelatin; or if thinner sections are desired, evaporate it 30 hours in a desiccator over anhydrous calcium chloride at 37° C., stopping the evaporation while the gelatin solution is still liquid.
3. Cool it in a refrigerator, cut out the tissue block, and harden it one day in 1% calcium chloride, 1% cadmium chloride, 10% formalin solution.
4. Cut frozen sections at 15μ from the 25% gelatin, or as thin as 5μ if the concentration procedure has been used.
5. Attach sections by floating onto slides previously coated with 2.5% gelatin, drained, and dried.
6. Expose slides to the fumes of concentrated formalin for ten minutes to harden the gelatin, and put back into calcium cadmium formalin until required.
7. Wash the slides to be stained by the Smith-Dietrich procedure for three minutes in water and
8. Place in cold 5% potassium bichromate solution in a Coplin jar and then place the jar in an oven at 60° C. (not 57° C.) for 48 hours.
9. Slides should be lifted out of the solution a few times during the first few hours to get rid of air bubbles.
10. Then take out the jar and allow it to cool.
11. Wash slides in several changes of distilled water, and
12. Stain five hours in a modified Kultschitzky's hematoxylin (hematoxylin 1 Gm., distilled water 98 cc., sodium iodate 0.2 Gm., glacial acetic acid 2 cc.) at 37° C. The hematoxylin forms a resistant black lake with the chromium held by the lipins.
13. Normally one differentiates in Weigert's 1% borax, 2.5% potassium-ferricyanide solution for 15 hours; for Golgi substance, only 8 hours.
14. Then wash slides five minutes in running water and mount in glycerol gelatin.

In vitro tests indicate that kephalin and sphingomyelin are stained black. Lecithin is black in the presence of other Smith-Dietrich-negative lipoids, but not when alone. Galactolipins (cerebrosides) are gray. (J. R. Baker, Quart. J. Microscop. Sci. 85:1, 1944.)


1. Fix six hours in Baker's calcium chloride formalin (p. 33).
2. Transfer for 18 hours to 5% potassium bichromate containing 1% calcium chloride (CaCl₂).
3. Transfer to a second bath of 1% CaCl₂, 5% K₂Cr₂O₇ for 24 hours at 60°C.
4. Wash 6–18 hours in running water.
5. Cut frozen sections at 10µ directly, or after Baker's gelatin imbedding (p. 55). Harden the gelatin block 18 hours in calcium chloride formalin, and wash the gelatin block in running water for 30 minutes before sectioning.
6. The sections are then incubated one hour at 60°C in 1% CaCl₂, 5% K₂Cr₂O₇ solution and washed in several changes of distilled water (five minutes total).
7. Incubate in acid hematein solution for five hours at 37°C. [Acid hematein: Boil 50 mg. hematoxylin with 10 mg. sodium iodate (1 cc. of 1%) in 49 cc. distilled water. Cool, and add 1 cc. glacial acetic acid. Prepare fresh daily.]
8. Rinse in distilled water and leave 18 hours at 37°C. in borax ferrocyanide (250 mg. each of borax [Na₃B₄O₇·10H₂O] and of potassium ferrocyanide K₃Fe(CN)₆ in 100 cc. distilled water).
9. Wash in distilled water (4–5 changes, ten minutes total).
10. Mount in Farrant's or Kaiser's medium (pp. 109–110); or dehydrate, clear and mount in balsam.

Results: phospholipids: lecithin, kephalin, and sphingomyelin—dark blue to blue black. Galactolipids from brain—blue black to pale blue. Gelatin—black or brown. Mucin—dark blue to pale blue or brown.

Baker's Pyridine Extraction Test. Fix 20 hours in dilute Bouin's fluid: Saturated aqueous picric acid 50 cc., formalin (40% formaldehyde) 10 cc., glacial acetic acid 5 cc., water 35 cc. Extract one hour in 70% alcohol, 30 minutes in 50% alcohol, 30 minutes in running water. Dehydrate in two changes of pyridine at 20–25°C, one hour each, and extract for 24 hours in fresh pyridine at 60°C. Wash two hours in running water. Transfer to 1% CaCl₂ 5% K₂Cr₂O₇ at Step 2 of the acid hematein test and proceed as before with that test.

Results: Lecithin, kephalin, sphingomyelin, and galactolipids remain unstained. Mucin, gelatin, chromatins are stained black, blue-black, or dark brown. Erythrocytes stain black both with and without pyridine extraction. Mitochondria and myelin are positive without extraction, negative after pyridine. Nuclei stain after extraction, but not before.

Golgi Substance

Golgi apparatus was first described as an intricate anastomosing network of cytoplasmic strands and was first demonstrated by Golgi in nerve cells in 1898. It is demonstrable by impregnations with silver, with osmium tetroxide, or both. The technics are fickle and often fail, and considerable experimentation may be necessary to obtain optimal results with any given type of ma-
terial. For these reasons its study in routine pathologic material would be a matter of considerable difficulty, especially since it requires separate fixation of special tissue blocks.

Ramón y Cajal’s uranium silver method is recommended by Cowdry for young animals. Thus:

1. Fix 8–24 hours in uranyl nitrate 1 Gm., formalin 15 cc., distilled water 100 cc.
2. Wash quickly in distilled water.
3. Impregnate 1–2 days in 1.5% silver nitrate.
4. Rinse in distilled water.
5. Reduce 12 hours in a freshly prepared solution of hydroquinone 2 Gm., formalin 15 cc., distilled water 100 cc., anhydrous sodium sulfite 150 mg. (This is Ramón y Cajal’s developer.)
6. Wash in distilled water. Dehydrate, clear, and imbed in paraffin, using a short alcohol or acetone schedule.
7. Deparaffinize sections and mount.

Da Fano’s cobalt silver method is of more general application. Thus, from Cowdry:

1. Fix in 1 Gm. cobalt nitrate \( \text{Co(NO}_3\text{)}_2 \cdot 6\text{H}_2\text{O} \), 100 cc. distilled water, 6–15 cc. formalin for 3–18 hours. Embryonic tissues require the smaller amounts of formalin. With ordinary adult tissues use 15 cc. Cartilage and small organs, such as those of mice, are fixed adequately in 3–4 hours; routine tissues, in 6–8 hours; central nervous tissues, in 8–18 hours.
2. Rinse in distilled water.
3. Impregnate 1–2 days in 1.5% silver nitrate (1% for very small, easily permeable fragments, 2% for fatty and central nervous tissues).
4. Rinse in distilled water.
5. Cut blocks thinner than 2 mm. Reduce in fresh Ramón y Cajal’s developer for 12–24 hours (supra).
6. Wash in distilled water 30 minutes. Dehydrate, clear, and imbed in paraffin. For greater permanence, bring sections to water, tone 1–2 hours in 0.1–0.2% gold chloride, wash, and counterstain with alum carmine.

Results: Golgi apparatus, black. This technic is essentially that given by Cowdry.

Elftman’s Aoyama Silver Method Variant for Golgi Substance. Elftman (Anat. Rec. 106:381, 1950 and letter, May 5, 1950) recommends an Aoyama Golgi-body technic for the selective demonstration of Sertoli cells. Fix mouse testis 18–24 hours in neutral 10% formalin (carefully decanted from the excess calcium carbonate) to which is added 1% \( \text{M/23} \) of cadmium chloride \( \text{CdCl}_2 \cdot 2\frac{1}{2}\text{H}_2\text{O} \). Rinse in two changes of distilled water. Immerse for
16 hours in 1.5% silver nitrate. Rinse quickly in two changes of distilled water. Reduce five hours in Ramón y Cajal’s developer (p. 324) or in a similar fluid containing instead of 2 Gm. just 1 Gm. hydroquinone, 15 cc. formalin, 100 cc. distilled water and 150 mg. sodium bisulfite (NaHSO₃). Wash, dehydrate, clear, and infiltrate and imbed in paraffin as usual.

If desired, counterstain by the azure eosin method (p. 118) or by some other similar Romanovsky technic.

Sertoli-cell cytoplasm blackens, as does Golgi material in the spermatogenic-cell series.

Elftman comments that both he and Baker find a five-hour reduction more than ample in this and the DaFano technic (p. 324). Elftman has had poor success in applying this procedure to paraffin sections. Frozen sections might be tried.

Elftman later (Stain Technol. 27:47, 1952) found prior fixation productive of irregularities, and introduced gold toning to confer greater resistance to subsequent histochemical procedures.

**Elftman’s Direct Silver Method for Golgi Substance.**

1. Place small blocks of fresh tissue in 15% formalin containing 2% silver nitrate (pH 4) for two hours. (Adjust with a drop or two of acetic acid if necessary.)
2. Rinse five seconds with 15% formalin or distilled water.
3. Immerse in 15% formalin containing 2% hydroquinone for two hours.
4. Complete fixation by an additional overnight treatment with 15% formalin.
5. Dehydrate with alcohols, clear, and infiltrate and imbed in paraffin and section as usual.
6. Sections may be deparaffinized and mounted at this point for preliminary examination. Otherwise deparaffinize and hydrate as usual.
7. If necessary, reduce density of silver deposit by oxidizing in 0.1% iron alum for a few minutes under microscopic control.
8. Wash in distilled water.
9. Tone in 0.2% acid gold chloride for 10 or 15 minutes.
10. Rinse in distilled water and remove silver chloride in 2% sodium thiosulfate, two minutes.
11. Wash five minutes in running water.
12. Counterstain as desired: e.g., the periodic acid Schiff procedure (p. 123), the allochrome method (p. 357), a Mallory anilin blue variant, the Van Gieson stain, etc.
13. Dehydrate, clear, and mount in synthetic resin.

**Results:** Golgi elements are blackened; but as Elftman cautions, not all that is black is Golgi material. Omission of the gold toning leaves the silver
deposits much more susceptible of oxidative fading, either by subsequently employed histochemical reagents or by the mounting medium.

The Osmium Tetroxide Method. Cowdry recommends especially Ludford's (J. Roy. Microscop. Soc. 46:107, 1926) variants of the osmium tetroxide methods. Thus:

1. Fix thin blocks 18 hours in Mann's osmic sublimate fluid (p. 42).
2. Wash 30 minutes in distilled water.
3. Impregnate three days at 30° C. in 2% osmium tetroxide; or one day in 2% osmium tetroxide at 35° C., one day in 1%, and one day in 0.5%.
4. Wash one day in water at 30° or 35° C.
5. Dehydrate, clear, and imbed in paraffin.
6. Cut sections at 3-5 μ; deparaffinize and mount.

Results: Golgi apparatus, yolk, and fat are blackened. Other structures remain yellow to brown.

By treatment with turpentine the blackening is gradually removed from yolk and fat, perhaps completely in 10-15 minutes, leaving the Golgi apparatus black (Lee, Baker). If mitochondria are not blackened they may be counterstained (Ludford) with Altmann's aniline acid fuchsin, heating to steaming and letting stand for 30 minutes. Then wash in water and differentiate with 100% alcohol. Or one may counterstain nuclei with safranin, crystal violet, or neutral red for a few minutes in dilute (1:1,000?) aqueous solutions. Again dehydrate and differentiate with alcohol.

Baker's Sudan Black. Baker (Quart. J. Microscop. Sci. 90:293, 1949) revised his 1944 method (ibid. 85:1, 1944) cited in my first edition, by introducing a chromation after formaldehyde fixation, less vigorous than that of Ciaccio. The method differs from Ciaccio's in that no fat solvent test is applied. Neutral fats are colored in the same way as Golgi bodies; but Baker now states that the latter do not stain with Sudan IV, reversing his previous position. The technic:

1. Fix blocks 3 mm. or less in thickness for one hour in 10% formalin containing 0.7% sodium chloride and kept over marble chips.
2. Transfer directly to a fresh formalin bichromate mixture containing 88 cc. 2.5% K₂Cr₂O₇, 7 cc. 10% NaCl and 5 cc. neutral formalin (kept over marble chips), and let stand for five hours.
3. Transfer directly to 5% potassium bichromate for 18 hours at room temperature, and then place in 60° C. paraffin oven for 24 hours.
4. Wash six hours in running water.
5. Infiltrate at 37° C. for 18 hours (or any convenient longer period) in 25% gelatin containing 0.2% sodium parahydroxybenzoate.
6. Cool, block, and harden in formalum, a solution of 20 cc. formalin,
4 Gm. potassium alum and 80 cc. water, for 18 hours, or any convenient longer period.

7. Section at 8–10 μ on freezing microtome.

8. Transfer section to 70% alcohol.

9. Stain 2½ minutes (30 seconds to 4 minutes) in a saturated solution of Sudan black. (Boil 0.5 Gm. in 100 cc. 70% alcohol under a reflux condenser for ten minutes, or saturate by shaking at intervals for several days.)

10. Wash five seconds in 70% alcohol; and then one minute in 50% alcohol.

11. Wash in water, sinking section below surface.

12. Counterstain in Mayer’s carmalum for 2–4 minutes.

13. Rinse in distilled water and wash in two large changes of water.


Results: Solid Golgi bodies and outer parts of hollow ones, dark blue; Golgi vacuoles, colorless; cytoplasm, pale gray-blue to colorless; chromatin, red or pink; neutral fats (triglycerides), dark blue.

Characteristics of the Golgi Substance. The Smith-Dietrich procedure, as outlined on pp. 321–322, stains the Golgi substance gray to black if the differentiation procedure is shortened to eight hours.

The Golgi substance is negative to the Windaus and Schultz cholesterol tests (pp. 317–318). It is removed when fresh tissues are fixed in alcohol or other lipid solvents. After fixation in calcium formalin it withstands boiling water (30 minutes), ether (15 hours), 1 N hydrochloric acid at 57° C. (4 hours), boiling 100% alcohol and boiling ether (30 minutes each), or ether alcohol hydrochloric acid at 50° C. for 5 hours; but not 30 parts xylene + 70 parts glacial acetic acid at 57° C. for 15 hours. (The last was devised as a myelin solvent.) It resists the usual paraffin embedding technic, thus resembling myelin after formalin fixation.

Elftman reported at the 1953 meeting of the Histochemical Society that the Golgi substance was peracetic acid Schiff positive (p. 310). In the discussion which followed I brought out that the periodic acid Schiff reaction of the Golgi substance reported by Gersh (Arch. Path. 47:99, 1949) and attributed by him to glycoprotein, and the peracetic-acid Schiff reaction of this substance were probably both due to unsaturated fatty acids and their oxidation products (J. Histochem. & Cytochem. 1:387, 1953).

After formalin fixation the phosphatides kephalin, sphingomyelin, and lecithin likewise resist solution in ether, acetone, or 100% alcohol, but may be extracted by pyridine, by a 30–70 xylene acetic mixture, or by a sequence treatment in saturated aqueous sodium oleate or ricinoleate (24 hours) followed by 30 minutes each in boiling 100% alcohol and boiling ether. Kepha-
lin and lecithin are blackened by osmication and withstand decolorization by turpentine, while sphingomyelin does not.

Hence the Golgi substance probably consists of kephalin, lecithin, or both, at least in the snail spermatocytes investigated by Baker.

Myelin

Procedures apparently allied to the Smith-Dietrich method are the traditional myelin methods. With these methods formalin fixation and subsequent chromate treatment are required, although prolonged fixation with chromate alone may suffice. In one variant, chromation after paraffin imbedding has been shown to be futile. Any lipoid not rendered insoluble by formalin would be removed by the solvents employed in the course of paraffin section preparation, and no further change of lipoid could be effected by the chromate.

After fixation and chromation, tissue may be sectioned frozen, in nitrocellulose or in paraffin. Sections are then stained with oxidized hematoxylin until heavily overstained, and then differentiated until other structures than myelin sheaths are decolorized. Both iron-alum and iron-chloride premixed hematoxylin solutions of the type containing no added acid or excess of iron salt are used. These yield black or blue-black sheaths. Others use alkalinized or acid hydroalcoholic oxidized hematoxylin solutions. Two methods use sequence iron chloride or iron alum technics for staining the myelin with hematoxylin. Decolorizing is done with various acids, iron alum, alkaline ferricyanide solutions, or ferric chloride or perhaps two of them.

Benda's methods employed either a simple hydroalcoholic hematoxylin solution or a neutral alum hematoxylin (Delafeld's or Boehmert's) in sequence after 2.5% iron alum mordanting.

There has been much insistence on naturally aged and ripened alcoholic stock solutions of hematoxylin, but some recognized methods use fresh solutions. With premixed ferric salt technics, either chloride, sulfate, or alum, the ferric salt itself promptly oxidizes the hematoxylin, and I have been unable to discern any advantage in aged as compared with fresh stock solutions of hematoxylin in the premixed type. Since the re-use of hematoxylin solutions is often urged in sequence technics, it seems evident that part of the oxidation of these is consequent on carrying over small amounts of ferric iron into them.

In the lithium carbonate and acetic acid methods it would seem necessary that the hematoxylin solutions be previously oxidized, as no oxidant is included. Nevertheless Mallory prescribed a fresh 0.75% aqueous hematoxylin solution made by heating in the Pal variant. Schmorl specified a hematoxylin solution at least three months old for the lithium-carbonate methods; and Baker, in his use of Kultschitzky's acetic hematoxylin for the Smith-Dietrich procedure, ripened it with sodium iodate (p. 78). Apparently the acetic acid hematoxylin stains myelin and cell nuclei alone, and the functions of the following baths in lithium carbonate solution and in acid are to blue and to
further differentiate the hematoxylin stains. It is to be noted that the lithium-carbonate hematoxylin very promptly removes paraffin sections from the slides, as well as glass marking ink. I have summarized several of the common methods in the accompanying table (p. 330).

**Lillie's Variant of the Weil-Weigert Method.** I have found the following variant of Weil's modification very useful: Material should be fixed two days in 10% formalin or in Orth's fluid and then transferred to 2.5% potassium bichromate to complete a total of four days' chromation. Then dehydrate with graded alcohols, clear in benzene or gasoline, and imbed in paraffin. Bring paraffin sections to 80% alcohol.

1. Transfer to a mixture of equal volumes of 4% iron alum and a 1% alcoholic hematoxylin solution (1–5 days old only) and stain in paraffin oven at 55–60° C. for 40 minutes.
2. Wash in water and decolorize one hour in 0.5% iron alum. Old formalin material will decolorize more promptly, and it may not be possible to obtain satisfactory decolorization of cells and matrix before the myelin also decolorizes. Fresh material may sometimes require as much as 1% iron alum, but usually 0.5% suffices.
3. After the iron alum, wash in water and blue in 1% borax, 2.5% potassium ferricyanide solution for ten minutes.
4. Wash in water, and counterstain five minutes in a 1:1,000 solution in 1% acetic of safranin O (C. I. No. 841) or 6B (C. I. No. 843).
5. Dehydrate, clear, and mount through an acetone, acetone + xylene, xylene sequence in synthetic resin.

**Results:** Blue black myelin, yellow to brown to black erythrocytes, red nuclei and tigroid, pink background.

Material fixed in formalin, alcoholic formalin, or any formalin mixture and imbedded in paraffin without chromation will give fairly good myelin by this method, but the demonstration of fine fibers requires chromation before exposure to lipoid solvents. Strangely enough, chromate fixatives not containing formaldehyde are ineffective in the usual 1–3 day fixation periods. It is to be noted that the older writers required 2–3 weeks' hardening in Müller's fluid, and that Endicott found about three weeks' treatment with chromate necessary to render certain unsaturated fats insoluble and myelin positive in vitro. It is further to be noted that Kaufmann and Lehmann insisted that the chromation in the Smith-Dietrich procedure be done at 60° C. at least. This again follows a formaldehyde fixation.

**References for Myelin Technics.** Weigert's iron chloride hematoxylin method is taken from his article in Ehrlich's *Encyklopädie*, pp. 937–944, and is quoted essentially without change by Mallory. Pal's method I have taken from Weigert's article, from Schmorl, and from Mallory. Kultschitzky's and Wolters' technics are as given by Romeis, Schmorl, and Mallory. Wright's technic is given as included in the 8th edition of Mallory and Wright's *Patho-
### Table 20
**Schedules for Myelin Stains by Various Methods**

<table>
<thead>
<tr>
<th></th>
<th>Weigert</th>
<th>Pal</th>
<th>Kulschitsky</th>
<th>Wolters</th>
<th>Wright</th>
<th>Spielmeyer</th>
<th>Weil</th>
<th>Lillic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fixation</strong></td>
<td>Formalin or Orth 2–3 d.</td>
<td>Formalin 2 d. or Müller 2–3 wk.</td>
<td>Formalin 2 + d.</td>
<td>Formalin 2 d. or Müller 2–3 wk</td>
<td>Formalin 2 + d.</td>
<td>Formalin 2 + d.</td>
<td>Formalin 2 + d.</td>
<td>Formalin or Orth 2 d.</td>
</tr>
<tr>
<td><strong>Washing</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 hr.</td>
<td></td>
</tr>
<tr>
<td><strong>Washing</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd mordant</td>
<td>Weigert 2nd 1 d. 37°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Section method</strong></td>
<td>Graded alcohols to</td>
<td>Graded alcohols to</td>
<td>Graded alcohols to</td>
<td>Frozen sections</td>
<td>Frozen sections</td>
<td>Graded alcohols to paraaffin</td>
<td>Graded alcohols to paraaffin or frozen sections</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cellloid</td>
<td>cellloid</td>
<td>cellloid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hematoxylin method</strong></td>
<td>Combined, Weigert's neutral iron p. 80</td>
<td>Combined, Weigert's lithium p. 332, No. 5</td>
<td>Combined, Kulschitsky's acetic p. 332, No. 8</td>
<td>Combined, Wolters' acetic p. 332, No. 10</td>
<td>Sequence, 5 min. 10% FeCl₃ and Wright's p. 332, No. 11</td>
<td>Sequence, 6 min. 2–5% iron alum and Spielmeyer's p. 332, No. 12</td>
<td>Combined, Weil's iron p. 80</td>
<td>Combined, Lillic-Weil p. 329, Step 1</td>
</tr>
<tr>
<td>Staining time Temperature</td>
<td>24 hr. 15°-20° C.</td>
<td>6-48 hr. 15°-20° C.</td>
<td>12-24 hr. 15°-20° C.</td>
<td>24 hr. &quot;Warm&quot;</td>
<td>30 min. 20° C.</td>
<td>10-24 hr. 20° C.</td>
<td>15 min. 50° C.</td>
<td>40 min. 56° C.</td>
</tr>
<tr>
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<td>---------------</td>
</tr>
<tr>
<td>Washing</td>
<td>30-60 min. tap</td>
<td>Water and 2-3 drops sat. sq. LiSO₄</td>
<td>Müller few sec.</td>
<td>Rinse</td>
<td>Rinse</td>
<td>Rinse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st differentiator</td>
<td>Weigert's borax ferricyanide 15-30 min.</td>
<td>0.25% KMnO₄ 15-20 sec.</td>
<td>Kulchitsky's decolorizer 3-4 changes 4-12 hr.</td>
<td>0.25% KMnO₄ 20-30 sec.</td>
<td>10% FeCl₃ brief</td>
<td>2.5% iron alum micro. control</td>
<td>4% iron alum to gross differentiation</td>
<td>0.5% iron alum 1 hr.</td>
</tr>
<tr>
<td>2nd differentiator</td>
<td>Phl's bleach few sec.</td>
<td>Phl's bleach 30 sec to 3 min.</td>
<td></td>
<td></td>
<td>Weil's borax ferricyanide</td>
<td></td>
<td>Lillie's borax ferricyanide 10 min.</td>
<td></td>
</tr>
<tr>
<td>Washing</td>
<td>24 hr. tap</td>
<td>Thorough</td>
<td>Thorough</td>
<td>Thorough</td>
<td>2 changes dist. 1-2 hr. tap.</td>
<td>Water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Counterstain</td>
<td>(Carmine optional)</td>
<td></td>
<td></td>
<td></td>
<td>Acetic safranin, 5 min., wash</td>
<td>Alcohol, xylene, or carbol-xylene, balsam</td>
<td>Alcohol, acetone-xylene, xylene, clarite</td>
<td></td>
</tr>
<tr>
<td>Mounting procedure</td>
<td>Alcohol, aniline-xylene or carbol-xylene, balsam</td>
<td>95% alcohol, terpineol, or origanum oil, xylene, balsam</td>
<td>95% alcohol, xylene, balsam</td>
<td>95% alcohol, terpineol, or origanum oil, xylene, balsam</td>
<td>95% alcohol, carbol-xylene or xylene (blot), balsam</td>
<td>95% alcohol, xylene, balsam</td>
<td>Acetone, acetone-xylene, xylene, clarite</td>
<td></td>
</tr>
</tbody>
</table>

Table 21

**FORMULAE OF REAGENTS USED IN VARIOUS MYELIN TECHNICS**

<table>
<thead>
<tr>
<th>No.</th>
<th>Technic</th>
<th>Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Formalin</td>
<td>A 10% dilution in water of 40% formaldehyde solution.</td>
</tr>
<tr>
<td>2.</td>
<td>Weigert’s 1st mordant</td>
<td>Potassium bichromate 5 Gm. Chromium fluoride 2.5 Gm. Distilled water to make 100 cc.</td>
</tr>
<tr>
<td>3.</td>
<td>Weigert’s 2nd mordant</td>
<td>Cupric acetate 5 Gm. Chromium fluoride 2.5 Gm. Glacial acetic acid 5 cc. Distilled water to make 100 cc.</td>
</tr>
<tr>
<td>4.</td>
<td>Weigert’s borax ferricyanide</td>
<td>Borax (Na₂B₄O₇·10H₂O) 2 Gm. Potassium ferricyanide 2.5 Gm. Distilled water to make 100 cc.</td>
</tr>
<tr>
<td>5.</td>
<td>Weigert’s lithium hematoxylin</td>
<td>Hematoxylin 0.75-1.0 Gm. Alcohol 10 cc. Distilled water to make 100 cc. Saturated aqueous lithium carbonate 1-2 cc.</td>
</tr>
<tr>
<td>6.</td>
<td>Müller’s fluid</td>
<td>Potassium bichromate 2.5 Gm. Sodium sulfate crystals 1 Gm. Distilled water to make 100 cc.</td>
</tr>
<tr>
<td>7.</td>
<td>Pal’s sulfite oxalic bleach</td>
<td>Potassium sulfite 0.5 Gm. Oxalic acid 0.5 Gm. Distilled water 100 cc.</td>
</tr>
<tr>
<td>8.</td>
<td>Kulitschitzky’s hematoxylin</td>
<td>10% alcoholic hematoxylin, aged 6 mos. 10 cc. Glacial acetic acid 2 cc. Distilled water to make 100 cc.</td>
</tr>
<tr>
<td>9.</td>
<td>Kulitschitzky’s decolorizer</td>
<td>1% potassium ferricyanide 10 cc. Saturated aqueous lithium carbonate 100 cc.</td>
</tr>
<tr>
<td>10.</td>
<td>Wolter’s hematoxylin</td>
<td>Hematoxylin 2 Gm. Alcohol to dissolve 10-20 cc. Glacial acetic acid 2 cc. Distilled water to make 100 cc.</td>
</tr>
<tr>
<td>11.</td>
<td>Wright’s hematoxylin</td>
<td>Was an extempore solution of a few crystals in 15 cc. distilled water.</td>
</tr>
<tr>
<td>12.</td>
<td>Spielmeyer’s hematoxylin</td>
<td>5% alcoholic hematoxylin (aged) 4 cc. Distilled water 36 cc.</td>
</tr>
<tr>
<td>13.</td>
<td>Weil’s borax ferricyanide</td>
<td>Weigert’s formula 50 cc. Distilled water 50 cc.</td>
</tr>
<tr>
<td>14.</td>
<td>Lillie’s bichromate</td>
<td>Potassium bichromate 5 Gm. Water 100 cc.</td>
</tr>
<tr>
<td>15.</td>
<td>Lillie’s borax ferricyanide</td>
<td>Borax 1 Gm. Potassium ferricyanide 2.5 Gm. Distilled water 100 cc.</td>
</tr>
<tr>
<td>16.</td>
<td>Lillie’s acetic safranin</td>
<td>Safranin O (C. I. No. 841) 100 mg. Glacial acetic acid 1 cc. Distilled water to make 100 cc.</td>
</tr>
</tbody>
</table>

In frozen sections, myelin of peripheral nerves is stained vividly red to red-purple by the periodic acid (pp. 123, 125, 310) and peracetic acid (p. 310) Schiff procedures. These reactions are obtained (though less vividly) in material fixed with formaldehyde and chromated before dehydration and embedding.
While the methods for collagen, reticulum, basement membranes, and elastin are different in some respects, it is profitable to discuss them together, since it is often desirable to stain two types of fibers simultaneously.

Four basic processes exist for the selective demonstration of basement membranes and collagen and reticulum fibers: the silver impregnation from alkaline solution, the staining with acid anilin dyes from strongly acid solution, the phosphotungstic and phosphomolybdic acid hematoxylin methods and the periodic acid leucofuchs in method.

The Silver Methods

The silver methods purport to differentiate between reticulum and collagen fibers. The former are colored black; the latter brown, lavender, and gray in varying shades and tones. These methods are often uncertain in their action. One learns by experience to discern whether or not a given preparation has afforded a reasonably complete demonstration.

On account of the high alkalinity of the silver solutions, paraffin sections are often partially or completely loosened from the slides. To combat this tendency Masson used a gelatin glue which I have found no more uniformly successful in keeping sections on than was Mayer's albumen glycerol. Mallory, however, recommended it highly.

Masson (J. Tech. Methods 12:75, 1929) dissolved 50 mg. gelatin in 25 cc. distilled water and floated out sections on a large drop of this placed on the slide on a warm plate. When the sections were smooth, the excess gelatin water was drained off, and the section was blotted dry with filter paper and placed in a large, closed, moist chamber with formaldehyde vapor at 40-50° C. for several hours to overnight. Shorter periods are said to be adequate for other than alkaline silver methods.

With the Foot silver carbonate method Mallory recommended alcohol as
the final diluent of the silver solution in place of water, to prevent sections from floating off the slides in the alkaline silver solution.

Personally I prefer to use routine paraffin sections which are deparaffinized with xylene and transferred to 100% alcohol. They are then soaked 5–10 minutes in 1% collodion. The collodion is then drained for one minute and the preparations are next immersed in 80% alcohol for five minutes, and then transferred to water.

The Bielschowsky-Maresch method, the Perdrau-Da Fano, one Foot variant, the Wilder method, the Gomori variant, and a new method of ours depend on silver oxide (or hydroxide) dissolved in ammoniacal solution. The Del Rio-Hortega method and Foot's and Laidlaw's variants use ammoniacal solutions of silver carbonate. Except Foot's two technics, which specified Zenker material but work well after formalin fixation, and Laidlaw's, which specified Bouin's fluid as an alternative method, all methods specify formalin fixation. The details of manufacture of the ammoniacal silver solution vary somewhat, but in all except our new method one produces a precipitate from silver nitrate with sodium or potassium hydroxide, with ammonium hydroxide and then sodium hydroxide, or with lithium or sodium carbonate. In all, one then barely dissolves the precipitate with ammonium hydroxide except in Gomori's, in which one back-titrates with more silver nitrate. In all, the re-solution in ammonium hydroxide is done in quite concentrated solution; and, except in Laidlaw's method, the solution is considerably diluted for use.

In the following formulae solutions are expressed in Gm. per 100 cc. of solution, except in the Bielschowsky-Maresch and Del Rio-Hortega formulae, where it is presumed that the continental European custom of expressing solutions in percentage by weight was followed. Thus the 10% silver nitrate solution with a specific gravity of 1.088 contained 10.88 Gm. per 100 cc. in Bielschowsky's laboratory, while according to American custom 100 cc. of 10% silver nitrate solution contains 10 Gm.

**Maresch's Bielschowsky Silver Solution** *(Zentralbl. f. allg. Path. u. path. Anat. 16:641, 1905).* Add 5 drops of 40% sodium hydroxide (4 Gm. NaOH, 6 Gm. H₂O) (this would be about 0.3 cc. and would contain about 172 mg. NaOH, the equivalent of 731 mg. AgNO₃) to 10 cc. 10% (10 Gm. AgNO₃, 90 Gm. H₂O) silver-nitrate solution (1088 mg. AgNO₃). The brown precipitate is then redissolved by constant shaking while adding 28% ammonia water drop by drop (perhaps 1 cc.). It is best to leave the last few granules undissolved, as an excess of ammonia inhibits the impregnation. Dilute to 25 cc. with distilled water for use. Use once.


**Perdrau** *(J. Path. & Bact. 24:117, 1921)* used Da Fano's Silver Solution which I have cited from **Bailey and Hiller** *(J. Nerv. & Ment. Dis. 59:337,*
1924). Add 2 drops of 40% sodium hydroxide (40 Gm. NaOH in 100 cc. solution) to 5 cc. of 20% (20 Gm. in 100 cc. solution) silver nitrate and 28% ammonia drop by drop (about 1.1 cc.) with constant shaking until precipitate is almost or just dissolved. Dilute to 50 cc. Use once.

Foot's Silver Oxide Solution (J. Lab. & Clin. Med. 9:777, 1924). Add 20 drops of 40% sodium hydroxide to 20 cc. 10% silver nitrate. Dissolve the brown precipitate by adding strong (28%) ammonia water drop by drop with constant shaking until only a few granules remain. About 2 cc. (theoretically 1.7 cc.) is required. Dilute to 80 cc. with distilled water. Use once. Assuming 17 drops per cc. for the sodium hydroxide solution, an exact equivalent of the silver nitrate is used in this variant.

Wilder's Silver Oxide Solution (Am. J. Path. 11:817, 1935). Add 28% ammonia water drop by drop (about 0.5–0.6 cc.) to 5 cc. 10.2% silver nitrate until the precipitate is dissolved. Then add 5 cc. 3.1% sodium hydroxide and redissolve the precipitate with a few drops of ammonia water. Make up to 50 cc. with distilled water.

Gomori's Silver Oxide Solution (Am. J. Path. 13:993, 1937). To 20 cc. 10% silver nitrate add 4 cc. (3.3–5 according to Gomori) 10% potassium hydroxide. (This amount precipitates 1–1.5 Gm. of the total 2 Gm. silver nitrate as oxide.) Then add 28% ammonia water drop by drop to dissolve the precipitate completely. Then again add 10% silver nitrate drop by drop until the precipitate formed dissolves easily on shaking. Dilute the solution with an equal volume of distilled water. It can be kept stoppered for two days.

The end point in the addition of silver nitrate is not clear in the above directions, which are as Gomori originally published them, and as they were copied by Mallory. Gomori writes me (1945) that he adds silver until the precipitate dissolves only on vigorous shaking or even to faint permanent opalescence. In the last case he again adds 1 drop of about 2% ammonia water to clear.

Lillie's Silver Oxide Method (Stain Tech. 21:69, 1946). Place one volume of 28% ammonia water in a small flask and add rapidly 7 to 8 volumes of 10% silver nitrate solution, shaking the flask constantly. Then cautiously add more silver nitrate, shaking until the brown precipitate completely dissolves between each addition. Continue the addition of silver nitrate until a faint permanent opalescence is produced. This takes a total of 9–10 volumes of silver nitrate solution. Dilute the faintly opalescent solution with an equal volume of distilled water. Use the solution by placing 2 cc. on each slide, and discard. The solution is good for 1–2 days after mixing. For other purposes I now make this solution directly with 5% silver nitrate, using about 38 cc. to reach the faint-turbidity point with 2 cc. ammonia. No further dilution is made.

Krajian employed the silver solution produced by adding concentrated ammonia water to 10% silver nitrate solution until the precipitate is almost
completely dissolved. This should yield essentially the same mixture as the preceding 10% silver solution.

**The Del Rio-Hortega Silver Carbonate Solution** (Cited from Romeis).

To 5 cc. 10% silver nitrate add 15 cc. 5% sodium carbonate. Then carefully add (28%) ammonia water drop by drop (about 0.4 cc.) with constant shaking until the precipitate is just dissolved. Then add 55 cc. distilled water. The solution may be kept a long time in brown glass.

**Foot and Menard’s Silver Carbonate Solution** (*Arch. Path.* 4:211, 1927).

To 10 cc. 10% silver nitrate add 10 cc. saturated (about 1.3%) aqueous lithium carbonate solution. (The solubility of lithium carbonate is less in hot water than in cold; do not heat to saturate.) Wash the precipitate three times with distilled water by decantation. Add 25 cc. distilled water and then add 28% ammonia water drop by drop (about 0.8 cc.) until the precipitate is almost dissolved, shaking vigorously the while. Make up to 100 cc. with distilled water. Mallory modified this by making the final dilution to 100 cc. with 95% alcohol instead of water and adding a few more drops of ammonia water to dissolve the resultant precipitate.


Dissolve 12 Gm. silver nitrate in 20 cc. distilled water in a 250 cc. graduate. Add 230 cc. saturated (1.33%) aqueous lithium carbonate solution. Shake well and let precipitate settle to the 70 cc. mark. Carefully decant supernatant fluid, refill with distilled water, shake, and again let settle to 70 cc. and decant, repeating to a total of three or four washes. Again let settle to 70 cc., decant, and add 28% ammonia water with constant shaking until almost clear (about 9.5 cc.). Dilute to total volume of 120 cc., filter through a Whatman 42 or 44 filter, and store in stock bottle. This solution may be kept for months and may be filtered and re-used a dozen times or more.

**A modification of Foot’s silver carbonate solution** used for years in this laboratory is prepared thus: To 5 cc. 10% silver nitrate add 5 cc. saturated (16–20%) aqueous sodium carbonate. Let settle and wash five times with 35–40 cc. portions of distilled water by decantation. Then add 15 cc. distilled water and 28% ammonia water drop by drop (about 0.4 cc.) with constant shaking until precipitate is almost dissolved. Dilute to 50 cc. with distilled water.

The relative virtues of sodium, potassium, and ammonium hydroxides as precipitants are difficult to evaluate, and all solutions should contain diammine silver, hydroxyl, nitrate, and ammonium ions as well as the potassium or sodium ions that may have been added. The alkalinity is controlled with fair precision by limitation of the excess ammonia to the amount just sufficient to form the diammine silver radical.

The use of lithium or sodium carbonate in the Foot, the Laidlaw, and our variants of the Del Rio-Hortega method seems to be a matter of indifference, since all three of these methods carefully wash the silver carbonate free of soluble salts before forming the diammine compound.
General Considerations of the Technics. The technics follow a quite uniform general pattern, with quite pronounced variations in duration of individual steps and in concentration of some of the reagents.

