Fixation and Processing

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Fixation

Fixation is the single most influential factor in the long sequence of steps between procurement of the specimen and coverslipping the stained slide; nearly any other step can be reversed to ameliorate a problem. Tissues can be reverse-processed and then reprocessed if a mistake or breakdown occurs in tissue processing. Most stains can be removed and reapplied to correct problems with intensity or specificity. Bubbles under the coverslip can be removed simply by removing and resetting the coverslip. In sharp contrast to these examples, errors in fixation are permanent. On the positive side, properly fixed tissue is nearly impervious to abuse during tissue processing and slide preparation. Understanding the role and mechanism of fixation is crucial to producing quality slides and interpreting artifacts. Important aspects can be grouped under four rules.

Rule #1 is that fixatives denature macromolecules; ie, fixation changes the shape of large molecules. This rule is the basis for the varied functions of fixation and why fixed specimens look the way they do under the microscope. Thus:

a. Fixation kills cells because denatured molecules can no longer engage in life-supporting chemical reactions.
b. Fixation prevents autolysis because biological activity of the specimen's enzymes is destroyed as their shape is altered.
c. Fixation prevents microbial attack because substrates are no longer recognizable in their new conformation.
d. Fixation firms the tissue (making it easier to gross and to section) because denatured molecules form new intramolecular and intermolecular bonds.
e. Fixation changes the tissue's receptivity to stains and histochemical procedures. In most cases the influence is positive because procedures have been optimized to work with fixed tissue, but prominent negative examples exist (eg, masking of antigenic sites necessary for immunohistochemical staining).

Rule #2 is that different fixatives produce their own morphological patterns. That is an objective fact that does not imply good or bad. Whether we like what we see is a subjective matter predominantly based on our individual training. Many chemicals act as fixatives in that they denature macromolecules, but few produce "acceptable" results because each creates its own unique pattern of changes visible at the level of the light microscope. We speak of "formaldehyde patterns" (implying "good") versus "alcohol patterns" ("bad") in describing how a specimen appears under the microscope. Some observers give high preference to mercuric fixatives over neutral buffered formalin (NBF) for lymphoid tissues, and picric acid for gastric biopsies, because of the extra-sharp images they produce. Defining "good" fixation, then, is difficult because of varying personal preferences. However, there are well-documented and accepted minimum staining criteria that specify well-defined nuclear patterns, epithelial cell membranes, and cytoplasmic staining exhibited by well-fixed tissues.[1,2]

Rule #3 is that fixation is a chemical reaction that is not instantaneous. Its rate is dependent upon the chemical nature of the fixative solution and its temperature. Closely correlated with this is Rule #4, which says that a fixative must be present for any reaction to occur. This self-evident notion is so frequently ignored that it warrants discussion. Raw specimens are not freely porous objects. Fluids of any sort take time to diffuse into the mass. If there are numerous intercellular channels, as in lymph nodes, movement is faster than if cells are tightly adherent to one another, but penetration still is not instantaneous. Most specimens present membrane barriers that must be crossed each time the fluid moves into the next cell. Because membranes have fatty interiors, aqueous fixatives penetrate poorly. Alcoholic versions of common fixatives (alcoholic formalin, alcoholic zinc formalin, and alcoholic glyoxal) are able to penetrate much faster.

In most cases fixation increases permeability, but some fixing agents (eg, mercuric salts) create such tight

Table 1.1. Fixation/Processing Errors on the 2003-2004 HQIP H&E Challenges

Tissue H&E	% Fixation Delayed	% Fixation Incomplete	% Excessive Dehydration	% Poor Processing	% Cell Shrinkage	% Formalin or Mercury Pigment	% Nuclear Bubbling	% Excessive Decalcification	% Poor Section Orientation
Breast	2	14	1	9	0	2	9	NA	0
Skin	0	10	1	5	0	1	7	NA	2
Lymph Node	7	30	5	7	3	3	0	NA	0
Lung	7	11	2	3	2	6	5	NA	0
Uterus	11	19	0	3	1	1	27	NA	0
Colon	15	20	0	3	2	1	8	NA	2
Bone Marrow	5	16	1	11	2	1	3	8	0
Liver	8	12	5	6	1	0	8	NA	0
Average	**7**	**17**	**2**	**8**	**1**	**2**	**8**	**NA**	**0.5**

intermolecular bonds that diffusion may be impeded, and the fixative cannot penetrate all the way into the specimen. While alcoholic solutions of aldehyde fixatives do not seem to affect permeability adversely, plain alcohol used for dehydration certainly does.

