

***PROCUREMENT, PROCESSING, AND STAINING TECHNIQUES  
FOR DERMATOPATHOLOGIC SPECIMENS***

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It is an unfortunate reality that many dermatologists and dermatopathologists have only a rudimentary knowledge of the effects of biopsy technique and tissue processing, regarding the final results that will be obtained in stained microscopic sections of the specimen in question. All too often, one is faced with a sample of skin that has been obtained crudely, fixed badly, or mishandled in the histology laboratory, making morphologic interpretation of it needlessly complex. These faults typically occur not through willful neglect of proper methodology, but rather because of ignorance of the sequence of steps that constitute the science of histotechnology. Most trainees in dermatology or dermatopathology are not well-versed in the details of this laboratory discipline, making them totally dependent on the expertise of their technicians (or lack of it).

Accordingly, this chapter will provide an outline of recommendations for the procurement and subsequent handling of skin biopsies. Potential reasons for poor results are also included.

### **BIOPSY TECHNIQUES IN DERMATOLOGY**

The specific procedures that are used in performing biopsies of cutaneous lesions are usually left to the discretion of the attending clinician. This provision is not a problem if the operator has been adequately educated on the specific advantages and disadvantages of various techniques, as applied to specific classes of skin lesions. However, it may prove to be a disaster if the dermatologic surgeon is inexperienced in such matters. Enzyme histochemistry, immunohistology, or electron microscopy-- all of which are greatly affected by nuances in tissue preservation-- may be necessary in some instances to obtain a firm diagnosis. Because the clinician may not be able to anticipate these possibilities before obtaining the tissue sample, a predetermined routine should be followed in doing so (1).

There are basically four generic categories of procedures that may be used in any given case. These include punch biopsies, using circular dermatomes of several sizes; shave- or excisional biopsies done with a scalpel; electrosurgical excisions; and laser-mediated biopsies. In choosing one of these options, the operator should be cognizant of the two opposing "forces" that affect his or her final decision on this matter. On the one hand, the patient is often preoccupied with the cosmetic effects of a biopsy, and this typically induces the surgeon to limit the size of the sample as much as possible. The opposing consideration is represented by the degree of difficulty with which the microscopic diagnosis is made by the pathologist-- a factor that is often predictable by the amount of material that will be required to study the disease process adequately.

The cardinal rule to be remembered on this topic is that a properly-done biopsy is virtually never cosmetically deforming, if it can be accomplished in an outpatient setting by a competent operator. In contrast, specimen inadequacy and marked artifactual changes in tissue are problems relating to faulty procedure that account for the great majority of diagnostic obstacles that dermatopathologists encounter. There is nothing quite so aggravating for the clinician (and the patient) as to be informed that a second biopsy will be necessary because of these deficiencies, adding additional expense, time, and anxiety to the situation at hand.

As an example of these contentions, it is well-known that malignant hematolymphoid proliferations and certain metastatic carcinomas are composed of extremely fragile cells that are exquisitely susceptible to the compressive or shearing effects of some biopsies (2). Moreover, it

is probable that several cubic millimeters of tissue will be necessary for the complete pathologic characterization of such lesions. Hence, a small shave- or punch biopsy specimen would be predictably unsuitable in these circumstances. When in doubt, the clinician should contact the pathologist before the procedure is done, and inquire about recommended handling of the tissue sample and its minimally acceptable size based on the likely diagnostic possibilities.

Other procedures causing reproducibly detrimental physical effects on cutaneous specimens are represented by electrocautery and laser excision. These methods enjoy wide clinical usage at present because of their ease of performance and the limitation of surrounding tissue damage that they afford. Nonetheless, lesional cells in the specimen are often rendered unrecognizable because of widespread thermal coagulation, precluding histologic interpretation altogether. It should therefore be obvious that cauterizing techniques must be avoided for diagnostic purposes.

Several adjunctive pathologic studies (see below) require the availability of specimens that have been handled in a special manner (**Table 1**). Again, these can be obtained prospectively following preprocedural consultation with the laboratory.

#### **IDENTIFICATION AND ORIENTATION OF THE BIOPSY SPECIMEN**

There is nothing quite so exasperating for the pathologist as to receive a specimen that is unoriented, and for which no anatomic location is given on the request form for pathologic examination. A lack of meaningful clinical history or a failure to list potential clinical diagnoses often compound such omissions. These problems usually cannot be solved by the dermatopathologist, and they typically require a laboratory visit by, or a telephone conversation with, the responsible physician. In many instances, it would be medicolegally dangerous to attempt a morphologic interpretation in the absence of such information, and this practice is censured strongly. On occasion, a specimen may be received which is so poorly labeled that the identity of the patient is in question. Such a submission should never be accepted by the laboratory unless the clinician is willing to provide written documentation verifying its origin, and accepting exclusive medicolegal responsibility for its interpretation.

If a skin lesion is a suspected malignancy for which a diagnostic biopsy is also intended to be a complete excision, the clinician should provide some means of identifying the superior, inferior, medial, and lateral borders of the tissue sample. This can be accomplished by attaching sutures of differing lengths or types to the specimen, and sending a corresponding “map” of the tissue to the laboratory along with the dermatopathology request form (3). Alternatively, indelible (e.g., tattoo) ink of various colors can be affixed to the borders of the specimen and identified accordingly. As a minimal requirement-- for example, in very small excisional biopsies-- at least one pole of an elliptical or circular tissue fragment should be labeled by such means.

The clinician should be discouraged from attempting to prosect the specimen further before it is examined by the pathologist, except in very well-defined settings. When they are improperly performed, transections of ellipses and bisections of punch biopsy samples often confound subsequent orientation steps and may mechanically damage the lesion that is intended for study. The only acceptable reason for undertaking further clinical manipulation of the tissue sample is that of preparing cellular “touch” preparations in examples of suspected hematology disease. The latter can be obtained if the operator bisects a lesion at its bulkiest

point and touches the cut surface of the tissue gently to glass slides in a serial fashion (2). When this is done, special care should be taken subsequently to orient both halves of the resulting two-part specimen for the pathologist. Moreover, all air-dried or fixed touch preparations must be labeled with the patient's name, his or her date of birth, and the date on which the procedure was performed.

### **PREPARATION OF FROZEN SECTIONS**

The widespread use of the Mohs chemosurgical technique in dermatology has made the interpretation of frozen tissue sections an important facet of this specialty (4). Most Mohs surgeons are well trained in the procedural aspects of the frozen section (FS) method. However, these will be reviewed briefly in this section for the benefit of other practitioners.

The purposes of obtaining FS examination are twofold; it may be used to secure a rapid diagnosis for a lesion with unknown histologic attributes, or the technique may be employed to confirm that margins of excision are uninvolved by the pathologic process in question. Because of the potential distortion of morphologic detail that this procedure may induce, the first of the cited applications is not one that should be used frequently. With respect to the analysis of excisional margins, the operator must be certain to supervise the orientation and labeling of all specimens, as outlined above. This makes the availability of indelible ink an absolute requirement in the Mohs laboratory.

Following such steps, one must be certain that the tissue sample is small enough to assure rapid and uniform freezing, and ease of sectioning with the cryomicrotome ("cryostat"). The specimen is usually placed in a small pool of gelatinous, water-soluble mounting medium (e.g., Cryogel<sup>®</sup>) that has been applied to a precooled Teflon or metal "chuck." After making sure that the tissue is properly oriented on the flat surface of this implement, it is then totally covered with additional mounting medium, fashioned into a circular pledget. Immediately thereafter, best results are obtained if the chuck is immersed in a bath of isopentane suspended in an outer container of liquid nitrogen. These devices are available commercially, and they allow for virtually instantaneous freezing of the mounting medium with minimalization of ice crystal formation. The latter eventuality is undesirable, because entrapment of ice in the specimen (caused by slowly decreasing temperature) will cause significant microanatomic distortion and may interfere with microscopic interpretation. For this reason the utilization of metal cooling "plates," which are incorporated into many cryostats by their manufacturers, is not recommended as a means whereby initial freezing is accomplished. However, these plates are indeed acceptable for maintaining the chucks in a frozen state while sections are being cut.

The microtome in any cryostat must be affixed in such a manner that uniform sections of reproducible thickness (approximately 10 microns) can be prepared. Regular maintenance regarding the sharpness and integrity of microtomy blades is essential to this process. After "facing" the frozen block with the blade-- to obtain a smooth, flat tissue surface-- the operator cuts a "ribbon" of several individual sections that can be kept flat by manipulation with a camel-hair brush. These are then apposed to acid-cleaned glass slides which have been kept at ambient temperature, causing the tissue to adhere to them quickly. To eliminate concerns about the subsequent loosening of this bond, slides that have been pre-coated with albumin, poly-L-lysine, or a chrome-alum gel may be utilized (5).

Most FS laboratories employ a brief (30-60 second) fixation step immediately after

mounted sections are prepared, in Copland jars containing absolute acetone or 95% ethanol. The slides may then be stained with hematoxylin and eosin, a “polychromatic” or metachromatic reagent such as methylene blue, or other reagents. Following dehydration in graded alcohols and xylene, a synthetic mounting medium is placed over the tissue, and a glass coverslip is applied. Additional of a few drops of xylene to the mounting medium will slightly lessen its viscosity and help to prevent the entrapment of air bubbles under the coverslip.

Alternatively, the surgeon may wish to keep some unstained frozen sections for future studies, such as those involving immunohistochemistry. This aim is best served by removing slides from the acetone or alcohol fixative and placing them promptly in a -20 or -70 Centigrade freezer. They can be kept in such devices indefinitely for further analysis at a later date.

Specific problems connected with poor microtomy technique will be considered subsequently in this discussion. However, the most common difficulty that is seen in the FS area can be ascribed to improper calibration of the cutting interval between successive sections. Overly-thick sections may result in consumption of the tissue before a suitable slide is obtained for microscopic examination; on the other hand, it is extremely hard to obtain very thin sections without causing them to fold on themselves or shred. Thus, it is imperative that the cryostat be checked frequently to make certain that it is set up properly from a technical viewpoint. Also, there is no substitute for practice and experience on the part of the operator, in regard to procurement of optional FS preparations. The labeling of specimens subjected to FS examination should be no different than that used for other tissue samples. The remnant tissue should be placed in a plastic cassette that is suitably impregnated with the accession number of the case (preferably using a Cas-Mark<sup>R</sup>-type labeler), and kept together with corresponding paperwork for transmittal to the histology laboratory. Under no circumstances should unlabeled frozen tissue be allowed to accrue in the FS laboratory, lest disastrous errors in identification should occur.

### **FIXATION OF BIOPSY SPECIMENS**

Questions that are often asked of the pathologist concern the choice of one fixative solution over another for the preservation of various cutaneous specimens. There is no “universal” fixative in dermatopathology, because skin samples may be used for an ever-growing number of investigative analyses, many of which demand that special processing measures be applied in order to procure optimal results. Selected immunohistologic studies, electron microscopy, and genotypic assessment represent three advanced modalities of pathologic evaluation that are associated with specific fixation requirements. Laboratory specialists are continuing to develop procedural modifications to lessen the need for such provisions, but they still do exist.

In the most optimistic of scenarios, it would be best to submit all skin biopsies in their fresh state in physiologic saline solution, and for the pathologist to subdivide these specimens into several parts for future diagnostic eventualities. Nevertheless, this is often not practical for two main reasons. Firstly, outpatient specimens are commonly submitted over long distances from the dermatopathology laboratory, increasing the likelihood that unfixed tissue will undergo autolysis before it is received. Secondly, cutaneous biopsies are rarely generous in size, making judiciousness in the selection of special studies an important point. The latter issue again emphasizes the wisdom of preprocedural consultation with the pathologist, if unconventional

evaluations are desired.