The iodine-sodium thiosulfate sequence in the two Foot technics is to be regarded simply as the usual treatment for mercury-fixed tissues, and I have always omitted it with the carbonate method on formalin-fixed material. The function of this iodine-thiosulfate treatment in Laidlaw’s variant is not clear, but Laidlaw seems to have regarded it as an integral part of the Mallory bleach. The thiosulfate in any case should be thoroughly washed out before proceeding with the next step.

Weigert’s Bleach. The sequence treatment with potassium permanganate and oxalic acid, or a substitute for it, appears in all the variants after the Bielschowsky-Maresch and the Del Río-Hortega. Its introduction into diamine silver methods is due to Perdrau (J. Path. & Bact. 24:117, 1921) and appears to be purely pragmatic. Omission of the permanganate step results in failures of impregnation, and omission of the oxalic acid step causes irregularities of impregnation, so that both excellent impregnations and failures are obtained on similar material. Perdrau, Foot, Laidlaw, and Wilder prescribed 0.25% potassium permanganate for 10, 5, 3, and 1 minute respectively, Gomori 0.5–1.0% for 2–1 minute, and I have used 0.5, 0.33, and 0.25% solution for 2–5 minutes. In any case the sections are colored fairly deep brown, and the reaction is probably completed in a few seconds. Wilder’s substitution of a one-minute 10% phosphomolybdic acid treatment probably serves the same purpose, as the hexavalent molybdenum of the molybdic acid is easily reduced to a lower valence. The washing after this step serves to remove the excess reagent. The reducing agent in Foot’s methods, and Laidlaw’s and our variants has been 5% oxalic acid, varying in time thus: 15–30 minutes in Foot’s oxide method, 10 minutes in his and our carbonate methods, 3 minutes in Laidlaw’s, and 2 minutes in our oxide method. Actually the brown permanganate stain disappears in a few seconds in this reagent, and what benefit may inure from further exposure is difficult to discern. Gomori used a 1–3% potassium metabisulfite (K₂S₂O₅, which hydrates to KHSO₅ in water) solution for one minute and Perdrau used Pal’s 0.5% oxalic acid, 0.5% acid potassium sulfite (KHSO₅) solution until the sections were white. Wilder prescribed a one minute bath in 1⁄4 dilution of concentrated hydrobromic acid to follow the permanganate step and stated that this could be omitted if phosphomolybdic acid was substituted for permanganate. The hydrobromic acid removes the brown permanganate stain instantly. Couteiro and Freire oxidize 40 minutes in 4% chromic acid solution, Gridley uses a 15-minute bath in 0.5% aqueous periodic acid (H₃IO₆). The latter should lead to greater constancy of results, since periodic acid carries oxidation of 1,2-glycols and 1,2-hydroxyl-amines only to the aldehyde stage, while permanganate and chromic acid both produce aldehyde and destroy it.
<table>
<thead>
<tr>
<th></th>
<th>Marches</th>
<th>Perdras</th>
<th>Fout</th>
<th>Wilfer</th>
<th>Gomorri</th>
<th>Lilliee</th>
<th>Gridley</th>
<th>Kranen</th>
<th>Cucircte &amp; Freite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fixation</strong></td>
<td>Formalin or alcohol</td>
<td>Formalin</td>
<td>Zenker or formalin</td>
<td>Formalin, Zenker, or Helly</td>
<td>Formalin</td>
<td>Formalin or Orth</td>
<td>Formalin, Zenker, Bouin, alcohol, Carnoy</td>
<td>Formalin (?)</td>
<td>Formalin, alc. form., Helly, Orth, Carnoy, Gendre</td>
</tr>
<tr>
<td><strong>Section method</strong></td>
<td>Wash several hours, cut frozen sections</td>
<td>Wash 1 d. tap, 1 d. dist. water, Frozen or paraffin sect.</td>
<td>Paraffin sections</td>
<td>Paraffin or loosened or mounted frozen or paraffin sections</td>
<td>Paraffin sections</td>
<td>Paraffin sect. well dried</td>
<td>Frozen sect. 7-10 μ</td>
<td>Frozen (12-15 μ) or paraffin 6 μ</td>
<td></td>
</tr>
<tr>
<td><strong>Iodine</strong></td>
<td>0.5% alc., 5 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Only for Zenker</td>
<td></td>
</tr>
<tr>
<td><strong>Wash</strong></td>
<td>Water</td>
<td>Tap water</td>
<td>Tap water</td>
<td>Tap water</td>
<td>Tap and dist. water</td>
<td>Distilled water</td>
<td>Dist. water 3-5 min., 2-3 ch.</td>
<td>Dist. water 3-5 min., 4-5 ch.</td>
<td></td>
</tr>
<tr>
<td><strong>Oxidant</strong></td>
<td>0.25% KMnO₄ 10 min.</td>
<td>0.25% KMnO₄ 5 min.</td>
<td>0.25% KMnO₄ 1 min. or 10% phosphomolybdate acid 1 min.</td>
<td>0.5-10% KMnO₄ 1-2 min.</td>
<td>0.5% KMnO₄ 2 min.</td>
<td>0.5% H₂O₂ 15 min.</td>
<td>15 cc 0.37% KMnO₄ + 5 cc 28% NH₄OH 5 min.</td>
<td>45% CrO₃, 50 min.</td>
<td></td>
</tr>
<tr>
<td><strong>Wash</strong></td>
<td>Dist. water</td>
<td>Tap water</td>
<td>Rinse tap</td>
<td>Tap water</td>
<td>Distilled water</td>
<td>Dist. water, few sec.</td>
<td>Dist. water 3-5 min., 4-5 ch.</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td><strong>Reducer</strong></td>
<td>Pal's bleach (p. 239) until white</td>
<td>5% oxalic acid 15-30 min.</td>
<td>10% HBr 1 min. (see p. 237) or none</td>
<td>1-3% K₂Cr₂O₇ 1 min.</td>
<td>5% oxalic acid 2 min.</td>
<td>None</td>
<td>2% eosine to decolorize</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td><strong>Wash</strong></td>
<td>Dist. water</td>
<td>Dist. water 5 min.</td>
<td>Dist. water</td>
<td>Tap, several min.</td>
<td>Tap water</td>
<td>2 changes dist. water</td>
<td>Dist. water</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td><strong>Sensitizer</strong></td>
<td>2% AgNO₃ 24 hr.</td>
<td>2% AgNO₃ 24 hr.</td>
<td>2% AgNO₃ 48 hr.</td>
<td>1% uranyl nitrate 5 sec.</td>
<td>2% iron alum 1 min.</td>
<td>1/50 hq. ferr. chlor. 2 min. or 37% H₂O₂ 2 min</td>
<td>2% AgNO₃ 30 min.</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Wash</td>
<td>Dist. water less than 5 min.</td>
<td>Dist. water, brief 1-3 sec</td>
<td>Dist. water, 2 ch.</td>
<td>Dist. water, 2 ch.</td>
<td>Top, 3 min.</td>
<td>Top, 3 min.</td>
<td>Top, 3 min.</td>
<td>Top, 3 min.</td>
<td>Top, 3 min.</td>
</tr>
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<td>-------------</td>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Sheet</td>
<td>2 min, 25°C, 5 min, 23°C</td>
<td>30 min, 25°C, 5 min, 23°C</td>
<td>5 min, 25°C, 5 min, 23°C</td>
<td>5 min, 25°C, 5 min, 23°C</td>
<td>5 min, 25°C, 5 min, 23°C</td>
<td>5 min, 25°C, 5 min, 23°C</td>
<td>5 min, 25°C, 5 min, 23°C</td>
<td>5 min, 25°C, 5 min, 23°C</td>
<td>5 min, 25°C, 5 min, 23°C</td>
</tr>
<tr>
<td>Developer</td>
<td>Dist. water quick 3 min.</td>
<td>Dist. water quick 3 min.</td>
<td>Dist. water quick 3 min.</td>
<td>Dist. water quick 3 min.</td>
<td>Dist. water quick 3 min.</td>
<td>Dist. water quick 3 min.</td>
<td>Dist. water quick 3 min.</td>
<td>Dist. water quick 3 min.</td>
<td>Dist. water quick 3 min.</td>
</tr>
<tr>
<td>Gold chloride</td>
<td>1/3% formula, 30 min.</td>
<td>1/3% formula, 30 min.</td>
<td>1/3% formula, 30 min.</td>
<td>1/3% formula, 30 min.</td>
<td>1/3% formula, 30 min.</td>
<td>1/3% formula, 30 min.</td>
<td>1/3% formula, 30 min.</td>
<td>1/3% formula, 30 min.</td>
<td>1/3% formula, 30 min.</td>
</tr>
<tr>
<td>Sodium thiosulfate</td>
<td>5% water, 15-40 sec.</td>
<td>5% water, 15-40 sec.</td>
<td>5% water, 15-40 sec.</td>
<td>5% water, 15-40 sec.</td>
<td>5% water, 15-40 sec.</td>
<td>5% water, 15-40 sec.</td>
<td>5% water, 15-40 sec.</td>
<td>5% water, 15-40 sec.</td>
<td>5% water, 15-40 sec.</td>
</tr>
<tr>
<td>Mounting</td>
<td>Alcohol, glycerin, benzol</td>
<td>Alcohol, glycerin, benzol</td>
<td>Alcohol, glycerin, benzol</td>
<td>Alcohol, glycerin, benzol</td>
<td>Alcohol, glycerin, benzol</td>
<td>Alcohol, glycerin, benzol</td>
<td>Alcohol, glycerin, benzol</td>
<td>Alcohol, glycerin, benzol</td>
<td>Alcohol, glycerin, benzol</td>
</tr>
</tbody>
</table>

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Table 23
SCHEDULES FOR DIAMMINE SILVER-CARBONATE RETICULUM METHODS

<table>
<thead>
<tr>
<th></th>
<th>del Rio-Hortega</th>
<th>Foot-Menard</th>
<th>Laidlaw</th>
<th>Lillie</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fixation</strong></td>
<td>Formalin</td>
<td>Formalin or Zenker</td>
<td>Bouin or Formalin</td>
<td>Formalin</td>
</tr>
<tr>
<td><strong>Section method</strong></td>
<td>Frozen sections</td>
<td>Paraffin sections</td>
<td>Paraffin sections, Wash Bouin 20 min., formalin 5 min.</td>
<td>Paraffin sections collodionized</td>
</tr>
<tr>
<td><strong>Iodine</strong></td>
<td>0.5% alc., 3 min.</td>
<td>1% alc., 3 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Wash</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Thiosulfate</strong></td>
<td>0.5% aq., 5 min.</td>
<td>5% aq., 3 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Wash</strong></td>
<td>Tap water</td>
<td>Tap water</td>
<td>Tap water</td>
<td></td>
</tr>
<tr>
<td><strong>Oxidant</strong></td>
<td>0.25% KMnO₄, 5 min.</td>
<td>0.25% KMnO₄, 5 min.</td>
<td>0.25% KMnO₄, 5 min.</td>
<td></td>
</tr>
<tr>
<td><strong>Wash</strong></td>
<td></td>
<td></td>
<td>Tap water</td>
<td></td>
</tr>
<tr>
<td><strong>Reducer</strong></td>
<td>5% oxalic acid 10 min.</td>
<td>5% oxalic acid 3 min.</td>
<td>5% oxalic acid 10 min.</td>
<td></td>
</tr>
<tr>
<td><strong>Wash</strong></td>
<td>Tap water</td>
<td>Tap water, 10 min.</td>
<td>Tap water</td>
<td></td>
</tr>
<tr>
<td><strong>“Sensitizer”</strong></td>
<td>Dist. water</td>
<td>Dist. water</td>
<td>Dist. water</td>
<td>Dist. water</td>
</tr>
<tr>
<td><strong>Silver</strong></td>
<td>1-2 min. 45-50° C. p. 336</td>
<td>10-15 min. 37° C. p. 336</td>
<td>5 min., 50° C. p. 336</td>
<td>5 min., 60°-70° C, until golden brown p. 335</td>
</tr>
<tr>
<td><strong>Wash</strong></td>
<td>Dist. water, rinse</td>
<td>Dist. water, rinse</td>
<td>Dist. water, rinse</td>
<td>Dist. water, rinse</td>
</tr>
<tr>
<td><strong>Developer</strong></td>
<td>1% formalin until yellow</td>
<td>20% formalin 5 min.</td>
<td>1% formalin several changes 3 min.</td>
<td>20% formalin 2 min.</td>
</tr>
<tr>
<td><strong>Wash</strong></td>
<td>Thorough, tap</td>
<td>Dist. water, rinse</td>
<td>Tap water</td>
<td></td>
</tr>
<tr>
<td><strong>Gold Chloride</strong></td>
<td>0.2%, 30 sec.</td>
<td>0.2%, 5 min.</td>
<td>0.2%, 10 min.</td>
<td>0.2%, 2 min.</td>
</tr>
<tr>
<td><strong>Wash</strong></td>
<td>Tap water</td>
<td>Dist. water</td>
<td>Tap water</td>
<td></td>
</tr>
<tr>
<td><strong>Sodium Thiosulfate</strong></td>
<td>5%, brief</td>
<td>5%, 2 min.</td>
<td>5%, 10 min. several changes</td>
<td>5%, 2 min.</td>
</tr>
<tr>
<td><strong>Wash</strong></td>
<td>Tap water</td>
<td>Tap water</td>
<td>Tap water</td>
<td></td>
</tr>
<tr>
<td><strong>Counterstain</strong></td>
<td>Van Gieson</td>
<td>Hematoxylin Van Gieson</td>
<td>As desired</td>
<td>Hematoxylin Van Gieson</td>
</tr>
<tr>
<td><strong>Mounting</strong></td>
<td>95% alcohol, carbol-creosote-xylene, xylene, balsam</td>
<td>Alcohols, xylene, balsam</td>
<td>Alcohols, xylene, balsam</td>
<td>95% alcohol, acetone, acetone-xylene, xylene, salicylic balsam</td>
</tr>
</tbody>
</table>

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Sensitizers. Maresch employed a 24 hour bath in silver nitrate solution, with no antecedent oxidation. Wilder substituted 1% uranyl nitrate for five seconds. Gomori prescribed one minute in 2% iron alum as preferable to several other metal-salt impregnations tried by him. I have substituted a two-minute bath in a 1:50 dilution of U.S.P. ferric chloride solution as equivalent, and have found a number of oxidant solutions also successful: 3% H$_2$O$_2$, 1% NaIO$_3$, 1% iodine, or a 1% acetic acid, 5% potassium bichromate mixture. After Orth fixation this sensitizing step is better omitted. Since ferric chloride treatment also sensitizes connective-tissue fibrils so that they more or less specifically reduce the methenamine silver of the argentaffin cell method (p. 166) it would appear that there may be actual metal-organic compounds formed, in which the silver later replaces the metal. Aldehyde sites do not appear to reduce ammine or methenamine silver complexes selectively, perhaps because the aldehyde is bound with amino groups under alkaline conditions.

However, aldehyde sites, engendered by permanganate or periodic acid, may well operate to initiate silver deposition from the 2% silver nitrate of the Maresch, Perdrau-Da Fano, Foot, and Gridley methods and thus afford nuclei for further deposit in the alkaline ammine silver solutions. Gomori found that the same effect could be achieved in ten minutes with 10% silver nitrate, and noted that thorough washing in distilled water after sensitization was preferable to the brief rinses prescribed by the Bielschowsky-Maresch and the Foot silver-oxide technics. Bailey and Hiller washed not over five minutes with distilled water, using the Perdrau-Da Fano method.

Sensitizing agents have not been commonly employed in diammine silver carbonate technics. All technics prescribe washing with distilled water before the silver bath.

Silver Bath. Times in the silver oxide baths vary thus: one minute at room temperature prescribed by Gomori and Wilder, the 2–30 minutes' interval of the Bielschowsky-Maresch, Foot's 30 minutes, and Perdrau's 40–60. These vary in concentration in grams of silver nitrate used to make 100 cc. of solution thus: 4 Gm. for the Bielschowsky-Maresch; 4–5 Gm. for our oxide method and the Gomori; 2.5 Gm. for the Foot; 2 Gm. for the Perdrau-Da Fano; and 1 Gm. for the Wilder. With carbonate methods concentrations are 10 Gm. for Laidlaw's; 1 Gm. for Foot's and ours; and ½ Gm. for Del Río-Hortega's; and times and temperatures are respectively five minutes at 50° C., 10–15 minutes at 37° C., 3–5 minutes at 60–70° C. and 1–2 minutes at 45–50° C. Sections turn yellow to brown during silvering by the carbonate methods and in the original Bielschowsky-Maresch oxide technic. With the other oxide methods they remain unchanged in color until developed. Though little correlation is seen among time, temperature, and concentration, nevertheless, when the same variant is employed, increase in temperature accelerates the process, and variation of dilution alters the time requirement in the expected direction.
Most technics prescribe a quick rinse of 1-10 seconds in distilled water between the silver bath and the reduction bath. Wilder used alcohol, and Del Río-Hortega carried sections direct from his weak silver bath to weak formalin.

**Reduction.** All methods use formaldehyde in concentrations varying from a 30% dilution of the concentrated 37-40% formaldehyde down to 1%. Gridley used 30% formalin for three minutes; Couceiro and Freire the same for five minutes; Bailey and Hiller 20% formalin for 30 minutes for the Da Fano technic used by Perdrau; Maresch for 5-30 minutes; Foot’s carbonate method for five minutes; ours for two minutes; Foot’s oxide method used two changes of 5% over a 30-minute period; Gomori required 10-20% for three minutes; Laidlaw 1% for three minutes; Del Río- Hortega 1% until yellow; and Wilder a 0.03% uranyl nitrate, 1% formalin solution for one minute. Actually reduction occurs visibly in the first few seconds after adding formalin, and no further darkening is evident after 30 seconds or so. I have used a two-minute period in 10% formalin for the oxide variant, and in 20% formalin for the carbonate.

Washing after the formalin is usually in tap water, though Wilder and Laidlaw prescribe distilled water.

**Toning.** All technics use a toning in yellow auric chloride (which is 1:500) until violet according to Bailey and Hiller in Perdrau’s method, one minute in Wilder’s method, 30 seconds in Del Río-Hortega’s, five minutes in Foot’s carbonate method, ten minutes in Laidlaw’s, two minutes in our carbonate and oxide variants. Foot’s silver oxide method used 1% for one hour, while the Bielschowsky-Maresch technic called for 5 drops of 1% solution with 1 drop of glacial acetic acid in 10 cc. distilled water until the sections turned violet, and Gomori used either 1:500 or 1:1,000 for ten minutes. Couceiro and Freire used 0.1% for three or four minutes, controlling microscopically; and Gridley employed 0.5% for five minutes and insisted that golden brown tones be replaced by pale yellowish gray (taupe) or lavender. Actually the sections change color in a few seconds and probably even Wilder’s one minute interval is longer than necessary.

Following gold toning usually a brief wash or rinse in tap or distilled water is recommended. Gomori prescribes a second reduction with 1-3% potassium metabisulfite for one minute, followed by another wash.

**Fixing.** Then all methods remove unreduced silver by means of sodium thiosulfate. Gomori prescribes 1-2% for one minute, the rest use 5% for 15-60 seconds (Bielschowsky-Maresch), brief (Del Río-Hortega), 1-2 minutes in Wilder’s, two minutes in Perdrau’s and Foot’s two cited methods and our carbonate and oxide variants, and Couceiro and Freire’s method, five minutes in Gridley’s technic, and ten minutes with Laidlaw’s strong silver carbonate method.

**Counterstains, etc.** Thorough washing follows in all technics and then counterstains are inserted by Foot, Wilder, Del Río-Hortega, and our car-
bonate and oxide variants. Usually a brief hemalum stain, tap-water bluing, and a 45–60 second Van Gieson technic are recommended, and have been quite successful in our hands. If Gomori's iron alum sensitizer or our iron chloride bath is used before the silver oxide bath, an iron hematoxylin effect is produced. This I find too dense and prefer to omit the hematoxylin with this variant. I have had very interesting results by following the reticulum method with a Ziehl-Neelsen acid-fast stain.

Alcohol dehydration, clearing with xylene, carbol xylene, or carbol creosote xylene and inclusion in balsam are recommended. With our collodion film technics we find it advisable to complete the dehydration with acetone (thus removing the collodion and any precipitate which may have formed on it) and then pass through acetone and xylene into two or three changes of xylene.

The following reticulum technic is based on Wilder's, Gomori's, and our variants of the Bielschowsky-Maresch method.

Place 1 cc. 28% ammonia water in a small flask. Add 10% silver nitrate, the first 7 or 8 cc. fairly rapidly, the rest of the 9–10 cc. total cautiously, shaking between each addition to clear the brown clouds of silver oxide until a faint permanent opalescence remains. Then add an equal volume of distilled water. This diammine silver hydroxide solution can be used for one or two days, and is discarded after using once.

The procedure of impregnation is as follows:

1. Fixation in formalin or Orth's fluid. Paraffin sections through two changes of xylene and two of 100% alcohol into
2. 1% collodion in ether and 100% alcohol (equal volumes) for 5–10 minutes.
3. Drain one minute.
4. 80% alcohol five minutes.
5. Rinse in tap water.
6. 0.5% potassium permanganate two minutes.
7. Wash in water.
8. 5% oxalic acid two minutes.
9. Wash in water.
10. Wilder's 1% uranyl nitrate 5–10 seconds, or Gomori's 2% iron alum one minute, or liquor ferri chloridi 1:50 in distilled water two minutes, or 3% hydrogen peroxide two minutes. With Orth-fixed material omit this step.
11. Wash three minutes in running water and then rinse in two changes of distilled water. Wilder omitted this wash, but it does not interfere even after uranyl nitrate.
12. Lay slides face up on glass rods over a large pan and deposit on each about 1.5–2 cc. of the diammine silver hydroxide. Let stand three minutes, and decant and
13. Rinse quickly in distilled water.
14. Reduce two minutes in 10% formalin.
15. Wash three minutes in running water.
16. Tone two minutes in 0.2% acid gold chloride (HAuCl₄).
17. Rinse in tap water.
18. Fix two minutes in 5% sodium thiosulfate.
19. Wash in tap water.
20. Counterstain as desired. For example, acetic alum hematoxylin (p. 76) two minutes, tap water two minutes, Van Gieson’s picrofuchsin (p. 346) one minute. Differentiate in two or three changes of 95% alcohol. Or stain by Ziehl-Neelsen for acid-fast organisms (p. 380). Or stain five minutes in 0.1% safranin, thionin, or toluidin blue, differentiate one minute in 5% acetic acid, wash well in tap water.
21. Complete dehydration and decollodionization in three changes of acetone, clear with one change of acetone and xylene (50:50) and two or more changes of xylene. Mount in clarify or other synthetic resin.

The Hematoxylin Methods

Mallory’s phosphotungstic acid hematoxylin stains nuclei, centrioles, spindles, mitochondria, fibrin, fibrils of neuroglia, and the so-called myoglia and fibroglia and the contractile elements of striated muscle blue; collagen, reticulum, elastin, cartilage, and bone matrix are yellowish to brownish red.

For staining of mitochondria Mallory prescribed (p. 185) a prolonged oxidation with ferric chloride before dehydration of the formalin-fixed blocks, and prolonged the staining interval. For other elements the following general technic is prescribed. The solution is the same in either case and is prepared by dissolving 1 Gm. hematoxylin and 20 Gm. phosphotungstic acid in 1,000 cc. distilled water. This ripens in several weeks and the naturally ripened product is thought to be best. However, Mallory states that the addition of 177 mg. potassium permanganate will ripen it at once.

Zenker fixation was prescribed by Mallory. Earle has told me that he has had satisfactory staining of material fixed in buffered formalin, without subsequent mercury treatment. Peers (Arch. Path. 32:446, 1941) stated that sections of formalin-fixed material should be brought to water as usual and then mordanted three hours at 57° C. (paraffin-oven temperature) in saturated aqueous mercuric chloride solution. Sections thus mordanted are then treated just as though Zenker fixation had been employed, thus:

1. Iodine in 95% alcohol (0.5%) five minutes.
2. 0.5% sodium thiosulfate five minutes (or 5% for one minute).
3. Wash in tap water.
4. 0.25% potassium permanganate five minutes.
5. Wash in water.
6. 5% oxalic acid five minutes (Mallory 10–20 minutes).
7. Wash in running water 1–2 minutes.
8. Stain in phosphotungstic acid hematoxylin overnight (12–24 hours).
9. Dehydrate rapidly in 95% and 100% alcohol or in acetone, clear with a 50% mixture of the dehydrating agent and xylene, then two changes of xylene. Mount in balsam or clarite.

Lieb (Arch. Path. 45:559, 1948) introduces mordanting in ferric ammonium alum after Step 7 thus: Rinse in distilled water, mordant one hour in 4% iron alum, rinse in tap water and in distilled water and stain in the hematoxylin solution 2–24 hours. The color of normally blue staining elements is intensified by this treatment.

Linder (Quart. J. Microscop. Sci. 90:427, 1949) has described a hematoxylin method for collagen, reticulum, and capillary basement membranes. It appears to be limited in its usefulness to material fixed with mercuric chloride fixatives. The hematoxylin solution requires ten weeks’ ripening to stain basement membranes well, though collagen and reticulum are stained by the fresh solution. The lake retains its staining potency up to at least seven months. I have had no experience with this method.

Thomas’s phosphomolybdic acid hematoxylin was made by Linder as follows.

Dissolve 2.5 Gm. hematoxylin in 49 cc. dioxan and 1 cc. aerated water. Dissolve 16.5 Gm. phosphomolybdic acid in 44 cc. distilled water and 11 cc. ethylene glycol or glycerol, and filter. Mix equal volumes of the two solutions and ripen the mixture at (British) room temperatures for ten weeks. The similarity of the solution to Mallory’s phosphotungstic-acid hematoxylin is noteworthy.

The Linder-Thomas Phosphomolybdic Acid Hematoxylin Technic.

1. Fix in 9 volumes saturated aqueous mercuric chloride and 1 volume commercial formalin.
2. Imbed in paraffin by usual technics, section at 3μ, deparaffinize and hydrate as usual.
3. Treat with Lugol’s iodine solution (I:KI:H₂O = 1:2:100) for one minute.
4. 5% sodium thiosulfate one minute.
5. Wash in distilled water.
6. Barely cover section with phosphomolybdic acid hematoxylin and stain for ten minutes.
7. Wash with distilled water.
8. Dehydrate, clear and mount.

Results: Collagen, reticulin, and basement membranes of pulmonary capillaries, deep violet; red corpuscles, purple; nuclei, blue but lightly
stained; cytoplasm, bluish. A counterstain with 0.02% acriflavin in 1% acetic acid for 30 seconds gives a greenish background that contrasts well.

The Acid Anilin Dye Methods

The general subject of collagen staining with acid anilin dyes I have reviewed (J. Tech. Methods 25:1, 1945) and the reader may find many details and methods there which I have not found space for in this account.

The selective collagen stains with acid anilin dyes apparently depend on the selectivity of collagen for certain acid dyestuffs from fairly strongly acid solutions. With one group of methods a mixture of anilin blue, methyl blue, indigocarmine, acid fuchsin, or other dyestuffs is made in appropriate proportion with picric acid, which acts both as the acidifying agent and as a counterstain for muscle, cytoplasm, and other materials. In another group the counterstain precedes the fiber stain and the latter is mixed with or preceded by an acid such as phosphotungstic, phosphomolybdic, or picric acid, or even hydrochloric.

The first group are the simplest in application, and are widely used also as general stains.

Picroindigocarmine was the first differential connective-tissue stain reported (by Jullien in 1872). In Ramón y Cajal's (Rev. Cien. Med. Barcel. 22:97, 1896; Histology, 1933) modification it is still widely used. In this technic one first stains nuclei red with carmine, then washes in water and stains 5-10 minutes with a solution of 250 mg. (originally 333 mg.) indigocarmine (C. I. No. 1180) in 100 cc. saturated (1.2%) aqueous picric acid solution. Rinse in weak (perhaps 0.5%) acetic acid and dehydrate and differentiate in absolute alcohol. Connective tissue is blue green, muscle greenish yellow, nuclei red.

An iron hematoxylin stain, such as Weigert's (p. 81), for 3–6 minutes can be substituted for the carmine, and a sequence of 95% and absolute alcohol is just as satisfactory for differentiation and dehydration. This method on the whole is less selective and less complete for fine fibers than its methyl blue or acid fuchsin counterparts.

Van Gieson's mixture consists of acid fuchsin (C. I. No. 692) and picric acid. (See J. Tech. Methods 25:1, 1945, for literature.) The usual proportion is 5 cc. 1% acid fuchsin (50 mg. of dye) to 95 cc. saturated aqueous picric acid solution. The proportion of acid fuchsin can profitably be raised to 100 mg. per 100 cc. picric acid solution (Weigert). Addition of 0.25 cc. concentrated hydrochloric acid to the last mixture sharpens the differentiation, so that muscle is purer yellow and collagen deeper red. Ünna's variant, containing 250 mg. acid fuchsin, 1.5 Gm. nitric acid (the P. G. 1884 was 30%, sp. gr. 1.185, and the 1.5 Gm. would equal 0.5 cc. of present-day concentrated [70%] acid, sp. gr. 1.42), 10 cc. glycerol, 90 cc. water and picric acid to saturation, gives deep crimson collagen and bright yellow muscle.

The technic: Bring sections to water as usual.
1. Stain five minutes in Weigert's acid iron chloride or other similar hematoxylin (p. 81).
2. Wash in water.
3. Stain five minutes in the picrofuchsin mixture.
4. Dehydrate and differentiate with two or three changes each of 95% and 100% alcohol.
5. Clear with a mixture of 100% alcohol and xylene followed by two or three changes of pure xylene. Mount in polystyrene.

Picro-methyl blue mixtures were used by Dubreuil, Curtis, Ohmori, and others. I find that 100 mg. methyl blue (C. I. No. 706) or anilin blue (C. I. No. 707) per 100 cc. saturated aqueous picric acid solution is a suitable proportion (J. Tech. Methods 25:1, 1945).

1. Stain first with iron hematoxylin (Weigert's acid iron chloride) for five minutes.
2. Wash with water.
3. Stain in picro-methyl blue or picro-anilin blue for five minutes.
4. Differentiate briefly in 1% acetic acid or directly in 95% alcohol.
5. Then 100% alcohol, 100% alcohol and xylene, two changes of xylene, and mount in polystyrene.

Results: Connective tissue, including much reticulum, mucosal, and renal glomerular basement membranes, is deep blue; muscle and cytoplasms, varying shades of yellowish green to gray; mucus, pale blue; nuclei, black.

Violamine R (C. I. No. 759) is a good substitute for acid fuchsin in the Van Gieson methods, yielding a similar color picture, with less tendency to fade than with acid fuchsin. Naphthol blue black (C. I. No. 246) can be used in place of methyl blue, giving a very precise dark bluish-green color to collagen. It is one of the most selective and precise collagen stains that I have encountered. For the best contrasts, use 20-40 mg. of this dye to the 100 cc. of saturated picric acid solution.

Masson's Van Gieson variant (J. Tech. Methods 12:75, 1929) introduced a plasma stain with 2 cc. saturated aqueous metanil yellow (C. I. No. 138) diluted to 100 cc. in 1% acetic acid between the iron hematoxylin and the picrofuchsin. Thus:

1. Stain nuclei black with Regaud's iron hematoxylin (p. 79).
2. Wash.
3. Differentiate in ½-saturated alcoholic picric acid.
4. Wash 15 minutes in running water.
5. Stain five minutes in the acetic metanil yellow.
6. Rinse in distilled water.
7. Mordant five minutes in 3% potassium bichromate.
8. Stain 30-60 seconds in 1% acid fuchsin in saturated aqueous picric acid.
9. Rinse quickly in distilled water.
10. Differentiate five minutes in 1% acetic acid.

I see no particular advantage to this method over an acidified Van Gieson stain such as Unna's, in which there are fewer steps.

**Biebrich Scarlet-Picro-Anilin Blue.** In 1940 I reported a similar method (Arch. Path. 29:705, 1940).

1. Weigert's acid iron chloride hematoxylin for five minutes in place of Regaud's.
2. Wash.
3. 0.2% Biebrich scarlet (C. I. No. 280) in 1% acetic acid for four minutes in place of the metanil yellow.
4. Wash, omitting the chromation, and
5. Stain for 4-5 minutes in 0.1% anilin blue in saturated aqueous picric acid, instead of in picro-acid fuchsin, and
6. Transfer directly to 1% acetic for three minutes.
7. Dehydrate, clear, and mount in polystyrene.

**Results:** Connective tissue was stained blue, including renal glomerular stroma, basement membranes, and much reticulum; erythrocytes, orange scarlet; muscle, pink; cytoplasm, pink to gray; nuclei, gray to black; mucus, light blue.

Further experimentation with this method corrected some of its defects and apparently increased its completeness of staining connective tissue (J. Tech. Methods 25:1, 1945), thus:

**Picro-Naphthol Blue Black.** Bring sections to 80% alcohol as usual.

1. Stain 6 minutes in acid iron hematoxylin (Weigert's or similar formula).
2. Wash in tap water.
3. Stain five minutes in: brilliant purpurin R (C. I. No. 454) 0.6 Gm., azofuchsin G (C. I. No. 153) 0.4 Gm., glacial acetic acid 1 cc., and distilled water to make 100 cc.
4. Rinse in water.
5. Stain 1-5 minutes in: naphthol blue black (C. I. No. 246) 50-100 mg., saturated aqueous picric acid 100 cc.
6. Differentiate directly in 1% acetic acid for two minutes.
7. Dehydrate, clear and mount by an alcohol, alcohol xylene, xylene, Clarite sequence.

**Results:** Dark green collagen, reticulum, basement membranes, and renal glomerular stroma; pale greenish blue mucus; brown to greenish brown epithelial cytoplasm; light brown muscle; red erythrocytes.
Since with fresh samples of brilliant purpurin R and azofuchsin G it may be necessary to adjust the proportions so as to maintain the brown-red balance between muscle and erythrocytes, it is well to prepare these two dyes in 1% solutions in 1% acetic acid and mix them in 6:4 proportion for use. This permits of ready variation to increase or decrease the red. Anilin blue or methyl blue at the same concentration can be used in place of naphthol blue black with only slightly inferior results, and somewhat better demonstration of mucus.

Fast Green-Van Gieson. A similar method giving red connective tissue, gray-green cytoplasm and muscle, green erythrocytes, and brown nuclei is done thus (J. Tech. Methods 25:1, 1945).

Technic: Bring sections to water as usual.

1. Stain six minutes in an acid alum hematoxylin (p. 76).
2. Wash in tap water.
3. Stain four minutes in 0.1% aqueous fast green FCF in 1% acetic acid.
4. Wash in 1% acetic.
5. Stain ten-fifteen minutes in 0.2% acid fuchsin in saturated aqueous picric acid.
6. Wash two minutes in 1% acetic acid.
7. Dehydrate, clear, and mount through alcohols, alcohol and xylene, xylene to polystyrene.

Results: Nuclei brown; collagen, reticulum, and basement membranes, deep purplish red; ocular-lens capsule and Descemet's membrane, a deeper red purple; muscle, grayish or yellowish green after formalin fixatives and a deeper and purer green with nonformalin fixatives; cytoplasm, ellipsoids of retina, and red corpuscles, green; Paneth-cell granules, sometimes red; hypophyseal alpha granules, light green; beta granules, brownish red; adrenal cortex cells, green; medulla cells, pink to gray pink except after chromate fixatives when they are a darker and grayer green than cortex cells. Mucus is almost unstained.

In Step 3 I have substituted 0.3% wool green S (C. I. No. 737) with good results. The greens are bluer. In Step 4 0.2% violamine R (C. I. No. 758) or 0.1-0.5% ponceau S (C. I. No. 282) may be substituted for the acid fuchsin with good results.

The Phosphomolybdic and Phosphotungstic Acid Methods

These include technics in which the acid and the fiber stain are used in sequence as in the Mallory 1900 method, the Heidenhain and Masson variants; and those in which the acid and the fiber stain are used together, as in the 1905 and 1936 Mallory methods. Often Zenker, Helly, Regaud, Bouin, or other mercurial or picric acid fixations are prescribed for these methods, and if the material has been fixed with formalin, mordanting in Zenker's or
Bouin's fluid or in saturated mercuric chloride or picric acid solution is prescribed, either of blocks of unimbedded tissue or of sections before staining. I have found saturated mercuric chloride in water and saturated picric acid in alcohol acceptable means of accomplishing this end. The effect of these fixations and aftertreatments is to enhance the staining of cytoplasm and to render connective tissue somewhat more difficult to stain fully.

While there has been considerable dispute both as to the relative efficiency of phosphomolybdic and phosphotungstic acids and as to the proper concentration and exposure time, it seems to make little difference whether one uses one, the other, a combination of both, or neither, so long as sections are treated with acid before and during the fiber stain. I have used picric acid, ferric chloride, and other reagents successfully in place of phosphomolybdic acid (J. Tech. Methods 25:1, 1945).

In the neighborhood of 50 variants of the Mallory technics have been published, and the tendency of recent years seems to have been in the direction of greater complexity of method and introduction of more visually controlled differentiations. While I do not question the efficacy of such methods in the hands of those who have had long experience with them, they are difficult of execution for the new or occasional user and require a familiarity with the results to be attained which is not general among other than specially trained technicians.

Of the group, the most widely used have been Mallory's 1905 and 1936 variants, Heidenhain's 1916 method, and Masson's 1928-1929 methods.


1. Zenker fixation.
2. Usual iodine and thiosulfate sequence.
3. Wash in water.
4. Stain five minutes in 0.5% acid fuchsin.
5. Drain and transfer to: anilin blue (C. I. No. 707) 0.5 Gm., orange G (C. I. No. 27) 2.0 Gm., phosphomolybdic acid 1 Gm., distilled water 100 cc. Stain 10–20 minutes.
6. Dehydrate and differentiate in several changes of 95% alcohol, then 100% alcohol, xylene and balsam.

**Mallory's 1936 Method** (Stain Technol. 11:101, 1936). As above, but use 1 Gm. phosphotungstic acid in place of the phosphomolybdic in the anilin blue stain. Stain with this for 20–60 minutes or longer. In 1936 Mallory recommended 0.25% acid fuchsin for 30 minutes, but in his 1938 text he reverted to a 1–5 minute stain in 0.5%, as in the 1905 technic.