Rules #3 and #4 dictate that adequate time be given for the fixation process (penetration + chemical reaction). Beyond that, no physical encumbrances should be introduced during the handling of specimen. Squeezing with forceps introduces localized artifacts because penetration is hindered at the sites of tissue damage. More commonly, forcing oversized chunks of tissue into a cassette inhibits or prevents penetration by any fluid and may render processing an exercise in futility. The tissue will remain unfixed, processing fluids will not dehydrate or clear, and the block will not section. If such a disaster is then trimmed thinner and reprocessed, sections may be possible, but the tissue will be rotten. There is no excuse for overly thick specimens.

Processing

If tissue is completely fixed, processing problems are less likely to occur. However, today's laboratory practices requiring ever faster turnaround times can result in incompletely fixed specimens, so proper processing is imperative to minimize the overall detrimental effect. The most common problems in processing are caused by processing both biopsy and large tissue specimens simultaneously on the same processing program. This leads to overprocessing and excessive dehydration of the biopsy tissues and/or underprocessing and incomplete dehydration of the larger specimens. These tissues should be processed separately and on different schedules. As stated in the previous section under "Fixation," tissues must be cut as thin as possible, with the optimum thickness being no more than 3 mm, or about the thickness of a nickel. A problem that has been encountered frequently within the last decade or so is one that leads to very poor staining of the nucleus. In the United States this problem is referred to as smudginess or blue halo effect, and in the United Kingdom as "nuclear meltdown." This is most often caused by incomplete dehydration prior to clearing, but using too much heat on the processor will also cause this same poor staining pattern.

Heat should be used only for the paraffin, and that should be at a temperature just above the melting point of the paraffin. These problems will be discussed more completely in the problem-solving section of this chapter. The most common fixation and processing problems on the 2003–2004 National Society for Histotechnology/College of American Pathologists (NSH/CAP) HistoQIP (HQIP) program hematoxylin and eosin (H&E) challenges are shown in Table 1.1. Percentages have been rounded to the nearest whole percent. The special stain challenges are not included because the problems are not always as apparent on special stains.

What Should Be Seen in a Well-Fixed, Well-Processed Specimen Stained With Hematoxylin and Eosin

- Nuclei should show a variety of chromatin patterns, with a crisp blue nuclear membrane. There should not be any nuclear bubbling, smudginess, or fading.
- The cell cytoplasm should be well preserved and should stain well with eosin.
- There should not be any artifactual spaces between the individual cells.
- There should not be any cell shrinkage.

A well-fixed and well-processed section of small intestine is demonstrated in Figure 1.1.[3] The nuclei show a variety of chromatin patterns, with crisp nuclear membranes. There is no cell shrinkage; the cell membranes are sharply defined, and the mucin droplets in the goblet cells and the brush border of the epithelium are also well preserved. This tissue was fixed for 7 hours in zinc-formalin.

Excellent fixation and processing of lymphoid tissue is illustrated in Figure 1.2. There is excellent nuclear detail in the germinal center, with eosinophilic nucleoli seen in several nuclei. Nuclei with a variety of chromatin patterns can be seen, and no artifactual spaces are seen around the lymphocytes. This tissue was received in neutral buffered formalin early in the morning, sectioned, put into cassettes, and fixed until 6:00 pm, when it was put on the processor on an overnight schedule. The first three stations on the processor are alcoholic formalin.

A number of the most common fixation and processing problems are discussed and demonstrated in the following pages. It should be noted that in many instances fixation and processing problems have multiple and varied outcomes, so it is not always possible to correlate one problem with only one solution.

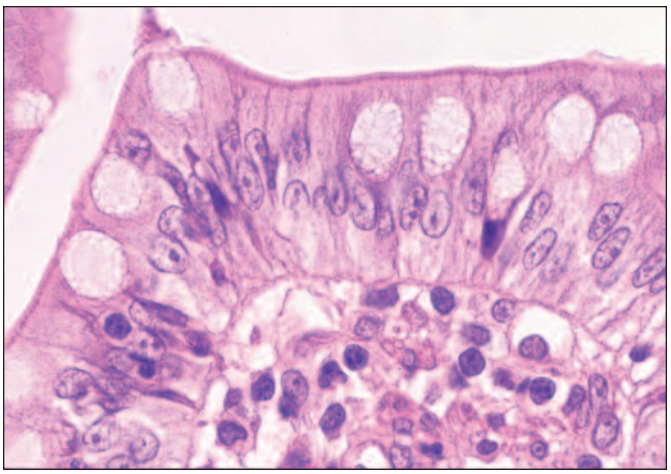

Figure 1.1. A well-fixed and well-processed section of small intestine is demonstrated in this image. A variety of chromatin patterns can be seen in the nuclei, and the nuclear membranes are crisp and sharply defined. No cell shrinkage is noted.