#### Fixatives Used for “Routine” Histopathologic Examination

In the great majority of cases, the clinician requesting histologic examination of a skin biopsy is interested in a “traditional” interpretation based on microscopic findings as seen with the hematoxylin and eosin stains. With this stipulation in mind, most laboratories have advocated the use of formalin as the fixative of choice. Nonetheless, the following sections will briefly review the chemical characteristics of preservative solutions in a broader sense, so that exceptions to the above-cited situation may be addressed.

#### **General Considerations**

The preservative effects of certain chemicals have been recognized for thousands of years, dating back to the ancient Egyptians. On an empiric basis, therefore, various fixatives have been employed to preclude bacterially-mediated putrefaction of human tissues since the inception of pathology as a discipline.

In the last century, detailed studies of these agents have elucidated the probable mechanisms responsible for these beneficial effects (7-10). In addition to antibacterial effects, fixatives also enhance the differences in refractive indices between dissimilar tissue constituents, allowing for greater resolution upon light microscopy. Moreover, they augment the affinity that chemical dyes have for particular cellular elements. It is now known that chemical fixatives may be divided into two broad categories-- coagulating and non-coagulating-- with respect to their effects on proteins, which form the framework of virtually all cells. Further subdivision into aqueous and non-aqueous agents, as well as additive or non-additive preservatives, is also possible (11).

**Non-coagulative** fixatives are the most widely-used, and these include formaldehyde (called formalin when prepared in aqueous dilution and paraformaldehyde when employed in polymeric form), glutaraldehyde, acetic acid, potassium dichromate, and osmium tetroxide. On the other hand, acetone, alcohols, chromium trioxide, mercuric chloride, and picric acid exemplify the **coagulative** preservatives. In the process of denaturation and coagulation, a network of altered protein is formed in tissue; in contrast, non-coagulative agents act to produce a stable intracellular “gel.” Acetone and alcohol are the major non-aqueous reagents, with most others being soluble in water. **Additive** fixatives react with tissues by combining with them chemically, whereas **non-additive** reagents (primarily alcohols and acetone) do not.

Various combinations of these chemicals (e.g., formalin-alcohol or Carnoy’s solution [a mixture of ethanol, chloroform, and acetic acid]) are sometimes utilized as fixatives that are intended to augment the stainability of predefined tissue components. Moreover, metal salts-- such as those containing zinc and mercury-- may be added to aqueous solutions, as in zinc-formalin or “B5” fixative (a mixture of mercuric chloride, sodium acetate, and formalin). The apparent effect of the latter agents is to stabilize complexes that are formed by nucleic acid and protein, yielding improved preservation of nuclear detail. By convention, many fixatives are named for the laboratory investigators who devised them. Thus, one may encounter such designations as Bouin’s, Hollande’s, Zenker’s, Helly’s, Zamboni’s, Orth’s, and Carnoy’s solutions. Most of these are mixtures of chemicals in different classes or showing differing effects on proteins, with or without metal salts. Selected reagents in this list will be alluded to later in this discussion.

One important concept to be borne in mind is that all fixatives induce chemical artifacts in tissue sections. This effect has two potential ramifications for pathologists. Firstly, we all become inured to the artifacts that we are accustomed to seeing with routine use of a particular preservative solution; indeed, we may even employ such changes as histodiagnostic features. Changing the fixative one uses will also alter the tissue artifacts, often leading to interpretative confusion with any given staining method. Secondly, one artifact produced by a preservative may be desirable, whereas others are detrimental. For example, B5 solution yields excellent nuclear detail on hematoxylin and eosin stains, but it virtually destroys the integrity of some cellular proteins that may be the targets of immunohistochemical studies (12). Lastly, the optimal period of fixation varies greatly from one solution to another; tissue placed in formalin may be allowed to remain in it for days with no compromise of morphologic features, whereas specimens in B5, Zenker's, and Bouin's fixatives must be transferred to other chemical solutions after predefined periods of time to avoid a serious loss of cellular definition (7,8). Thus, the ultimate choice of a preservative solution is not one to be made indiscriminately.

### Specific Fixatives

**Formalin:** Formalin represents a 37 to 40% aqueous solution of formaldehyde, the latter of which is marketed commercially in the United States. Because the former reagent is characteristically used at a 10% dilution, the final formaldehyde concentration is 3.7 to 4%. Various other chemicals have been added to formalin to alter its stability and preservative capabilities, including calcium chloride, calcium carbonate, ammonium bromide, sodium chloride, sodium phosphate, sodium hydroxide, and absolute ethyl alcohol. Among these mixtures, that consisting of formalin, distilled water, and monobasic/dibasic sodium phosphate is the most widely employed and is known as "10% neutral buffered formalin" (NBF). Paraformaldehyde is a polymerized form of formaldehyde admixed with methanol; it is generally employed as a fixative for specialized immunohistologic procedures, particularly when combined with periodate and lysine ("PLP" solution) (13).

Although it is a general-purpose fixative and yields good morphological detail when prepared properly, NBF does have some disadvantages in tissue pathology. Firstly, any solution containing formaldehyde is potentially carcinogenic, and levels of formalin vapor in the ambient air of the laboratory must be measured regularly by governmental mandate. The maximum permissible exposure limit for any individual employee is 1 part per million over an 8 hour period, as established by the Occupational Safety and Health Administration (14). Secondly, poorly prepared NBF, which has been buffered erroneously and has a pH outside of the physiologic range, may cause unwanted precipitates of "black acid hematin pigment" in tissue sections. The latter has a dark particulate appearance, and may simulate microorganisms on a histologic slide. These two possibilities can be distinguished through the use of polarization microscopy, because hematin pigment is birefringent whereas microorganisms are not (11). Thirdly, NBF that is allowed exposure to ambient air for prolonged periods of time (as with large "batches" that are diluted for use in the gross laboratory) will develop high levels of formic acid. The latter is detrimental to protein substructure, and may accentuate the formation of methylol bonds between polypeptides. This effect can "mask" proteinaceous epitopes which correspond to the targets of immunohistologic antibody reagents (15). Lastly, formalin has a limited capacity for penetration of bulky pieces of tissue, and specimens fixed in it must be no thicker than 4 to 5

millimeters.

Despite these drawbacks, formalin is inexpensive and widely available, and it therefore ubiquitously employed as the fixative of choice for clinical specimens. The above-cited failings of this preservative can be prevented by careful technique in its preparation, adherence to environmental monitoring requirements, and application of proper prosection and fixation techniques for the submission of tissue sections. Some laboratories prefer to use NBF-ethanol (mixed in equal volumes), because it affords a greater degree of tissue penetration than formalin alone.

B5/Zenker's/Helly's Solutions: B5, Zenker's, and Helly's solutions were introduced because of their superiority over NBF in the preservation of nuclear detail (7,8). They are fixatives based on the inclusion of mercuric chloride, with or without sodium acetate, potassium dichromate, sodium sulfate, acetic acid, and formaldehyde as additional constituents. Because of the excellent morphologic detail that is achievable with these solutions, many laboratories prefer them for the routine preparation of hematoxylin and eosin-stained sections. Nevertheless, there are three distinct disadvantages of B5, Zenker's, or Helly's reagents, as compared with NBF. Tissue sections must be removed from the former three fixatives after no more than 8 hours, and placed into 70% ethanol; if this is not done, specimens will become extremely brittle and virtually impossible to section (11). Also, the presence of mercuric chloride will cause deposition of pigment in microscopic preparations, which must be removed with iodine before final staining procedures are done. Lastly, mercury-based solutions are powerful coagulating agents and therefore damage many cytoplasmic proteins. This effect commonly renders tissue sections unsuitable for a variety of immunohistochemical studies (12).

Bouin's Solution: Bouin's fixative is again based on formaldehyde as a major component, together with picric and acetic acids in aqueous solution. Like B5, this reagent affords excellent preservation of nuclear morphology, but suffers from the same failings pertaining to brittleness of tissue, pigment deposition, and adverse effects on cytoplasmic polypeptides. In addition, Bouin's-fixed specimens acquire a yellow color (because of the effects of picric acid) that must be removed by post-fixation washing in alcohol and lithium carbonate. Bouin's fixative is preferred for visualization of delicate mesenchymal tissues, because of its superior differentiating abilities in regard to these elements (11). Accordingly, some "stromal" special stains (such as the Masson trichrome method [see below]) are best performed on specimens preserved in this solution.

Acetone and Alcohols: Acetone and alcohols are rapidly-acting fixatives with good penetration of tissue. They also afford better preservation of some cytoplasmic enzymes than formaldehyde-based solutions do, in paraffin sections. However, two major disadvantages attend the use of these organic reagents. They cause striking shrinkage of tissue because of their dehydrating effects, thereby altering morphologic details appreciably. Also, acetone and methyl or ethyl alcohols are relatively expensive, and they require special storage and inventory procedures because of possible use of laboratory workers as inebriants. In current practice, these agents are usually applied only in the fixation of touch preparations and are not commonly utilized in the processing of biopsy specimens. Similar comments apply to Carnoy's solution, which is constituted by ethyl alcohol, chloroform, and acetic acid.

Decalcifying Solutions: Decalcification of tissue specimens is not commonly required in



dermatopathology. However, on occasion, a biopsy sample may contain obvious foci of calcium salts, as suggested by clinical findings or difficulty in performing the biopsy procedure. In these circumstances, two main methods exist for the removal of such minerals from the specimen. One employs simple acids (hydrochloric or nitric), which rapidly solubilize calcium deposits. The other technique is based on the ability of certain chelating agents-- such as ethylenediaminetetraacetic acid (EDTA)-- to accomplish this task. The second of these methods is much "gentler" and does not cause the loss of microscopic detail that acid decalcification may incur. Fixation is allowed to progress in concert with decalcification with both acidic or EDTA reagents, because they are commercially marketed as mixed solutions containing formaldehyde. Glutaraldehyde: Glutaraldehyde is similar in chemical activity to formaldehyde; both cause cross-linkage of proteins in tissue (7). However, glutaraldehyde penetrates specimens very slowly, making the size of the tissue sample a critical determinant of fixation with this reagent. Moreover, 2 to 4% glutaraldehyde (representing the usual working concentration) has a propensity to cause brittleness of specimens that are immersed in it for more than 2 to 3 hours; transfer to a buffer solution is absolutely necessary after this point. For these reasons, among others, glutaraldehyde is not used often for the preservation of biopsy samples that are intended for light microscopy. However, it is the preferred fixative for electron microscopy, wherein specimens are very small and limited "hardening" of tissue may actually be morphologically advantageous.

### **Other Factors Influencing Fixation**

As outlined by Carson (11), there are several other considerations in the fixation of tissue besides one's choice of preservative solution. These include temperature, size of the sample, the volume ratio of tissue to fixative solution, the duration of fixation, and the pH of the solution.

Recently, the rapid but controlled elevation of temperature with microwave ovens has been utilized as an independent means of fixation, by coagulation of tissue proteins. Surprisingly, this process appears to have little adverse effect on staining characteristics, even with immunohistologic methods. However, it must be emphasized that careful control is the key to thermal fixation; overheating may completely destroy the specimen if it is allowed to reach an extreme level (e.g., over 65 degrees C). In a more conventional context, there are really no compelling reasons to employ fixative solutions at one temperature versus another.