**Results:** With both Mallory's variants collagen fibrils are deep blue; cartilage and bone matrix, mucus, amyloid and some hyaline materials, varying lighter shades of blue; fibrin, nuclei, glia fibrils, "fibroglia and myoglia" and axis cylinders, red; erythrocytes and myelin, yellow.
Heidenhain's "Azan" variant (Ztschr. wiss. Mikroskop. 32:361, 1916) gave red nuclei and erythrocytes, orange muscle, reddish glia fibrils, blue mucin, and dark blue collagen and reticulum, including glomerular stroma.

The technic: Fix in Zenker, Helly, Bouin, or Carnoy.

1. Stain 30–60 minutes in a covered dish at 50–55° C. and then 1–2 hours at 37° C. in: 0.25–1.0 Gm. azocarmine B (C. I. No. 829) in 100 cc. cold water and 1 cc. glacial acetic acid; or 100 cc. water saturated by boiling with 0.1 Gm. azocarmine G (C. I. No. 828), cooled and acidified with 1 cc. glacial acetic acid.
2. Wash in distilled water.
3. McGregor (Am. J. Path. 5:545, 1929) inserts the step of differentiating in 0.1% aniline in 95% alcohol and rinsing in 1% acetic acid in 95% alcohol.
4. Heidenhain then mordants 30 minutes to 3 hours in 5% phosphotungstic acid.
5. Rinse in distilled water.
6. Stain 1–3 hours in a 50–33% (Mallory: 25%) dilution of a stock solution of anilin blue 0.5 Gm., orange G 2 Gm., glacial acetic acid 8 cc., distilled water 100 cc.
7. Rinse in water.
8. Dehydrate and differentiate in 95% alcohol; then 100% alcohol, xylene and balsam as usual.

I have not used this method, regarding a 3–9 hour technic as too cumbersome for frequent use, and having found some of the picric acid and hydrochloric acid methyl blue methods fully effective. The periodic acid leucofuchsirn technic (pp. 123, 357) is suggested for kidney and lung.

Masson's Trichrome Stain (J. Tech. Methods 12:75, 1929) also followed a sequence procedure. Thus: Fix in Bouin's or Möller's (Regaud's) fluid or premordant formalin or alcohol material with Bouin's or Möller's fluids respectively. Paraffin sections are fastened to slides with Masson's gelatin (p. 333).

1. Stain with Regaud's iron hematoxylin (p. 79) and
2. Differentiate to a pure nuclear stain in % saturated alcoholic picric acid solution.
3. Wash 15 minutes in running water (three minutes is adequate to remove the picric acid).
4. Stain five minutes in 2 parts 1% "xylidene ponceau" (ponceau 2R, C. I. No. 79) and 1 part 1% acid fuchsirn (C. I. No. 692), both in 1% acetic acid.
5. Rinse in distilled water.
6. Mordant five minutes in 1% aqueous phosphomolybdic acid.
7. Drain and stain five minutes in 2.5% anilin blue (C. I. No. 707) in 2.5% acetic acid or in 2% light green SF (C. I. No. 670) in 1% acetic acid.

8. Return anilin blue stains to the phosphomolybdic acid for another five-minute period. (I find this step unnecessary.)

9. Take the anilin blue stains from the phosphomolybdic acid and the light-green stains directly from the stain and differentiate two minutes in 1% acetic acid. Dehydrate, clear, and mount by an alcohol, xylene, balsam sequence. Masson recommends Curtis's salicylic acid balsam or natural acid balsam. I find xylene Clarite better. Polystyrene should serve well.

Results are similar to those of other Mallory stains, depending on the colors of the plasma and collagen stains used.

Other writers have substituted the simpler Weigert's acid iron chloride hematoxylin (p. 81) for Regaud's. Fast green FCF and wool green S (C. I. No. 737) have been found (Lillie, J. Tech. Methods 25:1, 1945) to be good substitutes for light green, giving green and blue-green collagen respectively and staining mucus in paler tones of the same colors. Biebrich scarlet (C. I. No. 280), Bordeaux red (C. I. No. 88), chromotrope 2R (C. I. No. 29) in the reds, brilliant purpurin R (C. I. No. 454) as a brown are good substitutes for the ponceau 2R and acid fuchsin mixture. Saturated alcoholic picric acid and saturated aqueous mercuric chloride are good premordants for formalin material.

In the past decade or so there have appeared a considerable number of single-solution methods giving the general color effects of the Mallory anilin blue connective-tissue stain. Several of these have been applied mainly to the staining of smears of desquamated epithelia and notably in the study of the variations of vaginal contents during the oestrus cycle, in the diagnosis of uterine cervical carcinoma and the like. Some are applicable also to sections of paraffin- and celloidin-imbedded material.

In the Papanicolaou (J. Lab. Clin. Med. 26:1200, 1941; Science 95:438, 1942; J. Nat. Cancer Inst. 7:357, 1947) technics for staining smears for cancer diagnosis, ether alcohol (50:50 or 1:2) or isopropyl alcohol fixation (two minutes) is usually recommended. Smears are then brought through descending alcohols to water and stained 5-10 minutes in acid or alum hematoxylin; washed, blued and dehydrated through ascending alcohols; counterstained 30-100 seconds in a phosphotungstic acid orange G solution in alcohol (OG-5, 6, or 8); washed in three changes of alcohol; stained in an alcoholic solution of light green SF, Bismarck brown, and eosin Y for ½ minutes (designated as EA 25, 31, 36, 50, or 65); and washed three times in 95% alcohol; then 100% alcohol, alcohol xylene, two changes of xylene, and balsam.

Some of these technics are presented in quite elaborate detail. Gates and Warren (Handbook for Diagnosis of Cancer of the Uterus, etc., Cambridge, Harvard University Press, 1947), for example, prescribe dipping five times into each of the descending and ascending graded alcohols.
<table>
<thead>
<tr>
<th>Light green SP (C. I. No. 670)</th>
<th>0.5% in 95% alc.</th>
<th>0.5% in 95% alc.</th>
<th>0.5% in 95% alc.</th>
<th>Phosphotungstic acid crystals</th>
<th>sat. aq. (ca. 1%) sol.</th>
<th>Lithium carbonate</th>
<th>drop</th>
</tr>
</thead>
<tbody>
<tr>
<td>EA25</td>
<td>44</td>
<td>45</td>
<td>45</td>
<td>170</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>EA31</td>
<td>50</td>
<td>45</td>
<td>45</td>
<td>170</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>EA436</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>200</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>EA50</td>
<td>45(0.24%)</td>
<td>cc</td>
<td>cc</td>
<td>200</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 24: Composition of Parancilau EA Solutions
The orange G solutions are 0.5% in 95% alcohol and contain respectively (for OG-5, 6 and 8) 25, 15, and 10 mg. phosphotungstic acid per 100 cc.

The composition of the EA solutions is tabulated on p. 353.

The last two solutions are mentioned quite casually. EA50 appears to have been a commercial designation of EA36, and Papanicolaou refers to the use of EA36 or EA50 in one of his technics without making it clear whether they were the same or different. EA65 is mentioned as containing half as much light green (Gradwohl) as EA36. Papanicolaou (ca. 1947) also speaks of diluting EA36 with an equal volume of 95% alcohol, with increase in transparency of the preparations.

The Shorr (Science 94:545, 1941) stains are also used principally for smears. Shorr also fixed in ether alcohol for two minutes; then stained one minute in “S-3,” washed (ten dips each!) in 70%, 95%, and 100% alcohol, cleared in xylene, and mounted. Foot (in Pathology in Surgery, Philadelphia, J. B. Lippincott Co., 1945, p. 11) brought smears (or sections) to water, stained in alum hematoxylin, washed, blued and counterstained five minutes in his modified Shorr stain, and carried through ascending alcohols to xylene and balsam or Clarite.

<table>
<thead>
<tr>
<th>Table 25</th>
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<tbody>
<tr>
<td><strong>Composition of Shorr Stains</strong></td>
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<tr>
<td></td>
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<tr>
<td><strong>Shorr 1941</strong></td>
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<tr>
<td><strong>Foot 1945</strong></td>
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</tr>
<tr>
<td>Biebrich scarlet C. I. No. 280</td>
</tr>
<tr>
<td>Anilin blue WS C. I. No. 707</td>
</tr>
<tr>
<td>Orange G C. I. No. 27</td>
</tr>
<tr>
<td>Fast green FCF</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
</tr>
<tr>
<td>Phosphotungstic acid</td>
</tr>
<tr>
<td>Phosphomolybdic acid</td>
</tr>
<tr>
<td>Alcohol</td>
</tr>
<tr>
<td>Water</td>
</tr>
</tbody>
</table>

The Cason stain (Stain Technol. 25:225, 1950) is recommended for staining connective tissue in paraffin sections. Cason dissolves successively in 200 cc. distilled water, 1 Gm. phosphotungstic acid, then 2 Gm. orange G, 1 Gm. anilin blue and finally 3 Gm. acid fuchsin.

Paraffin sections are deparaffinized and hydrated as usual, removing mercury with the iodine thiosulfate sequence if necessary, stained five minutes, dehydrated through graded alcohols, cleared in xylene and mounted in balsam. Mendelson (Stain Technol. 26:1951) modifies the method for celloidin sections as follows: Hydrate as usual, stain 15–20 seconds only, and rinse quickly in distilled water. Decolorize until celloidin is colorless in 3 cc. aniline,
12 cc. chloroform, and 85 cc. 95% alcohol; rinse in 7:1 95% alcohol-chloroform mixture, clear in terpineol-xylene (1:3) and two changes of xylene, and mount.

A similar single-solution stain giving muscle and cytoplasm in pink; with blue collagen, reticulum, and hypophyseal \( \beta \)-granules is our buffered Mann stain (p. 191).

Gomori \((\text{Am. J. Clin. Path.} 20:661, 1950)\) finds that one-solution trichrome mixtures can be made from blue or green acid triphenylmethylene or diphenyltriphenylmethane dyes as collagen stains with red sulfonated azo or disazo dyes as plasma stains, and acetic and phosphotungstic or phosphomolybdic acids. Phosphotungstic acid tends to intensify the plasma stain; phosphomolybdic, the fiber stain; and alcohol weakens the plasma stain. Pretreatment with hot Bouin’s fluid intensifies staining of muscle and plasma. Water washing extracts plasma stains more than fiber stains, whereas acetic rinsing makes the preparation more transparent without altering the color balance. Gomori recommends the following technic:

Gomori’s Trichrome Stain.

1. Fix smears in alcohol or alcohol ether. Imbed tissue blocks in paraffin and section at 3–5\( \mu \).
2. Bring smears or sections to water as usual.
3. Stain nuclei five minutes with alum hematoxylin (Delafield, Harris, Lillie-Mayer, or Ehrlich, p. 76).
4. Wash in water.
5. Stain 5–20 minutes in
   - Chromotrope 2R 0.6 Gm.
   - Fast green FCF 0.3 Gm.
   - Phosphotungstic acid 0.6 Gm.
   - Glacial acetic acid 1 cc.
   - Distilled water 100 cc.
6. Rinse in 0.2% acetic acid
7. Dehydrate and clear by usual alcohol xylene sequence.
   Mount in polystyrene, Permount, HSR, or other synthetic resin.

Results: Connective tissue, green; muscle and cytoplasm, red; nuclei, gray-blue.

Like other fast green methods of the Masson type, it gives a rather diffuse and incomplete picture of the more finely fibrillar stroma. Much of the reticulum of liver and spleen is difficult to discern.

The Hydrochloric Acid Methods

An HCl-Orange G-Methyl Blue method of mine (loc. cit.) omits all so-called mordants and yields deep-blue reticulum, fine collagen fibrils, renal
glomerular stroma, and basement membranes; blue to brown coarse collagen bundles, lighter blue epithelial mucus, orange pink erythrocytes, orange to gray cytoplasm, orange yellow muscle, and black nuclei. Brush borders of renal epithelium sometimes stain differentially blue. Anilin blue can be used instead of methyl blue.

Formalin material is used. Paraffin sections are brought to water.

1. Stain six minutes in Weigert's or similar acid iron hematoxylin.
2. Wash in water.
3. Then stain ten minutes in 1% aqueous phloxin B (C. I. No. 778).
4. Wash two minutes in 1% acetic acid.
5. Stain ten minutes in methyl blue (or anilin blue) 100 mg., orange G 600 mg., concentrated hydrochloric acid (37%) 0.25 cc., distilled water 100 cc.
6. Wash in 1% acetic acid five minutes.
7. Stain ten minutes in 1% acetic acid.
8. Dehydrate and clear by an acetone, acetone and xylene, xylene sequence, and mount in Clarite.

The HCl-Biebrich Scarlet-Methyl Blue variant of this technic giving a denser over-all stain with more brilliant colors may be done as follows (loc. cit.):

1. Stain paraffin sections of formalin-fixed material six minutes in Weigert's acid iron chloride hematoxylin.
2. Wash in water.
3. Stain five minutes in a 1% solution of Biebrich scarlet in 1% acetic acid.
4. Wash two minutes in 1% acetic acid.
5. Stain five minutes in a 0.5% dilution of concentrated hydrochloric acid containing 0.5% methyl blue or anilin blue or 0.2% naphthol blue black. Satisfactory results can also be attained by reducing the methyl blue to 0.1% or the naphthol blue black to 0.05% in the same concentration of acid, if the staining time is prolonged to 20–40 minutes.
6. Dehydrate with 95% and 100% alcohol, clear with 100% alcohol and xylene mixture and two changes of xylene, and mount in clarite.

Results: Muscle, red with darker cross striae; erythrocytes, scarlet; cytoplasm, gray pink; brush borders of renal epithelium, sometimes blue or green; mucus, pale blue or blue green; collagen, reticulum, basement membranes and renal glomerular stroma, deep blue or blue black.

Periodic Acid Oxidation Methods

A fourth method for the demonstration of collagen, reticulum and basement membranes is the periodic acid leucofuchsin method (p. 123). This procedure is apparently quite unrelated chemically to the silver methods or
to the acid aniline dye collagen methods. Basically it is supposed to depend on
the presence of hydroxyl groups or one hydroxyl and one primary or second-
ary amine group on adjacent carbon atoms. It now appears that this reaction
in the case of collagen and reticulum (and gelatin) is due in small measure
to the presence of hydroxylysine in the protein molecules, and, more largely
to the presence of a closely bound, non-glucosamine saccharide (Bangle and

The periodic acid Schiff reaction of collagen and reticulum is quite readily
blocked by sulfite treatment after periodic acid oxidation; that of basement
membranes, somewhat less so. Immersion after periodic acid for a two-hour
period in 0.05 M NaHSO₃ followed by a ten-minute bath in Schiff reagent
results in positive reactions of glycogen, starch, cellulose, most mucins, renal
casts, yeasts, lens capsule, Descemet's membrane, rod acromeres, and vitreous
coagulum. The connective tissues including collagen, reticulum, basement
membrane, bone and cartilage matrix, zymogen granules, and thyroid colloid
fail to react.

Acetylation with a 40% acetic anhydride 60% pyridine mixture before
periodic acid oxidation prevents the aldehyde formation in most substances,
if adequately prolonged. Cartilage matrix and starch resist acetylation the
longest, 18–24 hours at 25° C. being required to block this reaction. Con-
trarwise, much shorter periods are adequate to prevent the reaction in
collagen, reticulum, and basement membranes (two hours). Glycogen and
the mucins are intermediate in their requirements.

The reaction of renal reticulum and glomerular basement membranes with
the periodic acid leucofuchsin technic is not abolished by 16 hours digestion
in 1:4000 bull-testis hyaluronidase at pH 5 and 37° C., but the metachro-
natic staining of umbilical-cord matrix was completely destroyed by the
same solution in two hours.

The usual periodic acid Schiff procedure used after the above chemical
treatments is given on p. 123. A useful variant specifically valuable for the
differentiation of the connective tissues is the allochrome procedure (Am. J.
Clin. Path. 21:484, 1951). This method colors most collagen and reticulum
bright blue; lens capsule, suspensory ligament, Descemct's membrane, renal
tubule basement membranes, and many other epithelial basement mem-
branes, capillary basement membranes, and part of the medial stroma of
arteries deep red. It depends on the use of a picric-methyl blue counterstain
after a usual periodic acid Schiff sequence. The technic follows.

Lillie's Allochrome Connective Tissue Method.

1. Deparaffinize and hydrate paraffin sections through xylene, alcohols,
   and water as usual. Treat with iodine and thiosulfate (p. 94) if re-
   quired for removal of mercury precipitates. Wash frozen sections with
two changes of water to remove excess formaldehyde.
2. Oxidize ten minutes in 0.03 M (0.69%) KIO₄ in 0.3% nitric acid. Equivalent solutions are 0.68% H₅IO₄ in water and 0.83% Na₂IO₅ in 0.4% nitric acid. (1 cc. conc. or 70% HNO₃ contains about 1 Gm. HNO₃.)

3. Wash five minutes in running water.

4. Immerse ten minutes in Schiff reagent (p. 156).

5. Wash 1, 2, and 2 minutes in three changes of 0.5% sodium metabisulfite.

6. Wash five minutes in running water.

7. Stain two minutes in Weigert's acid iron chloride hematoxylin (p. 81).

8. Wash four minutes in running water.

9. Stain six minutes in saturated aqueous picric acid containing 40 mg. methyl blue (C. I. No. 706) or anilin blue WS (C. I. No. 707) per 100 cc.

10. Dehydrate and differentiate in two changes each of 95% and 100% alcohol. Wash in 50:50 alcohol xylene mixture and clear in two changes of xylene. Mount in synthetic resin: polystyrene, Permount, HSR, Clarite, Depex, or the like.

**Results:** Nuclei, black, gray, or brown; cytoplasms and muscle cells, gray green to greenish yellow; most reticulum, blue; ring fibers of splenic reticulum, typically red purple; basement membranes of glomerular, renal, and many other capillaries, red purple; medial stroma of arteries, red with fewer blue fibrils; epithelial basement membranes, characteristically red purple in the kidney, where they are relatively thick, perhaps deep violet in other organs where they may be quite thin; but usually one can find at least small areas where an inner red lamina is slightly separated from an adjacent blue reticulum fibril. Amyloid varies from fairly deep to quite pale purplish red to lavender. Arterial hyalin colors orange red to purple red. Fibrin is red to orange. Bone matrix colors gray orange; cartilage, red purple. The collagen of areolar tissues is colored bright blue; that of denser masses may retain some coarse pink or greenish yellow fibers among the predominant blue or greenish-blue fibers. Glycogen, starch, cellulose, Descemet’s membrane; lens capsule and suspensory ligament; bacterial, yeast, and mold chitins; epithelial mucins and that of acute torulosis; thyroid and hypophyseal colloids; follicle fluid and zona pellucida in the ovary, prostatic secretion and corpora amylacea; intestinal melanosis and adrenal and ovarian lipofuscin pigments—all retain the red purple colors of the Schiff reaction. Renal brush borders remain purplish pink, hyaline or colloid droplets color orange, and casts color deep red purple.

**Elastic Fibers**

Though elastic tissue in arterial walls is often stained brilliantly pink with azure-eosin methods and the like, elastic fibers are less easily discerned in
the derma, the pulmonary parenchyma, and other locations. In fresh material containing collagen and elastin fibers, the former are distinguished by their wavy course and their pronounced swelling and clearing in dilute acids such as acetic, citric, and oxalic. Elastic fibers remain as slender, straight fibrils anastomosing at intervals in such preparations. Normally elastic fibrils stain with acid dyes, but may be rendered basophilic by treatment with chromic acid or chromates. They quickly blacken with osmium tetroxide. In the presence of ferric salts they stain with basic fuchsin with or without resorcin. From acid solution they are selectively stained by the weak acid orcein. Selective staining from a hydroalcoholic solution of Victoria blue (4R probably, C. I. No. 690; but possibly B, C. I. No. 729) after chromo-osmic fixation has been reported (Lee).

Elastic fibers in some species, notably rodents, color red purple with Schiff reagent in a variety of procedures in which this reagent is employed: The Feulgen nucleal and plasmal reactions; the 1,2-glycol reactions after oxidation with periodic acid, chromic acid, potassium permanganate, lead tetraacetate, and sodium bismuthate; the ethylene reactions with performic and peracetic acids; and finally, with brief (ten-minute) exposures to Schiff reagent alone. A brief (30-minute) treatment with 5% phenylhydrazine before periodic or peracetic acid prevents the red coloration of elastica in these methods, and prevents their direct Schiff and Feulgen reactions as well. It is indicated that the reactive material is an aldehyde insoluble in fat solvents.

Verhoeff's procedure is an overstaining with an iodine ferric chloride hematoxylin mixture, followed by a ferric chloride differentiation (Mallory). It seems to be quite permanent. I have seen a section stained by Verhoeff nearly 50 years ago which is still excellent. The elastic tissue was deep violet, nuclei blue violet, the acromeres of the rods and cones a good violet.

Verhoeff's Method, According to Mallory. Fix in Zenker or in formalin, imbed in paraffin or celloidin, bring sections to 80% alcohol. Do not iodize before staining.

Dissolve 1 Gm. hematoxylin in hot 100% alcohol, 20 cc. Add 8 cc. 10% ferric chloride solution. Mix and add 8 cc. Lugol solution containing 2% iodine and 4% potassium iodide. This solution is best fresh, but can be used for two or three weeks. Immerse sections in this mixture for 15 minutes or more, until quite black.

Differentiate a few seconds in 2% ferric chloride, observing microscopically in water. If overdifferentiated, restain at once. Wash in water; then in 95% alcohol to remove the excess iodine; then again in water. Counterstain in 0.5% aqueous eosin, dehydrate in alcohols, clear in origanum oil, mount in balsam.

Results: Elastin, black; nuclei, blue black; collagen, fibrin, glia, and myelin, pink; erythrocytes, orange red. The acromere staining noted above is probably a lipoid staining.
The Taenzer-Unna Acid Orcein Method. Bring paraffin sections to 70% alcohol by the usual sequence.

1. Stain 1–18 hours at room temperature or 10–30 minutes at 30–37° C. in a freshly filtered solution of 1 Gm. orcein (natural or synthetic) in 100 cc. alcohol of 65% (Stutzer), 70% (Mallory, Romeis), 75% (Taenzer), 80% (Merk), 90% (Kornhauser, from Conn and Darrow), 100% (Cowdry, Schmorl, Lee) to which 1 cc. of official P.G. or concentrated hydrochloric acid is added. (Stutzer, Taenzer, and Merk are cited from Ehrlich's *Encyklopädie*.)

2. Wash in alcohol (70–100%) and in water, either first.

3. Counterstain briefly in polychrome methylene blue (Unna), 1:1,000 azure A or toluidine blue for one minute, or alum hematoxylin for 3–6 minutes, if desired.

4. Dehydrate and clear by an alcohol or acetone xylene sequence and mount in balsam or Clarite.

One may further add a connective-tissue stain of Van Gieson or Mallory type, or a picroindigocarmine. The foregoing technic is a composite from the various modern texts.

**Fraenkel's Method** (Schmorl).

1. Stain nuclei red with (Orth's) lithium carmine. Differentiate in hydrochloric acid alcohol (1%).

2. Stain 24 hours: Of a stock solution of 1.5 Gm. orcein, 120 cc. 95% alcohol, 60 cc. distilled water, 6 cc. nitric acid, add sufficient to a 3% alcoholic hydrochloric acid solution to give a dark-brown color. This is the staining solution.

3. Differentiate in 80% alcohol.

4. Stain 10–15 minutes in 0.25% indigocarmine in saturated aqueous picric acid solution.

5. Rinse in 3.5% acetic acid.

6. Dehydrate quickly in 95% and 100% alcohol, clear in oil or xylene—I suggest the 100% alcohol + xylene 50:50 mixture first, then two changes of xylene. Mount in balsam (or Clarite).

**Results:** Red nuclei, dark brown elastin, blue green collagen, greenish yellow muscle.

**Romeis's Method.** Bring paraffin sections to 70% alcohol.

1. Stain one hour in 1% orcein, 1% hydrochloric acid, 70% alcohol.

2. Wash thoroughly in two changes distilled water.
3. Stain heavily with Ehrlich's acid hematoxylin, acid hemalum, or three minutes in Hansen's iron alum hematoxylin.*

4. Wash ten minutes in distilled, tap, and distilled water.

5. Stain in 60 cc. 0.1% acid fuchsin in saturated aqueous picric-acid solution to which is added 0.25–0.3 cc. 2% acetic acid.

6. Rinse in 60 cc. distilled water containing 2.5 cc. of the acidified picric-fuchsin solution above, for not more than 2–4 seconds.

7. Blot dry.

8. 95% alcohol one minute.

9. 100% alcohol three minutes, then clear with xylene and mount in balsam.

**Results:** Black to red brown elastica, yellow muscle, bright red collagen, dark brown nuclei.

Weigert's acid iron chloride hematoxylin can probably be used in place of Hansen's formula, and any alum hematoxylin would undoubtedly serve. The addition of the few drops of 2% acetic acid (pH probably above 2.0) to the Van Gieson stain already at pH 1.95 seems rather futile; and it seems that the technic could be simplified by direct transfer from the Van Gieson mixture to alcohol.

**Resorcin Fuchsin**

Another important group of elastin stains are the iron resorcin lakes of basic fuchsin, crystal violet, and other basic dyes. Weigert (*Centralb. allg. Path. 9:289, 1898; Ehrlich's Encyklopädie*) directed thus: To 200 cc. 1% basic fuchsin solution add 4 Gm. resorcin and boil until dissolved. Then add 25 cc. liquor ferri sesquichlorati P.G.† (the modern U.S. official solution of iron chloride is the same) and boil 2–5 minutes longer. Cool and collect the precipitate on a filter. Take up the precipitate from the sides and bottom of the original vessel as well as from the filter paper with 200 cc. 95% alcohol (by boiling as necessary). Add 4 cc. concentrated hydrochloric acid and filter, washing the filter through afterward with enough fresh alcohol to restore the total volume to 200 cc.

Weigert directed 15–30 minutes' staining in this solution, followed by alcohol differentiation and clearing in xylene. This gave black elastic fibrils on a pale violet background.

One may use 2 Gm. crystal violet in 200 cc. water in place of the basic fuchsin. In this case 0.5–2 Gm. dextrin should be added according to French (*Stain Technol. 4:11, 1928; also personal communications*). The resulting

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*Dissolve 10 Gm. iron alum and 1.4 Gm. ammonium sulfate in 150 cc. distilled water and pour into a solution of 1.6 Gm. hematoxylin in 75 cc. distilled water. Heat and boil not more than 30–60 seconds. Cool, and keep in a nearly full bottle. Stain 1–10 minutes and differentiate in 2–3% sulfuric or 0.5–1% acetic acid; or for pure nuclear staining add 2/3 to ½ volume of 1% sulfuric acid to 1 volume of stain.

stain is green. Or if 1 Gm. basic fuchsin and 1 Gm. crystal violet are used, a deep blue-green color is achieved. If 2 Gm. safranin is used, elastic fibrils are stained brownish red. The resorcin used should be fresh and crystalline. When heating the alcohol to dissolve the precipitate a closed electric hot plate or a steam table is preferred because of the fire hazard. Romcis specified the hydrochloric acid as the P.G. official 25%, sp. gr. 1.126; Ehrlich, as concentrated. Most writers have followed the latter, and some even add additional hydrochloric acid for staining.

Usually this procedure is combined with other stains which, because of the high acidity and alcohol content of the resorcin fuchsin stain, must follow it rather than precede. Silver impregnations for reticulum, however, should precede the elastic tissue stain. In this case the crystal violet compound is preferable to resorcin fuchsin, because the green contrasts better than blue black with the black silver deposit. The more common combinations are with fibrin or collagen stains. Some simply precede the elastin stain with a carmine stain for nuclei.

For combination with staining of tubercle bacilli, one first stains as usual with hot (one hour at 55–60° C.) carbol fuchsin (pp. 380–381), washes off in water, and then stains 20–30 minutes in the acid alcoholic resorcin fuchsin solution, which decolorizes cells and other structures at the same time. Then differentiate in alcohol, counterstain five minutes in 0.1% methylene blue in 1% acetic acid, dehydrate with alcohol or acetone, and clear with a 50% xylene mixture with 100% alcohol or acetone followed by two changes of xylene. Mount in Clarite.

Hornowski’s Combined Elastin Van Gieson Staining. First stain 30–60 minutes in resorcin fuchsin, differentiate in 95% alcohol, stain 5–10 minutes in Weigert’s acid iron hematoxylin, wash in water, stain five minutes in 0.1% acid fuchsin in saturated aqueous picric acid, dehydrate and differentiate with three changes of 95% alcohol, two of 100% alcohol, and clear with one change of xylene 100% alcohol 50:50 mixture and 2–3 changes of xylene. Mount in Clarite or balsam. This is essentially Hornowski’s (1908) method (Abstr. Ztschr. wiss. Mikroskop. 26:128, 1909).

The Volkman-Strauss Method. Others have combined the resorcin-fuchsin with one of the anilin blue methods, such as the Volkman-Strauss (Ztschr. wiss. Mikroskop. 51:244, 1934) combination with the Mallory-Heidenhain procedure (see p. 351); and no doubt picroanilin blue, picromethyl blue and similar procedures, with or without a red plasma stain, could be used (pp. 348–349).

An elastic tissue-fibrin combination is sometimes useful and such a method is quoted by Schmorl. A variant of this which I have found useful is quoted:

Elastin-Fibrin Technic. Bring paraffin sections to 95% alcohol. (If mercury-fixed, treat with 0.5% iodine five minutes, rinse, then 5% sodium thiosulfate two minutes, and wash thoroughly in water; then briefly in 95% alcohol.)
1. Stain 20-40 minutes in resorcin fuchsin or the resorcin fuchsin crystal violet. Mercury-fixed tissue should be stained 1-2 hours.
2. Wash in 95% and 80% alcohol.
3. Stain 20 minutes in carmine (p. 84), five minutes in acid iron hematoxylin (p. 81) or ten minutes in 1% Bismarck brown R in 1% acetic acid.
4. Wash in water.
5. Stain 2-3 minutes in aniline methyl violet (saturated methyl violet in distilled water 9 cc. and 1 cc. saturated methyl violet in 20% aniline, 80% alcohol mixed at time of using). See also pp. 369, 370, 372; any methyl violet or crystal violet solution will serve.
6. Rinse quickly in 0.9% aqueous sodium chloride solution.
7. Flood with Weigert's (p. 92) iodine, pour off, and again flood, giving total exposure of 20-30 seconds.
8. Rinse in water.
10. Clear and decolorize in equal volumes of aniline and xylene as long as a fresh drop of mixture is colored violet by the section.

Results: Nuclei, red, gray or brown; elastin, blue black; fibrin, violet; Gram-positive bacteria, blue black.

Resorcin Fuchsin Oil Red O. The resorcin fuchsin method may also be combined with a fat stain if a suitable quantity of the stock stain is diluted with water to bring its alcohol content down to 60%. The frozen sections are stained 1-2 hours in a covered dish, then rinsed with 60% alcohol, and stained with oil red O for five minutes (dilute 3 cc. of stock saturated isopropanol solution with 2 cc. distilled water; let stand 7-8 minutes, filter, and use at once). Then wash in water, counterstain 3-5 minutes in 0.1% aqueous Janus green B (C. I. No. 133), and wash one minute in 5% acetic acid. Wash in water, float onto slides, and mount in gum syrup. Or one may use an alum hematoxylin nuclear stain for five minutes, wash, and mount.

Gallego's Iron Fuchsin

A technic which I have used for elastic fibers and collagen is a modified Gallego procedure in which I have substituted picroanilin blue for the picroindigocarmine of the original. Otherwise, I have converted Gallego's drops into cubic centimeters and slightly altered the proportions of the reagents in the iron mordant. With this technic nuclei are gray to black; collagen and reticulum, deep blue; muscle, greenish to orange yellow; calcium, reddish to purplish brown; mast-cell granules, deep red; cartilage, purple to violet; mucus, blue violet; cytoplasm, olive to brown; elastic fibers, purple to red.
1. Stain paraffin sections six minutes in Weigert's acid iron chloride hematoxylin (p. 81).
2. Wash in water.
3. Mordant 30 seconds in a fresh mordant composed of 200 cc. distilled water, 1.5 cc. concentrated nitric acid, 1.0 cc. 40% formaldehyde, and 1.5 cc. official (U.S.) iron chloride solution added in the order given. (To avoid immediate interaction of the reagents, they are mixed only after dilution.)
4. Rinse in water.
5. Stain five minutes in a fresh dilution of 3 cc. carbol fuchsin in 50 cc. 0.2% acetic acid. (I have on occasion found it necessary to increase the carbol fuchsin to 4 cc.)
6. Rinse in water.
7. Return to the mordant for two minutes.
8. Rinse in water.
9. Stain one minute in 0.1% anilin blue in saturated aqueous picric acid solution. Gallego used Ramón y Cajal's picroindigocarmine (p. 346).
10. Rinse in 0.1% acetic acid. Dehydrate and clear by an acetone, xylene sequence, and mount in Clarite or balsam.

This method was published by German (Am. J. Clin. Path., Tech. Suppl. 3:13, 1939) and was also included in Langeron; it was attributed by them to Gallego. The above modification is essentially in the form in which I contributed it to Conn and Darrow, with emendations.

Gomori's Aldehyde Fuchsin Method for Elastic Fibers (Am. J. Clin. Path. 20:665, 1950). Dissolve 0.5 Gm. basic fuchsin (C. I. No. 677) in 100 cc. 70% alcohol. Add 1 cc. concentrated hydrochloric acid and 1 cc. U.S.P. paraldehyde. In 24 hours the mixture becomes a deep violet and is ready to use. Store at 0–5° C.

Avoid chromate fixations. Formalin and Bouin's fluid give colorless backgrounds, mercury fixatives a pale lilac.

1. Deparaffinize and hydrate paraffin sections as usual.
2. Treat 10–60 minutes with 0.5% iodine.
3. Decolorize 30 seconds with 0.5% sodium bisulfite.
4. Wash two minutes in water.
5. Transfer to 70% alcohol.
6. Stain in aldehyde fuchsin: for elastic fibers 5–10 minutes; for pancreatic-islet cells 15–30 minutes; for hypophysis ½–2 hours. Rinse in alcohol (70%) and inspect microscopically from time to time.
7. Wash in several changes of 70% alcohol.
8. Counterstain with hematoxylin and orange G (p. 115) or with the Masson trichrome or Mallory-Heidenhain method (pp. 351–352). The
latter are preferred for hypophysis. Fast green FCF or light green SF should be substituted for the anilin blue in these methods.

9. Dehydrate, clear, and mount through an alcohol, xylene, synthetic resin sequence.

**Results:** Elastic fibers, mast-cell granules, gastric chief cells, beta cells of pancreatic islets, and certain of the hypophyseal beta granules—violet to purple. Alpha granules of the hypophysis stain orange red, delta granules green to greenish blue, and chromophobe cells present pale gray-green cytoplasm. Collagen is stained green, using the fast-green Mallory-Heidenhain variant.
Chapter 17

Bacteria, Protozoa, and Other Parasites

For the study and demonstration of these, a variety of special methods are prescribed: some use the usual fixed material, but some perhaps function better with special fixations.

Preparations of Smears and Films

In place of sections or to supplement them it is often desirable to utilize spread films or smears of tissues, blood, and exudates. Films are preferable for the study of the cytology of the blood, the red bone marrow, the spleen pulp, and various inflammatory exudates. For the demonstration of bacteria, protozoa, and rickettsiae they are often preferable to sections. For the demonstration of scarce blood protozoa, thick blood films are made and hemolyzed before or during staining so that a relatively large volume of blood can be scanned quickly for the presence of malarial plasmodia, trypanosomes, leishmaniae, and even microfilariae.

Endicott's Marrow Smear Method. For the staining of spleen and marrow Endicott's (Stain Technol. 20:25, 1945) technic for the preparation of films is recommended. Dip a capillary pipet into a tube of human serum or plasma and take up a column about 10 mm. in length. Then immediately place the point of the pipet in the red marrow or spleen pulp and aspirate about 2 mm. of tissue. Blow the tissue and serum onto a clean slide near one end, and mix thoroughly by repeated aspiration and expulsion with the same pipet. Finally, leaving the drop on the slide, smear it in the usual manner by placing another slide against the first at an angle of 30–45° over the drop and between it and the center of the first slide. Then draw the slide back toward the drop until it makes contact and the fluid spreads along the acute angle between the two slides. Then push the second slide along the first away from the original site of the drop so that the fluid film follows in the
acute angle. The thickness of the film can be regulated by varying the angle of contact; the more acute the angle, the thinner the film.

**Impression Films.** Useful tissue films can also be prepared by simply pressing a clean slide lightly on a freshly cut surface of the tissue in question. These films are called *impression films*. The method is often used for brain.

**Thin blood films** are simply prepared by depositing a small drop of blood near one end of the slide and drawing it along in the acute angle made by a second slide as above.

**Thick blood films** are made by depositing several large drops of blood near one end of a clean slide and spreading them with a glass rod, the corner of a clean slide, or a match into a circular area about 10-15 mm. in diameter. It is often useful to make a thick film on one end of a slide and a thin film on the remaining two thirds. Since the value of thick films depends on the removal of the hemoglobin from the red corpuscles by hemolysis in distilled water or some other agent, one should be careful with such combined films not to allow the fixative to come in contact with the thick film before hemolysis.

**Smears of thick pus** may perhaps require dilution with serum (as on p. 366 for spleen and marrow smears). Thinner exudates can be smeared as is blood. Relatively clear or even turbid fluids may require centrifugation and resuspension of the sediment in a small drop of serum or serous exudate for the preparation of satisfactory films. With serous inflammatory exudates of relatively high protein content the supernatant fluid is satisfactory for resuspension of the sediment; but with urine and spinal fluid serum is a more satisfactory diluent.

**Centrifugates.** According to Arcadi (*J. Urol. 61:814, 1949*) centrifuged urinary sediments may be smeared on slides, air-dried, and then fixed by immersion for one minute in 99% isopropyl alcohol. After this fixation he stained two minutes in alum hematoxylin (Harris’s, p. 76) washed in water, counterstained one minute in 2% aqueous eosin Y, washed 15 seconds in water, dehydrated with isopropyl alcohol, cleared in xylene, and mounted in Clarite, dammar, or other suitable resin.