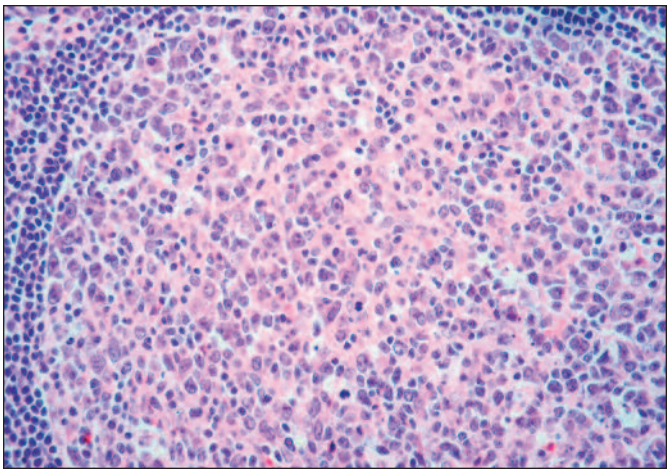

Figure 1.2. Excellent fixation and processing is demonstrated on this section of lymphoid tissue. The nuclear detail is excellent in the germinal center, with a variety of chromatin patterns seen. Eosinophilic nucleoli can be seen in several nuclei.

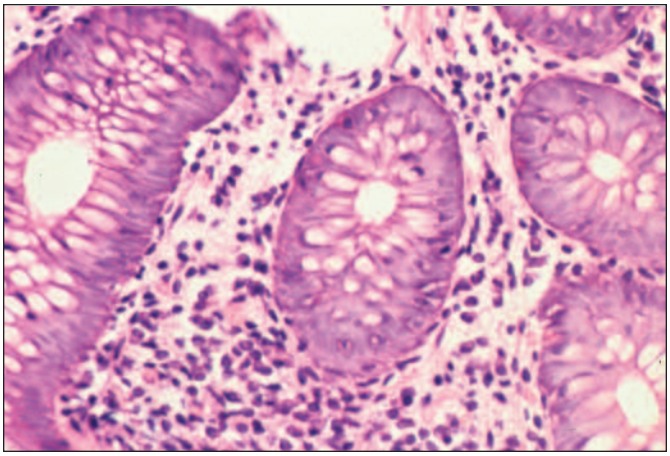

Figure 1.3. Some of the nuclei are very faded and have almost totally disappeared in this section of intestine, while others are very pyknotic. This is a manifestation of early autolysis or delayed fixation.[3]

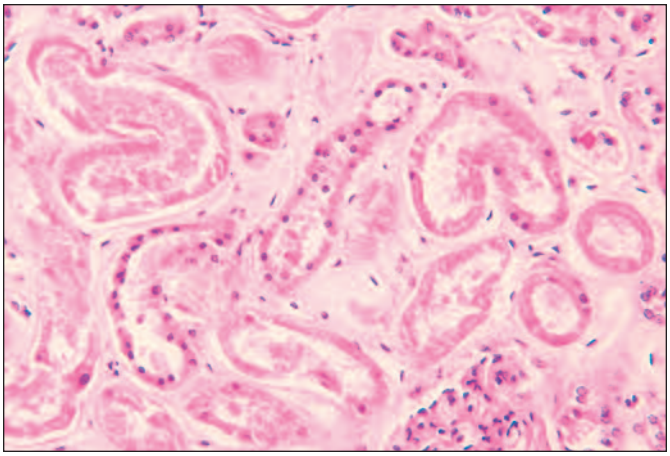

Figure 1.4. The effects of mild autolysis can be seen in this section of kidney. No nuclei remain in some of the tubules.

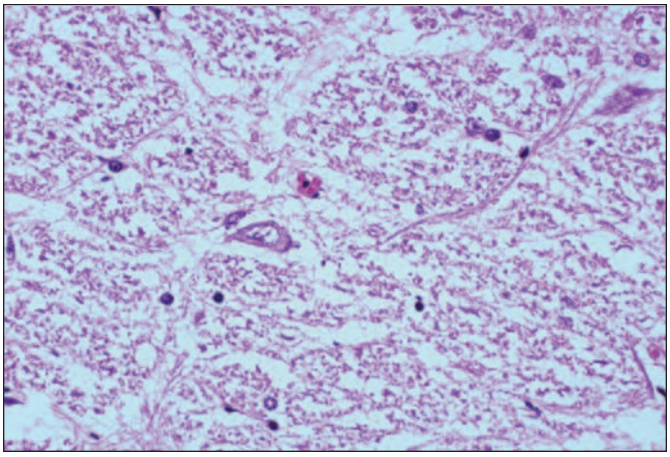

Figure 1.5. A section of central nervous system tissue demonstrates the results of delayed fixation, with marked disruption of the normal morphology.