Specimen size is, on the other hand, a potentially crucial factor affecting quality of fixation, and this determinant goes hand in hand with the volumetric relationship between a tissue sample and the solution in which it is immersed. Large, extremely thick specimens will be inadequately penetrated by most fixatives, allowing autolysis to proceed unchecked in their central areas. This results in eventual loss of the unfixed foci during microtomy, yielding microscopic sections that resemble doughnuts. Because penetration is facilitated by minor thermal or mechanical currents in the fixative solution, large specimens that are covered with an inadequate volume of preservative will predictably be underfixed. An experienced histotechnologist typically detects this problem upon attempting microtomy of the tissue, and will "run the specimen back" for more prolonged fixation and reprocessing. However, this consumes additional time and should be unnecessary.

As noted at several points in the foregoing discussion, there is a maximum recommended period of fixation with several preservatives, over which unwanted changes reproducibly occur in

tissue biochemistry. Overfixed specimens are difficult to cut and often demonstrate alterations in morphologic definition or antigenic integrity. On the other hand, underfixation allows bacterial putrefaction to proceed, similarly damaging the tissue sample. Specimens that are immersed in the most commonly used preservative-- NBF-- should ideally be processed further within 8 to 12 hours (12).

The pH of fixatives is not critical for light microscopy, except that certain unwanted pigmentary deposits may be seen with unduly acidic preservatives. Nonetheless, hyperacidity is extremely detrimental to cellular ultrastructure, and also to the maintenance of tissue antigenicity (11,12). For these reasons, it would be wise to control pH within the physiologic range during fixation, in the event that electron microscopy or immunohistology are necessary diagnostically.

### **Tissue Processing and Preparation of Microscopic Sections**

Because most commonly-employed fixatives are aqueous in nature, the next step in tissue processing is usually that of dehydration and "clearing" (removal of all water from the specimen). Graded solutions of ethanol are used for this purpose, and these must be changed frequently to maintain their desiccating properties. A variety of clearing agents are available, but the most common are xylene and limonene derivatives. In likeness to the alcohols, such reagents may be contaminated by water with repeated use and should be monitored closely for this problem.

Xylene is inexpensive and does not leave a residue on glassware or other instrument parts in the histology laboratory. In light of these virtues, it is the most popular clearing agent. However, xylene fumes are potentially toxic to technologists, making careful storage, controlled disposal, and environmental monitoring mandatory. In addition, we have found that xylene may damage the protein substructure of certain fragile tissue antigens (12). Limonene-type clearing agents are derived from plants, and are therefore biodegradable. They have a strong odor-- like that of lemons or oranges-- which is alternatively perceived as pleasant or noxious by various technologists. Other disadvantages of limonenes are that they leave a residue on mechanical tissue processors and may sometimes interfere with the adherence of tissue sections to glass slides. Nevertheless, we prefer them over xylene in immunohistochemistry because they do not damage proteinaceous tissue antigens. Moreover, the microtomy of specimens that have been cleared in limonenes is said to be much easier than that encountered with xylene (11).

In the relatively early days of histotechnology, all dehydration and clearing steps were done by hand. Over the past 50 years, however, a variety of automatic tissue processors have been engineered and marketed. These are used widely at present, and may be divided into two main groups-- "open" and "closed." Open processors mechanically transfer baskets containing tissue cassettes from on "station" (chemical bath) to another, on a computer-driven schedule. The latter may be altered by the operator to change the time of dehydration, clearing, or other steps. Closed instruments vary the solutions to which each specimen basket is exposed by pumping chemicals in and out of fixed chambers, again according to a programmed schedule. In other words, open processors move specimens, whereas closed processors move chemical solutions.

Each of these two types of instruments has advantages and disadvantages. Open processors show a low incidence of reagent contamination from one station to another, but they are subject to the mechanical "hangup" of specimen baskets in transit. Closed processors do not

suffer from the latter drawback, but they are subject to chemical carryover from one reagent pumping step to another. This potentially compromises the dehydration-clearing sequence. On balance, individual experience on the part of technologists and pathologists ultimately determines which type of processor will be chosen.

### **EMBEDDING AND SECTIONING OF BIOPSY SPECIMENS**

The final stations in any tissue processor infiltrate all specimens with paraffin or another wax-based embedding medium. Thereafter, the technologist removes each biopsy (one at a time) from its metal or plastic cassette and proceeds to embed it in a rectangle of additional liquid wax, with attention to the proper orientation of the tissue sample. The pathologist may direct this process by notching or inking one or several surfaces of the specimen, and providing a “map” in accompanying paperwork that indicates whether these should be placed face-down, face-up, or in parallel with the lateral aspects of the cassette. Such provisions are usually necessary only with large pieces of tissue. In other cases, technologists accustomed to handling skin biopsies will, as a matter of routine, orient the epidermis perpendicularly to the bottom of the cassette mold and facing one of its long sides. If several pieces of tissue are included in the same block, these are best arranged diagonally.

The embedding step is a potential source of great irritation to the pathologist if it is done by an inexperienced or careless laboratory worker. With few exceptions, cutaneous specimens that are oriented improperly (e.g., with the skin surface facing the bottom of the cassette) cannot be interpreted microscopically, necessitating that the block be remelted and re-embedded. This takes time, and in the process of facing the poorly-oriented specimen for preparation of initial sections, valuable tissue may be lost. Another advisable tenet concerns the orientation of the epidermis within any given block at the embedding step. All fragments of each specimen should be placed in wax in such a way that the microtome blade will first meet the dermis when the paraffin block is cut, and, as far as possible, so that the skin surface is parallel to the edge of the blade. These provisions go a long way towards avoiding microtomy artifacts (16).

In order to circumvent embedding difficulties, some pathologists have taken to pre-embedding small skin biopsies in agar before they are put in cassettes for fixation. This does assure proper orientation, but agar will not “fix” in the same manner that tissue does, nor will it respond similarly to dehydration, clearing, and infiltration by wax. All of these factors may cause the tissue to “pop” free of the surrounding agar after embedding and during tissue sectioning, defeating the purpose of the agar impregnation step altogether. Therefore, we do not advocate this procedure, rather preferring to educate all of our technologists on the details of orientation during wax embedding. Even a very small cutaneous biopsy can be appropriately configured in the wax block, with the use of a magnifying lens or dissecting microscope to aid in finding the epidermal surface.

Paraffin is still the most widely-utilized embedding medium, but some laboratories have opted to employ “Carbowax” as a substitute. The latter compound is a water soluble wax, making dehydration and clearing of the tissue unnecessary and allowing for direct infiltration of formalin-fixed tissue with embedding medium in the tissue processor (11). This element of simplicity is attractive, but Carbowax has its drawbacks. One concerns the dissolution of the embedding medium when microtomed tissue “ribbons” are placed in a water bath prior to mounting them on glass slides. This unwanted eventuality makes it difficult for the technologist

to keep the tissue section flat, resulting in undesirable folds in the final stained slide. Secondly, we have noted irregularities in antigen preservation when Carbowax-embedded tissues are studied immunohistologically. The temperature of paraffin or Carbowax stations in the tissue processor, and at the embedding center, must be monitored closely. Overheating the wax will cause unwanted thermal artifacts in the tissue and compromise its cellular detail. Excessively cool wax fails to infiltrate the specimens adequately.

Another class of embedding compounds that is presently in vogue in some centers is represented by polymeric plastic resins such as glycol methacrylate or epoxy. Disadvantages of these compounds include the necessity of cutting corresponding tissue sections with a glass or diamond knife microtome, and the requirement for a “transitional fluid,” such as propylene oxide, to embed the tissue after dehydration and clearing (11). Moreover, plastic sections are difficult to stain with the same intensity as that seen in paraffin-embedded preparations. The main advantage of plastic media is that extremely thin, flat sections may be prepared by experienced microtomists, providing exquisite cellular detail. In addition, some enzyme-histochemical staining methods that otherwise require the use of frozen sections are possible with specimens embedded in epoxy or glycol methacrylate.

Histo-microtomy is a seemingly straightforward process, representing the cutting of serial paraffin-embedded sections with a tissue microtome. Nevertheless, this technique has many hidden traps that relate to the proper maintenance, calibration, and orientation of cutting blades; preparation of paraffin blocks; and dexterity of the technologist. Microtome blades that are dull or nicked will produce “chatter” or “venetian blind” artifacts in tissue sections. In addition, the “clearance angle” (between the tissue block and the microtome knife) is crucial to good technique. It should approximate 3 to 8 degrees. If the angle is too narrow, alternately thick and thin sections are cut, or they are folded on themselves (17-19). An excessive clearance angle causes chattered or otherwise hideous sections, and may preclude the ability of the technologist to obtain a tissue ribbon. Even worse are the effects of loose microtome blades or tissue blocks in the microtome chuck. These deficiencies may shatter the paraffin block entirely or deeply groove the tissue specimen. A block that is mounted crookedly in the microtome chuck will produce irregular ribbons, or cause individual sections in the ribbon to break free from one another.

Regardless of whether one uses paraffin or carbowax as an embedding medium, there is still a need to refrigerate tissue blocks before microtomy is attempted. This step hardens the wax slightly and allows for crisp sections to be cut. Warm blocks will yield wrinkled ribbons or cause successive sections to anneal to one another. In addition, failure to moisten the surface of blocked tissue suitably before cutting it yields an excessive number of knife marks or fragmented sections. The technologist can simply rub a wet finger over the block several times prior to microtomy, if the specimen is small. If it is large, and particularly if the tissue is heavily cornified, a wet piece of cloth or cotton soaked in 5% ammonium hydroxide may be applied for 2 or 3 minutes to rehydrate the tissue face (17).

Another problem that is sometime seen at this step is the tendency for ribbons to “fly” onto the knife blade. This is the result of static electricity between the wax or tissue and the metal blade, and also may be avoided by slightly moistening the knife and the block surface before each ribbon is prepared.

### **MOUNTING OF TISSUE SECTIONS**

The wax ribbon of serial tissue sections can be removed from the microtome knife as it is cut, by using a wooden tongue-depressor blade. In this process, the operator exerts slight traction on the end of the ribbon, stretching it gradually over the wooden blade, and subsequently depositing it on the surface of a warm water bath at the cutting station. The temperature of such flotation devices should be kept at 5 to 10 degrees C below the melting point of the embedding wax. If it is too hot, desiccated-looking sections will result: in contrast, cool flotation baths produce excessive wrinkling of the tissue.

To facilitate the process of obtaining a smooth, unwrinkled, paraffinized ribbon of tissue, it can be stretched by slight traction on its ends while floating in the warm water bath. Also, we have found that adding a few milliliters of ethyl alcohol to the water is beneficial in this regard. The ribbon must not be left in the bath for more than one or two minutes, or spurious overhydration of the tissue will be produced. This effect simulates the appearance of edema fluid microscopically (16). Because tissue sections do not adhere well to untreated glass slides, a bonding agent also must be a component of the water bath. Elmer's glue, albumin, and poly-L-lysine are all suitable additives of this type.