Undoubtedly other stains can be applied after this fixation technic.

Aspirated material and washings from hollow viscera may be fixed by mixing with an equal volume of 15% formalin and then centrifuging. Addition of alcohol to precipitate mucus may be necessary with gastric washings. The sediment can then be dehydrated after some hours in formalin, dealcoholized, and imbedded in paraffin. Paraffin sections may be prepared as usual and stained in a variety of ways. The method, according to Wollum *et al.* (*J. Nat. Cancer Inst. 12:715, 1952*) is superior to smears in cancer diagnosis, in avoiding thick areas, in preserving cell relationships of small tissue fragments, and in permitting multiple stains.

**Fixation.** Thin blood films and marrow films, tissue smears, and exudate smears should be fixed at once in methyl alcohol for 3-5 minutes and then
allowed to dry until it is convenient to stain them. Such films can be stained successfully for 2–3 weeks, whereas films not fixed at the time of taking soon deteriorate, so that staining becomes inferior.

Sometimes special procedures are better served by fixing still moist films by the vapor of osmium tetroxide or by gaseous formaldehyde (films are placed face down over a shallow vessel containing a 1–2% osmium tetroxide solution or a little concentrated formalin) or perhaps more conveniently, by depositing 1–2 cc. of strong formalin in a Coplin jar and then standing the films in it, film-end up, and putting on the lid.

In the case of quite thin smears, where focusing may be difficult, it may be useful to draw one or more lines on the slide with a grease pencil or, if the preparation is to be covered with a cover glass, with black marking ink to establish a readily visible plane of focus.

Thick blood films should ordinarily be dried in a place protected from dust, flies, and roaches for 18–24 hours before staining. Staining should not be delayed any longer after drying than necessary. If staining cannot be carried out within 24 hours, it is preferable to hemolyze with distilled or even tap water for 5–10 minutes and then fix five minutes in methyl alcohol.

Such films may be stained by a variety of methods, including simple solutions of basic anilin dyes, Gram's stain, the acid-fast stain, Macchiavello's stain, Goodpasture's, Giemsa's, Wright's, Leishman's, and many others. After staining they may be allowed to dry or mounted in Clarite.

In place of the ether alcohol mixture recommended by Papanicolaou (Diagnosis of Uterine Cancer by the Vaginal Smear, New York, Commonwealth Fund, 1945) Davidson, Clyman, and Winston (Stain Technol. 24: 145, 1949) highly recommend 3:1 mixtures of tertiary butyl alcohol with ethyl alcohol and with ethyl phosphate, and report excellent results with Papanicolaou's stain (p. 352).

Gram-positive Bacteria

Fibrin, certain hyalin droplets seen in degeneration of renal epithelium, and keratohyalin granules share to a greater or less extent the property of Gram-positive bacteria of retaining the dye complex formed by the action of iodine upon crystal violet when certain solvents are applied. Gram-positive bacteria, however, are more resistant to solvent extraction than are fibrin and hyalin droplets. Thus ethyl alcohol, acetone, and their mixtures usually leave Gram-positive bacteria blue black, and decolorize fibrin and hyalin droplets. Keratohyalin is intermediate in its resistance. Aniline and aniline xylene mixtures leave fibrin stained violet as well as bacteria.

Various other substances are usually added to crystal violet or methyl violet solutions in mixtures of water and alcohol. The dye concentrations are usually high and some solutions are apparently supersaturated when made. The function of the added phenol or aniline is not clear. I am not inclined to credit the alleged mordant action of these substances, since the Hucker-Conn
ammonium oxalate variant seems as good as any and is considerably more stable than the aniline water solutions, or even the phenol water solutions. Many of these solutions were formerly described as gentian violet solutions. Inasmuch as gentian violet seems to have been a variable mixture of dextrin, crystal violet (hexamethyl pararosanilin) and methyl violet (its tetra- and pentamethyl homologs), and crystal violet alone serves the same purpose better (Conn), this dye is here prescribed in all formulae.

**Ehrlich's Aniline Crystal Violet as Emended by Conn.** Crystal violet (C. I. No. 681) 1.2 Gm., dissolve in 12 cc. 95% alcohol and add 100 cc. freshly prepared and filtered aniline water made by shaking 2 cc. aniline in 100 cc. water. This keeps about two weeks. Weigert (Ehrlich's *Encyklopädie*) used methyl violet in a similar formula.

**Stirling's Aniline Crystal Violet, as Emended by Conn.** Crystal violet 5 Gm., 100% alcohol 10 cc., aniline 2 cc., distilled water 88 cc. This solution is quite stable.

**Nicolle's Carbol Crystal Violet, as Emended by Conn.** Crystal violet 1 Gm., 95% alcohol 10 cc., phenol 1 Gm., distilled water 100 cc. Dissolve the dye in alcohol, the phenol in water, and mix; or, perhaps easier, dissolve both in alcohol and add the water. Schmorl cites a carbol crystal violet containing 2.5 Gm. phenol, Mallory one with 3 Gm., both attributed to Nicolle. Both of these authors give the amount of gentian or crystal violet as 10 cc. of saturated alcoholic solutions. According to Conn this would be about 1 Gm. of most commercial samples.

**The Hucker-Conn Ammonium Oxalate Crystal Violet, Modified (Arch. Path. 5:828, 1928).** Crystal violet 2 Gm., 95% alcohol 20 cc., ammonium oxalate 800 mg., distilled water 80 cc. Dissolve the dye in the alcohol, the oxalate in the water, and mix. The solution keeps at least two or three years.

**Gram's Iodine.** Dissolve 2 Gm. potassium iodide in 2–3 cc. distilled water, dissolve 1 Gm. iodine crystals in this solution. Dilute with distilled water to 300 cc. for Gram's solution, or to 100 cc. for Weigert's.

**Gram Staining of Smears for Bacteria. Technic:**

1. Fix by quickly passing the smear face down through the blue flame of a Bunsen burner or alcohol lamp three times.
2. Stain with crystal violet 20–60 seconds. Acid-fast bacilli require at least 1–2 minutes, and perhaps it is safer to heat to 60–80° C. as well.
3. Wash in water. Conn blots off excess dye and does not wash.
4. Cover with Gram's iodine for 1–2 minutes or with Weigert's for 20–30 seconds.
5. Decolorize 30–60 seconds by dropping alcohol on the film or by agitation in 2–3 changes of alcohol until color clouds no longer come out and the film, if of an exudate, is largely decolorized. Instead of alcohol, one may use acetone from the dropping bottle. With this reagent decolorizing is complete in 5–10 seconds.
6. Wash in water.
7. Counterstain 30–60 seconds in a 0.1–0.5% solution of safranin O (C. I. No. 841), basic fuchsin (C. I. No. 677), Bismark brown Y or R (C. I. No. 331 or 332), or pyronin Y or B (C. I. No. 739 or 741).
8. Wash in water, dry, and examine in immersion oil.

I prefer the Hucker-Conn crystal violet formula, the Weigert iodine, acetone decolorization, and 0.2–0.5% safranin as counterstain.

Results: Gram-positive organisms are blue black; Gram-negative, red or brown, according to the counterstain used.

Gram Stain for Sections. The same technic may be applied to sections (Arch. Path. 5:828, 1928). Thus:

1. Bring paraffin sections through xylene and alcohols to water as usual.
2. Stain 30 seconds in Hucker-Conn crystal violet.
3. Rinse briefly in water.
4. Treat 20–30 seconds with Weigert's iodine.
5. Decolorize with acetone 10–15 seconds.
6. Wash in water.
7. Counterstain 30 seconds in 0.5% safranin.
8. Differentiate and dehydrate (10–15 seconds) with acetone from a dropping bottle.

Results: Gram-positive bacteria, blue black; nuclei, deep red; Gram-negative bacteria and fibrin, red; cytoplasm, pink.

Glynn's method (Arch. Path. 20:896, 1935) differs from the foregoing in using Nicolle's carbol crystal violet (p. 369) for two minutes, and in counterstaining (Step 7) first with 1:2,000 basic fuchsin in 0.002 N hydrochloric acid for three minutes and then for 30–60 seconds in saturated aqueous picric acid. Cytoplasm and red corpuscles are yellow; serum, fibrin, and collagen, pale pink; myelin, violet; and Gram-positive bacteria, blue black.

The Kopeloff-Beelman formula for crystal violet has given more intense Gram-positive reactions than the ammonium oxalate formula (Bartholomew and Mittwer, Stain Technol. 25:103, 1950). I cite the method from Conn and Darrow.

1. Hydrate sections or use air-dried films.
2. Stain five or more minutes in a fresh mixture of 4 parts 5% sodium bicarbonate and 15 parts 1% aqueous crystal violet solution.
3. Wash off with sodium hypoiodite. (Dissolve 2 Gm. iodine crystals in 10 cc. 1 N NaOH, and dilute to 100 cc.)
4. Immerse in or cover with hypoiodite for two minutes.
5. Wash in water, blot lightly and at once.
6. Decolorize with acetone or 30% ether acetone, dropping onto slide until color stops coming out (ten seconds or less).
   A higher proportion of ether (50%) slows the differentiation.
7. Counterstain 5–10 seconds in 2% safranin or 0.1% basic fuchsin.
8. Wash in water, dry, and examine. Sections can probably be dehydrated by dripping on acetone and xylene in sequence as in the Gram acetone technic (supra).

The Weigert Fibrin Stain

The Gram-Weigert technic devised by Weigert in 1887 for fibrin and bacteria differs from the Gram technics in that counterstaining, if any, must be done before the Gram reaction is carried out, and in that the differentiating agent is aniline, alone or weakened by admixture with xylene. Numerous variants have been suggested, chiefly in the counterstains used. Weigert, writing for the 1903 Enzyklopädie der mikroskopischen Technik, still recommended a carmine prestain. He stated that it was necessary to treat chromate-fixed material with oxalic acid before staining for fibrin, and suggested also the pretreatment with 0.33% potassium permanganate. This procedure is unnecessary for bacterial staining. On the other hand Weigert also stated that a simple aqueous methyl violet solution was adequate for fibrin staining, while for bacterial staining the aniline-methyl or crystal violet solution was to be preferred. Inasmuch as it is often desirable to have both fibrin and bacteria demonstrated in the same preparation, the technic adopted should conform to the above requirements for both.

Weigert's Technic (Ehrlich's Enzyklopädie).

1. Bring sections to water as usual.
2. Chromate-fixed material is treated briefly with 0.33% potassium permanganate, rinsed in water, bleached several hours in 3–5% oxalic acid solution, and then washed in water.
3. Stain with carmine and rinse in water.
4. Stain in Weigert’s methyl violet (or crystal violet) (p. 369).
5. Treat with Gram’s iodine.
6. Blot with filter paper and
7. Decolorize on the slide with repeated small portions of 50:50 aniline xylene mixture.
8. Wash thoroughly with xylene and mount in balsam.

Results: Gram-positive bacteria, blue black; fibrin, violet; keratohyalin, deep violet; other structures, red or pink. Some nuclei may be violet.

The foregoing technic is deficient in prescribing no times in the various reagents. Further, I now seldom use carmine as a nuclear stain, finding iron hematoxylin better. Fuchsin may be used when it is desired to stain Gram-
negative bacteria. It has sometimes been advantageous to precede with an elastic-tissue stain.

The Emended Weigert Fibrin Technics.

1. According to tradition, treat Zenker, Helly, and other mercury fixed-material with 0.5% iodine in 70% alcohol for 5–10 minutes. Wash in water, treat one minute with 5% sodium thiosulfate, and wash 3–5 minutes in running water. *I have omitted this step with satisfactory results.* Apparently the later iodine step removes the mercury.

2. Treat Orth, Moller (Regaud), Zenker, and other chromate-fixed material with 0.5% potassium permanganate for five minutes, rinse in water, bleach in 5% oxalic acid for 20–30 minutes, and wash in water. *I have omitted this step with quite satisfactory results.*

3a. Stain elastic fibers 30 minutes (if Zenker, 90 minutes) in Weigert’s resorcin fuchsin (p. 361), if concurrent staining of elastic fibers is desired. Wash in water.

3b. The periodic acid leucofuchsin reticulum method (pp. 123–124). Steps 1 to 9 can be used for concurrent red staining of reticulum.

4a. Stain nuclei with Orth’s lithium carmine (p. 84) five minutes, differentiate 2–4 minutes in 1 cc. concentrated hydrochloric acid and 99 cc. 70% alcohol. Wash in water.

4b. Stain nuclei 5–6 minutes in Weigert’s acid iron hematoxylin (p. 81) and wash in water.

4c. MacCallum’s variant (*J. Am. Med. Assoc.* 72:193, 1919): Stain 10–30 minutes in Goodpasture’s aniline carbol fuchsin: 30% alcohol 100 cc., basic fuchsin 0.59 Gm., aniline 1 cc., phenol 1 Gm.; wash and differentiate a few seconds to clear rose with 40% formaldehyde; wash in water; stain 3–5 minutes in saturated aqueous picric acid until purplish yellow; wash in water; differentiate briefly in 95% alcohol; wash in water. (It is not recommended that this variant be combined with the elastic-tissue stain.)

4d. Stain 10–30 minutes in carbol fuchsin (p. 379) and wash in water.

4e. The Feulgen nucleal reaction (p. 132) gives excellent effects, with red to purple nuclei.

5. Stain in Stirling’s or Ehrlich’s aniline crystal violet or in the Hucker-Conn (p. 369) ammonium oxalate crystal violet or in 1% methyl violet or crystal violet in water or in 20% alcohol for 2–3 minutes. (Mallory required 5–10 minutes. I have reduced the time to as little as one minute. For bacteria Weigert preferred the aniline formulas, and I have found the oxalate formula as well as simple solutions satisfactory. Fibrin is stained by any of them.)

6. Drain and blot with filter paper, or rinse quickly in 0.85% sodium chloride solution, or drain and drop on two or three changes of
7. The Gram-Weigert (p. 92) iodine potassium iodide solution (1:2:100) for 30 seconds.
8. Wash in water, wipe back and sides of slide, blot section dry with filter paper, and
9. At once drop on or immerse in a mixture of equal volumes of aniline and xylene, changing two or three times and blotting between changes to complete the dehydration, until violet stain clouds no longer come out of the section and differentiation is complete.
10. Rinse in two or three changes of xylene, or wash with xylene from a dropping bottle. Mount in Clarite or other resin.

**Results:** Nuclei are red or black according to the nuclear stain used; fibrin is violet; Gram-positive organisms are blue black. If a fuchsin nuclear stain has been used and the fixation was Helly or Orth, red corpuscles may remain a deep red, and nuclei are violet to red. Collagen may retain some violet, but in a lighter shade than fibrin. Keratohyalin granules and hair shafts often retain violet, just as they may retain red in Ziehl-Neelsen stains. Young intraepithelial rabbit coccidia (*Eimeria*) contain numerous blue-black granules between capsule and nucleus by this technic. In older, free forms these granules are more apt to be Gram negative, and with the acetone technic (p. 370) they are Gram negative in all stages. Mucus also may remain violet with Variant 4d above, but is Gram negative with the acetone technic. Sometimes glycogen is demonstrated by Gram-Weigert methods, as noted by Lubarsch (Ehrlich’s *Encyklopädie*). Hypophysial β granules are violet.

The usual fixations—formalin, Orth, Helly, and Zenker—seem quite satisfactory for both fibrin and Gram-positive bacteria; but with Bouin’s fluid fibrin may utterly fail to stain by the Weigert method even though the same blocks gave excellent (red) fibrin by Masson’s variant of Mallory’s anilin blue connective-tissue method. Gram-positive cocci, however, stained well. Variant (c) of Step 4, MacCallum’s, is designed to give red Gram-negative organisms as well. Variant 3b, periodic acid leucofuchsin, gives red reticulum. The violet fibrin contrasts well, if it can be stained successfully.

**Fungi**

A variant of Mallory’s of the foregoing for the demonstration of actinomyces seems worthy of note:

1. Stain paraffin sections of formalin or alcohol fixed material for 3–5 minutes in alum hematoxylin.
2. Wash in water.
3. Stain 15 minutes at 57° C. in 2.5% phloxine B (C. I. No. 778) or 5% eosin Y (C. I. No. 768).
4. Wash in water.
Stain in Ehrlich's or Stirling's aniline crystal violet (p. 374) for 5–10 minutes.

6. Wash in water.

7. Treat with Gram's iodine for one minute (or Weigert's for 20–30 seconds) (p. 92).

8. Wash in water and blot dry with filter paper.

9. Differentiate with several changes of aniline until no more color is removed.

10. Wash in three or four changes of xylene and mount in balsam (or Clarite).

Results: Mycelia blue, clubs red.

General on Fungi. The same method is useful for other ray fungi in mycetoma, Madura foot and allied conditions. Mycelia are often well brought out in light blue by azure eosin methods (p. 116) which are much better for study of tissue cellular reactions. The Bauer chromic acid leuco-fuchsin and the periodic acid Schiff methods (pp. 123, 124) can be recommended for mycelial fungi and yeasts in tissues. With the latter, it is desirable to suppress the background staining of collagen and reticulum by use of a sulfite blockade procedure (p. 158), by use of highly diluted (20–50 mg. fuchsin per 100 cc.) Schiff reagent, or by use of a triphenylmethane sulfonic acid dye counterstain as recommended by Kligman, Mescon, and DeLamater (Am. J. Clin. Path. 21:88, 1951) who used a brief counterstain with light green.

The allochrome method (p. 357) has given us brilliant results particularly with Cryptococcus neoformans (Torula histolytica), Coccioidoides immitus, Histoplasma capsulatum, the organisms of cutaneous blastomycoses, and various mycelial fungi have been successfully demonstrated by this method. Anthrax bacilli are well stained. The granules of mycetoma, actinomycosis, and botryomycosis are densely stained by this method; quite thin sections are required if any internal detail is to be discerned. All of these organisms retain the red purple.

Contrasts are better with the Bauer method or with the Kligman-Mescon-DeLamater periodic acid Schiff variant, but tissue morphology is much better shown by the allochrome method. In the Bauer method a brief nuclear stain with Weigert's acid iron hematoxylin is preferable to alum hematoxylin, as it seems less apt to overpower the red of the smaller fungi such as Histoplasma.

Cryptococcus neoformans is also well shown by simple brief stains with metachromatic dyes, such as 1:1000 toluidin blue or thionin for 30 seconds. Organisms are blue violet, the copious mucin of acute torulosis is red purple; cell nuclei and tigroid granules, deep blue; and mast-cell granules, violet to purple.

Use of safranin O in the Gram acetone technic (p. 370) gives red nuclei,
BACTERIA, PROTOZOA, AND OTHER PARASITES

Pink cytoplasm, orange-red and blue-black yeast cells, orange mucus, and orange-red cartilage and mast-cell granules.

Kligman, Mescon, and DeLamater (Am. J. Clin. Path. 21:88, 1951) prepare skin scrapings for examination for fungi by first applying a drop of Mayer's glycerol albumen to the surface of the lesion and then scraping with a blunt knife. The scales are then smeared on a slide, and the smear is fixed 30 seconds in 95% alcohol. Then pass through periodic acid (1% H₄IO₆) for one minute, and wash in water; then Schiff reagent for five minutes. Wash 5–10 minutes in 0.5% K₂S₂O₅ in 0.05 N HCl, using 2–3 changes. Wash in water, dry, and examine. The fungi appear red purple.

The internal filamentous structure of botryomyces granules should be well shown by this technic, as it is by the Bauer method (p. 124). The staphylococcal component is better demonstrated by Gram-Weigert variants, which do not, however, stain the filaments (J. Lab. & Clin. Med. 32:76, 1947).

Kligman's periodic acid fuchsin stain is probably derived from Arzac's, and may well depend on the aldehyde-binding capacity of the aryl amine of the basic fuchsin. The Schiff bases formed by the complexing of aldehyde with fuchsin are highly resistant to acid and alcohol decolorization. Kligman's technic has variants for smears and for sections (letter from Dr. Kligman Jan. 31, 1952).

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Kligman's Periodic Acid Fuchsin Method. Fix smears one minute in 95% alcohol. Deparaffinize sections and immerse briefly in 100% alcohol.

<table>
<thead>
<tr>
<th>Smears</th>
<th>Sections</th>
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<tbody>
<tr>
<td>1. Rinse in distilled water</td>
<td>no</td>
</tr>
<tr>
<td>2. Immerse in aqueous periodic acid</td>
<td>5%, 1 min. 1%, 10 min.</td>
</tr>
<tr>
<td>3. Wash in running water</td>
<td>no</td>
</tr>
<tr>
<td>4. Stain in 0.1% fuchsin in 5% alcohol</td>
<td>2 min. 2 min.</td>
</tr>
<tr>
<td>5. Wash in tap water</td>
<td>rinse</td>
</tr>
<tr>
<td>6. Immerse in a 0.5% tartaric acid, 1% zinc hydrosulfite solution</td>
<td>10 min. 10 min. &amp; 30 min. to 3 hrs.</td>
</tr>
<tr>
<td>7. Wash in tap water</td>
<td>rinse</td>
</tr>
<tr>
<td>8. Saturated aqueous picric acid sol.</td>
<td>2 min. 6 min.</td>
</tr>
<tr>
<td>9. Dehydrate in 95% and 100% alcohol, 10 seconds and 1 minute respectively, clear in two changes of xylene, one minute each, and mount in Clarite, HSR, or other synthetic resin or in Canada balsam.</td>
<td></td>
</tr>
</tbody>
</table>

The color of the positive reaction is somewhat more purple than a plain basic fuchsin stain. In a similar procedure utilizing 0.5 N alcoholic hydrochloric acid as the decolorizing reagent in place of the tartaric acid + zinc hydrosulfite mixture, I have interposed also a five-minute stain in Weigert's acid iron chloride hematoxylin before the picric acid step.

Gridley (Am. J. Clin. Path. 23:303, 1953) has modified the Bauer stain
for demonstration of fungi in tissues by adding counterstains with Gomori’s aldehyde fuchsin and metanil yellow.

Gridley’s Technic for Fungi in Tissue Sections.

1. Paraffin sections at 6 μ are deparaffinized and hydrated as usual. Rinse in distilled water.
2. Oxidize one hour in 4% chromic acid.
3. Wash five minutes in running water.
4. Immerse in Schiff reagent (0.5% fuchsin, p. 155) for 15 minutes.
5. Rinse in three changes of 0.5% sodium metabisulfite Na₂S₂O₅ in N/20 hydrochloric acid, two minutes each.
6. Wash 15 minutes in running water.
7. Stain 15–20 minutes in Gomori’s aldehyde fuchsin (p. 364).
8. Rinse in 95% alcohol and wash well in water.
9. Counterstain 2–5 minutes in 0.25% metanil yellow in 0.25% acetic acid.
10. Wash in water, dehydrate, clear, and mount in Permount.

Results: Hyphae, deep blue; conidia, rose to purple; elastin and mucin, deep blue; yeast capsules, deep purple; general background, yellow.

For staining nuclear chromatin in certain yeastlike fungi, DeLamater (Stain Technol. 23:161, 1948) recommends a procedure of acid hydrolysis and aldehyde mordanting followed by staining in basic fuchsin.

DeLamater’s Formaldehyde Fuchsin Method.

1. Fix (cultures) in Schaudinn’s fluid (p. 38).
2. Hydrolyze five minutes at 30° C., five minutes at 60° C., and then five minutes at 30° C. in 1 N HCl.
3. Wash in 1–3 changes of distilled water.
4. Mordant in 2% formalin four minutes. (2 cc. 40% HCHO + 98 cc. H₂O.)
5. Wash in distilled water.
6. Stain 15 minutes in 0.5% aqueous basic fuchsin acidified to 0.04 N with hydrochloric acid (to 50 cc. of stain add 2 cc. 1 N HCl).
7. Wash in distilled water.
8. Dehydrate and decolorize in graded alcohols.
9. Clear in xylene and mount in balsam or synthetic resin.

Results: Nuclei stain an intense magenta red, cytoplasm light pink.

The streptothrices are usually Gram positive and may be acid-fast, hence the technics for Gram-positive (pp. 369–373) or acid-fast (pp. 378–383) organisms may be used. Their reaction to the formaldehyde fuchsin methods of Goodpasture and of Fite I do not know.
Other mycelial fungi in tissues may be studied with Gram-Weigert, with azure-cosinate technics, and with the Bauer method.

The Alkali Method. A useful quick method for epidermal fungi is to scrape off material from the suspected area and macerate on the slide under a cover glass in 20% (Mallory) or 15% (Schmorl) sodium (or potassium) hydroxide solution. The epidermal cells are dissolved or cleared, leaving the fungal mycelia as refractile, perhaps branching, and often septate filaments and spores. Reduced illumination is often desirable for study of details by this method. This technic may be used for identification of the fungi of ringworm, favus, epidermophytosis, and the like.

**Diphtheria Organisms**

Christensen (Stain Technol. 24:165, 1949) recommends a method with sequence of acid toluidin blue, iodine, and safranin to replace Albert's method for diphtheria organisms. I have not tried Christensen's method.

**Christensen's stain for C. diphtheriae.** Use air-dried, heat-fixed smears of the usual Loeffler-medium cultures.

1. Stain one minute in 0.15% toluidin blue (52% dye content), 5 cc. glacial acetic acid, 2 cc. ethyl alcohol, and 100 cc. distilled water.
2. Wash with water and apply Albert's iodine solution (iodine 2 Gm., potassium iodide 3 Gm., water 300 cc.) for one minute.
3. Wash with water and counterstain with safranin.

**Results:** Cell bodies in light pink; protoplasmic striations in red or brownish red; metachromatic granules in black.

Excellent results may also be attained by simple, brief (1-2 minutes) staining in polychromed methylene blue. Loeffler's solution when aged several years performs excellently. For a freshly prepared solution I suggest 0.1% azure A in the same solvent. The orthochromatic color is blue violet, the metachromatic red purple. Loeffler's methylene blue: 0.3 Gm. methylene blue in 30 cc. alcohol + 1 cc. 1% potassium hydroxide and 90 cc. distilled water.

**Influenza Bacilli and Encephalitozoa**

The Goodpasture-Perrin (Arch. Path. 36:568, 1943) method for influenza organisms, encephalitozoa, and toxoplasmata:

1. Zenker or Orth fixation preferred, but Perrin found 10% formalin material postchromated with 2.5% potassium bichromate for two days quite satisfactory.
2. Paraffin sections to water as usual including iodine and thiosulfate sequence if Zenker material is used.
3. Stain in carbol aniline fuchsin: basic fuchsin 0.59 Gm., 30% alcohol
100 cc. aniline 1 cc., phenol 1 Gm.; for five minutes at 70° C.—steaming on hot plate.

4. Rinse quickly in tap water.

5. Decolorize with strong formalin (40% formaldehyde), a few drops at a time, until no more color is removed, 15–20 minutes.

6. Rinse in tap water.

7. Counterstain one minute in saturated aqueous picric acid solution.

8. Dehydrate with two changes of 95% and two of 100% alcohol. Clear with 100% alcohol and xylene followed by two changes of xylene. Mount in clarite.

Results: Encephalitozoa are blue black; the chromatin of toxoplasma is brownish red; cell nuclei are light red; cytoplasm, pinkish yellow; erythrocytes, bright yellow; influenza bacilli, blue.

The Wright and Craighead method as modified by Perrin (loc. cit., supra) for encephalitozoon and toxoplasma: Formalin fixation with 48 hours' postchromation in 2.5% potassium bichromate, or Orth fixation. Paraffin sections to water as usual.

1. Stain ten minutes at 70° C. in carbol fuchsin (p. 379).

2. Rinse in tap water.

3. Differentiate with concentrated formalin (40% HCHO) from dropping bottle until no more color is removed.

4. Rinse in tap water.

5. Stain four minutes in methylene blue 1 Gm., alcohol 20 cc., glacial acetic acid 0.5 cc., distilled water 80 cc.

6. Dehydrate with acetone, clear with 50:50 acetone xylene mixture followed by two changes of xylene. Mount in Clarite.

Results: Encephalitozoa are stained deep bluish red; cell nuclei and toxoplasmata, blue; cytoplasm, light blue to pink.

Acid-fast Bacteria

Fite's New Fuchsin-Formaldehyde. Except for the acid alcohol step contained therein, Fite's (Am. J. Path. 14:491, 1938) method for acid-fast bacilli is quite similar to the foregoing:

1. Stain paraffin sections 30–60 minutes or more at room temperature in: phenol 5 Gm., ethyl or methyl alcohol 10 cc., new fuchsin (C. I. No. 678) 1 Gm.; dissolve and add distilled water to make 100 cc.

2. Immerse in concentrated formalin (40% HCHO) for five minutes.

3. Decolorize nearly completely with acid alcohol (2 cc. concentrated hydrochloric acid, 98 cc. 95% alcohol).

4. Immerse again in concentrated formalin for a few seconds.

5. Counterstain with hematoxylin and Van Gieson's picrofuchsin. Dehy-
drate, clear, and mount through 95% and 100% alcohol, 100% alcohol and xylene, and two changes of xylene to Clarite.

**Results:** Acid-fast bacilli are stained violet; hair shafts, keratohyalin and mast-cell granules are decolorized. The behavior of ceroid with this stain has not been reported.

*Fite later considerably modified this technic* (National Institutes of Health technic files), thus:

1. Stain 12–24 hours at 60° C. or 1–2 days at 25° C. in new fuchsin 0.5 Gm., phenol 5 Gm., 100% alcohol 10 cc., distilled water to make 100 cc.
2. Wash in water.
3. Decolorize ten minutes in 2 cc. concentrated hydrochloric acid (37.5%) and 95% alcohol to make 100 cc.
4. Wash in water.
5. Treat in freshly redistilled formaldehyde of 5–30% strength, five minutes.
6. Wash in water.
7. Oxidize in 1% potassium permanganate 2–5 minutes.
8. Wash in water.
9. Reduce with 2% oxalic acid one minute.
10. Wash in water.
11. Alum hematoxylin two minutes.
12. Wash in water.
13. Stain three minutes in 0.1% acid fuchsin in saturated aqueous picric-acid solution.
14. Dehydrate with two changes of 95% alcohol and two of 100%.
15. 100% alcohol + xylene, then two changes of xylene and mount in Clarite or balsam.

**Results:** Acid-fast bacilli, blue; nuclei, brown; collagen, red; smooth muscle, yellow.

I believe Fite's staining time in the new fuchsin is unduly prolonged; but the long staining at room temperature, which he prefers, probably does no harm.

The **carbol fuchsin–methylene blue method** for acid-fast organisms: The stock carbol fuchsin is traditionally composed of saturated alcoholic solution of basic fuchsin (C. I. No. 677) 10 cc. and 5% aqueous phenol solution 90 cc. This solution keeps for years. Rosanilin chloride is soluble to about 6% in alcohol, pararosanilin chloride about 3.5% of commercial samples, 8.16% and 5.93% of the pure substances according to Conn. Neelsen (*Centralbl. med. Wiss.* 21:497, 1883; *Fortschr. d. Med.* 3:200, [footnote] 1885) prescribed first 0.75, later 1.0 Gm. fuchsin in 100 Gm. 5% phenol, adding "a little" alcohol, which he later specified as 10 Gm. Conn prescribes 300 mg. fuchsin, 10 cc. alcohol, 5 Gm. phenol, and 95 cc. distilled water. Kinyoun's
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(\textit{Am. J. Pub. Health} 5:867, 1915) formula—fuchsin 4 Gm., phenol 8 Gm., alcohol 20 cc.; heat to dissolve and then add water 100 cc.—is far stronger and is said to be a more energetic stain. This may well be true when the stain is freshly prepared, but it soon deposits a considerable quantity of excess dye, and thereafter is probably no better than other formulae.

Mallory recommends Verhoeff's formula. This is kept as a stock solution which is diluted at the moment for use. Dissolve 26.8 Gm. phenol (25 cc. melted crystals) in 50 cc. 100% alcohol. Add 2 Gm. fuchsin and heat at 37° C. with occasional shaking for 18–24 hours. Filter and store. For use dilute 1 cc. of stock solution with 6 cc. water. This represents a final dilution of 0.281% fuchsin, 9.53% alcohol, and 4.76% phenol. The main advantage seems to be in the permanency of the stock solution. In view of the usual stability of the usual formula, this advantage may be outweighed by the disadvantage of having to dilute it for use.

Fite (\textit{Am. J. Path.} 14:491, 1938) recommended new fuchsin (C. I. No. 678) and prescribed thus: 1 Gm. dye, 5 Gm. phenol, 10 cc. methyl alcohol; dissolve completely, and then add gradually with shaking enough distilled water to make 100 cc. Later he reduced the dye to 0.5 Gm. and used ethyl or methyl alcohol.

I now dissolve 25 Gm. phenol in 50 cc. alcohol, add and dissolve 5 Gm. fuchsin, and then dilute to 500 cc. with distilled water.

Carbol fuchsin solutions gradually form a dark-red caked deposit which fails to redissolve on warming and shaking. This deposition results in progressive weakening of the solution. Consequently positive controls should be used at frequent intervals to avoid false negatives from stain failure. While some batches of solution have remained effective for years, others become useless in as little as a year.

With any of the carbol fuchsin or carbol new fuchsin solutions the sections are heavily stained either by means of heat or by prolonged exposure, and then decolorized with acids, alcohol, or usually both. The heating methods occasion somewhat more section shrinkage and definitely more dye precipitation from evaporation. I have tried adding glycerol to carbol fuchsins to prevent drying while heating, but abandoned it because bacilli appeared less well stained. Fite's observation that previously heated and cooling or cooled carbol fuchsin stains more brilliantly than unheated, just as does hot carbol fuchsin, appears to indicate that supersaturation plays an important part in brilliancy of staining. This would also account for the greater vigor of staining obtained with the freshly prepared (and supersaturated) Kinyoun's solution.

\textit{Technic for Carbol Fuchsin and Methylene Blue.}

1. Bring paraffin sections to water as usual. (Smears are prepared as usual and heat-fixed.)
2. Stain 10 minutes at 70° C., 30 minutes at 55° C., 2 hours at 37° C. or 4-16 hours at 25-20° C., with any of the carbol fuchsins.

3. Wash in water.

4. Decolorize with 2 cc. concentrated hydrochloric acid + 98 cc. 95-70% alcohol. This ordinarily takes 20 seconds or more and may be extended to several minutes without harm.

5. Wash 2-3 minutes in running water.

6. Counterstain with acid hemalum (p. 76) for 2-5 minutes, or with 1% methylene blue or 1% Janus green B in 1% acetic acid, 20% alcohol for three minutes. (The latter two counterstains are suitable for smears.)

7. Wash in water. (At this point smears are dried in a warm air stream and examined directly in immersion oil.)

8. Dehydrate and clear with an acetone xylene sequence and mount in Clarite.

**Results:** Acid-fast bacilli, red; ceroid, red; nuclei, blue or green; mast-cell granules, blue violet with methylene blue, but unstained by hematoxylin. Red corpuscles are often pink in formalin material; and hair shafts and keratohyalin may retain more or less red.

By acidifying the counterstain the dense staining seen with ordinary methylene blue counterstains is avoided. This was at least part of the value of the Gabbett (Lancet 1:757, 1887) solution (2 Gm. methylene blue in 25 cc. concentrated sulfuric acid and 75 cc. distilled water: 48.8 Gm. sulfuric acid per 100 cc.) which was recommended for simultaneous decolorization and counterstaining. Gabbett did not specify the amount of methylene blue. This was supplied in the 1901 and later editions of Mallory and Wright. Its omission of alcohol was its most serious defect. Picric acid counterstains have been used by some workers, with good contrast, but give no tissue detail, fail to show leukocytes and non-acid-fast organisms, and, according to Fite, cause fading of the fuchsin stain.

In clearing of sections, carbol xylene should be avoided. It decolorizes nearly all of the previously well-stained tubercle bacilli and even impairs the acid-fast staining of ceroid.

Similar acid-fast stains may be achieved, with opposite color effects, by substituting night blue (C. I. No. 731) or Victoria blue R (C. I. No. 728), for fuchsin in my carbol fuchsin formula on p. 380, and using 0.1% safranin in 1% acetic acid as a counter stain. The technic follows that above. Acid-fast bacilli appear in dark blue, cell nuclei in red.

**The Fite-Faraco Oil Fuchsin Method.** Lepra bacilli sometimes fail to stain by the foregoing procedure. Fite recommends (from MS) the following modification of Faraco's procedure.

1. Deparaffinize with two changes of a mixture of 1 part of cotton seed, peanut, or olive oil with 2-3 parts of xylene. Fite states that various other light, nonvolatile oils can be used, including liquid petrolatum.
2. Drain, wipe the back and sides of slide, blot section with filter paper.
3. Treat with (aqueous) Weigert's iodine solution (p. 92) and with aqueous (5%) sodium thiosulfate solution to remove mercury precipitates, if necessary. If not, omit this step.
4. Wash in water.
5. Stain 15–20 minutes in Neelsen's or other carbol fuchsin of similar fuchsin concentration, at room temperature.
6. Decolorize for 1–2 minutes to a faint pink color with 1 cc. concentrated hydrochloric acid in 99 cc. 70% alcohol.
7. Wash in tap water.
8. Counterstain with methylene blue, wash in water.
9. Blot and let stand a few minutes to dry. Mount in Clarite or similar synthetic medium.

Results: Acid-fast bacilli red; other structures blue. I have had good results from this method with lepromata in which no lepra bacilli had been demonstrated by the previous method (p. 380).

Blanco and Fite (Internat. J. Leprosy 16:367, 1948) found that aqueous formalin fixation gave fair to good staining of lepra bacilli in biopsy material from human skin, using the Ziehl-Neelsen and oil fuchsin (above) technics. Zenker fixation gave excellent results, Bouin poor, and alcoholic 20% formalin fair.

For preservation of the acid-fast material in lepra bacilli, Wade (Stain Technol. 27:71, 1952) recommends Carbowax imbedding and sectioning (p. 52) and one of the oil fuchsin technics.

Hagemann's Phenol Auramine. Hagemann's (Münch. med. Wchnschr. 85:1065, 1938) technic of staining smears with phenol auramine, and examining with fluorescence microscopy (pp. 11–15) has been widely used. The principal advantages claimed are that organisms are readily discerned at lower magnifications than with Ziehl-Neelsen technics, and hence larger areas can be scanned in the same time. Use of magnifications as low as 180 × is claimed for scanning; but to determine morphologic characteristics 600 × was needed. Lempert (Lancet 247:818, 1944) used a 16-mm. (1/4 in.) objective for focusing the lamp and condenser system, and a 6-mm. (1/4 in.) objective for identification. The use of immersion objectives is unnecessary, but should one desire to use these for higher magnification, Lempert advises the use of glycerol for immersion, because of the fluorescence of cedar oil. Our white modified mineral oils (Shillaber's, Crown) are designated "low fluorescence" and "very low fluorescence" for high and low viscosities, and should be usable.

