CHAPTER

ONE

PRINCIPLES OF IMMUNOHISTOCHEMISTRY

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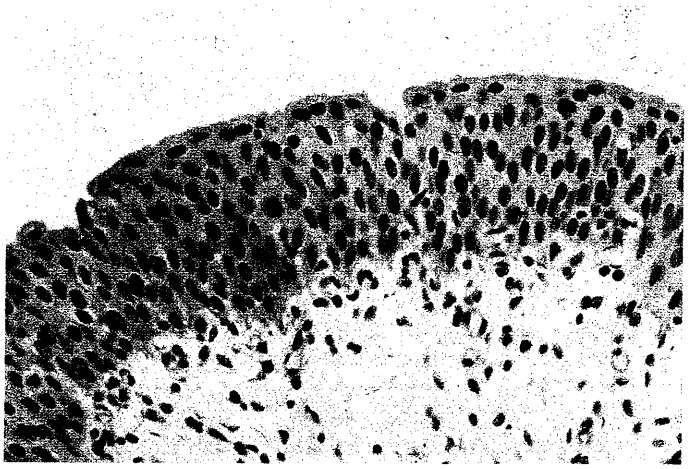


Immunohistochemistry involves the use of antibodies to locate antigens in tissue. Because molecules are restricted in distribution to specific cell types, the ability to identify molecules through antigenic sequences has become a powerful technique in diagnostic surgical pathology. In current diagnostic practice, antigens are most often localized to sections of tissue or to the surfaces of cells for visualization at the light microscopic level. Although there is no current role in diagnostic pathology for localization of antigens at the ultrastructural level, or for simultaneous immunostaining for multiple antigens, the potential of these special techniques is sufficiently great to warrant their inclusion in this chapter.

GOALS

The goal of immunohistochemistry is a more precise characterization of cells, infectious agents, and macromolecules than nonimmunologically based histochemical stains can usually provide. The following types of tissue characterization can be performed.

Differentiative features of morphologically poorly or undifferentiated cells, either neoplastic or hyperplastic, are determined. The precision of determining histogenesis varies with the cell and tissue type. With some differential diagnoses, the identification of a single antigen suffices, ie, localization of keratin is generally diagnostic of epithelial differentiation. In other cases, the tissue should be analyzed for multiple antigens because of the variability of expression of sets of antigens, ie, the distinction between adenocarcinoma and mesothelioma (Fig. 1.1).

A corollary is the hypothesis that batteries of antigens are specific for both cell type and tissue of origin. For example, the patterns of different keratins appear to be characteristic of epithelial cells of different tissues (Moll, *1982*; Cooper, *1985*). Specifically, lung adenocarcinoma lacks a 44 kd keratin that is immunohistochemically detectable in squamous-cell carcinoma (Banks-Schlegel, *1984*). However, current evidence suggests that characterization of carcinomas by their pattern of either immunoreactive or extractable keratins is not tissue specific. Ovarian surface carcinomas of similar histology have different patterns of immunoreactive keratins (Nagle, *1983*), and esophageal carcinomas differ in the types of extractable keratins (Grace, *1985*) (Fig. 1.2). Batteries of unrelated antigens may also differentiate tumors of different histogenesis, ie, of mesothelioma vs. adenocarcinoma (Battifora, *1985*).

Hyperplastic and neoplastic processes, typically of endocrine tissues, can be functionally characterized. For example, the cells comprising islet-cell tumors and pituitary adenomas can be identified by their hormonal content.

Quantification of elements in disease processes is performed. The relative percentages of cells expressing certain antigens correlate with tumor behavior, independent of staging and other histologic information. For example, an increased number of Langerhans cells in carcinomas may be associated with a better prognosis (Lauriola, *1984*), and an increased percentage of prostate carcinoma cells containing the *ras* sarcoma virus gene product p21 correlates with a higher Gleason's grade of differentiation (Hand, *1984*).