One of the most dangerous of all mistakes in the histology laboratory can take place when mounting sections from flotation baths. Friable tissue may "shed" small fragments that float free on the surface of the water, and these may be inadvertently picked up when mounting slides from subsequently processed, unrelated cases. Derisively known as "floaters," these rogue pieces of tissue commonly cause agonizing interpretative problems for the pathologist. For example, it is not difficult to envision a small piece of a malignant melanoma that may find its way onto slides of a melanocytic nevus, a ribbon of which is mounted subsequently in the same water bath. Technologists must be impressed with the tremendous medicolegal liabilities that such a mistake incurs, and they must routinely skim, or otherwise clear, the surface of the water bath between cases. An alternative source of floater-type artifacts is the "tongue blade metastasis," wherein tissue adheres to a wooden applicator stick that is used to float successively-prepared ribbons from two different cases (11). Needless to say, this practice is unacceptable.

With respect to optimizing the cost of slide preparation, we recommend that as many individual sections as possible should be mounted on one slide, from the same ribbon. It is not difficult for adept technologists to include 10 to 15 cuts of a specimen on each slide, arranged in a serial fashion. Also, in light of the limited size of most cutaneous biopsies, it is advisable to save any unmounted paraffin ribbons (with appropriate identification) from dermatopathology cases for one week after they are accessioned. Re-mounts can be prepared from these directly, without the need for further microtomy of the tissue block.

Finally, the identification of tissue sections must be scrupulously maintained throughout the remainder of their sojourn in the histology laboratory. Such a necessity is assured by having the technologist scratch the case and block numbers onto one end of all mounted glass slides with a diamond knife.

### **GENERAL STAINING AND COVERSLIPPING PROCEDURES**

Once the tissue has been affixed to glass slides, paraffin is removed prior to the staining procedure. This is accomplished by placing the sections in a carrier, heating them to 56 degrees C for at least one hour (to evaporate water from the glass slides), and immersing them for 3 to 5

minutes in each of three or four successive containers of a clearing agent (xylene or a limonene derivate; see above). Most histologic dyes penetrate tissue best if it has been rehydrated; thus, passage of the slides through containers of graded ethanol solutions (absolute; 95%; 70%) and distilled water is necessary before staining can be undertaken. The importance of proper paraffin clearance cannot be overemphasized, with regard to final results. If a sizable quantity of wax remains in the sections, dyes will not be able to penetrate them and impregnate constituent tissues.

The majority of diagnostic dermatopathology laboratories employ hematoxylin and eosin (H&E) stain as the stains of choice for microscopic interpretation. There are two principal procedures for applying these dyes-- the "progressive" method, and the "regressive" technique. The former is usually preferred, and it is characterized by sequential staining with hematoxylin (for roughly 15 minutes) and eosin (for 1 to 3 minutes). These steps are separated by application of ammonia water or lithium carbonate as "bluing" agents for hematoxylin, which is actually a chemical "lake" of hematein and a mordant such as ammonium aluminum sulfate (20). In addition, copious rinses with water must be assured after exposure of the tissue to hematoxylin and ammonia or lithium. The "progressive" H&E method is so-named because the technologist can monitor the depth of staining as it develops, and terminate each step appropriately. This procedure is desirable because it can compensate for the effects of various fixatives which enhance the uptake of either hematoxylin (formaldehyde, osmium, heavy metals) or eosin (picric acid). "Retrogressive" H&E staining refers to a technique wherein tissue is purposefully over-impregnated with hematoxylin and then modified to suit the operator by decolorization with dilute hydrochloric acid. Final results are much more difficult to control in this procedure, and it requires a highly experienced technologist to achieve good nuclear detail in the ultimate product.

In regard to eosin as a "counterstain" for hematoxylin, two points merit further mention. One is that eosin is a "differentiating" stain which dissimilarly impregnates tissues of varying chemical structure and molecular density. As much, it requires the same amount of technical attention as hematoxylin in a progressive H&E method. Different connective tissues should be stained to variable degrees in a properly-performed procedure of this type; however, overly-rapid passage of sections through ethanol solutions, after exposure to eosin, interferes with this effect and should be discouraged. Secondly, some workers prefer to use a combined eosin reagent (incorporating such dyes as phloxine or safran) to enhance its differentiating properties (11).

In addition to the H&E technique, there are many other specialized staining methods that are pertinent to pathologic diagnosis. These will be discussed specifically later in this chapter.

Inasmuch as most laboratories utilize Harris' hematoxylin-- which is insoluble in alcohol-- the final preparation of H&E- stained microscopic sections involves dehydration in graded alcohols and several changes of a clearing agent. Subsequently, a drop of synthetic mounting medium (such as "Permount") is placed over the tissue after blotting away excess clearing solution, and a glass cover slip is affixed. The latter steps sound simple enough, but they actually require some technical finesse. Experienced technologists often dilute the mounting medium slightly with a few drops of clearing agent, and also leave a very small amount of clearing solution on the glass surface surrounding the tissue. These provisions assure that the mounting medium will disperse evenly under the glass cover slip, to the exclusion of entrapped air bubbles. Excess mounting medium must be carefully blotted from the area around the attached cover slip,

because it may otherwise diffuse over the surface of the slide. This unwanted phenomenon results in “blobs” of Permount on the coverslips (interfering with microscopy), or on the sides of the glass slides (causing them to stick to other surfaces or to one another). Under no circumstances should newly-mounted sections ever be stacked on top of one another, or immediately placed in vertical slide boxes or small plastic carriers. These maneuvers make it virtually certain that sections will anneal to one another, to plastic surfaces, or to struts in slide boxes, as the mounting medium hardens. The latter process can be accelerated by brief and gentle warming in a drying oven into which forced air is pumped.

With regard to labeling, some laboratories attach gum-backed labels to coverslipped sections, on which corresponding case and block numbers are then written or typed. Others prefer to use slides with a “frosted” area on one end, where these numbers can be written in indelible ink. Whichever one of these procedures is followed, laboratory workers must be certain to check all labels against block designations and diamond knife-etchings on the slides. The crucial nature of this step cannot be overstated in reference to the dermatopathology laboratory, where gross characteristics of differing specimens are of little help in distinguishing one from the other. In other words, nearly all shave samples and 3- or 5-mm punch biopsies look alike when they are stained and mounted! If a mistake occurs that compromises the identity of any given case, the pathologist and responsible clinician should be informed immediately. Such errors commonly result in the need to rebiopsy the patients who are concerned, to avoid potential diagnostic disasters.

### **SPECIFIC “SPECIAL” HISTOCHEMICAL STAINS**

For purposes of differential diagnosis, it is often desirable to evaluate biopsy specimens for their content of such tissue constituents as glycogen, vicinal glycol, glycoproteins, mucosubstances, iron, melanin, calcium, lipofuscin, elastin, collagen, amyloid, muscular proteins, fat, nucleic acids, nucleoproteins, neural elements, specific enzymes, and microorganisms. These moieties comprise the targets of predefined “special” histochemical stains, in which the chemical affinities of various biological components for several dyes have been exploited. A comprehensive discussion of the specific reactions involved, the detailed methods behind such stains, and all of the circumstances in which they might be applied is clearly beyond the scope of our consideration herein. However, these topics have been well-covered in previous textbooks and review articles to which the interested reader is directed (**11,20,21-26**). General principles and pertinent brief summaries of selected histochemical methods are provided in the following sections.

General Principles: As summarized by Stoward (27), the conceptual bases of histochemical techniques can be divided into four groups. These include ionic reactions involving the association of cations and anions; the action of aldehydes with Schiff’s reagent (sulfuric acid + pararosaniline hydrochloride) to yield a colored product; the association of aromatic diazonium salts with aromatic constituents of proteins; and the mediation of reactions wherein dyes are precipitated in colored form by target tissue enzymes. Biochemical moieties that are not evaluable by these methods can be altered to make them so. For example, glycogen can be oxidized by periodic acid to allow it to combine with Schiff’s reagent (in the periodic acid-Schiff [PAS] procedure), whereas it would not otherwise be reactive with the latter compound.

Specific Histochemical Methods:**Glycogen, Glycoproteins, & Mucosubstances**

As just stated, the PAS procedure is used to visualize deposits of glycogen in tissue, as well as to label glycoproteins or mucinous cell products that contain vicinal glycol residues. In order to distinguish between simple glycogen and the latter chemical substances, one usually exposes two serial tissue sections to the PAS reaction. One of them is, however, modified by prior incubation with diastase or amylase; this step dissolves glycogen but does not interfere with the ability of sugars in glycoproteins or vicinal glycols in mucin to combine with Schiff's reagent and yield a bright red reaction product. Thus, pathologists commonly request that PAS stains be done "with and without" [diastase/amylase].

Glycogen is seen in normal skin in the acrosyringia of sweat ducts and in pilar epithelium (22). The detection of this polysaccharide may be important in the recognition of various cutaneous adnexal tumors, metastatic carcinomas of the skin, and clear cell acanthomas. It also plays a role in the distinction between poorly-differentiated epithelial neoplasms (which are potentially PAS-positive) and malignant hematopoietic tumors (usually PAS-negative) with which they may be confused.

The glycoproteins of greatest interest in the skin are those which are components of basement membranes, together with collagen type IV and laminin. They are well seen with the PAS technique after prior diastase digestion of tissue sections (PAS-D), and thickening of the epidermal basement membrane-- as seen in lupus erythematosus and other chronic interface dermatitides-- is often spectacularly visualized with this procedure. Characteristic alterations in the basement membranes of papillary dermal blood vessels in porphyria cutanea tarda also are demonstrable by using PAS-D stains.

Mucosubstances that react with PAS-D are commonly called "epithelial mucins" (more appropriately, glandular mucins), and they tend to contain sialic acid residues (28). As one would expect, connective tissue mucins are PAS-D-nonreactive. Hence, this method can be employed to identify selected constituents of the normal skin; apocrine glands are the only structures that produce glandular mucin, whereas dermal ground substance, blood vessels, papillae of anagen hairs, nerves, and intercellular intraepidermal spaces contain stromal mucosubstances. Stains in this category may distinguish cellular proliferations that produce glandular mucin (e.g., adenocarcinomas; Paget's disease) from those that synthesize "stromal" (mucopolysaccharide- or sulfated acid-rich) mucin (e.g., pseudoglandular squamous cell and basal cell carcinomas) (29). One more procedure that is sometimes used to recognize epithelial mucin-- the mucicarmine technique-- is not as well-characterized as PAS-D in regard to its biochemical modus operandi. Nevertheless, staining results with these two methods are largely equivalent (including the color of the reaction product), with the possible exception that mucicarmine is less sensitive.

If a distinction between the non-glandular mucins is desirable, the aldehyde-fuchsin stain (AFS) can be performed along with the PAS-D; colloidal iron; alcian blue; azure-A; and toluidine blue procedures. If it is done at a pH below 1.0, the AFS yields green-blue positivity in tissues that contain primarily sulfated acid mucosubstances (11). In contrast, acid mucopolysaccharides such as hyaluronic acid, heparin, and chondroitin/dermatan/keratin sulfates constitute the major components of other stromal mucins (23). All of these moieties are



potentially visualized with colloidal iron or alcian blue, both of which yield light blue positivity; azure-A, showing red reactivity; and toluidine blue, producing lavender or purple positivity. A side-benefit of toluidine blue is that it can be used to label mast cells in tissue, owing to the presence of heparan sulfate in mastocytic granules.

By manipulating either the tissue or the reaction conditions, further information may be obtained. Prior digestion of sections with hyaluronidase greatly decreases staining of ground substances with both the alcian blue or colloidal iron procedures. Pretreatment with neuraminidase will abrogate the labeling of sialomucins, but they are intensely stained with the alcian blue method when it is done in a milieu containing high concentrations of magnesium chloride at pH 2.5 (24). Similarly, decreasing the pH to less than 1 results in preferential alcian blue staining of sulfated glycosaminoglycans such as heparin or dermatan sulfate, but this same step abolishes the recognition of acid mucopolysaccharides by toluidine blue (23).