Hagemann dissolved 1 Gm. auramine (C. I. No. 655) in 100 cc. 5% phenol, Lempert used 300 mg. in 100 cc. 3% phenol and filtered after vigorous shaking and warming to 40° C. The technic follows:
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1. Stain heat-fixed films 8-10 minutes at room temperature in Lempert's phenol auramine.
2. Wash well in tap water.
3. Decolorize in two changes of two minutes each of hydrochloric acid alcohol. Lempert used 0.5 cc. concentrated hydrochloric acid, 0.5 Grm. sodium chloride, 25 cc. distilled water, and 75 cc. methanol. Fite used his usual 2 cc. concentrated hydrochloric acid and 98 cc. 95% alcohol mixture.
4. Wash well in tap water.
5. Treat for 20 seconds with 0.1% potassium permanganate solution.
6. Wash in water, dry at room temperature in an air current, and examine.

Results: The bacilli appear as bright yellow rods on a very dark red background. The method has not been found adaptable for histologic study. Part of the alleged superiority of the method disappears when one uses acetic methylene blue as a counterstain for Ziehl-Neelsen stains, either of smears or tissues. We have often readily found tubercle bacilli by this latter technic with an 8 mm. objective and the tissues and other non-acid-fast organisms are seen as well.

Blanco and Fite (Arch. Path. 46:542, 1948) have used a modified Jahnel procedure for the demonstration of lepra bacilli in tissues. While much too prolonged for regular diagnostic use, the procedure is said to afford a truer picture of the relations of the organisms to the tissues than most of the commoner methods.

Blanco and Fite's Silver Method for M. leprae.

1. Fix in 10% formalin for two or more weeks.
2. Soak blocks in pyridine 1-3 days.
3. Wash 24 hours in several changes of distilled water.
4. Immerse in 10% formalin four days.
5. Wash 24 hours in several changes of distilled water.
6. Treat with 95% alcohol 3-8 days, changing alcohol daily.
7. Transfer to distilled water until blocks sink.
8. Incubate at 37° C. in the dark in 0.5% silver nitrate for 5-8 days.
9. Wash ten minutes in distilled water.
10. Reduce two days in pyrogallol 4 Grm., formalin 5 cc., distilled water 95 cc.
11. Dehydrate, clear, and imbed in paraffin as usual.
12. Section, deparaffinize, and mount.

Results: Most lepra bacilli black, some brown. Melanin and keratohyalin are also blackened. Tubercle bacilli and spirochetes should also blacken by this method.
Bacterial Spores

Bartholomew and Mittwer (Stain Technol. 25:153, 1950) modified the bacterial spore stain of Schaeffer and Fulton (Science 77:194, 1933) with some simplification, thus:

1. Fix smears on slides by passing through flame
2. Stain in malachite green 3 times 20 times
3. Rinse in water 5%, 4 or 5 Sat. sol. (7.6%) times in 1 min. 10 min. cold
4. Stain in safranin 0.5%, 30 sec. 0.25%, 15 sec.
5. Rinse, blot dry, and examine

Results: Bacterial bodies, red to pink; spores, green.

Bacterial Cell Envelop

Hale (Lab. Pract. 2:115, 1953) stains bacterial cell envelopes by first mordanting unfixed smears for 5–10 minutes in 1% phosphomolybdic acid and then staining for a few seconds in 1% methyl green or 0.1% Janus green.

Bacterial Mitosis

DeLamater and Mudd (Exper. Cell Research 2:499, 1951; 504) demonstrate bacterial mitosis by the following procedure:

1. Cut a small slab of agar from a 2-hour-old 37° C. culture. Place on a small glass plate (slide), stand upright in a Coplin jar containing a little 0.5% osmium tetroxide, and cover the jar for five minutes.
2. Cut small fragments of the agar and press face down on cover glasses or slides to make impression preparations.
3. Hydrolyze six minutes in normal hydrochloric acid at 60° C. (preheated).
4. Rinse in distilled water.
5. To 10 cc. 0.25% aqueous thionin add 1 drop thionyl chloride, mix well, and stain preparations in this for two or more hours.
6. Rinse once in distilled water to remove stain particles, drain on filter paper, and
7. Immerse in 100% alcohol in jars surrounded by solid carbon dioxide to maintain a temperature below −50° C. Let stand 12 hours to complete dehydration.
8. Dip in fresh 100% alcohol at +25° C. Clear in xylene, drain on filter paper, and mount in synthetic resin.

The reaction of thionin (or azure A, which may also be used in the foregoing technic) in the presence of the HCl and H₂SO₃ formed by hydrolytic
decomposition of the thionyl chloride $\text{SOCl}_2$, is considered by the authors to be an aldehyde reaction. In a sense, the procedure parallels the Feulgen method, but uses an undecolorized thionin sulfurous acid complex in place of Schiff reagent.

**Gram-negative Bacteria and Rickettsiae**

Gram-negative bacteria and rickettsiae in tissues are generally best studied with stains of the azure-eosin type, such as Mallory's phloxine + borax methylene blue, Maximov's hematoxylin azure II eosin, or our buffered azure eosinate and Noccht azure eosin variants. Giemsa's blood stain in 1:40 to 1:50 dilution has been much used. Wolbach added 5 drops of 0.5% sodium carbonate to 100 cc. final stain mixture and differentiated after staining with colophonium alcohol. I prefer to buffer to a relatively acid level, say pH 4.0 for formalin material, and thus obviate the necessity for differentiation. Except for Wolbach's variant, these methods have been given under general methods (pp. 116–120).

**Wolbach's Giemsa Variant** *(The Etiology and Pathology of Typhus, Cambridge, Harvard University Press, 1922, pp. 13–14).* Fix thin slices of tissue 24–48 hours in Zenker's or Moller's (Regaud's) fluid (p. 45). Cut thin paraffin sections and take to water as usual.

1. Stain one hour in Giemsa's stain (pp. 386–388) 1 cc., methyl alcohol 1.25 cc., 0.5% sodium carbonate solution 0.1 cc. (2 drops), distilled water 40 cc.
2. Pour off and replace with two further changes of the same mixture during the first hour and leave in the third change overnight.
3. Differentiate in 95% alcohol containing a few drops of 10% colophonium alcohol.
4. Dehydrate with 100% alcohol, clear in xylene, and mount in cedar oil.

**Results:** Rickettsiae stain an intense reddish purple; nuclei, dark blue to violet; cytoplasm, in varying lighter blue shades; collagen and muscle, pale pink; erythrocytes, gray to yellow or pink. Further differentiation occurs after mounting in cedar oil; and exposure to sunlight for prolonged periods has been used to bring sections to the proper point. I have not used this method extensively, but would caution against identifying as rickettsiae the metachromatic granules of tissue mast cells, which also stain in redder shades than nuclear chromatin.

There are a number of methods used for the identification of rickettsiae in smears. Among these one of the most useful has been the Macchiavello technic *(Zinsser's Epidemiology and Immunity in the Rickettsial Diseases, Cambridge, Harvard University Press, 1940, p. 896).*

**The Macchiavello Method.** The following is Bengtson's variant, as used at the National Institutes of Health:
1. Fix thin films by passing quickly, face down, through a blue flame three times.

2. Stain five minutes in: basic fuchsin 0.5 Gm. (saturated), 0.1 M disodium phosphate 3.6 cc., 0.1 M sodium acid phosphate (NaH₂PO₄·H₂O) 1.4 cc., distilled water 95 cc. Filter. This buffer corresponds to pH 7.2 (p. 451).

3. Rinse rapidly with 0.5% aqueous citric acid solution.

4. Wash thoroughly in tap water.

5. Counterstain 1–2 minutes in a 0.1% aqueous methylene blue, or 10 seconds in a 1% solution.

6. Rinse with water, dry, and examine in immersion oil.

**Results:** Rickettsiae, red; cells and bacteria, varying shades of blue. Thus far this method has not been adapted to tissue sections.

The other method most often used is the Giemsa stain. With this one may either stain at pH 7.0–7.2 and then differentiate with faintly acid water until red corpuscles are pink, or one may stain at pH 6.5–6.0 and simply rinse and dry smears. Smears of blood, marrow, pus, or exudates should be spread thin so that in most areas cells lie separated from each other. These smears should be fixed at once with methyl alcohol or 100% ethyl alcohol for two and five minutes respectively.

Nyka (J. Path. & Bact. 67:317, 1945) recommends a relatively simple methyl violet-metanil yellow technic for typhus rickettsiae in mouse lungs:

Fix in 10% neutral formalin. The usual imbedding and sectioning procedures are presumed, and sections are deparaffinized and hydrated as usual.

1. Stain 30–60 minutes in 1:10,000 aqueous methyl violet.

2. Differentiate in weak acetic acid (0.03–0.04%: 2 drops glacial acetic acid in 100 cc. distilled water) until cell cytoplasm is decolorized.

3. Counterstain a few seconds in 1:10,000 aqueous metanil yellow.

4. Dehydrate and clear with an acetone xylene sequence.

5. Mount in synthetic resin. Gurr's Xam and the Media Manufacturing Centre's DPX4 are mentioned by the author. Our American synthetic resins such as Clarite, Permount, polystyrene, etc. will probably serve.

In the few preparations that I have seen stained by this method, rickettsiae are still difficult to distinguish from other basophilic granules and mast-cell granules.

**Blood, Tissue, and Protozoa**

Giemsa's stain is also widely used for the study of the morphology of blood, spleen, and marrow cells and for the identification of protozoan parasites such as trypanosomes, leishmaniae, plasmodia, and bartonellae.

The stain is best purchased from a reputable dye manufacturer. Insist on certification by the Biological Stain Commission. Either the prepared glyc-
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erol methanol solution or the dry mixed powder may be obtained. The latter should be dissolved in equal volumes of glycerol and methanol (methyl alcohol) at 800 mg. per 100 cc. of the mixed solvent. Although a small undissolved residue is found on dissolving Giemsa stains in amounts over 300 mg. per 100 cc., the solution increases in strength, staining capacity, and optical density with further addition of dye—the last in proportion to the total amount of dye added, up to 1.1 Gm. per 100 cc. Consequently the presence of a small residue is not to be taken as evidence of saturation of the glycerol methanol solvent. Quantities in excess of 1.1 Gm. per 100 cc. occasion no further increase in staining power nor optical density.

The best solvent appears to be an equal volume mixture of C. P. methanol and neutral C. P. glycerol. Special treatment of the methanol to render it acetone-free is expensive and of no discernible value. Glycerol may be of either 95% or 98% strength without affecting the quality of the stain. More important than traces of acid in the reagents are traces of alkali. A trace of acid actually acts more as a stabilizer of the azures and methylene blue against the alteration which occurs spontaneously in glycerol methanol solutions, and is readily overcome in staining by the use of the appropriate buffers. Traces of alkali, on the other hand, fairly rapidly convert methylene blue and azure B into lower azures and thiazoles and alter the staining effect profoundly.

The older German texts required equal weights of glycerol and methanol. This corresponds nearly exactly to 60 volumes of methanol and 40 of glycerol. Giemsa stain deteriorates somewhat more rapidly in this mixture than in the equal volume mixture. The 75:25 mixture used for MacNeal's stain appears to be little or no better for preserving the stain than plain methanol.

An equal-volume mixture of 100% ethyl alcohol and 98% glycerol appears to be as good a solvent for Giemsa stain as the corresponding methanol mixture, and to preserve the stain at least as well.

The recommended composition of Giemsa stain, using American dyes, is as follows: Methylene blue eosinate 4 Gm., azure B eosinate 5 Gm., azure A cosinate 1 Gm., and methylene blue chloride (85-88% dye content) 2 Gm. The mixture should be kept in a cool dry place, tightly stoppered.

It is not recommended that the eosinates be made in the laboratory, particularly not from commercial azure B, or from methylene blue unless special precautions are taken.

Commercial azure B is apparently a quite variable substance, containing varying proportions of methylene blue and azure A, often enough of one or the other to considerably modify the character of the Giemsa stain. Further, eosinates of azure B and of methylene blue are quite susceptible to partial demethylation ("polychroming") on drying even at moderate (55-60° C.) temperatures.

A quite constant, though perhaps crude, azure B eosinate is made by the following process, which is substantially that used by some of the American
Dissolve 10 Gm. methylene blue in 600 cc. distilled water. Add 7 cc. concentrated (96.5%) sulfuric acid (sp. gr. 1.84). Heat to boiling. Add 2.5 Gm. potassium bichromate previously dissolved in 25 cc. distilled water. Boil for 20 minutes. Formaldehyde is given off in the process. Cool to 10° C. Add gradually 21 Gm. sodium bicarbonate, allowing evolution of gas to cease between each addition. Dissolve 9 Gm. eosin Y (89–93% dye content) in 90 cc. distilled water. Pour about 80 cc. of this into the neutralized dye solution, agitating the while. Then cautiously add more eosin until the solution appears distinctly pale blue between particles of precipitate on splashing. Filter with vacuum on a large Buchner funnel. When the surface of the precipitate appears and just dries but does not crack, add 50 cc. distilled water, continuing the suction. When this is filtered off add another 50 cc. portion of distilled water and filter off; and then two successive portions of 95% ethyl alcohol as before. Then remove the filter and precipitate and dry on a larger sheet of filter paper on a warm plate at 35–40° C.

To make azure A eosinate proceed exactly as above, except that the quantity of potassium bichromate is doubled, that is, 0.5 Gm. per gram of methylene blue.

The Giemsa Stain for Films.

1. Thin films should be fixed as soon as taken in methyl alcohol for 3–5 minutes (see pp. 366–368 for preparation of films). Thick films are first hemolyzed and then fixed or stained without fixation.
2. Stain 40–120 minutes in 1 cc. Giemsa stain, 2 cc. stock buffer, and 47 cc. distilled water. For marrow a phosphate buffer (p. 451) of pH 5.8–6.0 is preferable; for blood cytology pH 6.4 or 6.5; for both blood and protozoa some prescribe pH 6.8; and for malaria survey work on thick films pH 7.0–7.2 is prescribed. For most things I use a pH 6.5 buffer.
3. Rinse in distilled water, dry, and examine. For thick-film malaria staining Wilcox uses a pH 7.2 stain and prolongs the distilled-water washing for some minutes. This takes out some of the excess basic dye deposited at the higher pH level. Use of a pH 6.5–6.8 buffer obviates this differentiation.

The 40-minute stain is adequate for blood work and for tertian and quartan parasites. For staining of Schüffner's granules or undulating membranes, longer staining up to two hours is often desirable. Platelets are often well demonstrated by these technics (better than by Wright's stain) with purple central granule and pale blue periphery.

Accelerated Giemsa Stain for Thick Films. Stain unfixed thick films, after one hour's drying, in: Giemsa stain 4 cc., acetone 3 cc., pH 6.5 buffer 2 cc., distilled water 31 cc. Stain 5–10 minutes, rinse in distilled water, dry, and
examine. By this brief staining one avoids the loss of thick films so often suffered with brief drying periods.

Methods for protozoa in sections are discussed on pp. 79, 120, 129, 133 and 385.

Wright's and Leishman's stains are compounds of eosin Y with methylene blue altered by the action of alkalies so as to contain a variable amount of azure B, azure A, and methylene violet. The stains are best procured as dry powders from commercial manufacturers, and should bear the certificate of the Biological Stain Commission.

As so certified, these stains usually consist chiefly of the eosinates of azure B and of methylene blue, regardless of the method of manufacture. Spectroscopically they should present two absorption bands of nearly equal density, the one at about 517 mμ (eosin) in a proportion of about 0.9:1.0 of the other, which represents the blue component, lying preferably between 652 and 658 mμ. A satisfactory stain with these characteristics may be made by mixing equal parts of the methylene blue and azure B eosinates as prepared for the Giemsa stain (p. 388).

The solvent recommended is C. P. methyl alcohol (methanol) which meets American Chemical Society specifications. Redistillation from silver oxide and sodium hydroxide to prepare a neutral, acetone- and aldehyde-free methyl alcohol, has been found to be unnecessary.

I prefer Wright's original prescription of 0.5 Gm. per 100 cc. methanol to the weaker solutions in vogue. Although some residue remains undissolved when as little as 150 mg. per 100 cc. is used, the solution increases in optical density and staining power up to 600 or 700 mg. per 100 cc., and it is probable that the solubility lies in that range.

One of the advantages usually urged for Wright's and Leishman's stains is that the undiluted stain may be used for fixation, and water then added for the staining period. I prefer to have films fixed at once at the bedside in methanol, and then later, on return to the laboratory, mix stain and water in a test tube in a proportion of 1 cc. stain to 3 cc. water, allowing 2 cc. total for each slide to be stained. The water should be buffered by addition of 1 cc. of M/15 phosphates at pH 6.5 for each 20 or 25 cc. water (p. 451). If it is necessary to use other than distilled or rain water, a larger amount of stock buffer may be needed, or a solid buffer (p. 451) may be added in such quantity as is found necessary under local conditions.

The Technic for Wright Stain.

1. Fix films in methanol for 2–3 minutes (or deposit 0.5 cc. stock stain on each slide and let stand two minutes).

2. Deposit 2 cc. of a 25% dilution with buffered water of the stock stain on each slide (or add 1.5 cc. water to the stock stain on the slide). Let stand 3–5 minutes.
3. Rinse in water, dry in an air stream (or with compressed air), and examine in immersion oil. Modified mineral oils preserve the stain if left on, while cedar oil decolorizes the blue and purple elements.

Lillie’s Wright Stain Technic. Superior results in regard to the sharpness of nuclear and parasite chromatin staining may be achieved by using a 1% solution in equal volumes of glycerol and methanol. Thus: Fix films 2–3 minutes in methanol. Stain five minutes in 4 cc. stock stain, 3 cc. acetone, 2 cc. M/15 pH 6.5 phosphate buffer, and 31 cc. distilled water in a Coplin jar. Rinse, dry, and examine. Such a mixture may be used at once for a second group of 10 slides with only slightly inferior results.

A slower, similar method uses 2 cc. of stock 1% stain, 2 cc. of pH 6.5 buffer, and 46 cc. water, and requires 20–30 minutes.

General Results of Romanovsky Stains on Films. With all of these Giemsa, Wright, Leishman, and similar Romanovsky stains, the cytoplasm of lymphocytes should be a clear medium blue; their nuclei, a deep purple to violet; chromatin of malaria parasites and of trophonuclei of trypanosomes, red purple; of blepharoplasts, a darker, perhaps more violet purple; undulating membranes, pink; cytoplasm of plasmodia and trypanosomes, light blue; nuclei of monocytes, lighter red-purple; their cytoplasm an opaque, faintly bluish gray; central granules of blood platelets, red purple; periphery, light blue; the granules of mast leukocytes are deep blue violet; eosinophil granules are orange pink; neutrophil granules, purple to violet. Bartonellae appear faintly blue with red-purple chromatin dots, and are best discerned when stained with Giemsa stain at pH levels between 6.2 and 6.8. Bacteria stain deep blue to violet. Diphtheroids may exhibit deeply stained bars and polar granules in a light-blue body. The color of erythrocytes varies with the pH of the diluting water from pink at pH 6.0 through yellowish pink at 6.5, pinkish or grayish yellow at 6.8, grayish yellow to greenish yellow or even gray blue at 7.0–7.2. Differentiation with distilled water which is often faintly acid or with very dilute (1:1,000 or weaker) acetic acid displaces the color of erythrocytes from the gray-blue toward the pink limit of the color series above. Eosinophil granules are often best stained when chromatin is much understained and pale blue in color.

Sudanophil Granules of Leukocytes

These granules occur in neutrophil and eosinophil leukocytes, monocytes, and probably basophil granulocytes as well, in general in much the same cells as show peroxidase and indophenol oxidase activities. They do not stain in the cold with the usual oil-soluble dye methods which suffice for the staining of true fats and lipoids. At temperatures of 20–25°C, they require prolonged exposures to the naphthol type oil-soluble dyes such as oil red O, Sudan III, and Sudan IV. Naphthylamine and aminoanthraquinone dyes act more rapidly, but still do not stain in the usual 5–10 minute intervals.
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used for lipoids. Reasonably rapid staining, 1–2 hours for oil red O for example, may be attained at 37° C., but elevation of temperature to 55–60° or higher appears to be positively deleterious in comparison with 37° C. staining.

After Savini’s and Sehrt’s papers (Wien. med. Wchnschr. 46:1964, 1921; Compt. rend. Soc. de Biol. 87:744, 1922; München. med. Wchnschr. 74:139, 1927) these granules were generally accepted as lipoid in nature, though a number of writers commented on the resistance of Sudan black stains to extraction with 100% alcohol or with xylene. Lillie and Burtner (J. Histochem. & Cytochem. 1:8, 1953) demonstrated their water-soluble nature, their resistance to extraction with a variety of fat solvents (aromatic and aliphatic hydrocarbons, pyridine, carbon tetrachloride, etc.), their unstainability with esterified Sudans (p. 304), and their capacity to react with alcohols and phenols; and concluded that they were not lipoid. Since they are readily rendered unstainable by alcohol of 60% concentration or higher at 60° C., staining is preferably done at 50% alcohol concentration. Indeed I have seen decolorization of previously well-stained preparations in the customary saturated 70% alcohol solutions of Sudan dyes.

Lillie and Burtner’s Technic for Staining Sudanophil Granules in Leukocytes.

1. Fix air-dried blood films ten minutes in 75% alcohol, in 75% alcohol containing 10% formalin, or with formaldehyde gas over a little strong formalin (p. 368). Wash briefly in water to remove excess formaldehyde.
2. Mix 0.5% oil red O in 100% alcohol or, better, 99% isopropanol with an equal volume of distilled water and place in oven or water bath at 37° C. Stain smears 2–4 hours. (Fewer but larger granules are shown by staining 30–60 minutes in dye mixture preheated to and kept at 60° C.)
3. Wash in water.
4. Counterstain five minutes in Lillie’s acetic hemalum or other alum hematoxylin.
5. Wash five minutes in running water.
6. Blow dry with air stream. Wash 1–2 minutes in xylene or acetone to remove dye precipitate. Mount in polystyrene with cover glass, or again blow dry and examine in immersion oil.

Results: Nuclei, blue; numerous fine to moderately numerous medium bright red granules in unstained to pale yellowish cytoplasm of neutrophil leukocytes; fewer similar granules in monocytes; granules of eosinophils largely pale gray-green to brownish yellow, usually with a few to moderately numerous bright red to orange-red, medium to coarse granules. Erythrocytes vary from greenish gray to very pale gray. Often erythrocytes and plasma
show globules to fine granules of red deposit, which is also highly resistant to solvents. This deposit varies from none to copious from one slide to another, and in different areas of the same slide. It seems to be uninfluenced by filtration of the stain mixture, and is extracted by alcohol of 60–90% in much the same way as the coloration of the leukocyte granules.

By substituting Sudan black B for oil red O in the foregoing technic the color of granules in the leukocytes is changed to greenish black, the granules of the eosinophils stain quite uniformly in dark gray-green, often appearing more lightly colored centrally. The erythrocytes assume varying shades of gray green, becoming dark gray to black if staining is prolonged, especially at 60° C. Staining time with Sudan black B is shorter than with oil red O. A half-hour at room temperature, 5–10 minutes at 60° C. (preheated), or 15 minutes at 37° C. suffice. Counterstains for nuclei are less satisfactory than with the hemalum of the oil red stain. A 2-minute stain in 0.5% safranin in 1% acetic acid is fairly satisfactory. Feulgen staining, as practiced on tissue sections (p. 132) gives rather pale colors.

Blood films fixed with formaldehyde (either gaseous or in aqueous solution) and stained with oil-soluble dyes for the demonstration of leukocyte granules are quite apt to show areas of partial detachment, wrinkles running in mosaic fashion, and irregular precipitates both of oil-soluble dye and of the hematoxylin counterstain. These precipitates and the droplets stained in red corpuscles may be quite difficult to remove, whether with alcohol, acetone, or xylene, after staining. They seem to be prevented in considerable measure by prior treatment with 60–80% alcohol, and are much less conspicuous in films fixed for ten minutes in 75% alcohol.

The sudanophil granules of polymorphonuclear leukocytes, though quickly destroyed by 60–100% alcohol at 60° C., resist 90% and lower alcohol concentrations for quite long periods at room temperatures and lower. They are water soluble at 60° C. after brief 75% alcohol fixation, but are rendered insoluble in water by mercuric chloride, lead nitrate, and formaldehyde.

Their sudanophilia is destroyed by treatment with ferric chloride, ferrous chloride, potassium bichromate, potassium permanganate, periodic acid, and hydrogen peroxide.

While it is uncertain whether Sudan staining demonstrates preexisting granules, or whether the granules simply represent deposition of dye complex at sites of reaction, it is convenient to refer to these sites as the sudanophil granules.

**Periodic Acid Schiff Reaction**

After alcohol fixations, a 10-minute oxidation (p. 123), 5 minutes' washing, 10 minutes in Schiff reagent, 5 minutes' washing in three changes of 0.5% sodium metabisulfite, 10 minutes' washing, 5 minutes in acetic hemalum as counterstain, and 5 minutes' washing in tap water, films are blown dry in an air stream and examined in immersion oil or mounted in synthetic resin.
Nuclei stain deep blue; erythrocytes, pale yellow to gray; cytoplasm of neutrophils, purplish red; granules of eosinophils, clear and unstained in foamy pink cytoplasm. Dark red-purple glycogen granules occur in lymphoid cells and sometimes in neutrophil leukocytes. Platelets contain purplish-red oval granules.

Pretreatment with diastase solutions weakens the cytoplasmic staining of neutrophil leukocytes, but does not destroy it. Glycogen is removed by diastase digestion (p. 275). The staining of platelets is unaffected. Treatment of films with water or pH 6 buffer weakens cytoplasmic staining of leukocytes only slightly.

For glycogen digestion tests, films fixed in formaldehyde gas or alcoholic formaldehyde solutions (p. 34) should be employed, since after simple alcoholic fixations, the proteolytic enzymes in the polymorphonuclear leukocytes selectively autolyze these cells, destroying first their cytoplasm and then their nuclei. This leucocyte protease is quite active at 60° C., and is not destroyed by ten minutes in boiling acetone, benzene, toluene, or xylene.

Acetylation for four hours at 60° C. in 40% acetic anhydride + pyridine mixture renders leukocytes and platelets periodic acid Schiff negative, and a 16-hour deacetylation in 20% ammonia water, 50% alcohol solution restores the reactivity of platelets and in part that of neutrophils. Benzoylation in 1:19 benzoyl chloride: pyridine (p. 161) is less successful at 25° or 37° C., and at 60° C., the reagent completely destroys the capacity of nuclei to stain with alum hematoxylin or Giemsa stain.

Fluorescence Microscopy. Primulin, berberin, and rivanol have been used for the demonstration of protozoan parasites in fluorescence microscopy. They give respectively blue, bright-yellow, and yellowish-green fluorescence to leukocyte nuclei; yellow, yellow, and bright yellow to leukocyte cytoplasm; and blue-white, golden yellow, and yellowish-green color to malaria parasites (*Haemoproteus, Plasmodium nucleophilum*, and *P. vivax*). One stains methyl alcohol fixed smears 2-5 minutes in saturated aqueous or alcoholic solutions of the fluorochromes. Parasites and leukocytes stand out as brilliantly fluorescent objects against a dark field and are readily discerned at 200 diameters under dry lens systems. Nothing is said about the behavior of blood platelets with this method, and most of Patton and Metcalf's work was done on the two avian parasites (*Science* 98:184, 1943). I have had no experience with this method.

Polyhydroxy Dyes. When Gomori's staining procedure for enterochromaffin cells with very dilute aqueous solutions of hematoxylin, brazilin, gallo cyanin or celestin blue is applied to alcohol- or formaldehyde-fixed human blood films, the granules of the eosinophil leukocytes are selectively stained. Gallo cyanin gives brilliant purplish-violet eosinophil granules with 48 hour staining in a 1/20,000 solution. The red corpuscles appear in pale yellowish gray, nothing else stains. With celestin blue at 1/20,000 for two days white cell nuclei appear in pink, red cells faint yellow, eosinophil granules blue
green, and neutrophils unstained. Hematoxylin gives blue-gray, brazilin, and alizarin pink, but the contrasts are inferior to those with the oxazin dyes.

Perhaps the most contrastful results are obtained with a 1/100,000 aqueous solution of celestin blue, staining 6 hours at 60° C. This yields light red-purple nuclei, blue-green cosinophil granules, unstained neutrophil cytoplasm and pale-yellow erythrocytes. It is necessary to employ alcoholic formaldehyde or formaldehyde-vapor fixation to avoid the autolytic digestion of the neutrophils which occurs in distilled water at 60° C.

In tissue sections a safranin counterstain may be used after 1/20,000 gallo cyanin (two days, 37° C.), but the staining is variable and generally less successful than in blood films.

Reticulocytes

Brecher (Am. J. Clin. Path. 19:895, 1949) prefers new methylene blue (C. I. No. 927, J30 in Conn, 5th ed.) to the more commonly used brilliant cresyl blue for the staining of reticulocytes in blood. Various lots of brilliant cresyl blue vary considerably in staining properties and spectroscopic characteristics, indicating differences in composition. As Conn states, brilliant cresyl blue is not used in industry and must be specially manufactured in small lots for biological staining. This fact undoubtedly explains the variations. New methylene blue, on the other hand, is manufactured by several manufacturers for textile dyeing and appears to be quite constant both in its absorption spectrum (λ = 630 - 632.5 mμ) and in staining performance.

Brecher's New Methylene Blue Technic for Reticulocytes.

1. Dissolve 0.5 Gm. new methylene blue (C. I. No. 927) and 1.6 Gm. potassium oxalate in 100 cc. distilled water.
2. Mix approximately equal drops of stain and of fresh or oxalated blood on a slide.
3. Draw up the mixed drop into a capillary pipet and let stand for ten minutes.
4. Expel the mixture in small drops on several slides and make thin smears as usual. Dry in air and examine with oil immersion.

Results: Erythrocytes are light greenish blue; reticulum, a deep blue and sharply outlined.

Deeper blue staining of erythrocytes indicates that an excess of dye was used. Generally the blood drop should equal or slightly exceed the stain drop in size.

As an example of a brilliant cresyl blue dry smear technic I quote the Cunningham-Isaacs technic from Conn and Darrow, 1948 (ID3-8):
Brilliant Cresyl Blue Method for Reticulocytes.

1. Dry a drop of 0.3% alcoholic solution of brilliant cresyl blue on a cleaned and polished slide or cover glass.
2. Deposit a drop of fresh blood 2–3 mm. in diameter on a similarly cleaned slide or cover glass.
3. Appose the stain-covered area on the first slide or cover glass to the blood drop and move this slide up and down hinge fashion until the dye film is all dissolved and the blood appears blue black.
4. Allow the two slides or cover glasses to cohere in parallel position and spread the blood drop.
5. Draw apart along the plane of contact and allow the two films to dry.

Results: Sharply stained blue reticulum and pale-blue erythrocytes. If desired the films may be counterstained with Wright’s or Giemsa’s stain by one of the usual technics (pp. 388–390). In this case the background is the usual one with these stains, and the reticulum appears deep blue.

Phase microscopy reveals a number of erythrocytes containing rods and granules which appear dark under dark-contrast phase illumination, and which Brecher (Bull. Internat. Assn. Med. Museums 30:99, 1949) finds to be associated with but not identical with the stainable reticulum in the same cells.

Since the presence of the hemoglobin largely obscures these rods and granules, hemolysis is a necessary part of the technic for their phase-contrast demonstration. Phenylhydrazine intoxication renders reticulocytes more numerous and more conspicuous under phase microscopy, perhaps because of in-vivo hemolysis, so that under these conditions they may be found without artificial hemolysis.

Brecher’s Technic for Phase Microscopy of Reticulocytes.

1. Mix a drop of blood with a drop of hypotonic ammonium oxalate solution (1.2%) on a clean cover glass.
2. Pick up the blood and cover glass by bringing a clean slide down on top of it.
3. Turn over and apply petrolatum U.S.P. (paraffinum molle B.P.) along the edges of the cover glass to seal the preparation. This is done conveniently from a 5 cc. syringe fitted with a large-bore needle.

Inclusion Bodies

Oxyphil inclusion bodies such as Negri bodies, Guarnieri bodies, herpes, varicella, and molluscum inclusions and the like are often quite well shown by azure-eosin (pp. 117–119) and phloxin azure sequence (p. 116) methods. I have seen them excellently demonstrated in hematoxylin eosin preparations
which had been mounted in Canada balsam for two or three years. Such old preparations appear to be distinctly superior to fresh stains for this purpose. The safranin O eriocyanine A method (p. 186) has given good results.

Most of the so-called specific Negri-body methods depend on balanced mixtures of two basic or two acid dyes, or on sequence procedures using an acid and a basic dye. The preferred material for Negri bodies is hippocampus and cerebellar cortex. The major lesions of rabies, however, are found in the brain stem.

Stovall and Black (Am. J. Clin. Path. 10:1-8, 1940) used acetone fixation and a sequence stain:

1. Stain two minutes in a 1% alcoholic solution of ethyl eosin (C. I. No. 770, sodium ethyl eosinate) adjusted to pH 3.0 with N/10 hydrochloric acid.
2. Rinse in water.
3. Stain 30 seconds in 10 cc. 1% methylene blue in 95% alcohol, 10 cc. M/5 acetate buffer of pH 5.5 (p. 450), and 20 cc. water.
4. Then differentiate in 0.38% acetic acid in water (19 drops in 60 cc.) until sections are brownish red.
5. Wash, dehydrate, and clear.
6. Mount in balsam.

Results: Negri bodies are brownish to pure red; nucleoli, pale blue; other structures, pink.

A variant of this method which we have used successfully:

1. In 90 cc. 100% alcohol or 94 cc. 95.5% alcohol, 3.25 cc. 1% acetic acid, and water to make 100 cc., dissolve 950 mg. ethyl eosin. Stain in this for two minutes.
2. Then wash in alcohol and in water.
3. Counterstain formalin material in 0.5% methylene blue in 25% alcohol; alcohol-fixed material, in alum hematoxylin.
4. Differentiate in 0.25% acetic acid for 2-5 minutes.
5. Wash, dehydrate, and clear.
6. Mount in balsam.

Gerlach's Method (Kraus, Gerlach, and Schweinburg).

1. Stain paraffin sections of formalin-fixed material in a fresh mixture of 3 cc. carbol fuchsin, 6 cc. Loeffler's methylene blue (about 0.35% in 22% alcohol containing 1:10,000 potassium hydroxide) and 50 cc. distilled water. Heat sections to steaming four times in four changes of this mixture.
2. Wash in water.
3. Differentiate and dehydrate in alcohols and clear in xylene.
4. Mount in synthetic resin or mineral oil; the stain fades rapidly in balsam.

*Results:* Negri bodies, red; chromatin and nucleoli, rather light blue.

Mann's methyl blue eosin technic (Kraus, Gerlach, and Schweinburg) is classical: Zenker fixation was prescribed. Paraffin sections are carried through 0.5% iodine and 5% sodium thiosulfate as usual and washed in water.

1. Stain 24 hours in 6 cc. 1% aqueous eosin (C. I. No. 768), 6 cc. 1% aqueous methyl blue (C. I. No. 706), and 28 cc. distilled water.
2. Wash in water and differentiate in 100% alcohol containing 4 mg. sodium hydroxide per 100 cc. (0.001 N; add 0.1 cc. 1% NaOH in alcohol to 25 cc. 100% alcohol.)
3. Wash in 100% alcohol.
4. Then wash in water containing a few drops of acetic acid—say 0.1%.
5. Dehydrate and clear through alcohols and xylene and mount in poly-styrene.

*Results:* Negri bodies and erythrocytes are stained red; nuclei and inner granules of the inclusions, blue.

Schleifstein's rapid method (*Am. J. Pub. Health, 27:1283, 1937*) (emended) prescribed a four-hour fixation in Zenker's fluid at 37° C., 30 minutes' washing in water, dehydration one hour at 37° C. in dioxane over anhydrous calcium chloride, infiltration one hour in dioxane-paraffin 50% mixture at 56° C. and in pure paraffin for one hour. In place of the dioxane schedule I suggest substitution of the rapid acetone benzene schedule (p. 62).

Schleifstein's stain consists of 1.8 Gm. basic fuchsin (rosanilin chloride) and 1 Gm. methylene blue, dissolved in 100 cc. glycerol and 100 cc. methyl alcohol. For use it is diluted 1:80 (Mallory, 1 drop to 2 cc.) with 1:40,000 potassium hydroxide.

1. Paraffin sections are brought to water as usual.
2. Steam gently for five minutes (70° C.? ) in the diluted stain.
3. Then wash in tap water.
4. Differentiate to a faint violet color in 90% alcohol.
5. Dehydrate rapidly with 95% and 100% alcohol, clear through 100% alcohol and xylene (50:50) and two changes of xylene. Mount in Clarite.

*Results:* Negri bodies, deep magenta red; erythrocytes, coppery red; nucleoli, blue black; cytoplasm, blue violet.

For various oxyphil inclusion bodies, notably Guarnieri and Kurloff bodies, the inclusions of infantile giant-cell pneumonia and others (but not Negri bodies) A. C. Lendrum (*J. Path. & Bact. 59:399, 1947*) recommends
his phloxin-tartrazine stain. Fluorane dyes are required, and of these phloxin B (C. I. No. 778) and rose bengal (C. I. No. 779) (Conn 6th ed.: R 31 and R 36 respectively) appear to be the best. Eosin Y and crythrosin are too readily extracted. Calcium chloride is used as an intensifier for the fluorane staining. As a differentiator Lendrum specifies the tartrazine NS of Imperial Chemical Industries, dissolved in Cellosolve (ethylene glycol monoethyl ether). It is presumed that this is C. I. No. 640 and that the tartrazines of other manufacturers will serve.

Lendrums's Phloxine-Tartrazine Method (emended).