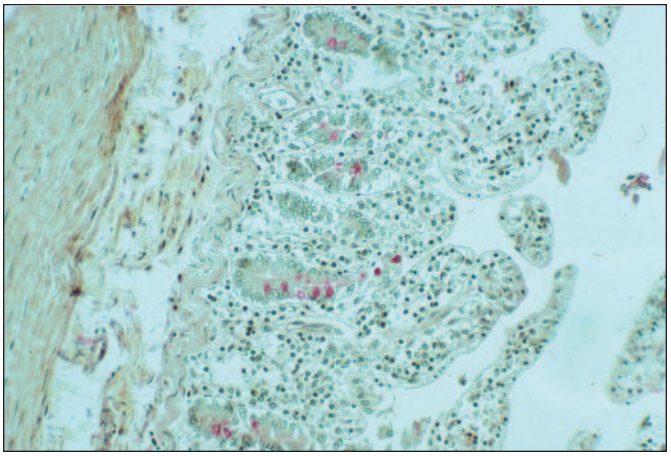

Figure 1.6. Except for the crypts, the epithelial layer has totally disappeared in this very autolysed section of small intestine. This is typical of delayed fixation of autopsy tissues, and sections of this type should not be used as control tissue.

Problems Encountered With Fixation and Processing

PROBLEM: Fixation Delayed

APPEARANCE: Nuclei may show a loss of chromatin, blue halo, fading, or complete disappearance (Figures 1.3 and 1.4). There may be cell shrinkage, disruption of the cytoplasm, and artifactual spaces around cells (Figure 1.5). If the delay is prolonged, some cells may completely disappear, such as the epithelial cells in intestinal specimens obtained at autopsy (Figure 1.6).

CAUSES:

- Specimens are obtained long after the blood supply has been compromised (eg, autopsy).
- The specimen is not opened so that fixative can come in contact with all surfaces (eg, uterus, small intestine, colon).
- The specimen is not thinly cut so that fixatives can penetrate more easily (eg, spleen, breast, organ resections, large tumors).
- Inadequate volume of fixative relative to the amount of tissue (20:1 minimum).

SOLUTIONS:

- Place specimens in fixative as soon as possible after the blood supply has been interrupted.
- Open specimens wherever possible. Gastrointestinal specimens should be opened, pinned to a cork or paraffin wax board, and placed in fixative. Uterus specimens should also be opened and placed in fixative. Lungs can be inflated with fixative by gravity flow.
- Slice specimens, such as spleen, breast, kidney, any organ resection, or large tumor, into thin slices and place in fixative.

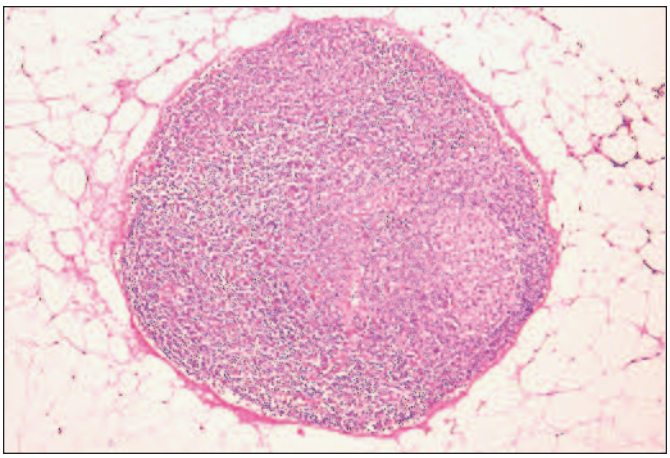

Figure 1.7. A section of an incompletely fixed lymph node shows slight disintegration in the water bath due to incomplete fixation. This node is completely surrounded by fat and was not bisected; thus the fixative had a difficult time penetrating.

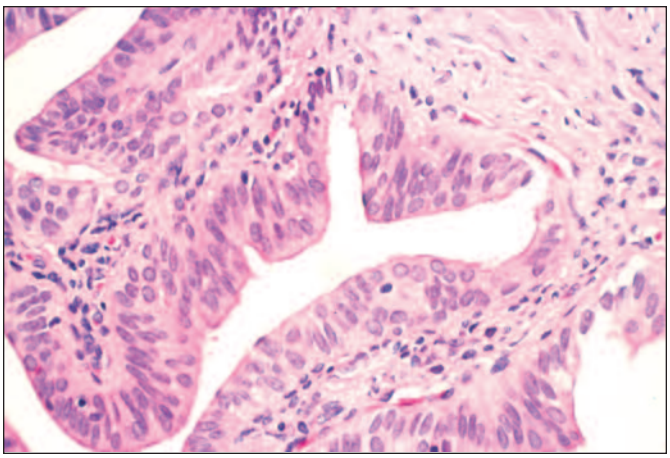

Figure 1.8. A section of fallopian tube demonstrates "smudgy" or "muddy" nuclei that result from incomplete fixation. The nuclear chromatin patterns would be much more apparent after a longer period of fixation.