The stromal mucins are important to the diagnosis of several dermatologic diseases. Follicular and focal mucinosis feature the deposition of colloidal iron/alcian blue-positive mucosubstance in the hair follicles or dermis, respectively. Myxedema, myxoid cysts, and lichen myxedematosus show regional or diffuse increases in the stromal mucin content of the corium. Necrobiotic processes may be separated from one another with the methods that have been presented in this section; the central areas of granulomas in granuloma annulare contain much more hyaluronic acid than do those of necrobiosis lipoidica or rheumatoid nodule (30). Lastly, cutaneous lupus erythematosus, dermatomyositis, and malignant papulosis all may demonstrate the augmentation of dermal stromal mucin (31).

Another exciting category of histochemical reagents is represented by the lectins; these proteinaceous products of various plants show preferential and differential affinity for particular carbohydrate residues (32) (Table 2). It is now known that some--such as peanut, soybean, and wheat germ agglutinins-- bind to sulfated or sialated mucins, whereas others (concanavalin-A and *Lens culinaris* agglutinin) do not (33). In addition, *Ulex europaeus* I agglutinin recognizes poly-L-fucose residues that appear to be integral components of a pan-endothelial determinant, and they also are seen in adnexal epithelium in patients of blood group O (34). Very few reports on cutaneous diseases have incorporated lectin histochemistry into the assessment of these processes (35), but it is anticipated that this situation will change rapidly in the near future.

### Amyloid

Amyloid is a peculiar tissue substance that is unique to pathological conditions. It is principally a fibrillar protein which forms rods that are of variable length and 7.5 to 10 nm in diameter, and that tend to aggregate extracellularly in a "tangled" configuration. A secondary, minor component of amyloid is sulfated proteoglycan, accounting for approximately 10% by weight (36). It accounts for the fact that amyloid has some chemical similarities to polysaccharides and certain mucosubstances, such as an affinity for iodine and metachromatic labeling with toluidine blue (11).

Detailed studies on linear amyloid fibrils have shown that they aggregate in "beta-pleated sheets" as seen by x-ray crystallography (36). This property has been exploited histopathologically, together with dyes that preferentially impregnate amyloid protein and proteoglycan. The latter stains, such as Congo red, Sirius red, Lieb's crystal violet, and thioflavine-T, are used to impart a red, magenta, or fluorescent yellow-green color to amyloid

deposits, respectively. Polarization microscopy of sections labeled with Congo red shows a characteristic “apple green” dichroism in amyloid deposits, because of its physicochemical attributes.

There are advantages and disadvantages to some of the above-cited methods. Sections stained with Congo red must be meticulously prepared at a thickness of 8 to 10 microns; if they are too thin or thick, the amyloid deposits will be a faint red or yellow upon polarization, rather than apple-green. We also have noticed that the use of unmatched polarizing microscopic lenses may cause similar problems. Lieb’s stain is soluble in organic compounds, necessitating that sections cannot be coverslipped in a conventional fashion. Glycerol has been employed as a mounting medium, but it allows for the diffusion of crystal violet from the amyloid with the passage of time. Modified Apathy’s medium (a mixture of acacia, cane sugar, water, sodium chloride, and thymol) is a suitable alternative, as are polymeric compounds such as Crystal Mount<sup>R</sup> (11). As stated above, the thioflavine-T procedure must be interpreted by fluorescence microscopy and is therefore not widely used.

Several biochemical classes of amyloid exist. These include one type derived from fragments of immunoglobulins in plasma cell dyscrasias (“AL” amyloid); another produced by the apparent modification of plasma proteins by macrophages in chronic inflammatory diseases (“AA” amyloid); a familial form (“AF” amyloid); and a fourth that represents altered neurohormonal cell products and is confined to the interstices of endocrine neoplasms. Regardless of which specific type of amyloid one encounters in an individual case, all of them show nearly identical staining properties (36).

### **Minerals and Pigments**

There are a number of minerals and pigments in tissue that have dermatopathologic relevance. These can be localized and identified with histochemical methods.

Iron is detected with Perl’s reaction, also known as the Prussian blue procedure. When loosely complexed with protein, ferric ions will combine with acidified potassium ferrocyanide to yield a deep blue product that is insoluble (20). Because of potential confusion with structures that hematoxylin may impregnate, Prussian blue tissue sections are counterstained with nuclear fast red.

Melanin is derived from tyrosine-containing compounds and it is typically bound to protein in tissue. This pigment is capable of reducing silver nitrate solutions to metallic silver, which is deposited in tissue sections as a black precipitate in the Fontana-Masson technique (24).

Alternatively, solutions containing ferrous ions can be used to form complexes with melanin, and these are subsequently detectable as a blue product with the Turnbull reaction (11). In the assessment of heavily-melaninized cutaneous lesions, it is sometimes necessary to bleach the pigment to allow for evaluation of cellular detail. This can be accomplished by treating tissue sections with 10% hydrogen peroxide for 1 to 2 days, or 0.25% potassium permanganate for 1 to 4 hours, followed by 1% oxalic acid (24). Lastly, one can capitalize on knowledge of the biochemical pathway whereby melanin is formed by melanocytes, in order to visualize non-pigmented cells of this type. The DOPA reaction involves the exposure of tissue sections to a dilute solution of 3,4-dihydroxyphenylalanine. Melanocytes that contain DOPA oxidase will convert this compound to melanin, which may then be detected by one of the above-cited methods. Unfortunately, the DOPA reaction mandates the use of frozen sections, inasmuch as

melanocytic enzymes are largely inactivated by routine tissue processing (37).

Calcium typically is hematoxylinophilic on H&E stains. In order to confirm its presence definitively in such conditions as calcinosis cutis and pseudoxanthoma elasticum, one of two procedures should be utilized-- the von Kossa technique, and the alizarin red method (21). The first of these employs a solution of silver nitrate; calcium carbonate will combine with the latter compound to form silver carbonate, which, upon exposure to ultraviolet light or sunlight, is reduced to elemental silver in a black precipitate. Unfortunately, formalin pigment will produce an identical result. This problem makes the alizarin-red technique, wherein the dye chelates directly with calcium ions to form an orange-red complex, preferred. Alizarin-red-stained sections are not counterstained, and the resulting calcium complexes show birefringence on polarization microscopy (11).

Uric acid deposits are seen in the skin in gouty tophi. These are difficult to visualize in sections that are fixed with aqueous solutions, because urates are dissolved in the course of routine processing. Alcohol-fixed tissues are therefore best used in this context. Uric acid reduces the silver ions in a 5% silver nitrate solution to their metallic form, giving a black precipitate over the gouty deposits (26). When employed as a part of the Gomori methenamine silver procedure, this reaction is useful diagnostically. Sections are counterstained with Light Green Solution.

### **Lipids**

Lipids in the skin and its adnexae may be of diagnostic importance in several settings. For example, it may be desirable to confirm the biochemical nature of apparent xanthomas, and the granulomas of necrobiosis lipoidica characteristically contain a central area of lipid deposition (30). Cholesterol emboli may affect the skin by causing microinfarction of it, and sebaceous tumors are definitively identified by the demonstration of cytoplasmic fat in the neoplastic cells .

An inviolate requirement for visualization of lipids in clinical specimens is that frozen tissue, or, at the very least, unembedded formalin fixed tissue must be available for study (29). This is because the alcoholic and clearing agent steps in tissue processors otherwise dissolve all fat before sections are prepared for microscopic analysis.

Three major staining techniques are applicable to this problem. The oil red O and Sudan black B methods utilize dyes that are miscible in fat and therefore impregnate it (24). These yield red and black products, respectively, as their names suggest. The third procedure is probably the best from a technical perspective, because it provides microscopic preparations of higher quality. This is the osmium tetroxide stain with periodic acid as a differentiating agent, wherein osmium fixes lipids and stains them black. Because this chemical is insoluble in organic compounds, frozen tissues labeled with 1% osmium can be post-fixed in formalin and processed routinely (11). The result is a histologic section with good cytologic detail, as opposed to the suboptimal architecture that one sees in frozen sections. Nevertheless, procedures employing osmium tetroxide do have a significant disadvantage. It is a very caustic and potentially toxic compound, and technologists must work in a fume hood to avoid such dangers. Disposal of the reagent is accordingly difficult as well.

### **Nucleic Acids and Nucleoproteins**

Before the advent of flow cytometry and in situ hybridization, histochemical methods

were developed to aid in assessing the amount of nucleic acid that malignant cells contain. The latter procedures are still useful in selected instances, particularly when coupled with morphometric (densitometric) techniques. There are two stains that are appropriately categorized as semispecific for nucleic acids-- the Feulgen method, and the methyl green-pyronine procedure.

The chemical basis for the Feulgen stain is predicated on the fact that controlled acid-hydrolysis can be used to digest purine bases in deoxyribonucleic acid (DNA), producing aldehyde groups that are capable of combining with the Schiff reagent (**11**). Therefore, the method is an indirect one requiring modification of the target compound. The reader will recall that Schiff conjugates yield a red color, representing the desired end product of the Feulgen stain. The hydrolytic step in this procedure is fixative-dependent; formalin yields that most consistent results and requires only short exposure of tissue sections to acid, whereas specimens preserved in heavy metal fixatives must be digested for a longer period of time. Bouin's solution is itself hydrolytic, and therefore renders tissue unsuitable for studies with the Feulgen reaction. Ribonucleic acid (RNA) is not sensitive to hydrochloric acid, and is not labeled in this procedure.

On the other hand, the methyl green-pyronine (MGP) technique depends on differential affinities of DNA and RNA for the two dyes used in this reagent. These affinities are presumably based on the tendency for methyl green to associate with large polymers (DNA), whereas pyronine intercalates with smaller polymeric compounds (RNA). As one would expect, nuclei are stained green with MGP, and the cytoplasm-- where the majority of intracellular RNA resides-- is red (**38**). It should be remembered that pyronine is not specific for RNA; this dye also labels keratin and the granules of mast cells or eosinophils. Therefore, neoplasms showing mastocytic, granulocytic, or keratinocytic differentiation are not reliably assessable with MGP. It is most often used in helping to confirm a plasmacytic or immunoblastic lymphoid phenotype in malignant lymphomas, where high levels of both DNA and RNA are expected.

Exclusive of immunohistochemical evaluation for cell phase-dependent nuclear antigens, or flow cytometric measurement of S phase populations, there is only one method for the estimation of actively dividing cells. It is an indirect one, and is known as the "Ag-NOR" procedure. This acronym stands for silver (Ag)- stained nuclear organizer regions, which are complexes of nuclear proteins and ribosomal RNA genes. They have been localized to five chromosomes in man, and tend to increase in number, size, and density in proliferative cell populations (**39**). Preliminary studies have suggested, for example, that cells in benign melanocytic lesions have few, compact Ag-NORs, whereas those in malignant melanomas tend to show multiple coarse intranuclear bodies of this type (**40**).