The percentage of tumor cells containing certain antigens may predict other disease. For example, medullary carcinoma of the thyroid characterized by large numbers of calcitonin-immunoreactive cells is a feature of multiple endocrine neoplasia syndrome I (MEN I); in contrast, sporadic medullary carcinomas contain only small numbers of calcitonin-positive tumor cells (Lippman, *1982*).

The apparent concentration of antigen in tumor cells may predict the aggressiveness of a tumor. Melanomas with increased S-100 immunoreactivity seem to have a worse prognosis (Rode, *1984*). The concentration of certain antigens predicts growth qualities of tumors. For example, the quantity of estrogen receptor predicts responsiveness to hormone therapy (McCarty, *1985*).

The ratios of cell types enable us to obtain more precise diagnosis of disease. For example, the relative number of plasma cell subtypes, identified by immunoglobulin heavy-chain subtype, helps to distinguish Crohn's colitis from ulcerative colitis (Bosman, *1979*).

The identification of infectious agents is performed. The diagnosis of medical and metabolic diseases by the distribution of molecules, eg, of immunoglobulins in renal and skin disease (see Chapter 10) and of α_1-antitrypsin in cirrhosis of the liver (see Chapter 7), is determined.

The precise cell and organelle localization of molecules is done most accurately with immunohistochemistry. For example, by immunohistochemistry the estrogen receptor has

PERCENTAGE OF TUMORS CONTAINING REACTIVE CELLS FOR VARIOUS ANTIGENS

	Antigen		
Tumor	**EMA**	**CEA**	**Leu-MI**
Mesothelioma	<10%	<10%	0
Adenocarcinoma	>90%	>90%	>50%

Figure 1.1. *The percentage of tumors containing a significant number of immunoreactive cells for each respective antigen—EMA (milk-fat globule protein, or epithelial membrane antigen), CEA (carcinoembryonic antigen), and Leu-M1. (From Otis,* 1987.*)*

been localized to the nucleus (King, *1984*). Biochemical assays of tissue extracts do not provide information about distribution of antigens. For example, the heterogeneity of distribution of proteins in the liver has been determined by immunohistochemistry (Gaasbeek Janzen, *1985*). Immunohistochemical information provides new criteria for diagnosing and evaluating disease.

This chapter will discuss the principles of all aspects of immunohistochemistry. Details of the methodology will be covered in Chapter 2.

ANTIGEN

Definition

An antigen is any molecule that has generated an antibody response (*anti*body *gen*erator). The part of the antigen that reacts with a given antibody is the specific antigenic determinant or epitope. A molecule typically has multiple different potential epitopes, although an antibody binds only one specific epitope.

Most antigens are proteins, although any type of chemical can be antigenic. For example, the antigen identified in neural tissues by the Leu-7 antibody is a carbohydrate (see Chapter 11), and some antigens recognized by lupus antibodies are phosphodiesters (Lafer, *1981*).

Antigenicity

Antigenicity is the presence of antibody binding activity. Because antigenicity is dependent on the physicochemical nature of the three-dimensional structure of the antigen, it is influenced by the chemical and physical forces of tissue processing. "Optimal" antigenic preservation implies retention of antigenicity that is identifiable with available antibodies. The possibility of altered antigenicity depends on the particular antigen and the effect of the various procedures of tissue handling—autolysis, fixation, and embedding.

Autolysis

Ischemia and cell death, which occur after tissue is devitalized, initiate the release of lysosomal proteolytic enzymes. By metabolizing cell components, these enzymes irreversibly alter their molecular structure and, hence, alter their antigenicity. To minimize autolysis of devitalized tissue, the endogenous proteolytic enzymes must be rapidly inactivated, either by fixation or by maintaining the tissue at a sufficiently low temperature.