- Bisect lymph nodes when appropriate and place in fixative.
- Place formalin container for holding cassettes on stir plates, and provide agitation to enhance the fixation and penetration process.
- Ensure that the volume of fixative is 15 to 20 times that of the tissue.
- Sort cassettes by specimen thickness and size for appropriate processing schedule.

PROBLEM: Fixation Incomplete

APPEARANCE: Nuclei may be muddy, or smudgy. Tissue components can separate easily on the flotation bath during microtomy. Tissue morphology is not well maintained (Figures 1.7, 1.8, and 1.9).

CAUSES:

- Tissue sections not allowed enough time in fixative.
- Inadequate amount of fixative relative to tissue volume.
- Sections grossed too thick for good penetration.
- Formalin solution is depleted.

SOLUTIONS:

- Ensure that enough time is allowed for good fixation (see "Comments").
- Ensure that the fixative volume is 15 to 20 times the tissue volume.
- Ensure that the grossed sections are thin, preferably no more than 3 mm thick.
- Change formalin solutions frequently throughout the process to prevent depletion.

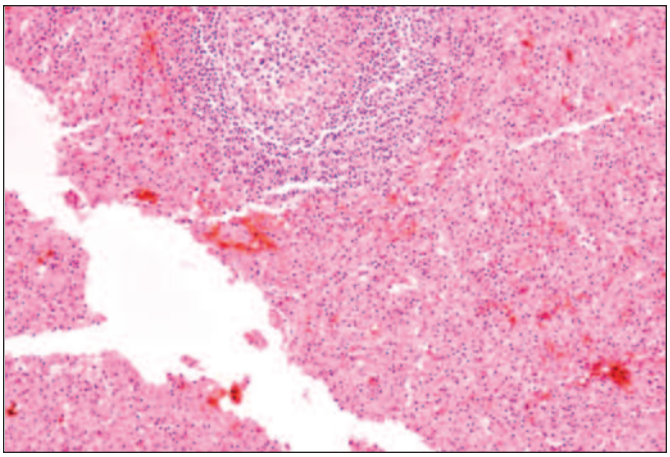

Figure 1.9. A section of spleen demonstrating the results of incomplete fixation. There is a large crack that occurred during flotation on the water bath due to the incomplete fixation. The white pulp also shows the poor cell adhesion due to the inadequate fixation. This problem will not occur with well-fixed tissue.

COMMENTS: Well-grossed sections of routine tissue should be fixed at least 8 to 12 hours to ensure at least adequate fixation; however, various authorities have stated that anywhere from 48 hours to 1 week is necessary for complete fixation. In a carefully controlled study, Dapson[3] found that artifact-free specimens could be produced with neutral buffered formalin fixation only if they were fixed a minimum of 30 to 40 hours, and that profound artifacts were apparent after 7 hours of fixative exposure. The use of alcoholic formalin on the tissue processor will help this somewhat, but enough exposure time is still needed between the tissue and the fixative solution. Excellent sections can be obtained after only 7 hours fixation in zinc formalin (see Figure 1.1).[3] Thin sections are critical in all instances for good fixation.

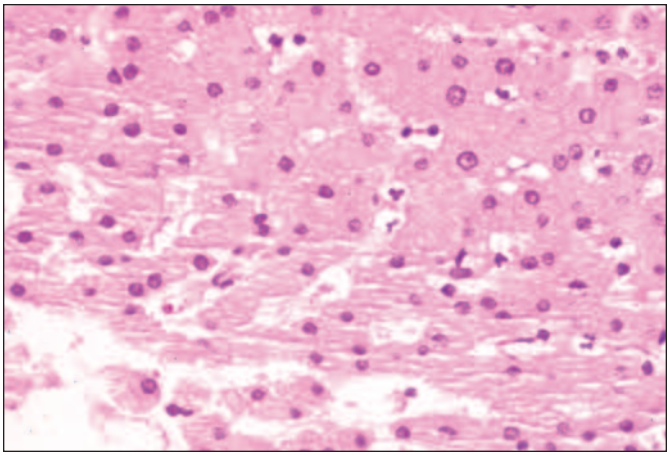

Figure 1.10. A liver biopsy showing the effects of over-dehydration. This artifact (chatter) is especially apparent at the edge of the tissue. This tissue also shows the effects of incomplete fixation, where cells present a homogenous appearance, with no nuclear detail and poorly stained hepatocyte cytoplasm.