1. Fix preferably in mercuric chloride formalin (9 parts saturated aqueous mercuric chloride solution and 1 part formalin) for 24 hours, dehydrate with iodized 70% alcohol and ascending alcohols, clear, and infiltrate and imbed in paraffin as usual.
2. Bring paraffin sections to water as usual, including iodine and sodium thiosulfate steps if tissue was not iodized before imbedding.
3. Stain as usual in Mayer's hemalum or in Weigert's acid iron chloride hematoxylin.
4. Blue and wash as usual.
5. Stain 30 minutes in 0.5% phloxine B or rose bengal in 0.5% (0.045 M) aqueous calcium chloride solution.
6. Rinse in water.
7. Differentiate with saturated tartrazine solution in Cellosolve, either briefly from a dropping bottle, or with strongly phloxinophil objects for as long as several hours in a Coplin jar.
8. Rinse in 60% alcohol, dehydrate with 95% and 100% alcohol, then 100% alcohol and xylene, and clear in two changes of xylene. Mount in balsam, Clarite or other suitable resin.

Results: Kurloff bodies in guinea-pig lung, Guarnieri bodies, inclusions of infantile giant-cell pneumonia, and others (but not Negri bodies) are stained (red) by the phloxin; collagen, yellow; nuclei, according to the hematoxylin stain selected.

I have not tried this method. It has been used by a number of British workers.

According to Wolman (Proc. Soc. Exper. Biol. & Med. 74:85, 1950) the elementary bodies of smallpox may be demonstrated by fixing smears of scrapings from incised recent papules or vesicles in ether and alcohol (50:50) for a few minutes, drying in air and staining by the Feulgen procedure (p. 132).

The strongly acidophil intranuclear inclusion bodies seen in some cases of chronic lead and bismuth poisonings may be acid-fast when stained by the Ziehl-Neelsen technic. Sections were stained in carbol fuchsin for three hours
at 56° C., washed in water and decolorized 3-5 minutes in 3 cc. conc. HCl: 97 cc. 70% alcohol and counterstained with Harris's alum hematoxylin. Those seen in lead poisoning failed to react to ammonium sulfide. (M. Wachstein, Am. J. Clin. Path. 19:608, 1949.)

Perrin and Littlejohn (J. Clin. Path. 3:40, 1950) detail a rapid method which may have some value in the cytologic examination of fresh sputum for tumor cells. Their carbol fuchsin methylene blue stain is composed of four volumes of a 0.5% methylene blue solution in 20% glycerol (by volume), mixed extempore with one volume of a 1% fuchsin solution in 10 parts alcohol to 90 parts 5% aqueous phenol solution. Purulent or bloody particles are picked out of the morning sputum, mixed thoroughly with a drop of the stain and warmed gently over a Bunsen burner for 30 seconds. The uniformly stained, sticky specimen is then covered with a large cover glass and spread by gentle pressure. Specimens may be examined at once, or allowed to stand a while. Staining continues, and in five or six hours becomes so intense as to obscure nuclear detail.

Improved nuclear detail was obtained by substituting 2% iodine green (C. I. 686) for the fuchsin methylene blue mixture. It is probable that the commoner, closely related dyes ethyl and methyl green can be used in the same way.

With the fuchsin methylene blue, cytoplasm of squamous and columnar cells stains bright pink; nuclei and bacteria, violet to purple. Leukocyte and lymphocyte cytoplasm is pale pink or green; that of macrophages, purplish red; and of plasma cells, violet to purple. Tumor-cell cytoplasm is red when well keratinized, and violet to blue green in more anaplastic cells.

**Spirochetes**

Spirochetes are generally demonstrated in tissues by one of the numerous reported silver methods. For fresh clinical material the dark-field technic is preferable, and this may be applied also to fresh autopsy material. For smears, various methods using Wright's and Giemsa's stains as well as silver methods and "negative" methods have been used.

The methods employing Wright's and Giemsa's stains either add a small quantity of an alkali carbonate or heat the staining solution or both. Heated stains are replaced 3-5 times and allowed to act 15 seconds to 3 or 4 minutes each time.


1. Fix films 15 minutes in 100% alcohol.
2. Dilute 1 cc. Giemsa stain with 38 cc. distilled water and 2 cc. 0.1% potassium carbonate. Stain smears 10-30 minutes.
3. Rinse, blow dry with compressed air or blot dry with filter paper, and examine in immersion oil.
Wright's (?) Rapid Method. Fix films 15 minutes in 100% alcohol or by passing thrice through a blue flame. Make a 1:40 dilution of Giemsa stain in distilled water. Flood slide with 2-3 cc. of this, heat to steaming, let stand 15 seconds, decant, and repeat flooding and heating five times more. Let cool for one minute the last time, rinse with distilled water, dry and examine.

This technic appeared in the 1908 edition of Mallory and Wright's Pathological Technic, apparently as an original modification of Wright's. Mallory later erroneously attributed it to Giemsa's paper in the Deutsche med. Wchnschr. 35:1751, 1909.

With both the foregoing methods spirochetes are stained dark purplish red.

Negative methods are derived from Burri's India-ink method, which itself, according to Conn and Darrow, has largely been abandoned on account of difficulties in obtaining ink free of bacteria. Dorner (Stain Technol. 5:25, 1930) introduced nigrosin for the same purpose, and Harrison (Brit. M. J. 2:1547, 1912) used collargol. Congo red posttreated with hydrochloric acid to turn it blue has been similarly employed, but Cumley (Stain Technol. 10:53, 1935) notes that after the slides are made they may again turn red or fade altogether.

Mallory recommends a 10-25% suspension of India ink in distilled water, which is to be autoclaved before use. Dorner prescribed dissolving 10 Gm. water-soluble nigrosin (C. I. No. 865) in 100 cc. distilled water by heating 30 minutes in a bath of boiling water. Harrison prepared a 5% suspension of collargol in distilled water. This is good for months.

Technic. Mix a bacteriologic loopful of exudate on a clean slide with a loopful of one of the above fluids and spread into a thin smear. Dry, and examine. Spirochetes and bacteria appear unstained in a dark-brown, gray, or reddish-brown background respectively for the ink, nigrosin, and collargol.

Silver Methods

Fontana’s method is the traditional silver method for smears:

1. Conn and Darrow prescribe heat fixation, Mallory a one-minute treatment with Ruge's 1% acetic, 2% formalin solution using several changes, followed by rinsing in water.
2. Steam 30 seconds in: Phenol 1 Gm., tannic acid 5 Gm., distilled water 100 cc. Use only a few drops.
3. Rinse 30 seconds in distilled water.
4. Steam (70-80° C.) 30 seconds in Fontana’s ammoniacal silver hydroxide: To 5% silver nitrate solution add 28% ammonia water drop by drop until the dark-brown precipitate just dissolves (about 0.5 cc. for 10 cc. of 5% silver nitrate), then add more silver nitrate, shaking to dissolve the brown clouds of silver oxide between drops, until a faint permanent turbidity is attained. Conn and Darrow state that this solution is good
for several months; but judging from experience with similar solutions for reticulum, it seems preferable to prepare a small quantity fresh each time.

5. Wash, dry, and mount in balsam.

Levaditi's method is the traditional method for spirochetes in blocks of tissue (Conn and Darrow):

1. Fix blocks about 1–2 mm. thick in 10% formalin for 1–2 days. Older material stored in formalin can be used.
2. Rinse in water and soak in 95% alcohol for one day.
3. Place in distilled water until the tissue sinks.
4. Impregnate in 2% silver nitrate at 37° C. for four days, changing the solution daily.
5. Wash in distilled water.
6. Reduce 48 hours in: 3 Gm. pyrogallic acid, 5 cc. 40% formaldehyde, and 100 cc. distilled water.
7. Wash in several changes of distilled water.
8. Dehydrate with graded alcohols, clear in cedar oil, and imbed in paraffin. Section at 5 μ. Deparaffinize with xylene and mount in balsam or Clarite.

Results: Tissues, varying shades of yellow and brown; spirochetes, black. The method is capricious and often fails.

The Warthin Technic. Of the numerous proposed single paraffin section methods, I have finally selected a modification of Warthin's (Am. J. Syph. 4:97, 1920) technic which has given us more consistent results than any previously tried. Faulkner (Stain Technol. 20:81, 1945) substituted an acetate buffer for Kerr's (Am. J. Clin. Path. 8:63, 1938) dilute citric acid solution which seems to control the process better. The technic:

1. Bring paraffin sections of formalin-fixed material to water as usual.
2. Wash in water buffered to pH 3.6 or 3.8 with a 1:25 dilution of stock Walpole's acetate buffer (p. 450).
3. Impregnate 45 minutes at 55–60° C. in a Coplin jar filled with 1% silver nitrate in water buffered to the same pH level.
4. While slides are incubating, heat and mix the developer solution. Make in advance a stock gelatin solution by dissolving 10 Gm. gelatin in 200 cc. distilled water buffered as above to the same pH, heating in paraffin oven for one hour. Add 2 cc. 1:10,000 merthiolate (sodium ethylmercurithiosalicylate) as preservative. Cool and store. Melt the stock gelatin solution, take 15 cc. and heat it to 60° C. Heat 3 cc. 2% silver nitrate in the same buffered water to 60° C. and add to the gelatin. Then add 1 cc. freshly prepared 3% hydroquinone in the same buffered water. Use the mixed developer at once.
5. Place slides face up on glass rods and pour on the warm developer. When sections become golden brown to grayish yellow and developer begins to turn brownish black, pour off and rinse with warm (55-60° C.) tap water, then with distilled water.

6. Dehydrate, clear, and mount.

Results: Underdevelopment gives a pale background and slender or pale spirochetes; overdevelopment gives thick spirochetes, dark background and precipitates; optimal development gives pale yellowish-brown tissue and black spirochetes. At the lower pH level, tissue staining is less; at the higher, organisms are denser. This method has performed well on syphilitic fetal liver, on yaws lesions, on Weil’s disease, on Vincent’s fusospiropllosis mouth lesions, and on other spirochetoses.

If desired, one may

6. Tone three minutes in 0.2% gold chloride.
7. Wash in water and counterstain with hemalum and eosin or by other methods.
8. Then dehydrate, clear, and mount as usual.

Trichinae

The Unfixed Muscle Spread. The usual technic for examination for trichinae in muscle is taken from Nolan and Bozicevich (Pub. Health Rep. 53:652, 1938). Take about 1 Gm. of fresh muscle in the aggregate, as small fragments snipped with scissors from various areas, best from the diaphragm near the tendinous portions. Lay these in rows on one or more 2 x 3 in. slides. Place a second slide on top and press the fragments out flat. Heavy spring paper clips may be used to supply the pressure, one on each side, or some heavy rubber bands. The muscle fragments should be pressed out to a state of transparency. Examine directly with a dissecting microscope or with the low power of the microscope with reduced illumination. The preparations do not keep.

The Formic Acid Method. Even more satisfactory crush preparations can be made after preliminary fixation in 20% formic acid for 12-24 hours. This method has the advantage of allowing removal of the material to the laboratory and working up at leisure. The muscle swells greatly. Small snips are cut off, teased, and crushed out to transparency. Stains may be applied, and permanent preparations mounted. My technic is this:

Cut small pieces of muscle from various locations at autopsy, especially diaphragm and intercostal muscles. Pectoralis and rectus abdominis can be used. Deltoid and gastrocnemius are favorable sites for biopsy. Masseter is also a good muscle for biopsy, if cosmetic considerations can be disregarded. Immerse these at once in a 1:3 or 1:4 dilution of concentrated (90%) formic acid in distilled water and leave overnight. Wash 30-60 minutes in
running water and transfer to a mixture of equal volumes of glycerol and of 50% alcohol for at least 18 hours. The material can be kept in this.

When desired snip off small fragments of muscle with scissors, taking at least a dozen from various pieces of muscle. Drop these into 30 cc. 1% acetic acid to which 0.3–0.6 cc. of Lillie’s acid hemalum (p. 76) has been added. Other hematoxylins can be used, if diluted in 1% acetic acid to give a final concentration of about 5–10 mg. hematoxylin per 100 cc. Stain overnight. Place the fragments on cover glasses in two rows of two or three pieces each and tease and crush gently with needles. Add a few drops of Apáthy’s gum syrup (p. 109). Lay the cover glass on a blotter on a flat surface. Put a slide down on top of the preparations and press down firmly with a slight rotatory motion, so as to crush and spread the muscle fragments. The excess gum syrup is squeezed out into the blotter. The preparations may be examined at once, using a 32-mm. objective for finding and an 8-mm. objective for detailed study; but they clear further on standing.

Muscle nuclei and cross striae are well shown; the nuclei of the giant cells surrounding recently encysted parasites are readily identified.

If the preparations acquire air bubbles after release of the pressure, run in a little more syrup from one edge, but do not press on the cover glass directly. It is likely to break. The syrup dries hard.

Use of very dilute safranin in dilute acetic acid solution in place of the hematoxylin yields more transparent preparations with a purer nuclear stain. Use a 1:1,000 to 1:10,000 safranin O (best, about 1:2,000) in 1% acetic and stain overnight as with the hematoxylin.

Muscle previously fixed in formalin does not yield satisfactory crush preparations.

The digestion technic, quoted from Bozicevich (Pub. Health Rep. 53:430, 1938) is more sensitive but requires more apparatus. Set up a 3-liter glass funnel with a large rubber tube on the stem. Into the open end of this tube fit a 15 cc. centrifuge tube with conical bottom. Between the bottom of the funnel and the centrifuge tube attach a screw clamp for closing the tube when the centrifuge tube is to be removed after the digestion is complete. Support the whole in a suitable circular hole in an inch plank. Lay in the funnel a 6-in. perforated porcelain plate. (See illustration from Bozicevich.)

Make up the digestion fluid by dissolving 15 Gm. pepsin in three liters of warm (40°C.) tap water. Add 21 cc. concentrated hydrochloric acid. Place this solution in the funnel with attached centrifuge tube.

Grind 70 Gm. of fresh muscle, preferably from the diaphragm; place the ground muscle a little at a time on a quadruple thickness of 40-mesh gauze or cheesecloth which is previously laid in the funnel over the porcelain plate. Place the whole apparatus in a 37°C. incubator and digest for 15–18 hours (overnight). During the digestion the living larvae are liberated from the muscle and pass through the gauze, settling into the bottom of the centrifuge tube. Then clamp off the rubber tube, remove the centrifuge tube, and take
up larvae with a pipet from the bottom of the tube. Most of the fluid may be decanted off first.

Valuable as the silver methods may be in the study of reactive gliosis, it is often even of more value to study brain tissues stained by general oversight methods such as the azure-cosin technics (pp. 116–120) which reveal cellular gliosis, perivascular infiltration, hemorrhages, necroses, bacteria, inclusion bodies, and tigrolysis. Use of diammine silver technics for reticulum may aid in establishing the relation of focal gliosis to blood vessels. I have successfully used the picric acid and hydrochloric acid methyl blue methods for this purpose. The periodic acid leucofuchsin and allochrome methods (pp. 123, 357) seem worthy of trial. Fat stains and myelin technics have their place. Perivascular and interstitial macrophages can well be studied with iron reactions as well as with fat stains.

Ependyma is quite well shown with the above general methods for cells and fibers. Some of the special glia procedures can be of value in the study of ependymitides. Chorioid plexus and their epithelium are well shown by the azure-cosin methods. The usual stroma methods of general histology are applicable to these essentially vascular and epithelial structures. Fat stains may be of value.

The meninges may require almost any of the methods applicable to the general tissues. The azure-cosin methods are useful for the cytology of exudates; the bacterial methods, collagen methods, pigment methods, fibrin, and other methods may all have their specific applications.

Generally the amateur has indifferent success with the metallic impregnation methods, but many workers have used them and secured useful preparations. Unfortunately special fixations and very fresh material are often required, and this limits their usefulness. I have endeavored to select methods which are adaptable to routine formalin-fixed material when possible, and have relied to a considerable extent on the selection made by Davenport, Windle, and Rhines for Conn and Darrow. These authors have supplied a number of valuable emendations, as well as modifications.
Generally collodionization of paraffin sections (p. 94) should be employed when alkaline silver solutions are to be used. Frozen or loose celloidin or nitrocellulose sections should be handled with glass needles during the metal impregnation stages. Paraffin-coated forceps [dipped in smoking hot (100-120° C.) paraffin] may be used for handling tissue blocks.

Glia Cells and Glia Tumor Cells

**General**

**Weil and Davenport** (quoted from Conn and Darrow) prescribe 10% formalin fixation for several days and paraffin sections at 10 μ.

1. Deparaffinize sections and bring through 100% alcohol into a 1.5% collodion solution in ether alcohol mixture for 2-3 minutes.
2. Drain partially and then hold horizontal face down with slight tilting movements, both lateral and longitudinal, until the remaining film congeals.
3. Then harden the film in 80% alcohol.
4. Impregnate 6-48 hours at 37° C. in 8 Gm. silver nitrate dissolved in 10 cc. distilled water and diluted with 90 cc. 95% alcohol.
5. Rinse quickly in 95% alcohol.
6. Reduce in 5 Gm. pyrogallic acid, 5 cc. 40% formaldehyde, and 95 cc. 95% alcohol for about one minute, more or less according to desired intensity.
7. Wash thoroughly in running water.
8. Tone 5-10 minutes in 0.2% aqueous gold chloride solution.
9. Wash in distilled water.
10. Fix in 10% sodium thiosulfate for one minute.
11. Dehydrate with alcohols; dissolve the collodion with 100% alcohol and ether, and clear in xylene. I suggest substitution of an acetone and xylene sequence.

**Results:** Pathological glia, gray to black; axis cylinders, black or gray; background, gray violet.

Addition of 0.25-0.5 cc. normal nitric acid* to the silver bath may be required to inhibit staining of normal glia cells.

**Weil and Davenport** (*ibid.*) also cite a modification of Stern's diammine silver hydroxide method for microglia and oligodendroglia. Fixation in 10% formalin and celloidin sections at 15 μ are prescribed.

1. Wash sections in distilled water. If it is desired to increase the relative density of impregnation of oligodendroglia, add 0.5 cc. 28% ammonia water to 100 cc. distilled water and soak three minutes.

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* Mix 63.4 cc. 70% nitric acid, sp. gr. 1.42, with 936.5 cc. distilled water.
2. Impregnate 10–20 seconds; the longer intervals favor the impregnation of oligodendrocytes, in a diammine silver hydroxide prepared by adding 10% silver nitrate to 2 cc. 28% ammonia water until a faint permanent opalescence is produced. This requires 18–20 cc. This solution is essentially identical with that used for reticulum in Lillie's technic (p. 335).

3. Transfer sections directly to fresh 15% formalin and agitate until deep brown. For oligodendrocytes use 10% formalin and commence agitation only after celloidin is blackened and section begins to turn brown.

4. Wash in tap water and dehydrate with two changes of isopropyl or normal butyl alcohol, clear in one change of a 50% mixture of the selected alcohol with xylene and two changes of xylene. Mount in balsam.

Oligodendrocytes

The following methods are designed especially for oligodendrocytes but often impregnate microglia as well.

Del Rio-Hortega's silver carbonate method, slightly altered from Conk and Darrow: Fix in Ramón y Cajal's formalin ammonium bromide (p. 34) for 12–48 hours.

1. Heat the block in fresh formalin ammonium bromide at 45–50° C. for ten minutes. Ramón y Cajal heated at 50–55° C.
2. Cut frozen sections at 15–20 µ.
3. Wash in 1:100 dilution of 28% ammonia water and then in distilled water.
4. Impregnate 1–5 minutes in an ammoniacal silver carbonate solution prepared as follows: To 5 cc. 10% silver nitrate add 20 cc. 5% sodium carbonate solution (a threefold excess) and then drop by drop add 28% ammonia water to barely dissolve the precipitate (about 0.4 cc. should be required). Add about 20 cc. distilled water and filter, bringing volume to 45 cc. Keep in a brown bottle.
5. Wash sections 15 seconds in distilled water.
6. Reduce 30 seconds in a 1:100 dilution of strong formalin.
7. Wash thoroughly in tap water.
8. Tone in 0.2% gold chloride until gray. Ramón y Cajal specified 10–15 minutes in cold (15° C.) solution; less time if warmer.
9. Fix in 5–10% sodium thiosulfate (2–5 minutes). Ramón y Cajal specified 5%.
10. Wash thoroughly in tap water, float onto slides, blot down with filter paper, dehydrate, and clear by a 100% alcohol xylene sequence, and mount in balsam or Clarite.

Results: With this method cytoplasm and processes of oligodendrocytes should be black, cell nuclei unstained, and general background gray. Since the exposure to the diammine silver carbonate is brief and at a lower tem-
perature than in the same author's reticulum method and a considerably
greater excess of sodium carbonate is present, reticulum should not be im-
pregnated.

Longer fixation than that prescribed is said to make the impregnation less
selective.

Penfield's variant (Conn and Darrow) of this method gives both oligo-
dendrocytes and microglia. By increasing the volume of the silver solution
above from 45 to 75 cc. with distilled water and by taking out sections after
20, 45, and 120 seconds in the silver, the rest of the technic being the same,
 microglia and processes are black, other glia cells dark gray to black, and
background pale.

Astrocytes

Large protoplasmic astrocytes are often well demonstrated with azure­
eo sin stains (pp. 116-120); and with Mallory's anilin blue (pp. 350-351) or
his phosphotungstic acid hematoxylin (p. 344) both astrocytes and glia fibrils
may be well shown. The classical method, however, is:

S. Ramón y Cajal’s Gold Sublimate Method. The following technic
follows Conn and Darrow for the most part.

Fix about 5 days (not less than 2 nor more than 25) in Ramón y Cajal's
formalin ammonium bromide (p. 34). Cut frozen sections at 15-30 μ, and
store in the same fixative.

1. Wash in two changes of distilled water.
2. Impregnate well-spread-out sections 3-4 hours in 5 cc. 1% yellow or
brown gold chloride, 5 cc. 5% mercuric chloride, and 30 cc. distilled
water. Ramón y Cajal used 5 cc. 1% gold chloride, 4-5 Gm. mercuric
chloride (saturated solution?), and 20-25 cc. water. Temperatures
around 25° C. are preferable; 18-40° C. is permissible. Check impregna-
tion from time to time by examination of a wet section under the micro-
scope. Astrocytes should appear dark with a relatively light background,
and sections acquire an overall purple coloration. When impregnation
is satisfactory,
3. Wash in distilled water and
4. Fix in 5-10% sodium thiosulfate solution (Na₂S₂O₃·5H₂O) for 5-10
minutes. Ramón y Cajal preferred a saturated solution with an added
2% of a normal sodium bisulfite solution.
5. Wash thoroughly in several changes of tap water, float onto slides, blot
down with filter paper, dehydrate with 100% alcohol, and clear with
xylene, blotting between changes. Mount in balsam or Clarite.

According to Globus (Arch. Neurol. & Psych. 18:263, 1937) material
stored in formalin for a long time may be used if one soaks frozen sections
24 hours in a 10% dilution of 28% ammonia water, rinses twice in distilled
water, and then immerses for 2-4 hours in a 10% dilution of concentrated (40%) hydrobromic acid. Following this treatment rinse in two changes of a 1:2,000 dilution of strong ammonia water, and proceed as above.

Achucarro’s tannin silver method according to Del Río-Hortega, emended slightly from Conn and Darrow: Fix ten days or more in 10% formalin (alkalinized to litmus with ammonia, according to Ramón y Cajal). Cut frozen sections (not over 10 μ; Ramón y Cajal).

1. Wash in distilled water.
2. 3% aqueous tannic acid at 50° C. for five minutes. Ramón y Cajal used 10% tannin, and cites Del Río-Hortega as using 3% tannin rather than tannic acid.
3. Wash in a 1% dilution of 28% ammonia water until sections become pliable.
4. Impregnate sections a few at a time in three changes of diammine silver hydroxide. The recommended solution is made by precipitating silver oxide from 30 cc. 10% silver nitrate by the addition of 2 cc. (40 drops) 40% sodium hydroxide drop by drop (a slight excess). The precipitate is then filtered out on hard filter paper and washed free of excess alkali with ten or more washes of distilled water. (Ramón y Cajal omitted this filtration and washing.) Then transfer precipitate to a 250 cc. flask with 50 cc. distilled water and dissolve by adding 28% ammonia water drop by drop (2-2.38 cc.; the latter is the theoretical quantity for Ag(NH₃)₂OH, presuming no loss of silver) avoiding any excess of ammonia, and preferably leaving a few granules undissolved. Dilute to 150 cc. with distilled water and store this stock solution in a brown glass bottle. For use, dilute 5 cc. with 45 cc. distilled water. (This would correspond to perhaps a 1:50 dilution of Lillie’s diammine silver-hydroxide solution as prepared for reticulum impregnation [p. 335].)

Sections become yellowish brown when sufficiently impregnated. The weak silver solution requires frequent renewal.
5. Wash in three changes of distilled water.
6. Tone 20 minutes at 40-45° C. in 0.2% gold chloride solution.
7. Wash in water.
8. Fix in 5-10% sodium thiosulfate (for 2-5 minutes). (Ramón y Cajal specified 5%.)
9. Wash in (several changes of) water. Float onto slides and blot down. Dehydrate and clear with a 100% alcohol xylene sequence, blotting between changes to keep sections in place and to accelerate clearing. Mount in balsam or Clarite.

Results: Protoplasmic astrocytes should be dark gray to violet; fibrils, black; connective tissue, pale; other tissue elements, reddish to violet. This method is considered less selective than the gold sublimate method.
Nerve Cells and Their Processes

Fox's Zinc Chromate Variant of the Golgi Technic, for formalin-fixed brain: (Stain Technol. 26:109, 1951).

1. Brain tissue should be fixed promptly in 10% formalin, using perfusion for large brains, and should be stored in 10% formalin for a minimum of 3-4 months; even better, 1-2 years. Cut in thin slices.

2. Chromate for two days in a solution of 6 Gm. zinc chromate dissolved in 100 cc. 4% formic acid. A solution of half this concentration may be used, but Fox prefers the stronger solution.

3. Remove and blot. Silver the surfaces by agitating each slice gently in 0.75% silver nitrate until all free surfaces are deep red. Then remove, pass a thread through the slice, and

4. Suspend in 0.75% silver nitrate in a large vessel for 48 hours, changing the solution at 24 hours. Before and after the last silvering, brush the surfaces rather vigorously with a camel's-hair brush to remove excess silver chromate crystals, and collect them on a paper towel or sheet of filter paper.

5. Carry through two 15-minute changes of 95% alcohol, two 15 minute changes of 100% alcohol, a 10-minute bath in xylene and a 10 minute bath in 50° paraffin and imbed.

6. Cut sections at 90-100μ and store serially in 95% alcohol. Complete sectioning of the entire block, for it is not infiltrated with paraffin and would shrink badly if allowed to dry.

7. Dehydrate sections with 100% alcohol and clear with several changes of xylene, washing off the loose crystals of silver chromate in the process. (Filter the xylene before re-using.)

8. Mount sections on slides and apply several coats of Permount. Let dry 5-7 days.

9. Moisten surface of Permount with toluene, apply cover glass and place flat lead weights on top, leaving the slide on a warming oven for a few hours.

Results: Background should be pale yellow with isolated cells and processes shown in black.

Celloidin infiltration and imbedding have been employed successfully by some authors. This, of course, necessitates more adequate alcohol dehydration and 3-4 days in nitrocellulose. The low-viscosity process (p. 68) is suggested, especially for eyes.


1. Fix in freshly prepared chloral hydrate bichromate formalin: K₂Cr₂O₇ 5 Gm., distilled water 90 cc. Add 10 cc. 40% formaldehyde and then
3 Gm. chloral hydrate. For fibrillary astrocytes fix 18-48 hours; for protoplasmic astrocytes, 12-17 hours; for oligodendroglia, 18-24 hours; for microglia, 12-18 hours. At 24 hours, rinse blocks in 10% formalin and place in fresh fixing solution if a second day is required.

2. Wash rapidly in 3-4 changes of distilled water.

3. Pass blocks individually through four baths of 25 cc. 1% silver nitrate, allowing about two minutes in each, handling with paraffin-coated forceps.

4. Impregnate 18-24 hours in 1.5% silver nitrate.

5. Rinse in distilled water and brush off surface precipitate.

6. Cut frozen sections at 60-100 μ. Collect in 30% alcohol (two changes).

7. Dehydrate in 95% alcohol and 100% alcohol, and clear in toluene. I suggest use of alcohol-toluene mixtures to facilitate the clearing.

8. Float onto slides, blot, cover liberally with toluene Permount, and apply cover glass.

**Neurofibrils, Axones, and Nerve Endings**

These methods generally require special fixations or fresh tissue, and hence are often not applicable in pathologic studies. Davenport’s method and Foley’s variant of Bodian’s protargol method utilize material fixed in 10% formalin.

Davenport (Arch. Neur. & Psych. 24:690-695, 1930) preferred nitrocellulose sections at 15-30 μ, but stated that paraffin sections could be used.

1. Celloidin or nitrocellulose sections are to be spread on slides and then immersed in 2% collodion and drained. Paraffin sections are to be passed through xylene and 100% alcohol into 2% collodion in ether alcohol mixture and drained.

2. When the collodion film has set, immerse in 80% alcohol for 5-10 minutes or as much longer as convenient.

3. Immerse sections in: 10 Gm. silver nitrate, 0.5 cc. normal nitric acid (see p. 455), 10 cc. distilled water, and 90 cc. 95% alcohol. Let stand until sections are light brown at 37-40° C. Formalin-fixed material usually requires about an hour. With other fixations longer intervals may be necessary. Avoid exposure to bright light.

4. Rinse in 95% alcohol.

5. Reduce in 5 Gm. pyrogallic acid, 5 cc. strong formalin, 100 cc. 95% alcohol, watching under the microscope. This may take as little as two minutes. The reduction may be slowed by adding more alcohol. To avoid precipitates, the preparation should be kept moving. Addition of 100 mg. glucose in 0.5 cc. water (0.125 cc. corn syrup, 0.375 cc. water) also aids in preventing precipitates. One 50 cc. portion of reducer is sufficient for at least 20 slides.

6. Rinse in two or three changes of 95% alcohol, agitating constantly.
7. Dehydrate with 100% alcohol, remove collodion with ether + 100% alcohol mixture, clear in xylene, and mount in balsam.

_results_: Nerve cells, yellow to brown; fibers, dark brown to black.

The Bodian copper protargol method, Foley's variant (Stain Technol. 18:27, 1943): Dehydrate and infiltrate with celloidin or low-viscosity nitrocellulose as usual, and section at 15–25 μ; or infiltrate 24 hours at 58° C. in 5% agar and cut frozen sections at 20–40 μ.

1. Soak 24 hours in 1 cc. 28% ammonia and 99 cc. 50% alcohol.
2. Drain and place in 1% protargol (silver albumose) in distilled water for 6–8 hours at 37° C. In the protargol solution, but not in contact with the sections, place a 200–300-mg. piece of 0.002 inch (50 μ) electrolytic copper foil which has been thinly coated with collodion. Use no metallic instruments.
3. Drain and transfer sections to a mixture of 50 cc. 1% aqueous protargol, 50 cc. 95% alcohol, and 0.5 cc. pyridine (0.1 to 2.0 cc.: higher quantities of pyridine accentuate impregnation of thin fibers; lower quantities, that of cell bodies and dendrites). Put in another piece of copper foil, very thinly coated with collodion. Incubate 24–48 hours at 37° C.
4. Rinse five seconds in 50% alcohol.
5. Reduce for ten minutes in the following reducer: Dissolve 1.4 Gm. boric acid in 85 cc. distilled water; then add and dissolve 2 Gm. anhydrous sodium sulfite; then add and dissolve 0.3 Gm. hydroquinone; then add 15 cc. C. P. acetone and mix well.
6. Wash in several changes of distilled water.
7. Tone ten minutes in 0.2% gold chloride in about 0.3–0.4% acetic acid (20 drops of glacial acetic to 100 cc.).
8. Wash in several changes of distilled water.
9. Reduce 1–3 minutes in 2% aqueous oxalic acid solution.
10. Rinse in distilled water.
11. Fix 3–5 minutes in 5% sodium thiosulfate solution.
12. Wash in distilled water.
13. Stain 18–24 hours in Einarson's chrome alum gallicyanin (p. 141).
14. Wash thoroughly in distilled water.
15. Mordant 30 minutes in 5% aqueous phosphotungstic acid and
16. Transfer directly to the following stain: Anilin blue 10 mg., fast green FCF 500 mg., orange G 2 Gm., glacial acetic acid 8 cc., distilled water to make 250 cc. (I fail to see the function of the minute amount of anilin blue.) Stain one hour.
17. Dehydrate and differentiate with 70–95% alcohol.
18. Complete dehydration with normal butyl alcohol, and clear in cedar oil.
   (Or use the isopropyl alcohol xylene sequence for dehydration and clearing.)
Results: Nerve fibers, neurofibrils, blue black; nuclei, variable: blue black if high pyridine concentration was used; tigroid, light blue; collagen, blue to green; myelin, yellow.

The essential part of the foregoing method is comprised in Steps 1 to 12. The counterstains can well be varied according to taste, as with other silver methods where gold toning and thiosulfate fixation are employed. For instance, an acid picrofuchsin stain, giving red collagen and yellow cytoplasm, might well provide better contrast between collagen and nerve fibers than the blue green prescribed.

Romanes (J. Anat. 84:104, 1950) recommends a very dilute silver chloride ammonia procedure for staining nerve fibers in paraffin sections. Fix in alcohol, Bouin's fluid, Carnoy's fluid, acetic alcohol or acetic alcohol formalin. Treat blocks for a few hours in 2 cc. "0.880 ammonia" (35.3%) in 98 cc. 70% alcohol. Decalcify if necessary, dehydrate, clear, and imbed in paraffin. Section and attach to slides with albumin fixative. Deparaffinize and hydrate as usual. The following directions are as emended by Powers (J. Dent. Research 31:383, 1952; and in letters).

1. Incubate at 58° C. for 16 hours in the dark in a fresh mixture of 2.9 cc. 0.1% silver nitrate in 100 cc. distilled water. Mix and add 1 cc. 0.1% sodium chloride and adjust to pH 7.8 with 0.7 cc. of a 1% dilution of 28% ammonia water. This fluid does not keep.

2. Drain and transfer without washing to 1% hydroquinone in 10% sodium sulfite (crystals, Na2SO3·7H2O or 5% of the anhydrous salt) for five minutes at 20° C.

3. Wash well in running water, and rinse in distilled water.

4. Tone in 0.5% gold chloride for two minutes or (Powers) 0.2% for ten minutes. Wash one minute in two changes of distilled water. Reduce not more than three minutes in 2% oxalic acid. Wash in running water. Fix 3–5 minutes in 5% sodium thiosulfate. Dehydrate, clear, and mount in unsaturated synthetic resin.

Results: Nerve fibers, purple to black; nuclei, red; neurofibrils, purple; keratin, yellow; bone cells, black. Raising the pH to 8.3 and introducing 5 Gm. fine copper wire gives black nerve fibers throughout. Powers replaces this step by premerodanting 18 hours at 37° C. in 0.5% cupric nitrate (Cu(NO3)2·3H2O).

Powers (loc. cit.) recommends use of both Romanes and Ungewitter silver variants for the demonstration of nerve fibers in dentine. For the Romanes method she prefers fixation for 1–3 days in 10% formalin containing 10% chloral hydrate; for the Ungewitter method three or more days' fixation in a Bouin fluid containing 1 Gm. trichloracetic acid, 75 cc. saturated aqueous picric acid and 25 cc. 37–40% formaldehyde.

After either fixation, wash 16–24 hours in running water, decalcify in
Evans and Krajian's formic acid citrate mixture (p. 427) or in 5% trichloracetic acid in 50% alcohol. Wash 24 hours in running water, dehydrate in successive 24-hour changes of 30%, 50%, and 70% ethyl alcohol, in a mixture of 95% alcohol and n-butanol, and finally in two 24-hour changes of n-butanol. Infiltrate 12–24 hours in hard paraffin (56–58° C. melting point), imbed, and section serially at 10 to 15 μ.

For the Romanes procedure, deparaffinize, hydrate, and mordant 18 hours at 37° C. in 0.5% cupric nitrate (crystals, Cu(NO₃)₂·3H₂O). Wash in four changes of distilled water, and carry through the Romanes-Powers procedure (p. 413). The copper mordanting reduces the amount of background staining and obviates the need for the copper wire in the silver solution.

For the Ungwitter method, deparaffinize and bring to 80% alcohol. Transfer directly to cold 1% aqueous silver nitrate containing 20–30% urea and 1–3 drops (0.1 cc.) per 100 cc. of a 1% picric acid, 1% mercuric cyanide solution. Incubate in a paraffin oven for 60–90 minutes. Rinse quickly in two changes of distilled water and reduce 3–5 minutes in a urea hydroquinone solution containing 1–2 Gm. hydroquinone, 20–30 Gm. urea, and 10 Gm. anhydrous sodium sulfite in 100 cc. distilled water, agitating gently for the first two minutes. Wash in five changes of distilled water and examine microscopically in 80% alcohol. If not adequately silvered, repeat silvering and reduction as before. Finally dehydrate, clear, and mount.

The Nonidez method (Am. J. Anat. 65:361, 1939) is highly recommended by Davenport, Windle, and Rhines in Conn and Darrow:

1. Fix 1–3 days in 25 Gm. chloral hydrate in 100 cc. 50% alcohol.
2. Drain and blot and place in ammoniated alcohol (95% alcohol 30 cc., 28% ammonia water 0.1 cc. or 2 drops) for 24 hours, changing once or more if there is much fat.
3. Distilled water five minutes.
4. 2% silver nitrate (aqueous) at 37° C. for 5–6 days, changing after two days or sooner if the solution becomes yellowish brown.
5. Wash 2–3 minutes in distilled water.
6. Reduce 24 hours in pyrogallic acid 2.5–3.0 Gm., 40% formaldehyde 8 cc., distilled water 100 cc.
7. Wash in six changes 20–30 minutes each of distilled water. Imbed, section, and mount.

Nonidez prescribed graded alcohols, amyl acetate clearing, and paraffin imbedding. Other methods can undoubtedly be used. This method is used for nerve endings, both peripheral and central, and for neurofibrils and axis cylinders generally. They appear in shades of brown.

Nauta and Gygax (Stain Technol. 26:5, 1951), observing that increasing amounts of NaOH in ammonia silver nitrate mixtures increase the sensitivity and decrease the selectivity of silver impregnation, explored the effect of varying mixtures on impregnation of degenerating axones.
For axone degenerations in the rat brain they recommend the following modification of the Glees method.