Fixation

The purpose of fixation is threefold: inactivation of autolytic mechanisms, with retention of tissue architecture; immobilization of molecules to prevent their artifactual relocalization to other cell compartments or to prevent diffusion from the tissue; and increase in tissue rigidity for possible sectioning without embedding.

The precision with which fixatives localize molecules varies with the antigen, the fixative, and the conditions of fixation. For example, inaccurate localization of secretory component (SC) to plasma cells may have resulted from prolonged (24 hr) incubations in immunostaining reagents of sections in which SC was not completely immobilized (Brandtzaeg, *1981*). In general, smaller antigens are more subject to such relocation. The degree of loss of antigens from tissue varies with the fixative. Protein losses of up to 6%, 8%, and 10% have been reported with 10% formalin, Carnoy's solution, and ethanol, respectively (Mays, *1984*). The condition of fixation may further minimize protein loss. Using phase partition (where the tissue is in the organic phase and the aldehyde fixative is in the aqueous phase), virtually no loss of protein occurs (Mays, *1984*).

The manner of fixation also affects the precision of localization. Conditions that accelerate fixation—heat, agitation, and the more rapid delivery of fixative to tissues, such as by perfusion—optimize localization of antigens. The patchwork pattern of immunostaining of immersion-fixed liver for several liver proteins suggests that different liver-cell populations synthesize different proteins. In contrast, the uniform staining of perfusion-fixed liver for these proteins suggests that all hepatocytes synthesize the proteins (Feldmann, *1985*).

A consequence of the chemical reactions of fixation is altered antigenicity. The degree to which antigenicity is altered depends on both fixative and antigen. Formaldehyde greatly alters the antigenicity of keratin intermediate filaments (Figs.

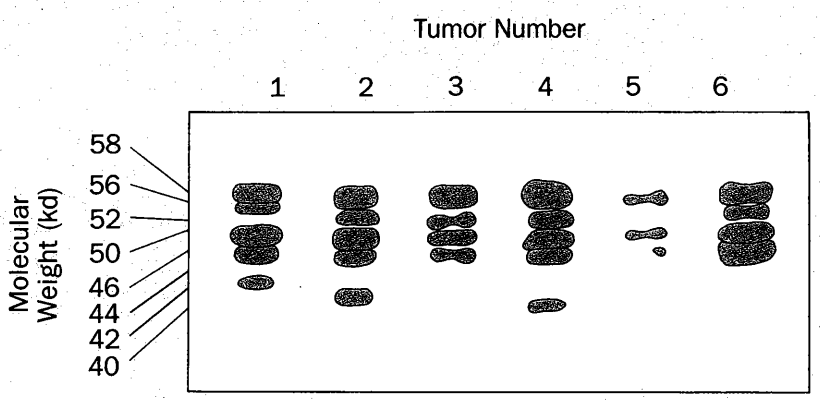


Figure 1.2. *Carcinomas of similar histology may produce different keratins, exemplified in this schematic of immunoblots from six esophageal squamous carcinomas. Although the production of keratins of molecular weights 56, 50, and 46 kd was common to the six tumors, only some of these synthesized additional keratins of molecular weights 58, 52, 44, 42, and 40 kd. Antikeratin antibodies specific to any of the latter three keratins would not have immunoreacted with all of the tumors. (From Grace,* 1985*).*

1.3—1.5); in contrast, neither formaldehyde nor ethanol changes the antigenicity of peptide hormones to readily available antibodies. Furthermore, the length of fixation correlates with the degree of alteration of antigenicity (Battifora, *1986*) (Fig. 1.6). A beneficial effect of alteration of protein structure is inactivation of endogenous enzyme activity, a potential source of false positivity.

The conditions of fixation also affect antigenicity. Fixation in an isotonic solution at neutral pH optimizes retention of native antigenicity.

The more common protocols and agents for fixation (Fig. 1.7) are as follows.