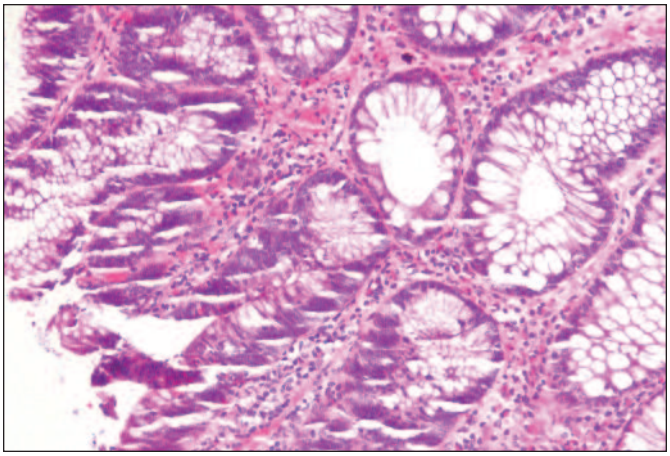

Figure 1.11. Gastrointestinal biopsy showing the effects of over-dehydration, with microchatter seen at the edge of the tissue. This section is also too thick.

PROBLEM: Tissue Excessively Dehydrated

APPEARANCE: Microchatter is most commonly seen at the edges of the tissue, especially biopsy specimens. Hairline cracks may also be seen in the tissue (Figures 1.10 and 1.11).

CAUSES:

- Too much time is allowed in the dehydrating solutions.
- Molecularly bound water is removed as well as the free water.

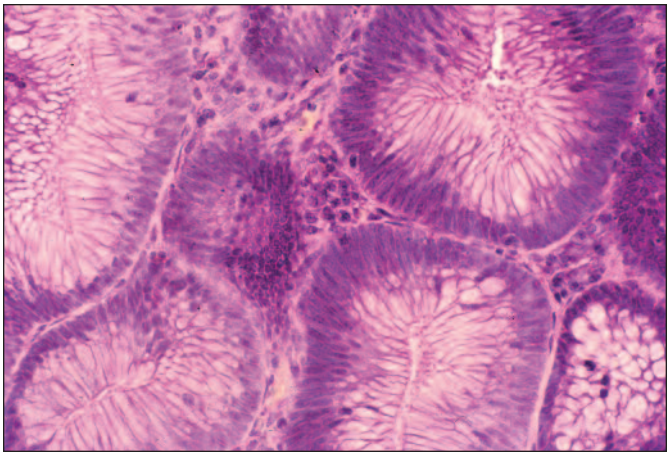

Figure 1.12. A gastrointestinal biopsy showing a marked lack of clarity and cellular detail. This is attributed to incomplete dehydration in the processor.

- Processing biopsy tissues on the same schedule as larger specimens.
- Processing biopsy tissues on a schedule that is too long.

SOLUTIONS:

- Decrease time in dehydrating solutions.
- Use a shorter processing schedule for biopsy tissues.
- Process biopsy tissues separately from large specimens.

PROBLEM: Tissue Poorly Processed

APPEARANCE: The section may appear cloudy, with the nuclei varying in staining properties. There is no chromatin definition in the nuclei, and some nuclei may appear very "washed out" (Figures 1.12 and 1.13).

CAUSES:

- Residual water remaining in tissues when they are placed in clearing agent.
- Fixative left in tissues when placed in clearing agent.
- Incomplete paraffin infiltration.
- Too much clearing agent in paraffin.
- Too much heat during processing.

SOLUTIONS:

- Ensure that the last alcohols are anhydrous.
- Ensure that fixative and alcohol droplets are not condensing on the processor chamber lid and then dropping into the processing chamber.

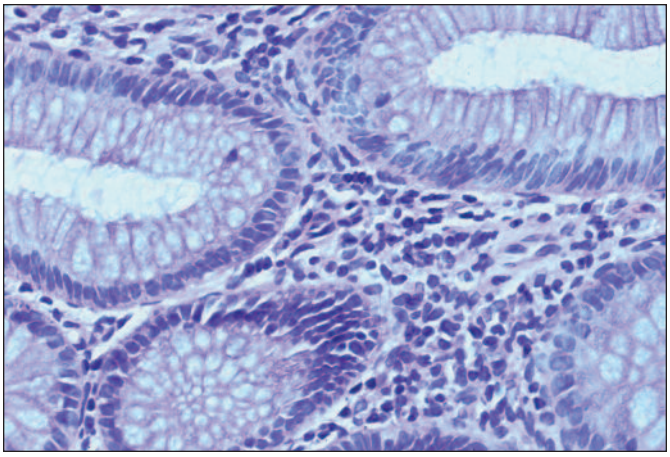

Figure 1.13. The washed out nuclei in this section are due to too much heat applied during processing. (Image courtesy of Richard Dapson.)