**Bielschowski-Glees Method for Degenerating Axones (Nauta and Gygax).**

1. Fix in neutral 10% formalin for 14 days to 6 months.
2. Cut frozen sections at 15–20 μ.
3. Soak sections 6–12 hours or more in 50% alcohol containing 1% of 25% ammonia water. Only handle sections with glass rod from Step 3 to Step 10.
4. Wash in three changes of distilled water.
5. Soak 24 hours in 1.5% silver nitrate solution in 5% aqueous pyridine solution (10 cc. lot is adequate). Transfer directly to Step 6.
6. Impregnate 2–5 minutes in a covered vessel in ammine silver: AgNO₃ 490 mg. in 20 cc. distilled water + 10 cc. 95% alcohol. Let cool and add 2 cc. 27–28% ammonia water and 1.5 cc. normal NaOH. (Emended slightly for American reagents.)
7. Transfer directly to a mixture of 45 cc. 10% alcohol, 2 cc. 10% unbuffered formalin and 1.5 cc. 1% aqueous citric acid (crystals) solution. The sections spread on the surface and turn golden brown rapidly.
8. Fix 1–2 minutes in 2.5% sodium thiosulfate.
10. Dehydrate with graded alcohols, clear with xylene, mount in synthetic resin—Caedax, Clarite, etc.


Brain tissue fixed in 10% formalin for 2–3 days may be used.

**Lassek's Lead Method for Axones.**

1. Imbed in paraffin, section, and hydrate section as usual.
2. Immerse in buffered lead nitrate at 37° C. for 1–96 hours.
   
   **Molar acetate buffer pH 4.7**
   5 cc.
   
   **5% aqueous lead nitrate solution**
   2 cc.
   
   **Distilled water**
   36 cc.
   
   At time of using add 50 mg. ascorbic acid.
3. Wash in distilled water.
4. Immerse in H₂S water or in 1% ammonium sulfide for 1–5 minutes.
5. Wash in water, counterstain as desired; 0.1% safranin or thionin in 0.1% acetic for five minutes is suggested. Rinse in 1% acetic.
6. Dehydrate, clear, and mount in balsam through alcohol xylene sequence. Axones should appear in brown.

**Methenamine Silver.** In the use of the Burtner argentaffin cell variant (p. 165) for the study of nerve-cell pigments I have seen in oversilvered preparations excellent demonstration of axones, along with generally blackened nerve-cell bodies. This occurs with formalin material after about 3–3½ hours' incubation at 60° C. or in about a half-hour less in material chromated before imbedding.

Prior methylation of formalin fixed material greatly accelerates the silvering of axones. I have seen excellent, perfectly black axones and brown to black tigroid substance after a 24 hour methylation at 60° C. in anhydrous methanol containing 0.8% concentrated hydrochloric acid (N/10). The silvering period in the same Gomori methenamine silver solution as above should be 1½ to 2½ hours at 60° C. This methylation completely inhibits the usual safranin counterstain, as it does all other basic aniline dye stains. If any counterstain is desired, it would be necessary to use the Feulgen procedure or alum hematoxylin for nuclei, since these nuclear stains are not prevented by methylation.

**Ranvier's Method.** The classical method for *motor end-plates* and other *peripheral nerve endings* is that of Ranvier. I have had some excellent preparations and some utter failures in a rather brief experience with this method. Of recent writers Carey has probably used this method with most consistent success, and I quote here his variant. (*Am. J. Path.*, 18:237-289, esp. 242, 1942):

Ranvier's Gold Chloride Method for Peripheral Nerve Endings (Carey).

1. Cut tissue (muscle) in 3–5 mm. slices and immerse in filtered fresh lemon juice for 10–15 minutes (they become transparent in 5–10 minutes, according to Ranvier), handling tissues with glass or paraffin-coated instruments.

2. Pour off lemon juice and add 1% gold chloride (HAuCl₄) without rinsing. (Ranvier rinsed in water.) Let stand 10–60 minutes until a uniform golden yellow tone is reached, not brown. (Ranvier gave 20 minutes' immersion and then washed in water.)

3. Transfer directly to a 25% dilution of concentrated (90%) formic acid and reduce in the dark for 8–12 hours (Ranvier used 20% for one day).

4. Wash in water and store in a 50:50 mixture of C. P. neutral glycerol and 50% alcohol.

5. Snip out a small fragment and tease gently in glycerol or crush out under a cover glass. Mount in glycerol sealed with Clarite or in glycerol gelatin.

*Results:* Nerve fibers and endings appear black; other tissues, in varying shades of red, purple, and violet.
Other writers have used a 20% dilution of formic acid in distilled water in place of the lemon juice, and reduced in a fresh portion of the same fluid for 24 hours. Tissues after spreading can be dehydrated, cleared, and mounted in balsam or Clarite. I have tried formic, acetic, and citric acids, and the last seems to give the cleanest impregnations. With these, 30–60 minutes in the gold chloride seems to be necessary. I have used Apáthy's syrup for mounting these preparations.

Various attempts have been made to apply this method to material previously fixed in formalin, but with poor success.

Rogers, Pappenheimer, and Goetsch (J. Exper. Med. 54:167, 1931) used a method employing fixed material and paraffin sections.

1. Fix in Boulin's fluid (p. 46) for three days or more.
2. Wash in 70% alcohol.
3. Dehydrate with 24 hours' bath in 80% and 90% alcohols each containing 2% of 28% ammonia water.
4. Complete dehydration with two changes, one hour each, of 100% alcohol. Clear with chloroform, xylene, cedar oil, or benzene. Imbed in paraffin. Cut sections at 10–30 μ.

Impregnation procedure:

1. Deparaffinize and pass through 100% alcohol. (At this point I suggest 5–10 minutes' immersion in 1% collodion in ether and alcohol. Then drain one minute and immerse 5–10 minutes in 80% alcohol.)
2. Soak one day in two changes of 90% alcohol, containing 1% strong (28%) ammonia water.
3. Rinse quickly in distilled water.
4. Immerse in 40% silver nitrate for 2–4 hours in the dark (I have used 20-minute to 2-hour immersions on post-picrated formalin material).
5. Rinse quickly in distilled water.
6. Reduce in a 20% formalin dilution for five minutes and in 5% formalin for one minute.
7. Drain and cover with warm (30–55° C.) diammine silver hydroxide (two or more changes) observing under the microscope until muscle appears brownish yellow and nerve endings are black. This diammine silver hydroxide is unlike the usual solutions in that it contains an excess of ammonia: to 4 cc. 20% silver nitrate add sufficient 28% ammonia water to redissolve the brown precipitate; then two drops (0.1 cc.) more; and dilute with 4 cc. distilled water (total volume nearly 10 cc.).
8. Rinse in distilled water.
9. Tone in 1 cc. glacial acetic acid + 5 cc. 0.33% gold chloride solution and bleach the connective tissue for 10–15 minutes.
10. Wash in water.
11. Reduce in 1% oxalic acid for five minutes or until sections are purple.
12. Rinse in water.
13. Fix in 5% sodium thiosulfate solution for five minutes.
14. Wash 3-5 minutes in running water.
15. Dehydrate with alcohol, remove collodion with alcohol ether mixture (or do both at once with acetone), clear in xylene, and mount in Clarite or balsam.

Results: Black nerve endings, background in reds and purples.

Ehrlich’s supravital methylene blue method is the other classical method for study of peripheral nerve endings. I have had no personal experience with this method, but have relied largely on Romeis, Lee, and Mallory for the following. The solution should be made from medicinal, zinc-free methylene blue. The dye should be dissolved in 0.9% sodium chloride of C. P. grade (0.6% for amphibia), not in Ringer’s or Locke’s or similar fluids, as these produce precipitates. The methylene blue is introduced into the tissues by intravascular perfusion, by instillation into body cavities, by injection into loose connective tissues, or simply by immersion of small fragments. Within the tissues the methylene blue is reduced to leucomethylene blue, and then reoxidized by atmospheric oxygen. Tissues must be taken as soon after cessation of circulation as possible. Human autopsy tissues are ordinarily not usable. Blood must first be washed out with saline solution before using the perfusion methods, though some use an intravital perfusion in living animals. The strength of methylene blue solution varies between 0.5% and 0.06%.

For perfusion and injection techniques use about 0.2% methylene blue at 37° C., and perfuse with sufficient volume to replace the salt solution used to wash out the blood; or inject with enough to distend the tissues moderately. After 30 minutes remove the tissues to be studied and place on glass wool moistened with 0.06-0.1% methylene blue in a loosely covered container in a 37° C. incubator. Tissue should be taken in small fragments or thin slices. Leave in the incubator until tissues become bluish—perhaps 15 to 120 minutes.

For the immersion technique immerse thin slices or small fragments of tissue in 0.1-0.25% methylene blue at 37° C. Romeis prefers to lay them on glass wool moistened with 0.05-0.1% methylene blue and to drip onto the surface of the fragments (which may measure 5-20 mm. in thickness) a 0.1-0.25% dye solution to flood the surface. Keep the preparations at 37° C. for 1-2 hours, observing under low magnification from time to time until nerves are stained blue.

Sectioning and Fixation. After staining by the variants above has reached its optimum, Romeis prescribes cutting fresh sections with a razor and immediate study; or fixation with ammonium molybdate according to Dogiel—a freshly prepared 5-8% solution, which is to be filtered if not clear. Thin
membranes are adequately fixed in 30-60 minutes. Thicker tissue sections may require up to 24 hours. This requires 50-100 volumes of solution. Then wash 1-6 hours in several changes of water according to size and thickness. Preparations are to be dehydrated rapidly in several changes of 95% and 100% alcohol during a total period of 10 minutes to 2 hours—again according to thickness. Alcohol tends to extract the stain. Clear in terpineol, changing once. Wash out in xylene, and mount in balsam (or Clarite).

Romeis also suggests the preparation of thick frozen sections after washing out the ammonium molybdate, with or without gelatin imbedding. Paraffin imbedding extracts the color badly.

Dogiel also proposed a fixation in saturated aqueous ammonium picrate solution (p. 87) for 2-24 hours, followed by mounting in equal volumes of glycerol and ammonium-picare solution.

Schabadash (Ztschr. f. Zellforsch. 10:221-385, 1930) proposed addition of 50-700 mg. resorcinol (or in some instances para-aminophenol) per liter of methylene blue, and buffering of the methylene blue solution with phosphates to various pH levels ranging from 5.7 for colon to 7.2 for isolated nerve. The methylene blue concentrations ranged from 150 to 350 mg. per liter for the most part. He fixed in a mixture of ammonium iodide or ammonium thiocyanate ("rhodanid") in ammonium picrate. The latter is to be impure and contaminated with enough "ammonium picraminicum" (picramate?) so that the crystals are large, orange needles, not the fine, yellow needles of the pure salt. Either a 2.11% solution of ammonium iodide or a 1.12% solution of ammonium thiocyanate is saturated with this ammonium picrate at 60° C., and then allowed to stand several days at room temperature. One fixes the stained preparations 3-6 hours at 35° C., and mounts directly in a mixture of equal volumes of C. P. glycerol saturated with ammonium picrate and of 10% gelatin solution. Thicker pieces may require preliminary clearing in saturated ammonium picrate glycerol.

Apáthy uses his gum syrup as a mounting medium for crushed or spread whole mounts.

Bethe proposes fixation in 5% ammonium molybdate containing either 0.25% osmium tetroxide or 1% chromic anhydride (CrO₃). In either case add 1 drop (0.05 cc.) of concentrated hydrochloric acid to 20 cc. of the fixative. Fix 4-12 hours in the osmium tetroxide variant, 45-60 minutes in the chromic; then wash, dehydrate quickly in alcohols, clear in benzene, and imbed in paraffin; or clear in xylene for direct mounting in Clarite or balsam (cited from Romeis).


Liang prescribed one-hour fixation in 1% acetic or formic acid, followed by two rinses in sulfurous acid of unspecified strength and 1-3 hours' immer-
sion in Schiff reagent (p. 155) until nerves are deep purple. He then washed in several changes of sulfurous acid, rinsed with distilled water, counterstained 15 minutes in 1% aqueous methyl green solution, dehydrated, cleared, and mounted as whole mounts. Or after the washing following the second sulfurous acid bath, he dehydrated, cleared, and imbedded in paraffin, sectioned, brought sections to water, stained five minutes in 1% methyl green, dehydrated quickly with alcohols, cleared, and mounted.

For the paraffin sections, an acid hemalum or iron hematoxylin picric acid counterstain can be used and renders other structures more visible. Elastic tissue of blood vessels sometimes stains red purple by this method, and myelin shows red-purple vacuolation figures around the solid red-purple axone.

Retina

Apparently three lipoid substances are demonstrable (Lillie, Anat. Rec. 112:477, 1952) in the rods and cones. One is relatively insoluble, is located in the acromeres, is apparently partly protein-bound, and is preserved in paraffin sections even after some partly alcoholic fixations; but is removed by alcohol ether and alcohol chloroform extraction from fresh unfixed tissue. It is best demonstrated by the periodic acid Schiff reaction after various aqueous fixations. It retains iron hematoxylin poorly in myelin type technics (pp. 194, 329), and best after formalin bichromate sequence fixations; and may be demonstrated with oil-soluble dyes in Ciaccio type technics (p. 320). It is thought that this lipid may be keratin.

The second lipoid substance, also located in acromeres, requires aqueous formaldehyde or bichromate fixations for its preservation in paraffin sections, and is best demonstrated by peracetic acid or performic acid Schiff technics after formalin bichromate sequence fixations. Conditions which produce Kolmer's droplets give also fine droplets between the rod acromeres which are performic acid Schiff positive. It is probably largely responsible for the positive myelin stains which are obtained after formalin bichromate combination or sequence fixations. It is assumed that this lipid also stains with fat stains in paraffin sections. That it is not identical with the periodic acid Schiff-positive material is indicated by its presence in Kolmer's droplets, and by its loss from sections first fixed in lead or mercury salts without chromates or formaldehyde and then dehydrated and imbedded by an alcohol-gasoline-paraffin sequence. This lipid is perhaps an unsaturated fatty acid lecithin.

The third lipoid is demonstrated by oil-soluble dyes and by iron hematoxylin methods after aqueous formaldehyde or chromate fixations, but not by the periodic, performic, or peracetic acid Schiff methods. It is localized in the ellipsoids and is associated with a strongly eosinophilic (p. 120, 349) basic protein, which is especially well shown after Carnoy and alcoholic formalin fixations.

Glycogen is demonstrated in the retina by the periodic acid Schiff method, removed by diastase digestion, and localized chiefly in the myoid segments of
rods and cones and in the zones of synapsis. Sometimes entire granule cells seem to be outlined by heavy glycogen deposits.

Myelin seems more difficult to demonstrate in the nerve-fiber layer of the retina than in the optic nerve or in the small oculomotor branches seen outside the bulb. It reacts with the performic acid Schiff as well as with hematoxylin methods and oil-soluble dyes.

For the demonstration of Kolmer’s droplets the preferably dark-adapted retina should be fixed 12–24 hours in Held’s fluid. This is a vague mixture composed of 4 volumes each of saturated aqueous potassium bichromate solution and of 10% formalin, with the addition of 1 volume of glacial acetic acid. By increasing the amount of acetic acid, droplets can be shown in relation to cones as well as to rods. After fixation, the tissues are washed overnight in running water, dehydrated through alcohols, and imbedded in paraffin, not celloidin. Staining is done with an iron hematoxylin procedure of the Heidenhain type, and preparations are differentiated, according to Kolmer, to the point where rod acromeres become colorless. At this point ellipsoids should still be rather darkly stained as well as the Kolmer droplets. According to Walls (Anat. Rec. 73:373, 1939) these lipoid droplets are artefactual, produced by high concentrations of acetic acid from acromere and pigment epithelial substances and oxidized to an insoluble state by chromation.

For the demonstration of rhodopsin (visual purple) fix retina in 2.5% platinic chloride solution for 12–24 hours (Romeis), dehydrate in alcohols, and imbed in paraffin as usual. The rod acromeres are colored an intense orange. This is essentially the method of Stern (Arch. f. Ophth. 61:561, 1905), who prescribed the use of animals dark-adapted for at least two hours and the use of red light only, during dissection, blocking, and fixation.

Other Ocular Structures

The vitreous humor contains a webwork of coagulum staining red by the periodic acid Schiff procedure and blue by the allochrome method. It colors blue with spirit blue in the Ciaccio type method using this dye (p. 320) but not with Sudan black B. It does not stain with basic thiazin dyes, either ortho- or metachromatically. It sometimes colors blue by the Lillie-Mowry ferric mamitol method (p. 288). Its staining is not evidently affected by bovine hyaluronidase chondromucinase preparations (p. 291).

The suspensory ligament of the lens merges into this vitreous web in periodic acid Schiff preparations, but like lens capsule and Descemet’s membrane, remains red in allochrome preparations.

Corneal connective tissue differs from that of the sclera and various ocular arcular tissues in its metachromasia to thionin, which may be demonstrable after lead nitrate or mercuric chloride fixatives (pp. 37–42), and in its pure deeper red color with periodic acid Schiff procedures when a picric acid counterstain is used.
Conjunctival epithelium may show quite pronounced alkaline phosphatase activity (p. 202) as well as moderate amounts of glycogen and some melanin. The conjunctival goblet-cell mucin is rather weakly stained by metachromatic dyes and alcian blue (pp. 285, 288), quite well by HIO₄ and CrO₅ Schiff procedures (pp. 123, 124).

The allochrome method (p. 357) often fails to distinguish a distinct red basement membrane, though one is often shown under the corneal epithelium and the retinal pigment epithelium over chorioid and ciliary.

Ocular pigments are considered on pp. 255, 256.
Bones, teeth, and other calcified tissues are sometimes studied by the preparation of ground sections of the macerated specimen (p. 428), but usually the removal of most of the calcium salts is required to permit the preparation of sections thin enough for the study of cellular detail.

Adequate fixation of hard bone is facilitated by sawing thin slices with a fine-toothed bone saw or hacksaw. This procedure produces a narrow zone of mechanical distortion adjacent to the saw cut. Mallory discarded the first half dozen sections; I have preferred to slice off this zone with a razor blade after decalcification and then section from the fresh-cut surface. Making such a cut through the center of a block further serves as an adequate test of completion of decalcification.

When the marrow of the cancellous bone is the primary objective of study, time may be saved and the acid damage to marrow cells reduced by cutting off the cortical bone as soon as the cancellous portion is soft enough.

Fixatives for bone and marrow should be chosen with the primary objective of study in mind, and are much the same as for soft tissues. Chromate fixatives such as Orth's, Kose's, and Möller's potassium bichromate formalin mixtures (p. 44) and formalin Zenker variants such as the Spuler-Maximow fluid (p. 39) are preferred by many for the study of marrow cells. They render the nucleic acids less susceptible to hydrolysis either by ribonuclease or by acids (pp. 139, 141), and engender moderate resistance even to normal hydrochloric or nitric acid.

When preservation of iron-bearing pigments is important, and for routine diagnostic purposes, formalin is the fixative of choice. The calcium phosphate of the bone substance serves as a fully adequate buffer to keep the pH level of the fixative probably above 6.0 within the tissue. If the action of the formaldehyde is not prolonged beyond the 2-3 days recommended for adequate fixation, nucleic acids are left quite susceptible to ribonuclease digestion or
hydrolysis by mineral acids. Hence formalin-fixed bone is preferably decalcified with formic acid or with buffer mixtures of pH 2.0 or higher.

For general diagnostic purposes, 5–7.5% nitric acid has been found effective over many years of trial. Recently the 8% hydrochloric, 10% formic-acid fluid of Richman, Gelfand, and Hill (Arch. Path. 44:92, 1947) has had considerable vogue (p. 427). This fluid is 1 N HCl containing 2.4 N HCOOH. It is a quite active and effective decalcifying agent. It will decalcify 1–2-Gm. pieces of compact bone in 12–24 hours at room temperature and in 6–8 hours at 37° C.

The effect of the electrolytic bath recommended by these authors for use with this fluid seems to be purely that of the heat produced by the passage of the current, since equally rapid decalcification occurs with external heating to the same temperature or in bones suspended in the electrolyte at the maximum possible distance from either electrode.

Decalcification at 55–60° C. is hazardous. Loss of calcium salts occurs rapidly and is followed promptly by swelling and hydrolysis of bone matrix, which soon results in complete digestion. This occurs in as little as 2½ hours in the 8% hydrochloric, 10% formic acid mixture and in 2–3 days in 5% formic acid.

Decalcification at 37° C. impairs alum hematoxylin staining and Weigert's iron hematoxylin staining of nuclei to some extent. Eosin stains of cytoplasm are well preserved. Feulgen staining of nuclei is unsuccessful, and azure eosin stains give pink cytoplasms and nuclei. Both the formic hydrochloric mixture and 5% formic acid produce these results. The latter also impairs Van Gieson and Masson staining of collagen and bone matrix. Mineral acid decalcification is 2–3 times faster at 37° C. than at 20° C.; formic acid is actually somewhat slower.

With decalcification at 15–25° C., either in mineral acids or in formic acid or in mixtures of formic and hydrochloric acids, satisfactory hematoxylin eosin, Van Gieson, Masson, and azure eosin stains may be obtained, if exposure to the mineral acids is not prolonged. Feulgen staining of nuclei is well preserved after formic acid decalcification at room temperature, but with mineral acids at 20° C. or even at 3° C. it is impaired or destroyed.

Altogether, for other than enzyme demonstration, 5% formic acid seems to be the best general decalcifying agent. It is far more economical of reagents than the Kristensen and other buffer mixtures and seems to be equally effective. The preferred procedure is to use two changes daily of about 25 cc. per Gm. bone, testing the removed fluid at each change to determine the presence of calcium ion by mixing 5 cc. of it with 1 cc. of 5% sodium or ammonium oxalate. When a negative test is obtained—wait several minutes for the development of delayed turbidity—the specimens may be considered decalcified. This test seems more objective and less liable to underdecalcification than the common practice of testing with a pin. It coincides well with the cessation of weight loss. It is equally applicable to decalcification with min-
eral acids, but it must be recalled that calcium oxalate will not precipitate from hydrochloric or nitric acid above about 0.7 N or 0.8 N final dilution. The concentration of stronger acid must be reduced by dilution or by addition of an amount of 2 N NaOH which will not quite neutralize; a drop of Congo red solution will give a color change at about pH 4–5.

Following this procedure 1–2 Gm. blocks of compact bone 3–4 mm. thick decalciﬁy in 4–5 days in 5% formic acid; cancellous bone, in 2–3. In 5% nitric acid it takes about half that time, and in the 8% hydrochloric, 10% formic acid mixture, even less: some 4–8 hours for cancellous and 12–24 for compact bone.

Trichloracetic acid is also a fully satisfactory decalciﬁying agent which permits just as satisfactory nuclear and marrow cell staining as does formic acid. However, on account of its high molecular weight (163) as compared with that of formic acid (46), much larger quantities (3.55 X) are needed for efﬁcient decalciﬁcation.

Sulfurous acid, available as a 5–6% solution of sulfur dioxide in water, is also a prompt decalciﬁying agent, but if its action is prolonged more than 48 hours, nuclear staining is seriously impaired.

After decalciﬁcation with nitric, formic, trichloracetic or sulfurous acid, tissues should be washed 18–24 hours in gently running water to remove acid, then hardened in 80% alcohol, dehydrated and imbedded as usual in parafﬁn or nitrocellulose. Frozen sections of decalciﬁed bone for the study of fats and myelinated nerves are readily prepared on the freezing microtome. In this case, naturally, all contact with alcohol or other fat solvents is to be scrupulously avoided.

Alcoholic solutions of acids are quite efﬁcient decalciﬁying agents. Solutions in 70% or 80% alcohol are quite slow: 0.5 N HCl took eight days at 25° C., compared with four days for 0.5 N HCl in 40% alcohol and two days for aqueous 0.5 N HCl. With formic acid 5% solutions took 3–5 days in water, about four months in 30% alcohol, and failed to decalciﬁy in several months in 80% alcohol. This occurs probably more because of the suppression of ionization by alcohol than because of insolubility of certain calcium salts in alcohol. Calcium nitrate, calcium chloride, and calcium bromide are listed as soluble to very soluble in alcohol; calcium acetate as slightly soluble; the formate as insoluble; but addition of 5% of either glacial acetic acid or of 90% formic acid to a saturated 70% alcohol solution of calcium chloride occasions no precipitation, though small quantities of oxalic or sulfuric acids give copious precipitates.

In the study of fractures, periosteal hemorrhages, and similar lesions where preservation of precise anatomic relationships is of more importance than cytologic preservation, it is often desirable to imbed in nitrocellulose either after or preferably before decalciﬁcation. Imbed before decalciﬁying to make sure that soft tissues are not displaced from hard.

For the gross opening of long bones in scurvy, rickets, and similar condi-
When imbedding the bone in plaster, its position may be marked by inserting toothpicks in the soft plaster to mark the plane of sawing. Allow the plaster to set for about an hour. Saw with a thin-bladed hacksaw. Then break off the plaster and wipe the surface with gauze or with a brush moistened in 5% acetic acid. The bones are usually greasy enough to separate readily from the plaster. If difficulty is encountered in this respect, the bones may be first dipped in light mineral oil before imbedding in plaster. I am indebted to Drs. J. H. Peers and T. H. Tomlinson for this hitherto unpublished note.

Mechanical agitation appears to have no great effect on the speed of decalcification, so that apparatus designed for this seems scarcely worth the trouble or expense.

The use of vacuum during decalcification does not materially expedite the process, but it does prevent the formation of large gas bubbles in the narrow in large pieces of bone. With small bones it has no evident value.

Prolonged, slow decalcification with fluids of relatively high pH level (5.0), such as 5–10% solution of ammonium chloride, ammonium nitrate, or potassium or sodium acid phosphate, gives excellent preservation of marrow cells after 2–3 weeks; but cortical bone is still hard at the end of three weeks.

Frank and Deluzarche (Bull. d'histol. appliq. 27:35, 1950) recommended for the decalcification of enamel and dentine in the crowns of human teeth first a primary fixation in 10% formalin followed by a 12-hour refixation in Bouin’s fluid. Then the crown is rinsed in water, in alcohol, and in ether, and coated with three layers of collodion on the enamel surface. Decalcification follows in a fluid composed of 2 Gm. nitric acid (sp. gr. 1.38) (1.23 cc. of our 69–70% acid, sp. gr. 1.42), 8 Gm. trichloracetic acid, 20 cc. Bouin’s fluid, and 70 cc. water. Maintenance of partial vacuum (about 100 mm.) during decalcification is recommended. Dehydration, clearing, and infiltration through graded alcohols, cedar oil, toluene, and paraffin should be fairly rapid.

Hahn and Reygadas (Science 114:462, 1951) report the use of a solution of sodium ethylene-diamine-tetra-acetate for demineralization of hard tissues. This solution is alkaline and the evolution of carbon dioxide is thereby prevented. Complex soluble electronegative calcium-containing ions are formed. If the ethylene-diamine-tetra-acetic acid is neutralized with quaternary sodium pyrophosphate instead of hydroxide, the demineralization effect for iron salts is increased. No further details as to concentration of solutions have been published.

Since iron salts are also removed along with calcium salts, this process is
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not recommended in those cases where demonstration of iron-containing pigments may be important.


Formulae for Decalcifying Fluids

1. 5% nitric acid.
   Concentrated (68–70%) nitric acid (sp. gr. 1.41) 50 cc.
   Distilled water 950 cc.
Older directions called for tenfold dilution of the Pharmacopeial acid, sp. gr. 1.25, assay about 500 Gm. HNO₃ per liter (Lange). Later some of the German writers prescribed 75 cc. of the 68% acid, sp. gr. 1.41, per liter, apparently neglecting the specific gravity in their computations. (Schmorl 1907, 1928, Roulet 1948, Romcis 1932, but corrected in 1948).

2. Von Ebner’s hydrochloric acid-sodium chloride mixture.
   Concentrated hydrochloric acid (sp. gr. 1.19) 15 cc.
   Sodium chloride 175 Gm.
   Distilled water 1000 cc.
During decalcification add 1 cc. of concentrated hydrochloric acid daily to each 200 cc. of the above mixture, until decalcification is complete.

3. Richman-Gelfand-Hill formic hydrochloric acid mixture.
   Concentrated formic acid (90%) 100 cc.
   Concentrated hydrochloric acid (38.8%, sp. gr. 1.19) 80 cc.
   Distilled water 820 cc.

4. 5% formic acid.
   Concentrated formic acid (90–92%) 50 cc.
   Distilled water 950 cc.

5. Kristensen’s fluid.
   1N sodium formate (6.8%)
   8N formic acid (see tables, p. 456)
   500 cc.

6. Formalin formic acid, according to Schmorl.
   Formic acid (90%) 500 cc.
   Formaldehyde (40%) 50 cc.
   Distilled water 430 cc.
Schmorl recommended this as working rapidly, without producing swelling. Nuclear staining was said to be well preserved.

   Sodium citrate crystals 10 Gm.
   90% formic acid 25 cc.
   Distilled water 75 cc.

8. Evans and Krajian’s fluid, Krajian’s variant.
   85% formic acid 100 cc.
   95% ethanol or 99% isopropanol 100 cc.
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Sodium citrate crystals 20 Gm.
Trichloracetic acid 1 Gm.
Distilled water 100 cc.

   Normal hydrochloric acid 540 cc.
   Molar sodium citrate solution 460 cc.
\[ \text{(29.4\% of the dihydrate, or 35.7\% of the } \text{Na}_3(\text{CO}_2)_2\text{C}_6\text{H}_4\text{OH} \cdot 5\\% \text{ H}_2\text{O)} \]

10. Lorch’s citrate HCl buffer of pH 4.4.
   Citric acid crystals 14.7 Gm.
   0.2 N sodium hydroxide 700 cc.
   0.1 N hydrochloric acid 300 cc.
   1\% zinc sulfate 2 cc.
   Chloroform 0.1 cc.

11. Normal acetate buffer of pH 4.5.
   Normal acetic acid 520 cc.
   Normal sodium acetate (8.2\% anhyd.; 13.6\% cryst.) 480 cc.
   Add 2 cc. 1\% zinc sulfate and 0.1 cc. chloroform.

   Normal citric acid (monohydrate 7\%)
   Normal ammonium citrate (anhydrous 7.54\%)
   Add 2 cc. 1\% zinc sulfate and 0.1 cc. chloroform.

Fine Structure of Bones and Teeth

Ground sections of bones and teeth are sometimes required for the study of bone lamellae and canaliculi, and dentine tubules and enamel prisms. These are made from macerated and dried bone.

To macerate bones and teeth, saw into fairly thin pieces, soak in several changes of water over a period of several months. Then wash out and dry thoroughly in air. Then grind sections until nearly transparent between two pieces of pumice stone with water, or between two pieces of plate glass with powdered pumice and water. When sections are thin enough wash in water and dry.

White (cited from Lee) recommended cutting moderately thin slices of bone or tooth, presumably previously macerated and dried, and soaking in ether for one or two days. Then soak in collodion colored red with basic fuchsin for two or three days. Then transfer to 80\% alcohol for another two or three days and finally grind down nearly to transparency by rubbing between two pieces of ground glass with water and pumice powder. Dry the surfaces and mount.

To mount ground sections, place on a slide a small fragment of solid balsam or Clarite and heat until it just melts. Do the same with a cover glass. Place the ground section in the resin on the cover glass and press down the resin area on the slide on top.

Results: Lacunae and canaliculi are filled with air or with the colored
collodion. Enamel requires preliminary etching with 0.6% hydrochloric-acid alcohol or weak aqueous picric acid and mounting in solid camosal* which has a lower refractive index (1.478).

Bone matrix in paraffin or nitrocellulose sections of decalcified bone stains pink with hematoxylin-eosin and azure-eosin methods (pp. 114–120). Remnants of cartilage in newly ossified bone are stained a deep purple with the latter. With Van Gieson’s picrofuchsin (p. 346) bone matrix is a deep red. Osteoid tissue is similarly stained, contrasting with the pink to yellow of cartilage in fracture callus. Anilin blue and methyl-blue collagen methods stain bone matrix only lightly.

Schmorl’s method is sometimes employed for the demonstration of lamellae and canaliculi in compact bone. Formalin fixation is recommended and should be followed by 6–8 weeks at 20° C. or 3–4 weeks at 37° C. in Müller’s fluid (p. 44). Then wash 24 hours in running water and decalcify according to preference. Schmorl recommends direct transfer from Müller’s fluid to hydrochloric acid alcohol: hydrochloric acid 2.5 cc., sodium chloride 2.5 Gm., distilled water 100 cc., 95% alcohol 500 cc. When decalcification is completed, wash in several changes of 80% alcohol. Then dehydrate and imbed in paraffin or celloidin. Cut thin sections.

1. Bring to water as usual.
2. Stain five minutes in half-saturated aqueous (0.125%) thionin. Staining may be accelerated and intensified by addition of a drop or two of ammonia water to the thionin.
3. Rinse in water.
4. Differentiate 1–2 minutes in 95% alcohol.
5. Again rinse in water.
6. Transfer to concentrated aqueous phosphotungstic acid. Differentiation is completed in a few seconds, but longer exposure is harmless.
7. Wash 5–10 minutes in water until sections are sky blue. Longer washing is harmless.
8. Fix the color in a mixture of equal parts of concentrated formalin and water for 1–2 hours.
9. Dehydrate in two changes of 95% alcohol and two of 100% alcohol and clear in one change of equal parts 100% alcohol and xylene and two changes of xylene. For celloidin sections substitute 99% isopropyl alcohol for the 100% ethyl alcohol in the three steps in which it is used.

Results: The walls of bone cavities and their processes are blue black; cells are diffuse blue; nuclei, only little darker than cytoplasm. Nuclear staining may be intensified by insertion of an alum hematoxylin stain after the formalin step. The method is excellent for teeth. Bone matrix is light blue.

* Camosal is an ordinarily liquid mixture of phenyl salicylate (salol) and camphor, in such proportion that a little camphor remains undissolved.
Cement lines between lamellae are readily seen. Fibrillar structure is distinct.

Powers, Rasmussen, and Clark (Anat. Rec. 111:17, 1951) have adapted the Romanes method (p. 413) for the demonstration of dentine tubules. They recommend fixation in a modified Bouin fluid composed of 75 cc. saturated aqueous picric acid, 25 cc. formalin (37% HCHO) and 1 Gm. trichloracetic acid; decalcification in 5% trichloracetic acid in 50% alcohol; and paraffin imbedding. Sections are deparaffinized, hydrated and mordanted 18 hours in 1% aqueous cupric nitrate solution (Cu(NO₃)₂·3H₂O?) at 37° C., washed thrice in distilled water, and put through the Romanes procedure. The solution used in Step 1 (p. 413) is adjusted by addition of 16 drops (1 cc.?) of 1% ammonium hydroxide (a 1% dilution of 28% ammonia water?).

Lillie's method of silver impregnation (Ztsch. wiss. Mikroskop. 45:380, 1928) with subsequent decalcification distinguishes osteoid tissue and new, uncalcified bone lamellae from older calcified lamellae, and in denser bone often gives a very sharp definition of lacunae and canaliculi. Gomori has used a basically similar method with similar results, but his is somewhat more complicated (Am. J. Path. 9:253, 1933).

Lillie's method:

1. Fix in 10% formalin for two days or more.
2. Wash thoroughly in several changes of distilled water.
3. Silver for 4-5 days at 37° C. in 2-2.5% silver nitrate solution.
4. Wash thoroughly in distilled water.
5. Decalcify in Ebner's sodium chloride hydrochloric acid mixture: concentrated hydrochloric acid 3 cc., saturated aqueous (95%) sodium chloride solution 100 cc., distilled water 100 cc. To this add 1 cc. hydrochloric acid daily until decalcification is complete.
6. Wash out acid in 2-3 changes of half-saturated sodium chloride solution over 4-5 days. If the salt solution becomes acid to litmus, add a few drops of dilute ammonia water to neutralize.
7. Wash 18-24 hours in running water, harden 1-2 days in 80% alcohol, imbed in paraffin or nitrocellulose. Section and counterstain with hemalum and eosin or, better, with Weigert's iron hematoxylin and Van Gieson's picrofuchsin (p. 346).

Results: Thin trabeculae of bone are completely blackened; thicker bone shows black outer lamellae, with perhaps an unsilvered lamella or two adjacent to the peri- or endosteum, and deep lamellae respectively pink or red according to counterstain, with black lacunae and canaliculi. Calcified areas in cartilage and other calcium phosphate deposits also blacken. Dentine is blackened; enamel partly so.

Actually, like the Von Kossa method, this demonstrates phosphates rather
than calcium salts, but since soluble phosphates are first washed out, it is essentially calcium phosphate that is demonstrated by both methods.

Feeding of madder or of alizarin (C. I. No. 1027) has been used for the marking of newly formed bone. In place of the rather uncertain feeding procedure one may give perhaps two intraperitoneal injections weekly, of 200 mg. per kg. (in rats) of alizarin red S (C. I. No. 1034) over a period of several weeks. Rats given more frequent injections presented evidence of renal damage. At autopsy bone laid down during and shortly after the period of alizarin administration is found to be stained red (Highman, unpublished data).

Unfortunately this red color is extracted or decolorized by most decalcifying agents.

Alkaline Phosphatase in Bone

For studies of alkaline phosphatase distribution in calcified bone, fixation in 70% ethyl or isopropyl alcohol for 24 hours at about 20–25° C. is recommended. Then decalcify at 0–5° C. in a buffer mixture of pH 4.5 or higher, using 5–10 cc. per 100 mg. bone and changing daily until oxalate tests for calcium are negative. Normal ammonium citrate + citric acid buffer is more effective than normal acetate buffer (Formulae 10, 9, p. 428); the one dissolves 777 mg. calcium as Ca₃(PO₄)₂ per 100 cc., the other 411 mg. Both decalcify considerably more rapidly than Lorch's mixture or the 0.1 M citrate and 0.1 M citrate and hydrochloric acid buffers (Formula. 8, and 1/10 dilution of Formula 7, p. 427, and the pH 4.5 mixture of the Horecker-Lillie buffer, p. 450), and appear to give adequate preservation of alkaline phosphatase activity. In the presence of an excess of bone, citrate buffers may give rise to copious calcium citrate precipitates which are deposited on the surface of the specimen as well as on the bottom of the container. The acetate buffers remain clear. Formate buffers of pH 4.5 are relatively poor calcium solvents, and tartrate ion produces a highly insoluble calcium salt. Neither of these can be recommended for this special purpose.