Freezing. Freezing optimizes retention of native antigenicity and, when done rapidly, precisely localizes antigen. The main disadvantages of immunostaining sections of frozen, nonfixed tissue are as follows:

1. There may be diffusion of antigens, especially of small molecules, to different cell compartments or even out of the tissue.
2. The inactivation of endogenous enzymes is reversible.
3. Structure may be significantly distorted by ice-crystal formation; ultrarapid freezing with dehydration can correct this problem (Livesey, *1987*).
4. There is labor intensiveness, especially with ultrathin cryosectioning (Tokuyasu, *1980*)
5. Frozen sections more easily detach from slides.

Alcohols. Alcohols and acetone fix antigens and enzymes by denaturation. Antigenicity is altered minimally. But the disadvantages are similar to freezing: translocation of antigens may occur, and endogenous materials are not inactivated. As with frozen tissue, ultrastructural preservation is poor.

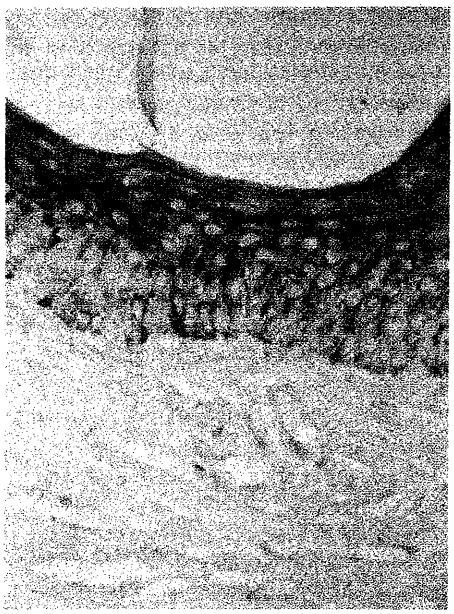


Figure 1.3. *Section of formaldehyde-fixed, paraffin-embedded skin immunostained with antikeratin antibodies. Note the variable immunoreactivity of keratinocytes, particularly in the basal cell layer.* (DAB-PO)

Aldehydes. The most common aldehydes used for fixation are formaldehyde (Fox, *1985*) and glutaraldehyde (Bullock, *1984*). Both fix by primarily covalently cross-linking proteins between α- and ε-amino groups.

Ultrastructural preservation is better with glutaraldehyde because the two aldehyde groups provide extensive cross-linking. Although tissue penetration is slow, (eg, 0.35 mm/hr in liver) (Hopwood, *1967*), cross-linking is rapid, providing complete fixation of tissue in close contact with the fixative.

In contrast, formaldehyde cross-links more slowly and therefore fixes more slowly; however, it penetrates tissues more rapidly. Furthermore, because it contains only single aldehyde groups, there is less alteration of antigenicity. Combinations of these two aldehydes can be used to maximize both ultrastructural preservation and retention of native antigenicity.

Formaldehyde can be combined with other fixatives. With picric acid, it improves fixation of basic proteins and histones. Tissue can also be fixed primarily with a combination of periodic acid (which oxidizes carbohydrate groups to aldehydes) and polymeric complexes of lysine and formaldehyde (Hixson, *1981*).

Osmium. Osmium tetroxide, which is a tetrapolar compound, cross-links lipids and proteins through chelation of multiple sites (Bullock, *1984*). Alteration of antigenicity from the native form is greatest with osmium, owning both to extensive cross-linking and to protein cleavage, although osmium can subsequently be removed by metaperiodate treatment (Hearn, *1985*).