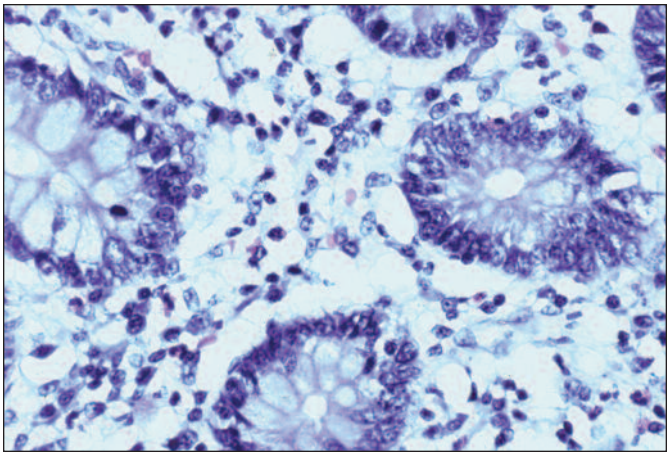

Figure 1.14. A section of gastrointestinal tract that demonstrates cell shrinkage as a result of inadequate fixation. (Image courtesy of Richard Dapson.)

- Have the processor checked for malfunctioning valves, sealant rings, or washers.
- Ensure that paraffin is changed on a regular schedule to prevent carryover or contamination from fixation and/or processing reagents.
- Ensure that all solution residues are completely cleaned from the retort (follow manufacturer's guidelines for periodic maintenance such as a warm water flush).
- Ensure that all processing solutions are kept at the lowest temperature possible; only paraffin should have heat applied.

COMMENTS: This artifact sometimes seems to come and go with no apparent reason. Although the cause of sections with this appearance has been attributed to several different things, including bad formaldehyde solutions, it is most often caused by incomplete dehydration of the tissue before the clearing step in processing, or condensation of the fixative on the processor lid followed by contamination of the succeeding reagents.[4] This problem is worse on small tissues, such as skin, endometrium, and gastrointestinal biopsies. It has been reported that this artifact can be partially reversed in tissues by heating with the BioGenex antigen retrieval solution,[5] or by boiling sections in any solution designed for heat induced epitope retrieval (HIER) or in just water.

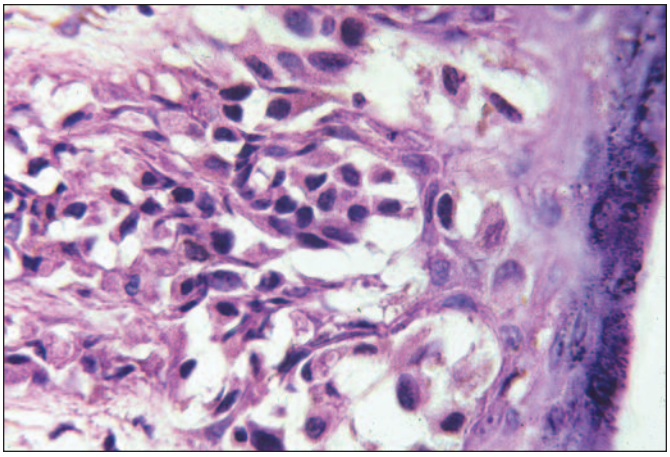

Figure 1.15. A section of skin containing a tumor. This tissue also shows cell shrinkage.

PROBLEM: Cell Shrinkage

APPEARANCE: Cells are shrunken and there are a lot of artifactual spaces around the cells. The nuclei may also appear pyknotic (Figures 1.14 and 1.15).

CAUSES:

- Inadequate fixation followed by excessive dehydration.
- Drying before submersion in fixative.

SOLUTIONS:

- Ensure adequate fixation before beginning processing.
- Ensure that specimens do not dry prior to immersion in fixative.