If it becomes impractical to continue daily changes and observations for completion of decalcification, the process may be interrupted by washing briefly in water 10–15 minutes, transferring to 80% isopropyl alcohol, and storing at −20° or −25° C. in a deep-freeze compartment until daily changes can be resumed. Then wash 10–15 minutes in water and return to the decalcifying fluid.

On completion of decalcification, wash overnight in running water and incubate six hours at 37° C. in 1% sodium barbital solution containing 75 mg. glycine per 100 cc. to reactivate the enzyme. Wash 2–4 hours in running water to remove glycine. Dehydrate through ascending alcohols and clear in lead-free gasoline or petroleum ether. A Technicon schedule (p. 64) may be used, but carriers and containers must be chemically free of metal and CO₃
ions. Infiltrate 10–15 minutes in paraffin at 58°C at 10–15 mm. mercury pressure in a vacuum oven.

If a vacuum oven is not available, preparations may be infiltrated with paraffin in 20–30 minutes after dealcoholization with isopentane (B. P. 28°C), ethyl ether (B. P. 37°C), or a low-boiling petroleum ether (Baker's special, B. P. 20–40°C). Or the usual 35–60°C petroleum ether may be used, but it will take longer to boil off. The specimens should be kept immersed in the paraffin by enclosing in wire gauze or in a Technicon capsule.

The paraffin sections are then handled for phosphatase demonstration according to the usual technics as for soft tissues. See p. 202, or below, for Lorch's special method for simultaneous demonstration of phosphatase and preformed phosphates.

General references.


1. Fix 12–24 hours in 80% alcohol, dehydrate in ascending alcohols, and clear and infiltrate in paraffin. I suggest the petroleum ether-vacuum infiltration sequence, or one of the variants on p. 203. Section at 8–10μ. The method is applicable only to developing bones which may be sectioned successfully without decalcification.

2. Deparaffinize and hydrate as usual.

3. Immerse in 2% cobalt nitrate for five minutes.

4. Wash in 3–5 changes of distilled water, 1–2 minutes each.

5. Immerse for 30 seconds in freshly diluted, 1% ammonium sulfide.

6. Wash five minutes only in tap water.

7. Incubate 20 minutes to 15 hours at 37°C in this substrate:
   - 2% calcium nitrate (Ca(NO₃)₂·4H₂O) 10 cc.
   - 2% magnesium chloride (MgCl₂·6H₂O) 10 cc.
   - 4% sodium β-glycerophosphate·5H₂O 10 cc.
   - 1% sodium barbital 70 cc.
   - Ammonium sulfide 1 drop.

8. Wash in 1% calcium nitrate.

9. Immerse for ten minutes in saturated aqueous gallamine blue (C. I.
No. 894, Conn's K-15) buffered to pH 7.0 (I suggest Michaelis's veronal sodium, p. 454).
10. Rinse 10–12 seconds in 0.5% sodium hydroxide.
11. Wash in water, dehydrate, clear, and mount as usual. Interpose counterstain if desired: safranin, methyl green, neutral red, eosin, or orange G.

*Results:* Sites of phosphatase activity are shown by purple calcium lake; preformed phosphates, by dark-brown cobalt sulfide.

**Phosphorylase**

Cobb (*A.M.A. Arch. Path.* 55:496, 1953) reports a technic for the histochemical localization of phosphorylase activity in young rat tibiae. The bones from freshly killed rats were split lengthwise and frozen in isopentane at −150° C. They were then desiccated in vacuo at −30° C. for at least three days. Next followed imbedding in vacuo for 15 minutes in the chloroform-soluble fraction of carnauba wax* and sectioning in this very hard wax without decalcification. Sections were dewaxed in chloroform.

**Cobb's Phosphorylase Technic.**

1. Dewax 10 minutes in chloroform, thus also partially inactivating the bone alkaline phosphatase.
2. Digest 90 minutes in amylase, saliva, or diastase at 37° C. to remove glycogen.
3. Soak overnight in distilled water to dissolve out all soluble bone salts.
4. Inactivate phosphoglucomutase by heating in a few drops of water in a moist chamber (in vitro this enzyme resists 65° C.).
5. Incubate 6 hours at 37° C. in pH 5.9 acetate buffer containing a "high" concentration of glucose-1-phosphate and an unstated amount of barium chloride to precipitate liberated phosphate ions.
6. Fix glycogen overnight in 100% alcohol. Stain glycogen by the periodic acid Schiff procedure (p. 123). Cobb used the Hotchkiss A (?) variant. For controls omit Steps 3 to 5 above. The excess over the controls represents glycogen newly formed as a result of phosphorylase activity.

Although I have not tried this method, and as published it lacks some necessary details, the rationale seems good and the procedure is worthy of trial.

* Carnauba wax is used industrially in furniture and floor waxes and should be procurable through the makers of these products.
Chapter 20

Various Special Procedures

Vascular Injections

The practice of vascular injection with colored masses has had wide application in the study of the circulatory system of various organs. The Berlin-blue and carmine gelatin masses are classical. They serve well for gross preparations and those intended for clearing and study under low magnification. With both of these some color loss occurs during paraffin imbedding, and a carbon mass has been found more satisfactory for strictly histologic purposes.

Carbon Gelatin Mass. Ashburn and Endicott, in this laboratory, have devised a carbon gelatin based on Mall’s statement that he used such a mass for study of the circulation of the liver. This mass must be freshly prepared each time, though it might be kept frozen for a few days if desired. The carbon particles are apparently held back by the capillaries so that either the afferent or efferent vessels may be injected without filling the other. This technic has been used in the study of lobular relationships of destructive and cirrhotic processes in the liver. The technic follows:

Dissolve 8 Gm. gelatin in 100 cc. warm water. Sift animal charcoal through a 100-mesh sieve and suspend 20 Gm. of the sifted charcoal in the gelatin solution in a conical glass of perhaps 200 cc. capacity immersed in a water bath at 45–50° C. While preparing the animal for injection keep the gelatin mass agitated with a small propeller-type glass stirrer driven by a small electric motor. The propeller is directed downward, and is kept fairly near the bottom of the conical glass.

Cannulae are made of glass with a slight bulbous enlargement on the end so that they can be tied in. It is preferable to have the opening on the side of the bulb, so as to avoid tears in the veins when introducing the cannula. At the same time, the opening must be distal to the constricted area about which the tie is made.

For injection of the hepatic veins Ashburn and Endicott remove the
thoracic and abdominal walls to expose the liver fully, tie off the vena cava below the liver, sever the portal vein, and insert the cannula into the right atrium and thence downward into the inferior vena cava. A ligature is then passed around the vena cava and tied proximal to the bulbous end of the cannula. Then wash out the blood from the liver with warm saline solution from a 20–50 cc. Luer syringe attached by a 15 cm. (6 in.) length of thin-walled rubber tubing to the cannula, carefully avoiding introduction of air. When clear saline solution flows from the portal vein, substitute another 10–20 cc. Luer syringe filled with the warm carbon gelatin and inject. In changing the syringes, carefully avoid air bubbles. The surface of the liver blackens quickly. Gentle digital massage of the liver surface during injection facilitates both removal of blood and penetration by the mass.

Injection of the portal system is done in a similar manner, by placing the cannula in the portal vein below the liver and opening the inferior vena cava above it.

With larger animals, or human organs, some type of gravity perfusion apparatus with a heating arrangement to maintain temperature is required. A manometer can readily be attached to the injection system with a T-tube and the pressure regulated by varying the height of the reservoir of the gravity apparatus.

Some workers use 0.5% ammonium oxalate and 0.75% sodium chloride in the fluid used for washing out the blood before injection. This tends to prevent clotting. The inclusion of 0.2% sodium nitrite, either in the washing or injection fluid, will promote vascular dilation.

Ashton (Brit. J. Ophth. 34:38, 1950) injects the retinal vessels with India ink. The long nerve end of the freshly enucleated eye is dipped in formalin for a few minutes. Then under a dissecting microscope, the optic nerve is cut across and a fine glass cannula is inserted into the isolated central artery. The vessels are first perfused for a few minutes with distilled water at about 500 mm. mercury pressure. This perfusion is followed by one with 10% formalin, and then by the India ink. The eyeball is then immersed in formalin for 12 hours before opening. While Ashton’s procedure was intended for gross display of the retina, Ashburn’s experience (p. 434) with the liver indicates that the material can be used for histologic purposes.

Fischer’s Milk Method (Centralbl. z. allg. Path. u. path. Anat. 13:977, 1902). Since these carbon masses do not penetrate the capillaries, and this is perhaps their greatest virtue, it is necessary to resort to other methods for demonstration of the capillaries. One may wash out with salt solution as above and then inject with milk or cream, tie off both afferent and efferent vessels, and fix in 7.5 cc. formalin, 1.5 cc. glacial acetic acid, and 100 cc. distilled water. Cut frozen sections and stain with Sudan III or IV (Fischer) or oil red O as usual (p. 303), and mount in Apáthy’s gum syrup (p. 109). Or one may use one of the carmine or Berlin blue gelatin masses.

Carmine gelatin masses are made by dissolving carmine in water with heat
and ammonia water, and then either first mixing with the gelatin solution and then neutralizing, or the converse. Overacidification with acetic acid is prone to occur and ruins the mass by producing a granular red precipitate. Mallory avoids this difficulty by driving off the excess ammonia by heat. I suggest the combination of gentle heat and blowing an air current over the surface of the carmine or carmine gelatin until ammonia is no longer detectable by odor or with moist litmus or nitrazine papers. Most of the directions for carmine gelatin masses are very vague as to the amounts of gelatin and of carmine in the final mixture. Robin alone among the authors cited in Lee prescribed 1 part gelatin and 7–10 parts of water for an aqueous gelatin mass, and 50 Gm. gelatin, 300 of water, and 150 of glycerol for a glycerol gelatin mass. He dissolved carmine in ammonia water and diluted it in glycerol and neutralized with acetic acid in glycerol, attaining perhaps a 3–4% stock solution of carmine, which was diluted with 3–4 volumes of either of the above masses.

**Berlin blue masses** we have generally found too pale for histologic use. Robin prescribed (A) 90 cc. (?) saturated aqueous (28–30%) potassium ferrocyanide solution plus 50 cc. glycerol; and (B) 3 cc. (30 cc.?) Pharmacopoeial (French) ferric chloride solution (about 26%) plus 50 cc. glycerol. These were mixed slowly, a few drops of hydrochloric acid added, and the mixture then combined with 3 volumes of one of his gelatin vehicles at 45–50° C. This is nearly a 15-fold excess of ferrocyanide. I suggest 17 Gm. crystalline potassium ferrocyanide, 85 cc. distilled water, and 50 cc. glycerol for solution A above; and 30 cc. U. S. or P. G. ferric chloride solution in B.

Or one may make a saturated aqueous “solution” of “soluble Berlin blue” at 60° C. and mix with 3 volumes of 12% gelatin solution at the same temperature.

Since glycerol masses especially tend to cause contraction of vessels, it is well to add 0.1–0.2% sodium nitrite either to the preceding saline solution or to the mass itself or to both.

After injection with any gelatin mass, quickly cool the injected organs and fix in 10% formalin. With Berlin blue masses, avoid alkali, since this tends to convert the ferric ferrocyanide into ferric hydroxide and soluble alkali ferrocyanide. The inclusion of a little ferric chloride in the dehydrating alcohol is suggested by Romeis.

**Corrosion Technics**

By substituting some such substance as rubber or neoprene latex for the injection mass and then digesting off the tissues with hydrochloric acid or artificial gastric juice, interesting casts of the vascular system may be obtained.

**Duff and More** (*J. Tech. Methods* 24:1, 1944) used such a method on adult human kidneys. Kidneys as soon as possible post mortem are washed with tap water at 75 mm. mercury pressure through a cannula tied into the
renal artery. After 30 minutes' washing, the pressure is raised to 150 mm. and washing continued for several hours (until the kidney becomes uniformly pale) but not over 12-15 hours. Then, leaving the cannula in place, disconnect from the water faucet and place in a covered dish at 4° C. for 6-12 hours to allow as much water to escape as possible. Then keep at 20-25° C. for about five hours before injection with neoprene. Completely fill the tubing with the neoprene before connecting to the cannula. Inject with air pressure at 150 mm. for 3½ minutes for normal kidneys. Injection of sclerotic kidneys may require five minutes' injection, or raising the pressure to 250 mm., or warming the kidney to 60° C. for 30 minutes before injection. Then disconnect and immerse the whole kidney in commercial hydrochloric acid at 56° C. for 24-36 hours, agitating gently from time to time until all the renal tissue can be removed by gently washing the cast in warm water.

The cast maintains its form floating in water, but collapses when removed. Small fragments of the vascular tree may be teased apart and cut off for microscopic study. The authors suggest Farrant's medium for examination under a cover glass of such fragments of the vascular tree. According to Lieb, neoprene casts should be stored in some mold-inhibiting fluid (J. Tech. Methods 20:48, 1940).

Similar corrosion methods have been used for injection of lungs through the bronchi, and simultaneous injection of the vascular system with neoprene latex of a different color can be done. Rigid plastics may be substituted for the latex.

McClenahan and Vogel (Am. Roentgenol. & Rad. Ther. 68:406, 1952) use an alloy of bismuth (44.7), lead (22.6), indium (19.1), tin (8.3), and cadmium (5.3), designated as “Cerrolow 117,”* which melts at 47.2° C.

Arteries of organs to be injected are ligated before removal from the body to prevent access of air. The injection system is cleared of air and the cannula is inserted into the artery and ligated in place under water. The organ is perfused with cool kerosene at 120-150 mm. mercury pressure until the return flow is free of blood clots. The organ and the alloy reservoir are then warmed for about 30 minutes in a water bath at about 50° C. The organ is so oriented that the metal enters from the lowest point, so as to avoid entrapping kerosene; and perfusion with metal is carried on until no more kerosene emerges from the efferent veins by maintaining a pressure nearly equal to the ante-mortem maximum. The organ is then chilled with cold water while pressure is still maintained. When cold, the organ is transferred to 20% potassium hydroxide (15% NaOH should serve) and the soft tissue is corroded off over a period of 24 hours, with two changes of alkali and periodic gentle washing in water.

Large casts tend to sag at room temperature. Imbedding in clear plastic and cold storage are suggested as means of keeping casts undistorted.

* Cerro de Pasco Copper Co., 40 Wall Street, New York, N.Y.
Injection apparatus for metal corrosion preparations. Hot and cold water faucet $A$, is over large sink $B$, with a water bath containing kerosene reservoir $F$, metal reservoir $G$, Y-tube $H$ with clamps (not shown) on each arm, cannula $I$, and ring stands $J$ to support reservoirs. Air pressure enters from inlet $C$, is measured by manometers $D$, and controlled by a maximum-pressure Starling escape valve $E$.

**Autoradiography of Radioactive Elements**

Recently a technic of study of distribution of so-called marked elements, or radioactive isotopes, has come into use. In brief, one selects a fixing fluid which on trial run does not extract the radioactive element as determined by examination of the fixing fluid with a Geiger counter after fixation.

Tissue from the organ under study is then fixed as usual in the selected fixative, imbedded in paraffin, and sectioned. Sections are floated onto slides in the usual manner. If dehydration and infiltration with paraffin has been adequate, the hazard of loss of radioactive material at this point is quite small. After drying, sections are deparaffinized with xylene, dipped briefly into 100% alcohol, immersed for 1–2 minutes in 0.5–1% collodion in ether and alcohol, drained, and dried in air.

Then the slide is placed on a piece of film or a photographic plate in the dark room, section side of the slide in contact with the emulsion side of the film or plate. For film, place a second blank slide on the back surface of the film. Film should be cut to the same size as the slide, so that it may be held firmly in place between the two slides. Fasten with cord, adhesive tape, or rubber bands, wrap in black paper, and put away for several days. If one
makes several such preparations, one piece of film may be developed daily until maximum contrasts are obtained.

These autoradiographs naturally are of the same size as the sections. They may be enlarged to a limited extent, depending on the fineness of grain of the photographic emulsion.

After the film has been removed, the slide may be stained as usual with any desired technic for comparison with the autoradiograph. It is suggested that the collodion film be allowed to remain throughout, and finally dehydrated and cleared by an isopropyl-alcohol xylene technic or by an alcohol, carbol xylene, xylene sequence.

The foregoing technic is essentially that used by Endicott for study of beta and gamma radiations.

Endicott's Alpha-Particle Method. In recent studies of the tissue distribution of the radioactive element polonium, which emits almost exclusively alpha particles, Endicott (Proc. Soc. Exper. Biol. & Med. 64:170, 1947) has devised a technic in which paraffin sections 5 \( \mu \) thick are floated directly onto photographic plates or films. A very fine-grained silver-bromide emulsion sensitive chiefly to alpha particles (Eastman alpha-particle plate No. 329, 489) was employed.

The technic: Fix in buffered neutral formalin (p. 34) for 48 hours. Decalcify bones in 5% aqueous formic acid and wash as usual. Dehydrate with acetone, clear with gasoline, and imbed in paraffin (Schedule III, p. 62). Section at 5 \( \mu \). Float sections in photographic darkroom under an amber safelight (for other emulsions use the safelight prescribed by the manufacturer) from cold water directly onto film or plate. Dry quickly with an air current and place in a light-tight container for the duration of the exposure period.

At the end of the exposure period deparaffinize with xylene (in a photographic darkroom), dry in an air current, and develop, harden, and fix in the fluids prescribed by the manufacturer for the emulsion used. Then wash two hours in filtered tap water. Stain two minutes in fresh Weigert's acid iron-chloride hematoxylin (p. 81), wash in running water five minutes, dehydrate with three changes of acetone, clear with 50:50 acetone xylene mixture and three changes of xylene, and mount in Clarite.

Particle tracks are visible as rows of black granules which appear bright under dark-field illumination. By a slight change of focus the stained section is brought into view. Preparations may be examined at 400 diameters under dark-field illumination to estimate the number of particle tracks for quantitation of the amount of radioactive material present. Higher magnifications, to 1,000 diameters or more, can be used for locating the exact source of individual straight-line particle tracks in the tissue cells by variation of focus.

The necessary exposure time has to be determined by trial in any individual series of experiments. Select for trial some tissue giving a medium radioactivity as determined by physical instruments, and take out and de-
velop section films at geometrically increasing intervals: for instance 1, 2, 4, 8, 16, 32 hours, and so on; and if closer interpolation seems indicated, also a series at 1/2, 3, 6, 12, 24, 48 hours, etc. The other tissues showing higher radioactivity can then be given correspondingly shorter exposures and vice versa.

Use of plasma and connective-tissue stains is not recommended on the film section preparations on account of the great affinity of the gelatin emulsion for these dyes. If necessary, alternate sections can be mounted on ordinary glass slides and stained in any manner desired for comparison.

Boyd and Levi's Beta-Particle Method (Science 111:58, 1950) for C\textsuperscript{14} \beta-particle tracks is quite similar. A special emulsion NTB2 of the Eastman Kodak Research Laboratories was utilized. Paraffin sections floated on water were picked up on photographic plates on the emulsion surface (in the dark), partially dried, placed in a black plastic slide box with anhydrous calcium chloride, and stored at 7\degree C. for a period amounting apparently to several days (one ten-day period was cited). After exposure the section was deparaffinized, hydrated as usual, developed 20 minutes in Eastman D19 developer, and fixed in 30\% hypo at 20\degree C. The tissue was then stained by hematoxylin and eosin and mounted as usual.

Campbell (Nature 167:274, 1951) finds an essentially similar technic applicable to the demonstration of tracks of low energy \(\beta\) particles, such as S\textsuperscript{35} (0.169 MEV) and Fe\textsuperscript{59} (0.46 MEV). He reduced cosmic-ray background effect by using plates immediately after manufacture and by heavy shielding during exposure.

Leblond's Liquid Emulsion Technic as Modified by Endicott and Endicott.

1. Stain paraffin sections as usual with a hematoxylin eosin technic (p. 114), with the ferrocyanide method for iron (p. 243), or with the Van Gieson method (p. 346).
2. Dehydrate with 100\% alcohol, dip in 1\% collodion in ether 100\% alcohol mixture, and drain vertically for one minute until dry. Then in the darkroom,
3. Pour 1 cc. melted photographic emulsion at 38\degree C. onto section, tilting section to spread emulsion evenly. Place at once on a cold metal or glass plate to chill and set emulsion. The plate should be perfectly horizontal. Use a spirit level to adjust.
4. Dry quickly in a dust-free atmosphere and keep in the dark long enough to yield a satisfactory autoradiographic image.
5. Develop and fix with the prescribed photographic solutions. Dry and mount under cover glass in balsam, Clarite, or the like.

The Endicotts used this method for study of P\textsuperscript{32}, I\textsuperscript{131} and Fe\textsuperscript{69} distribution. Ford (Nature 167:273, 1951) using a similar method for naturally radio-
active minerals in petrographic specimens, suggests hardening the emulsion by immersing for 15 minutes in 3% chrome alum at 16° C. after exposure and before developing. The chroming and the following ten-minute wash in running water are of course done in the dark.

The stripping film method of autoradiography (Endicott and Endicott, unpublished, 1951) is carried out as follows. Stain paraffin section as usual by the hematoxylin eosin technic (p. 114), dehydrating with 100% alcohol or acetone. Then soak a minute or more in Duco cement diluted 1:20 with ethyl acetate. Drain partly and while the cement is still wet strip the photographic emulsion off its base and apply it cellulose ester side down to the cement covered section, pressing down gently as the film is smoothed out. The cement sets rapidly and exposure is continued in the dark long enough to yield a satisfactory image. Then develop and fix in the prescribed photographic solutions. The exposure to hypo should not be prolonged more than 5–8 minutes. The film and section usually separate from the slide during this process. The film is then thoroughly dried between blotters and pressed down convex side down (section side down) onto a drop of xylene Clarite or other mounting medium on the slide. A second drop of the mounting medium is placed on the upper side of the film over the section, and the cover glass is applied.

I am indebted to Kenneth M. and Frances C. Endicott for the foregoing unpublished variations.

Microincineration

The practice of microincineration, according to Lison, dates back over a century to Raspail in 1833, and has been reintroduced at intervals several times de novo.

It is necessary to utilize some method of preparation of tissues which neither adds extrinsic mineral elements, as do chromates and mercuric salts, nor removes those mineral elements naturally present. Some have tried using frozen sections of unfixed fresh tissue, but these are difficult to prepare or to flatten on slides without some flotation procedure. The procedure of rapid freezing with liquid air (or easier, with one of the acetone carbon-dioxide technics used for freezing blood plasma) followed by desiccation in vacuo at —10° to —20° C. and infiltration with paraffin directly after desiccation offers theoretically the best tissue preparation. Paraffin sections can then be cut and attached to appropriate slides as usual.

Fixation with 100% alcohol conserves most mineral elements well, but gives indifferent to poor tissue fixation. The use of 10% formalin in alcohol is perhaps the best method. With 100% alcohol dehydration, clearing in benzene and paraffin infiltration affords losses of water-soluble salts perhaps as small as any.

The paraffin sections for incineration are to be floated on freshly filtered 95–100% alcohol on clean glass slides without albumen or other adhesive.
Alternate sections should be prepared as usual for ordinary histologic examination and topographic comparison with the ashed sections. Sections should preferably be cut at 3-5 μ.

Place the slides on quartz plates or on slides in an electric furnace. Raise the temperature gradually at first, taking ten minutes to reach 100° C.; then more rapidly to 650° C., reaching this temperature in another 25 minutes. These are Scott’s directions. Lison recommends the Schultz-Braun procedure of heating in flowing nitrogen until 500-530° C. is reached, then admitting air for a few minutes to burn off the carbon. By this method, chlorides are said to be better preserved than at the higher temperatures, and the formation of not easily combustible partial-oxidation products at lower temperatures is avoided.

When the oven is opened after incineration, remove the quartz plate and slides with heated forceps, to avoid rapid chilling and cracking of the glass. Then place the slides on an asbestos plate to cool. Cover the ashed section or spodogram (σπόδος ashes, γράμμα a drawing, a writing, or a picture) with a cover glass and fasten down the edges with paraffin, petrolatum, sealing wax, or a pyroxylin cement. Some sections may be sprayed with thin collodion (0.5-1% nitrocellulose in ether alcohol) for the purpose of making solubility tests and chemical reactions on the spodograms. This is not advised as the routine procedure.

The first examination is to be made with oblique illumination from a strong light. Dark-field condensers may also be used; and study under reflected light as practiced in mineralogic microscopy may be of value. Low magnifications are generally most profitable.

Sodium and potassium chlorides, tricalcium and magnesium phosphates are preserved as such; carbonates are converted into oxides such as sodium, calcium, and magnesium oxides. Dipotassium phosphate (K₂HPO₄) is converted into the pyrophosphate (K₄P₂O₇). Iron compounds appear as yellow to red ferric oxide (Fe₂O₃). Silica generally combines with calcium to form various silicates. Sulfur is either volatilized or converted into (or remains as) sulfates.

Identification of elements still remains difficult. Certain birefringent silica crystals remain identifiable. Silica appears as a white, doubly refractile crystalline mineral. It is insoluble in water. Calcium is seen as a white, singly refractile ash, almost insoluble in water. It responds to the gypsum and oxalate tests (p. 266). Magnesium is difficult to identify in the presence of calcium.

According to Alexander and Myerson, iron is present as yellow to red ferric oxide. It is to be distinguished from remaining carbon. The latter is black in direct transmitted light. Application of the HCl-potassium ferrocyanide reagent to the collodionized ash should give the Prussian blue reaction, though the use of this test is not mentioned by these authors.

Cell topography is often well maintained, but nuclei may not be evident.
The papers of Scott (Prot. 20:133, 1933; Am. J. Anat. 53:243, 1933; Anat. Rec. 55:75, 1933), of Cowdry (Am. J. Path. 9:149, 1933), and of Alexander and Myerson (Am. J. Path. 13:405, 1937) may be consulted. The last contains a bibliography of 124 titles, many of which deal with micro-incineration.
Chapter 21

Buffers, Buffer Tables, Normal Acids, and Alkalies

Since the previous edition we have expanded considerably our use of buffers, not only for staining procedures but also during fixation methods, in enzyme localization techniques, and in some decalcification procedures. Additional buffers in the acid and alkaline ranges have been found useful and are included here. Since the pH levels of buffer mixtures vary with their dilution, in a few cases figures for more than one dilution are included.

It is to be remembered that buffer mixtures are comparatively ineffective in preventing pH displacement when one of the salts is present in nearly pure form. The same phenomenon is observed with phosphates and citrates when the mixture is such that a mono- or dihydrogen salt would be crystallized in nearly pure form if the mixture were evaporated. Hence, where adjacent pH figures in any buffer series are relatively widely separated, and no other contraindications exist, it is preferable to use a different buffer series if the desired pH level is approximated by a number of consecutive readings in the tables.

Due regard must be given to the effect of the buffer ions on the other ingredients of the solution in which they are used. For example, acid phosphate buffers precipitate thionin. Tartrates precipitate calcium salts, as do the less acid phosphate buffers. Acetates and citrates can be used in acid silver solutions. Formates and citrates reduce permanganate solutions, but acetates and phosphates can be used. (Acetic acid reduces permanganates slowly at 95–100° C.)

In the alkaline range buffers are less satisfactory because of their avidity for carbon dioxide from the laboratory air.

The salts and acids used should be of reagent grade—those specially made for buffer use when available. Particular note should be taken of the
molecular weight specified on bottle labels. That weight should be used in preparing solutions, since some of the salts are available in a variety of states of hydration. It is often a matter of indifference whether a sodium or a potassium salt is used in a buffer mixture, providing that due account is taken of the change in molecular weight.

Distilled water is usually acid as prepared from block-tin stills, and re-distillation from glass does not seem to remedy this fault. Even freshly distilled or doubly distilled water boiled to expel carbon dioxide and cooled often gives pH levels between 5.0 and 6.0. However, the amount of acid present seems too small to affect seriously even hundredth-molar buffers, and we usually ignore the fact that fresh distilled water may measure pH 5.5. For most purposes singly distilled water of that pH level is quite satisfactory in buffers.

Buffers containing sodium citrate and acetate are particularly liable to mold growth. Strong stock buffers should be made up in 20–25% alcohol to inhibit this growth. When diluted 1:20 or 1:25 for use the alcohol concentration falls to an insignificant 1% or so.

In the acid range I have included new series of HCl + KH₂PO₄ at 1 M and 0.1 M, HCl + Na citrate at 1 M, 0.1 M and 0.01 M, a maleic acid + sodium maleate series at 0.1 N, an oxalic acid + sodium oxalate series at 0.1 M, a potassium oxalate + ferric chloride series at 0.1 M in addition to the Walpole acetic acid + sodium acetate, the Horecker-Lillie citric acid + sodium citrate, and the modified McIlvaine citric acid + disodium phosphate of the first edition. In the middle range, new data for 0.1 M, 0.067 M, and 0.005 M concentrations of the Sörensen phosphate series have been determined, and the dry salt mixtures have been recalculated (on a straight percentage basis for Na₂HPO₄ + NaH₂PO₄·H₂O and for Na₂HPO₄ + KH₂PO₄ mixtures) to give the stated pH at approximate M/15—about 0.94%—dilution. Komori's maleic acid trihydroxymethylaminomethane series has been added. In the alkaline range I have substituted the Sörensen borate buffers for the Clark and Lubs, and have inserted Sörensen's glycine, sodium chloride, sodium hydroxide buffer as well as a glycine sodium hydroxide series without sodium chloride for the Nadi oxidase reaction. Holmes's borate series and Komori's collidine hydrochloric acid series are retained from the first edition, for I have found occasional use for them. The McIlvaine series as given varies to some extent from that given by Clark, whose figures are based on the undiluted aqueous mixtures. The figures here are based on a 1:25 dilution of the stock 25% methanol solutions in distilled water, the concentration at which this buffer is used in azure-eosin stains.

New data were determined on a Beckman pH meter, corrected to 25° C., and slightly smoothed. I am indebted to P. Jones for these measurements.

New tables based on Lange's data for density and grams per liter of the significant constituent are presented for the ready preparation of normal acid and ammonia solutions. For chemical titration procedures, these solu-
tions must be standardized as usual, but for most histologic purposes they may be used as prepared.

References

G. Gomori: Personal communication.
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* Emended slightly from Lange 1949.

1 See special tables for preparation of normal solutions, pp. 455–456.
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DRY SALT MIXTURES

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Calculated on basis of 1% (approximately M/14) solutions from Sorensen M/15 data.

* The mixtures on this page were made by Mr. P. Jones, and read electrometrically on a Beckman pH meter. Readings are corrected to 25° C. and slightly smoothed. Phosphate buffers above 8.0 and below 5.3 are considered unreliable for histological use and readings are omitted.
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<td>5 5 9 31 6.86</td>
<td></td>
</tr>
<tr>
<td>5 5 10 30 7.20</td>
<td></td>
</tr>
<tr>
<td>5 5 11 29 7.50</td>
<td></td>
</tr>
<tr>
<td>5 5 12 28 7.75</td>
<td></td>
</tr>
<tr>
<td>5 5 13 27 7.97</td>
<td></td>
</tr>
<tr>
<td>5 5 14 26 8.15</td>
<td></td>
</tr>
<tr>
<td>5 5 15 25 8.30</td>
<td></td>
</tr>
<tr>
<td>5 5 16 24 8.45</td>
<td></td>
</tr>
</tbody>
</table>


Holmes’ Alkaline Buffer for Silver Salts

<table>
<thead>
<tr>
<th>pH</th>
<th>M/5 H₂BO₃⁻ Na₂B₄O₇·10H₂O M/20</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>18 2</td>
</tr>
<tr>
<td>7.5</td>
<td>17 3</td>
</tr>
<tr>
<td>7.8</td>
<td>16 4</td>
</tr>
<tr>
<td>8.0</td>
<td>14 6</td>
</tr>
<tr>
<td>8.2</td>
<td>13 7</td>
</tr>
<tr>
<td>8.4</td>
<td>11 9</td>
</tr>
<tr>
<td>8.7</td>
<td>8 12</td>
</tr>
<tr>
<td>9.0</td>
<td>4 16</td>
</tr>
</tbody>
</table>

M/5 = 12.4 Gm.  M/20 = 19.0 Gm.
<table>
<thead>
<tr>
<th>Sörensen's Borate Buffers</th>
<th>Gomori's 2,4,6 Collidine-Hydrochloric Acid*</th>
<th>L. Michaelis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N/10 HCl cc.</strong></td>
<td><strong>Borate cc.</strong></td>
<td><strong>NaOH cc.</strong></td>
</tr>
<tr>
<td>475</td>
<td>525</td>
<td></td>
</tr>
<tr>
<td>450</td>
<td>550</td>
<td></td>
</tr>
<tr>
<td>425</td>
<td>575</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>600</td>
<td></td>
</tr>
<tr>
<td>350</td>
<td>650</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>700</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>750</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>800</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>850</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>900</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>950</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>22° C.</strong></td>
<td><strong>30° C.</strong></td>
<td><strong>22° C.</strong></td>
</tr>
<tr>
<td>450</td>
<td>525</td>
<td></td>
</tr>
<tr>
<td>425</td>
<td>575</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>600</td>
<td></td>
</tr>
<tr>
<td>350</td>
<td>650</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>700</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>750</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>800</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>850</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>900</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>950</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>At 37° C. subtract 0.08 from the pH indicated for 23° C.</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Borate is 12.404 Gm. boric acid and 100 cc. N/10 sodium hydroxide diluted to 1 liter with distilled water.

* To dissolve the collidine, some of the HCl must be added to it. I suggest a mixture of 5 parts M/10 HCl with 25 parts M/5 collidine. Then use 30 cc. quantities of this mixture and decrease the indicated quantities of HCl by 5 cc.
<table>
<thead>
<tr>
<th>Specific Gravity</th>
<th>% H₂SO₄</th>
<th>Gm. H₂SO₄ per liter. 20° C.</th>
<th>CC = 49.04 Gm. for N/1 sol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.8337</td>
<td>95</td>
<td>1742</td>
<td>28.2</td>
</tr>
<tr>
<td>1.8355</td>
<td>96</td>
<td>1762</td>
<td>27.86</td>
</tr>
<tr>
<td>1.8364</td>
<td>97</td>
<td>1781</td>
<td>27.6</td>
</tr>
<tr>
<td>1.8361</td>
<td>98</td>
<td>1799</td>
<td>27.3</td>
</tr>
<tr>
<td>1.8342</td>
<td>99</td>
<td>1816</td>
<td>27.1</td>
</tr>
<tr>
<td>1.8305</td>
<td>100</td>
<td>1831</td>
<td>26.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Specific Gravity</th>
<th>% HCl</th>
<th>Gm. HCl per liter. 20° C.</th>
<th>CC = 36.47 Gm. for N/1 sol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1789</td>
<td>35</td>
<td>424.4</td>
<td>86.0</td>
</tr>
<tr>
<td>1.1837</td>
<td>37*</td>
<td>438.0</td>
<td>83.3</td>
</tr>
<tr>
<td>1.1885</td>
<td>38</td>
<td>451.6</td>
<td>80.8</td>
</tr>
<tr>
<td>1.1932</td>
<td>39*</td>
<td>465.4</td>
<td>78.4</td>
</tr>
<tr>
<td>1.1980</td>
<td>40</td>
<td>479.2</td>
<td>76.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Specific Gravity</th>
<th>% HNO₃</th>
<th>Gm. HNO₃ per liter. 20° C.</th>
<th>CC = 63.02 Gm. for N/1 sol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4048</td>
<td>68</td>
<td>955.3</td>
<td>66.0</td>
</tr>
<tr>
<td>1.4091</td>
<td>69</td>
<td>972.3</td>
<td>64.9</td>
</tr>
<tr>
<td>1.4134</td>
<td>70</td>
<td>989.4</td>
<td>63.8</td>
</tr>
<tr>
<td>1.4176</td>
<td>71</td>
<td>1006</td>
<td>62.7</td>
</tr>
<tr>
<td>1.4218</td>
<td>72</td>
<td>1024</td>
<td>61.6</td>
</tr>
</tbody>
</table>

*Figures for 37% and 39% HCl are interpolations.*

Figures in Column 4 are calculated from Lange's data as given in the first three columns. Where assay figures are stamped on printed concentrated-acid labels this figure should be used.

To prepare normal acid solutions measure the amount specified in Column 4 into a 1-liter volumetric flask and then add distilled water up to the liter mark. These instructions are intended to be sufficiently accurate for histologic purposes.

If the normal solutions are to be used for quantitative chemical work they must be standardized as usual for that purpose.
### Volume Equivalents of Molar Weights (Calculated from Lange, with Interpolations)

<table>
<thead>
<tr>
<th>Formic Acid</th>
<th>Acetic Acid</th>
<th>Ammonia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Per cent Assay</strong></td>
<td><strong>Spec. Grav.</strong></td>
<td><strong>Gm. acid per cc.</strong></td>
</tr>
<tr>
<td>84</td>
<td>1.1929</td>
<td>1.002</td>
</tr>
<tr>
<td>85</td>
<td>1.1953</td>
<td>1.009</td>
</tr>
<tr>
<td>86</td>
<td>1.1976</td>
<td>1.023</td>
</tr>
<tr>
<td>87</td>
<td>1.1994</td>
<td>1.030</td>
</tr>
<tr>
<td>88</td>
<td>1.2012</td>
<td>1.036</td>
</tr>
<tr>
<td>89</td>
<td>1.2028</td>
<td>1.050</td>
</tr>
<tr>
<td>90</td>
<td>1.2044</td>
<td>1.063</td>
</tr>
<tr>
<td>91</td>
<td>1.2059</td>
<td>1.077</td>
</tr>
<tr>
<td>92</td>
<td>1.2078</td>
<td>1.084</td>
</tr>
<tr>
<td>93</td>
<td>1.2099</td>
<td>1.090</td>
</tr>
<tr>
<td>94</td>
<td>1.2117</td>
<td>1.104</td>
</tr>
<tr>
<td>95</td>
<td>1.2140</td>
<td>1.111</td>
</tr>
</tbody>
</table>

456
An italicized page reference among multiple entries indicates the more important reference.

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