The Ag-NOR reagent is somewhat complicated to prepare and utilize. It consists of freshly-dissolved gelatin and formic acid, which are mixed with silver nitrate solution prepared with deionized distilled water. The final product is a colloidal suspension; it is applied to deparaffinized-rehydrated or frozen sections of tissue and incubated in the dark for 30 minutes. Ag-NORs are visible in the sections as distinct, dark, intranuclear bodies when the procedure is performed properly (**41**). However, slight variations in incubation time or reagent preparation can result in abundantly but nonspecifically-deposited silver, producing "dirty" slides that are difficult to interpret.

### **Mesenchymal Structures**

The skin contains several "connective" tissues and derivatives of embryonal

mesenchyme, which figure into the assessment of selected inflammatory, degenerative, and neoplastic diseases. These include collagen types, elastic tissue, muscle, and nerve, as well as fat and ground substances, which were discussed earlier.

One of the most widely used histochemical methods for the evaluation of these tissue components is the Masson trichrome technique. It is a mixture of three dyes-- Biebrich's scarlet; hematoxylin; and aniline blue-- as well as phosphotungstic or phosphomolybdic acid. The latter compounds alter the affinity of various mesenchymal tissues for the dyes, causing differential staining (11). Collagen and elastin are stained blue and muscle is red with the trichrome procedure, whereas nuclei are black and non-muscular cytoplasm is also red. Attention to fibrillation of the cytoplasm is necessary on high-power microscopy to distinguish myogenous from non-muscular cells.

Elastic tissue is best seen with Verhoeff's method (and a modification known as the Verhoeff-van Gieson method) or the aldehyde fuchsin elastic stain. In the first of these, a chemical "lake" of hematoxylin and ferric chloride is the principal staining reagent. Elastic tissue has a strong affinity for the latter complex and therefore resists decolorization better than other tissue components in this regressive staining technique. Elastic fibers are blue-black, collagen is red, nuclei are blue, and other tissues are yellow in the version of Verhoeff's method where van Gieson's reagent is used as a counterstain (24). This procedure is an excellent one for the demonstration of elastic tissue abnormalities, such as those seen in elastofibroma dorsi, pseudoxanthoma elasticum, and solar elastosis.

In the aldehyde fuchsin elastic stain (42), basic fuchsin is combined with hydrochloric acid and paraldehyde to yield aldehyde fuchsin. This reagent has the ability to form Schiff bases and shows a strong affinity for elastic fibers. The latter acquire a dark blue or purple color, and Light Green is usually used as a counterstain.

Nerve fibers may be visualized either through staining their neuronal processes, or the myelin surrounding them. Silver methods such as the Bodian stain are utilized to accomplish the former task, whereas the Luxol fast blue technique is employed to delineate myelin in tissue. The Bodian method is a complicated procedure using silver proteinate, gold chloride, hydroquinone, sodium thisulfate, hydrochloric acid, and aniline blue. It was designed as a regressive staining technique to selectively impregnate neuronal processes with elemental silver, yielding a black precipitate on these elements (43). Nuclei are black, and surrounding tissues are blue. In the Luxol fast blue procedure, sulfonated copper phthalocyanine is used in an acid-base reaction to replace the bases of lipoproteins in myelin. As its name suggests, this dye has a blue color in the final staining product (26).

Another cellular grouping of interest in dermatopathology is that called the "diffuse neuroendocrine system." It includes Merkel cells of normal skin, and "Merkel cell carcinomas." Silver impregnation techniques also are employed to delineate neurosecretory granules in neuroendocrine cells. The Grimelius and Churukian-Schenk procedures are useful in this context (44,45). Both of them are predicated on the knowledge that neurosecretory granules have the ability to bind silver ions (i.e., they are argyrophilic); a reducing substance is then applied to tissue sections to yield a precipitate of black elemental silver. Argyrophilic techniques are influenced by fixation. Formaldehyde-based solutions produce inconsistent positivity, whereas this is one situation where Bouin's fixative gives superior results. Also, melanosomes are

reactive with the Grimelius and Churukian-Schenk methods, necessitating concomitant performance of the Fontana-Masson stain before interpreting an argyrophilic cellular proliferation as neuroendocrine in nature.

### **Microorganisms**

The range of infectious agents that are evaluated in cutaneous diseases is broad. It includes conventional bacteria, spirochetes, mycobacteria, fungi, protozoan organisms, and nematodes (11). A complete discussion of the histochemical methods that are used to identify these microbes is not within the scope of this presentation. However, a summary of such techniques is given in **Table 3**.

### **Cytoplasmic Enzymes**

Similarly, a large number of cytoplasmic enzymes has been assessed in cutaneous lesions using histochemical procedures. In particular, phosphorylase, succinic dehydrogenase, leucine aminopeptidase, indoxyl esterase, and cytochrome oxidase are felt to be helpful markers of eccrine differentiation in adnexal neoplasia. On the other hand, acid and alkaline phosphatases, ATPase, beta-glucuronidase, acetate esterase, and monoamine oxidase are used as evidence for apocrine differentiation (46). The methodology for each of these techniques has been outlined in other sources (37,46), and will merely be referenced here. Most of them are based on the ability of any given enzyme to hydrolyze or oxidize a colorless insoluble compound to yield a colored precipitate. Appropriate counterstains are then applied to yield the final histological product.

The procedures just mentioned are not used much currently, because immunohistochemistry has largely superseded the application of enzyme histochemistry in dermatopathology. However, one that has not been cited is the naphthol AS-D chloroacetate method, also known as the von Leder stain. It is very useful in identifying immature granulocytes in suspected leukemia cutis cases, or, perhaps more importantly, in recognizing tissue mast cells. The red product in this stain derives from the inclusion of Fast Garnet as the active dye (47). Contrary to metachromatic techniques such as the toluidine blue stain-- which is also employed for the labeling of mast cells-- the von Leder procedure is not dependent on the integrity of mastocytic granules to produce a positive result.

### **IMMUNOHISTOCHEMICAL TECHNIQUES**

Immunohistochemistry is still regarded by some pathologists as a “new” technology. This is really not an accurate perspective, because immunofluorescence-- the prototype of immunohistologic procedures-- was introduced over fifty years ago (48). It is true that immunologic analysis of routine pathologic specimens did not gain wide acceptance until the 1970s (49), but this discipline has literally exploded in scope since then. The following sections will present a summary of various immunohistochemical procedures that are applicable to dermatopathology.

**Immunofluorescence Techniques:** Coons et al., were the first to describe the attachment of fluorescent chemical ligands to antibodies, as diagnostic reagents (48). The most widely-used of these moieties is fluorescein isothiocyanate (FITC), which produces yellow-green emission when excited by ultraviolet light. Immunofluorescence is best performed on frozen sections prepared from specimens kept in saline for short periods after procurement; samples that are flash-frozen immediately after biopsy; or tissue that has been transported in Michel’s medium. The latter solution allows for preservation of tissue-bound immunoglobulins for one or two days, owing to

its content of N-ethylmaleimide and ammonium sulfate **(50)**. These chemicals must be removed from the specimen by thorough washing in buffer prior to immunolabeling, or poor results will be obtained. Similarly, unbound serum proteins must be rinsed out of all skin biopsies with saline before immunofluorescent ligands are attached, to preclude the presence of high “background” staining in final microscopic preparations.

For “direct” immunofluorescent microscopy, cryostat sections are cut at 2 to 4 microns and mounted on adhesive-coated glass slides. They are then incubated at room temperature for 30 minutes with specific, FITC-labeled antisera (to immunoglobulins A, E, G, and M; fibrinogen; and complement fractions [such as C3]), rinsed, mounted in glycerol, and coverslipped. If the targets of the antibodies were affixed to the tissue in vivo, they will be visualized as fluorescent green deposits in the epidermis, the dermis, or both **(Figure 8)** by ultraviolet microscopy. Permutations and patterns of stains for immunoglobulins or other reactants are provided in other chapters of this text, and can be used to classify the immunologically-mediated diseases of the skin **(5)**.

Direct immunofluorescence has the major limitation that morphologic detail is poor, and it may be necessary to switch back and forth between phase microscopy and ultraviolet illumination to determine the cellular locale for the reactants. In addition, collagen in the tissue may autofluorescence and complicate the interpretation. Moreover, immunofluorescence preparations fade after several days, and photomicrography is necessary for long-term documentation of results. Paraffin sections are suboptimal substrates for this procedure, but they may occasionally be rendered suitable by pretreatment with proteolytic enzymes and 0.1% ammonium hydroxide **(12)**.

“Indirect” immunofluorescence does not employ patient tissues as analytes, but rather uses serum taken from patients who are presumed to have circulating antibodies to cutaneous components. Normal substrate skin can be obtained from volunteers (or from previous diagnostic cases showing no immunologic abnormalities), or cross-reactive tissues such as animal esophagus can be procured from commercial vendors. These are exposed to patient serum for 30 to 60 minutes at room temperature, rinsed, and incubated with FITC-labeled antisera as cited above. Positive results may indirectly confirm the presence of circulating antibodies to keratinocyte attachment plaques (in pemphigus), bullous pemphigoid antigen (BPA), epidermolysis bullosa acquisita antigen (EBAA), and other tissue complexes.

In order to definitively distinguish anti-BPA from anti-EBAA, the “saline split skin” procedure must be used in indirect immunofluorescence. Normal skin is soaked in 1 Molar saline according to a predetermined protocol **(52)**, causing the epidermal basement membrane to split between its laminae densa and lucida. Because BPA and EBAA are dissimilarly located in one or the other of these layers, immunofluorescent labeling for BPA is seen in the base of the artificial “blister,” and EBAA is localized to its “roof.”

Indirect Antibody Procedures: Because of the morphologically-suboptimal nature of immunofluorescence, pathologists were driven to develop alternative immunohistologic (IHL) procedures that could be applied to conventional (fixed, paraffin-embedded) specimens. Antigens are localized in such samples indirectly, but they can be seen with a high level of resolution and clarity. Several indirect techniques are available, as outlined below.

### **The Peroxidase-Antiperoxidase Method**

Sternberger and colleagues can be credited with devising the first practical IHL technique, the peroxidase-antiperoxidase (PAP) procedure (53). It is based on the ability to complex two immunoglobulin (Ig) G molecules to several horseradish peroxidase molecules in vitro, via the Fab fragments of the antibodies. Rabbit Igs are most often used for this purpose. A specific rabbit antiserum against an antigen of interest is first incubated with test tissues, followed sequentially by application of a generic anti-rabbit Ig and the Ig-peroxidase complex (Figure 9). Peroxidase is capable of oxidizing 3,3'-diaminobenzidine hydrochloride (DAB) or 3-amino-9-ethylcarbazole (AEC) to their insoluble forms in the presence of hydrogen peroxide. This step produces brown-black and red precipitates, respectively, allowing for localization of bound rabbit Ig/anti-rabbit Ig/rabbit Ig-peroxidase complexes in tissue.

We prefer DAB over AEC, because the former compound gives a denser product and is insoluble in organics. In contrast, AEC dissolves in clearing agents and Permount, and sections must be mounted with an aqueous medium or polymeric resin. Glycerol medium is suitable for short-term study of AEC-labeled sections, but the chromogen tends to diffuse into it from the tissue after several weeks. Alternatively, Crystal Mount<sup>R</sup> provides more permanent preparations (11).

The PAP method is still widely used, because of its relative simplicity and the low cost of the reagents. However, it does have two potential failings. One relates to a relative insensitivity for low antigen densities, which may be partially overcome by “reiterative” application of the PAP complex (12) (Figure 10). The other problem reflects the need to “quench” endogenous tissue peroxidase in specimens to avoid unwanted background staining.