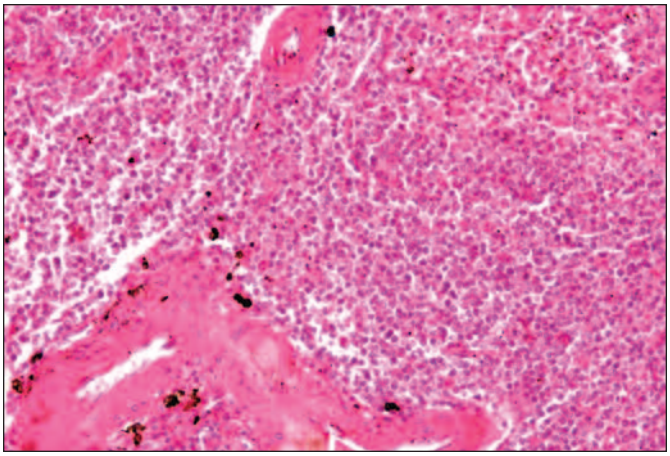

Figure 1.16. A section of spleen containing marked formalin pigment. This section is also not well fixed or well stained.

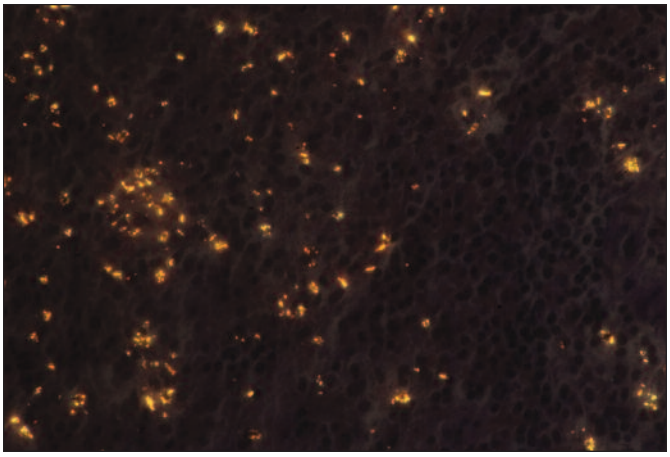

Figure 1.17. A section containing formalin pigment examined with polarization. This is a good method of checking for the presence of formalin pigment when it is not obvious on H&E-stained sections.

PROBLEM: Formalin or Mercury Pigment

APPEARANCE: Brown to black pigments in the tissue; formalin pigment is most prevalent in bloody tissues. These pigments usually lie on top of the cells, although rarely formalin pigment can occur within cells (Figures 1.16, 1.17, and 1.18).

CAUSES:

- Formalin solution has a pH below 5.5.
- Tissue is very bloody.
- Tissue has not been treated to remove mercury pigment.

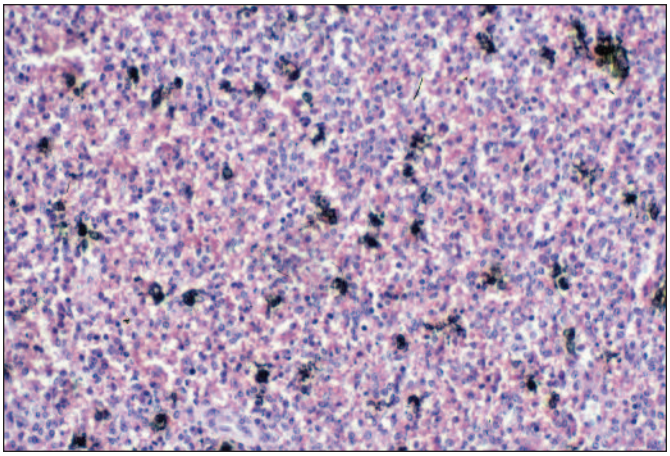

Figure 1.18. A section of spleen containing mercury pigment. This pigment is usually blacker, and the pigment deposition is larger than with formalin. Also, staining is usually not optimal when the pigment is not removed.

SOLUTIONS:

- Buffer formalin solutions to a pH of 6.8 to 7.4.
- Treat tissues with iodine and sodium thiosulfate to remove mercury pigment.

COMMENTS: It is almost impossible to totally prevent formalin pigment from forming in very bloody tissues or in sections that have been decalcified and not washed well in running water; however, the use of buffered formalin solutions will go a long way in preventing this pigment from forming. Formalin pigment has the ability to reduce silver solution and therefore will stain with some silver techniques. It can be removed, when necessary, by treating the sections with an alcoholic solution of picric acid. Mercury pigment is easily removed with iodine and sodium thiosulfate and should not be present in the stained sections.

PROBLEM: Nuclear Bubbling

APPEARANCE: Nuclei look as if they contain soapsuds; the chromatin pattern is disturbed and bubbly (Figures 1.19 and 1.20).

CAUSE:

- Incomplete fixation with formalin.

SOLUTION:

- Ensure that the specimen is fixed adequately before processing.

COMMENTS: Although this artifact has traditionally been thought of as a fixation artifact, it also can occur when sections are picked up from the flotation bath and not drained well before they are dried in a hot dryer or in the microwave oven.