### **The Avidin-Biotin-Peroxidase Complex Procedure**

Hsu et al., introduced the avidin-biotin-peroxidase complex (ABC) method as an alternative to the PAP technique in 1981 (54). The former procedure employs secondary, generic anti-species antibodies that are labeled by chemical attachment of biotin to their Fc fragments. This provision allows the biotin to bind to commercially-synthesized tertiary complexes of avidin, biotin, and horseradish peroxidase (Figure 11). These aggregates contain several-fold more peroxidase molecules than tertiary reagents in the PAP method.

The slightly greater cost of the ABC technique is more than offset by its higher degree of sensitivity, relative to PAP. Moreover, “second-generation” ABC kits have now been introduced with even more peroxidase molecules in the tertiary complexes, and the PAP and ABC procedures can be done in sequence (the “ABPAP” method) to further amplify sensitivity (12) (Figure 12). The only real disadvantage of ABC reagents is the continued need to quench endogenous tissue peroxidase, as noted above.

### **The Alkaline Phosphatase-Anti-Alkaline Phosphatase Method**

To avoid the need to inactivate native peroxidase in clinical specimens, the alkaline phosphatase-anti-alkaline phosphatase (APAAP) procedure was developed in the middle 1980's (55). Its principle is basically the same as that described for the PAP technique, except that the intestinal isozyme of alkaline phosphatase (AP) is used as the chromogenic enzyme instead of peroxidase, and Fast Red is substituted for DAB as the chromogen in APAAP. Because intestinal-type AP is not seen in most human tissues (including the skin), background staining is negligible and there is no need to quench endogenous catalytic agents (12). This is a distinct



advantage in certain diagnostic situations. For example, a biopsy specimen containing numerous neutrophils or erythrocytes (with considerable cell-bound peroxidase) would be expected to show high background labeling with the PAP or ABC procedures. In contrast, the APAAP method yields a very crisp result in such samples.

Disadvantages of APAAP are few, but may be troublesome nonetheless. It has been our experience that the reaction steps take longer to complete than those of the ABC or PAP methods, and the eventual precipitate of the Fast Red is not particularly dense. Lastly, APAAP is more expensive than the other techniques.

Choice of an Immunohistologic System: One serious problem confronting pathologists is that which concerns non-uniformity of methodology in immunohistochemistry. It may be safely said that, in general, the overall results of the PAP, ABC, and APAAP procedures should be comparable over a broad range of immunoreactants. Nonetheless, a huge body of disparate, often-contradictory information exists in the literature on immunohistologic findings in human diseases. This has resulted from many idiosyncratic approaches to such specific procedural details as antibody selection, antibody concentration, periods of incubation, and reaction temperatures. Attention to reports emanating from reputable diagnostic immunohistochemistry laboratories shows that they do, in fact, employ similar methods and reagents, with consonant results. Hence, this methodology should be emulated by responsible scientists in order to assure reproducibility of the technique. There is no room for “mavericks” in immunohistology (56).

Preprocedural Considerations in Immunohistology: Contrary to what many pathologists prefer to believe, simply changing IHL methods is not the solution for poor results that may be obtained with this technique. Instead, the most common problems in this realm relate to improper handling of specimens before they reach the histology laboratory, or inadequate attention to non-immunologic reagents that are used in these procedures (12).

### **Tissue Procurement**

One of the best adages for the budding immunohistochemist to remember is also one of the crudest-- “garbage in, garbage out.” It is amazing how often pathologists expect immunohistologic studies on “tortured” tissues to yield definitive diagnostic results, even though an interpretation was impossible by routine H&E studies because of processing distortions! Specimens that have been frozen and thawed, crushed, allowed to autolyze, fixed badly, or harshly decalcified are predictably poor substrates for immunologic analysis, and little can be done to retrieve their complete antigenic integrity.

Preservation difficulties are among the most prevalent in this context. Overfixation in formalin (e.g., for more than 24 hours) causes “masking” of many antigens by protein cross-linkage via methylol bonds (57). Heavy metal solutions, and those containing picric acid, denature many cytoplasmic proteins and severely limit the scope of immunohistologic studies. On the other hand, under-fixation promotes autolysis, with similar loss of antigenicity. The best approach is to carefully control the preparation of NBF that one uses for all specimens, with limitation of the fixation time to under 12 hours whenever possible; formalin-alcohol is also a viable option to improve tissue preservation. Specimens should be prosected so that submitted sections are no more than 3 to 5 mm thick, to allow for optimal penetration of fixatives.

Many immunohistochemists have applied digestion procedures with such enzymes as trypsin, pepsin, or pronase to counter the effects of overfixation in formalin. It is true that these

agents (as well as ficin, papain, DNase, and bromelain) are capable of restoring the immunoreactivity of several cellular markers (12). These include intermediate filament proteins, factor VIII-related antigen, CD30 molecules, collagen type IV, and others. However, the use of proteases may damage other determinants, and it must, in any case, be tailored to the antigens of interest. For example, the exposure of immunoreactive sites in S100 protein is enhanced by bromelain, but they may be digested entirely by ficin! Obviously, these agents must be applied after careful thought and familiarity with the pertinent literature (58).

### **Non-immunological Reagents**

As mentioned earlier in this discussion, some clearing agents-- even those as “innocuous” as xylene-- may damage tissue antigens. Embedding procedures with paraffin that has been heated to above 60 degrees C, or the alternative use of Carbowax, also have deleterious effects on immunohistochemistry.

Without any hesitation, we would also say that buffers used in this facet of histology are the greatest source of technical difficulty in our experience. These must be scrupulously prepared with respect to ionic constituency and pH, and such factors should be monitored carefully during storage of the solutions. The target value for buffer pH is the narrow physiological range of 7.2 to 7.4. Inappropriately acid reagents may preclude primary antibody binding totally, and overly basic solutions may result in extreme degrees of background labeling. Buffers with excessive ionic strength commonly cause nonspecific precipitation of DAB and AEC (12), yielding virtually uninterpretable preparations.

Selection of Primary Antibody Reagents: There are literally hundreds of antibodies in the catalogues of commercial vendors, many of which are applicable to diagnostic immunohistochemistry. However, these “menus” are often fraught with pitfalls for the unwary customer. Several antibodies have been developed for exclusive use in frozen sections, such as those applied to the study of hematolymphoid proliferations (e.g., reagents in the Leu series). Unless one asks for data from the manufacturer on optimal reaction conditions, sensitivity, and specificity, a large sum of money and a great deal of time may be invested in the workup of useless antibodies. Reagents that fail to demonstrate their stated characteristics should be promptly returned to the supplying firm, and payment for them can be appropriately withheld.

Also, an often-asked question concerns the “superiority” of monoclonal antibodies over heteroantisera, for use as primary reagents in diagnostic immunohistochemistry. It is true that hybridoma products commonly yield “cleaner” staining results, but this may be at the expense of sensitivity or specificity. All pathologists should attempt to assess the latter performance factors “in house,” by studying prior specimens from their own files that have been thoroughly characterized for their spectrum of immunoreactivity. The “sausage block” method is an efficient means of attaining this goal. In this procedure, strips of tissue from 50 to 70 sectioned paraffin blocks are rearranged in a predetermined fashion in one conglomerate block (59,60). The latter may be structured to contain many examples of one tumor type (e.g., 50 examples of malignant melanoma), or, more commonly, it is configured to include 4 or 5 grouped examples of 10 of 15 neoplasms (5 melanomas, 5 basal cell carcinomas, 5 lymphomas, etc.) (Figure 13). Any new antibody can be applied to sections of the sausage block, and “scored” in regard to its specificity and sensitivity for a particular tissue type.

Another caveat pertaining to antibody selection refers to the analysis of any given

diagnostic case. In an attempt to save money, many pathologists still attempt to “confirm” an H&E interpretation with one immunohistologic reaction. For example, they may study a tumor for its expression of S100 protein to support a diagnosis of malignant melanoma. This approach is deplorable. As we have learned more and more about the scope of distribution of many antigens, it has become clear that very few are tissue- or tumor-specific. In the above-cited instance, a poorly-differentiated metastatic carcinoma of the breast could also be reactive for S100 protein, and it could show histologic synonymity with malignant melanoma as well (61). Thus, immunohistology should always be applied exclusively in a multi-antibody panel setting. The combinations of reactivity patterns that are expected in dermatologic diseases are impossible to outline here. However, they have been considered in depth in other publications (62-64), and are mentioned in context in the remaining chapters of this text.

**Positive and Negative Controls:** All immunohistologic techniques must include a positive (method) and negative (tissue) control (12). Positive controls are, as noted above, archival specimens that have been well-characterized immunologically in the past. These serve to assure that the immunohistochemical procedure, as a unit, has been performed properly on any given day, and that antibody reactivities are constant over time. Negative controls are represented by sections of a study case that are stained immunohistologically after substitution of nonimmune sera for primary antibodies. The purpose of this step is to assure oneself that the tissue does not spuriously (nonspecifically) bind immunoglobulin from a particular animal source. Hence, nonimmune rabbit sera are substituted for specific primary rabbit antisera in a properly-done negative control. If the negative control looks “positive,” a tissue-related problem exists that will interfere with reliable interpretation of results.

In this age of increasing medicolegal accountability, it is highly desirable to keep written records of control reactivities over time, in the immunohistochemistry laboratory. This aids in the surveillance of reagent performance, assessing the competence of technical personnel, and maintaining documentation of quality assurance practices.

### **ELECTRON MICROSCOPY**

Electron microscopy has, in many people’s minds, been rendered “obsolete” by the availability of immunohistology and in situ hybridization. This is a fatuous contention. Ultrastructural studies still do have a potentially major role to play in the localization of immune complexes in skin biopsies, the identification of diagnostically-helpful cellular constituents, and the detailed localization of antigens (in combination with immunohistology) (65).

Appropriate fixation of tissue for electron microscopy necessitates prompt immersion of carefully-handled (un-crushed) specimens in 2 to 4% phosphate-buffered glutaraldehyde; the tissue blocks must be no larger than 2 cubic millimeters (11). Fixation occurs optimally at 4 degrees C, and should be allowed to proceed for a minimum of 2 hours. This step is followed by post-fixation in osmium tetroxide, dehydration in graded alcohols, and the embedding of each block in one of a number of polymeric resins. The latter include Epon, Spurr resin, and Lowicryl, which are “cured” by incubation at 60 degrees C for 8 to 10 hours (66).

Thereafter, “thick” (1 mm) sections are cut, mounted on glass slides, and stained with a polychromatic dye such as methylene blue. The pathologist should review each thick section with the responsible ultrastructural technologist, and indicate those that are suitable for further study. These specimens are then subjected to ultra-thin (60 to 70 nanometer) sectioning with a

diamond knife microtome, after which they are mounted on round copper grids and stained with uranyl acetate and lead citrate. Formvar-- a plastic resin-- can be added as a thin coating over the mounted ultrathin grid sections to provide additional support prior to staining (67).

Proper polymerization of the supporting resin and attention to microtomy technique are crucial to preparation of suitable specimens for electron microscopy. Incomplete resin curing, dull microtome knives, and overly-thick ultrathin sections all are responsible for wrinkles in the final tissue preparations. These are even more troublesome in ultrastructural studies than in routine light microscopy, because the electron beam that is used to illuminate the specimen will literally melt grids that have folds in the tissue.

Lowicryl is the resinous mounting medium of choice for immunoultrastructural analysis, in which tissue grids are incubated with antibodies that have been labeled with electron-dense gold or ferritin particles (68). This resin allows immunologic reagents to penetrate the specimen well, whereas Epon and Spurr do not.

### **IN-SITU HYBRIDIZATION**

In situ hybridization (ISH) procedures are, in many ways, similar to those used in immunohistochemistry. However, instead of employing antibodies as diagnostic reagents, ISH techniques use segments of single-stranded nucleic acid ("probes") that are carefully prepared in vitro. These are tagged with a radioactive moiety such as tritium, or with biotin. The binding of radiolabeled probes to tissue specimens is visualized by autoradiography (a similar process to the development of photographic images), and biotin-labeled reagents are localized via the ABC procedure (69,70). It should be noted at this point that tissue must be affixed to glass slides with a special adhesive-- aminoalkylsilane (11)-- to prevent its loss during this "harsh" procedure.

Either DNA or RNA probes can be utilized, depending upon whether one wished to determine the presence of nuclear or cytoplasmic (protein-related) nucleic acids in the target specimen. Prior, controlled heating of the tissue is necessary in DNA ISH techniques, to cause denaturation of the double-stranded nucleic acid. RNA ISH is particularly demanding technically, because RNase is a ubiquitous enzyme that is found in sweat and sebum. Therefore, glassware must be meticulously cleaned, and the technologist must be careful to wear surgical gloves to avoid contact of his or her skin surfaces with the specimens. Strict attention to the ionic strength and chemical constitution of all reagents is required to avoid inactivation of the probes, or, alternatively, their nonspecific attachment to the specimen being analyzed (70).

The sequence of nucleotides in each probe is structured in such a fashion that it is complementary to portions of the target nucleic acid in the tissue sample. If annealing occurs, DNA probes will show nuclear autoradiographic or ABC-positivity, whereas RNA reagents label the cytoplasm preferentially.

At present, ISH procedures are limited in their diagnostic applicability. They are most often used to evaluate the presence of viral (e.g., human papillomavirus) nucleic acid sequences in cutaneous biopsy specimens. However, they have substantial research value because ISH is capable of demonstrating the nucleic acid counterparts of cellular protein markers, even if the latter are not being actively synthesized. This technique can therefore be viewed as a definitive confirmatory method, vis-a-vis immunohistochemistry. As additional practical information accrues on such topics as oncogene amplification and mutation in cutaneous diseases, it is anticipated that ISH will acquire more diagnostic and prognostic utility.

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**Table 1: Specimen Processing in Dermatopathology**

<u>Pathologic Technique</u>	<u>Recommended Fixative</u>	<u>Processing Time</u>	<u>Comments</u>
Conventional Histology	NBF or FA*	1 day	Tissue should be sectioned at 2-3 mm for good fixation
Immunohistology	NBF or FA**	2-3 days	Technique can be applied to frozen or fixed sections
Electron Microscopy	2% phosphate-buffered glutaraldehyde	3-4 days	Tissue must be minced into 1 or 2 mm cubes
Immunofluorescence	None, if tissue is flash frozen; 95% ethanol or acetone for touch-preparations; Michel's medium for transportation	1-2 days	Tissue can be held in Michel's medium for up to 48 hrs. Frozen tissue must be kept at -70 degrees C until use
<u>In situ</u> hybridization	NBF or FA for DNA studies; Frozen tissue preferred for RNA studies	1 week	DNA studies can be done on frozen or fixed tissue

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NBF = Neutral buffered 10% formalin    FA = NBF-Ethanol (50:50)

\* Tissue for routine histology can be fixed in B5 or Bouin's solutions to improve nuclear morphology, but these preservatives require special processing and compromise immunohistology

\*\* Certain tissue antigens (e.g., light chain immunoglobulins) are detectable only by frozen section immunohistochemistry

Table 2: \*

## Lectins used in pathological diagnoses

Common abbreviations	Source	Nominal saccharide specificity
Glucose/mannose group		
Con A	Jack bean ( <i>Canavalia ensiformis</i> )	$\alpha$ Man > $\alpha$ Glc $\geq$ GlcNAc
LCA*	Lentil ( <i>Lens Culinaris</i> )	$\alpha$ Man > $\alpha$ Glc > GlcNAc
PSA	Pea ( <i>Pisum sativum</i> )	$\alpha$ Man > $\alpha$ Glc = GlcNAc
N-Acetylglucosamine group		
BSA II	Bandeirea simplicifolia seed	$\beta$ - and $\alpha$ -GlcNAc
GSA-II	Griffonia seed ( <i>Griffonia simplicifolia</i> )	$\alpha$ - and $\beta$ -GlcNAc
DSA	Jimson weed ( <i>Datura stramonium</i> )	GlcNAc ( $\beta$ 1, 4-GlcNAc) <sub>1-3</sub> = Gal $\beta$ 1, 4-GlcNAc
PWM	Pokeweed ( <i>Phytolacca americana</i> )	GlcNAc ( $\beta$ 1, 4-GlcNAc) <sub>1-5</sub> = Gal $\beta$ 1, 4-GlcNAc <sub>2-5</sub>
STA	Potato ( <i>Solanum tuberosum</i> )	GlcNAc ( $\beta$ 1, rGlcNAc) <sub>1-4</sub>
UEA-II	Gorse seed ( <i>Ulex europaeus</i> II)	L-Fuc $\alpha$ 1, 1-Gal $\beta$ 1, 4-GlcNAc > GlcNAc ( $\beta$ 1,4-GlcNAc) <sub>1-2</sub>
WGA	Wheat germ ( <i>Triticum vulgaris</i> )	GlcNAc( $\beta$ 1, 4-GlcNAc) <sub>1-2</sub> $\geq$ GlcNAc > Neu5-Ac
N-Acetylgalactosamine/galactose group		
BPA	Bauhinia purpurea seed	$\alpha$ and $\beta$ GalNAc > $\alpha$ and $\beta$ Gal
BSA 1-B <sub>4</sub>	Bandeirea simplicifolia seed	$\alpha$ -D-Gal > $\alpha$ -D-GalNAc
DBA	Horse gram ( <i>Dolichos biflorus</i> )	$\alpha$ -D-Gal-NAc >> $\alpha$ -D-Gal
GSA-1	Griffonia seed ( <i>Griffonia simplicifolia</i> )	$\alpha$ GalNAc > $\alpha$ -D Gall-A <sub>4</sub>
HPA	Edible snail ( <i>Helix pomatia</i> )	GalNAc $\alpha$ 1, 3GalNAc > $\alpha$ GalNAc
LBA	Lima bean ( <i>Phaseolus lunatus limensis</i> )	GalNAc $\alpha$ 1, 3[L-Fuc $\alpha$ 1, 2] Gal $\beta$ GalNAc
MPA	Osage orange seed ( <i>Maclura pomifera</i> )	$\alpha$ GalNAc > $\alpha$ Gal
PNA	Peanut ( <i>Arachis hypogaea</i> )	Gal $\beta$ 1, 3 GalNAc > $\alpha$ and $\beta$ Gal
RCA 1	Castor bean ( <i>Ricinus communis</i> )	$\beta$ Gal > $\alpha$ Gal $\geq$ GalNAc
SBA	Soybean ( <i>Glycine max</i> )	$\alpha$ and $\beta$ GalNAc > $\alpha$ and $\beta$ Gal
SJA	Japanese pagoda tree ( <i>Sophora japonica</i> )	$\alpha$ and $\beta$ GalNAc > $\alpha$ and $\beta$ Gal
VVA	Hairy vetch ( <i>Vicia villosa</i> )	GalNA $\alpha$ 1, 3Gal = $\alpha$ GalNAc $\alpha$ GalNAc
WFA	Wisteria seed ( <i>Wisteria floribunda</i> )	GalNAc $\alpha$ 1, 6Gal > $\alpha$ GalNAc > $\beta$ GalNAc
L-Fucose group		
AAA	Orange peel fungus ( <i>Aleuria aurantia</i> )	$\alpha$ L-Fuc
LTA	Asparagus pea ( <i>Lotus tetragonolobus</i> )	$\alpha$ L-Fuc > L-Fuc $\alpha$ 1, 2 Gal $\beta$ 1, 4 GlcNAc $\geq$ L-Fuc $\alpha$ 1, 2 Gal $\beta$ -1, 3GlcNAc
UEA-1	Gorse seed ( <i>Ulex europaeus</i> )	$\alpha$ L-Fuc
Sialic acid group		
LFA	Slug ( <i>Limax flavus</i> )	$\alpha$ Neu 5Ac > $\alpha$ Neu 5Gc
LPA	Horseshoe crab ( <i>Limulus polyphemus</i> )	Neu 5Ac (or Gc) $\alpha$ 2, 6GalNAc > Neu 5Ac

\* Not to be confused with Leukocyte Common Antigen.

Abbreviations: Man - Mannose, Glc - glucose, Ac - acetyl, GlcNAc - N-acetyl glucosamine, Gal - galactose, GalNAc - N-acetylgalactosamine, Neu - Sialic acid (neuraminic acid), Fuc - fucose, Gc - Glucuronic acid.

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Table 3: Histochemical Methods for Identification of Microbes

<u>Class of Organism</u>	<u>Stains</u>	<u>Biochemical "Targets"</u>	<u>Comments</u>
<b>"Conventional" Bacteria</b>	Brown-Hopps modification of Gram stain	Peptidoglycans and lipopolysaccharide in bacterial walls	Superior to Brown-Brenn method
	Hotchkiss-McManus version of PAS technique	Polysaccharides in fungal walls	Diastase digestion suggested
<b>Fungi</b>	Grocott's methenamine silver nitrate procedure	Polysaccharides in fungal walls	Also will stain <u>Nocardia</u> and <u>Actinomyces</u>
	Mucicarmine method	Mucinous material in capsules of <u>Cryptococci</u>	Only used for <u>C. neoformans</u>
	Biotinylated lectins (soybean and succinylated wheat germ)	Polysaccharides in fungal walls	Requires detection by ABC technique
<b>Mycobacteria</b>	Kinyoun's method	Complex lipids in bacterial walls	Does not label "atypical" Organisms; Will also stain <u>Nocardia</u>
	Fite's technique	Complex lipids in bacterial walls	Preferred method for <u>M. leprae</u> and other "atypical" organisms
	Auramine-rhodamine procedure	Unknown	Requires fluorescent Microscopy
<b>Spirochetes</b>	Warthin-Starry technique	Argyrophilic proteins in bacterial walls	

Table 3 (Continued)

	Dieterle method	Argyrophilic proteins in bacterial walls	
<b>Nematodes</b>	Grocott's methenamine-silver-nitrate technique	Polysaccharides in chitinous coat of organisms	Organisms usually seen well with H&E
	PAS-D method	Glycoproteins in chitinous coat of organisms	Organisms usually seen well with H&E
<b>Rickettsiae</b>	Giemsa or methylene blue technique	Unknown	Specific identification requires Immunohistology
<b>Leishmania</b>	Giemsa or methylene blue techniques	Unknown	Specific identification requires Immunohistology
<b>Viruses</b> ( <u>H. simplex</u> ; cytomegalovirus; Orf; Varicella-Zoster virus)	Methyl green-pyronine Y method	Viral DNA or RNA	Used for confirming generic nature of viral inclusions; Specific identification requires Immunohistology

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PAS = Periodic acid-schiff method PAS-D = PAS with Diastase

H&E = Hematoxylin and Eosin

DNA = Deoxyribonucleic acid RNA = Ribonucleic acid