Mercuric Compounds. Mercuric salts provide good light microscopic fixation (but bad ultrastructural preservation) with typically better retention of antigenicity than do pure al-

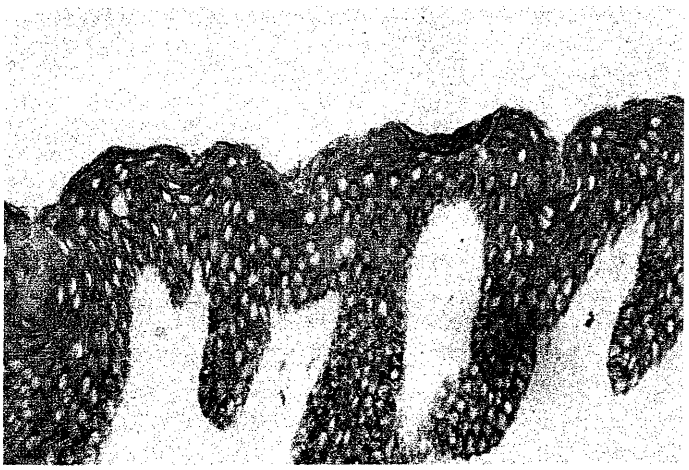


Figure 1.4. *In contrast, all keratinocytes of ethanol-fixed, paraffin-embedded skin immunoreact with the same antibodies. This indicates that formaldehyde fixation, rather than the process of paraffin embedding, has degraded immunoreactivity.* (DAB-PO)

dehyde fixatives. Because mercuric chloride penetrates tissue only slowly and may shrink tissue markedly, it is often combined with other fixatives:

1. B5 is a formalin–mercuric chloride mixture, neutralized with sodium acetate.
2. Zenker's is a solution of mercuric chloride, potassium dichromate, and acetic acid. Black mercuric precipitates, which can potentially confuse interpretation of immunostains, may be removed.

The factors that influence choice of fixative include: efficiency; preservation of light-microscopic morphology; preservation of native antigenicity; preservation of ultrastructural morphology; and retention of proteins.

In a diagnostic setting, efficiency and preservation of light microscopic morphology are dominant considerations. Accordingly, formaldehyde is the common fixative. However, when the goal is to maximize the precise localization of antigen, the particular fixative used and the method of fixation should be carefully considered. Because the preservation of immunoglobulins (either free, cell-bound, or as immune complexes) and of hematopoietic cell surface antigens is labile and is also dependent on fixation conditions (Warnke, *1979*; Jacobsen, *1980*), we are able to optimize retention of antigenicity by freezing tissues that might be analyzed for these antigens.

Embedding

Both the conditions of embedding and the type of embedding medium potentially alter antigenicity. Heat, which is necessary for infiltration of tissues by paraffin and which is a product of polymerization of methacrylates and epoxy resins, can po-

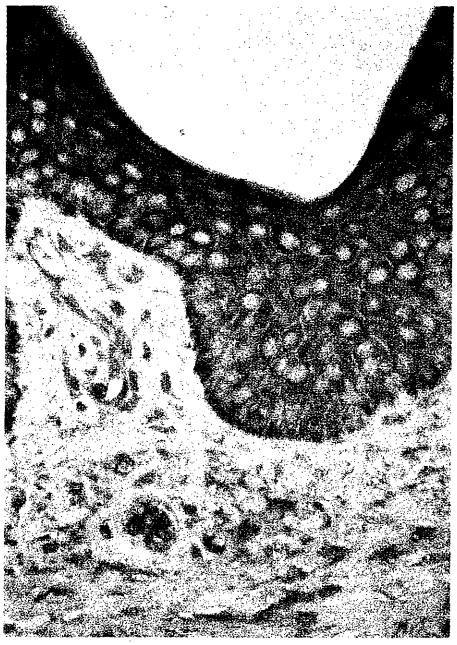


Figure 1.5. *A section of formaldehyde-fixed, paraffin-embedded skin incubated with Pronase-restored uniform immunoreactivity to keratinocytes. This section is a parallel section to that in Figure 1.3. "Digestion" of tissue sections with such an enzyme can restore intermediate filament reactivity.* (DAB-PO)

DURATION OF TRYPSIN DIGESTION FOR OPTIMAL IMMUNOSTAINING

	Duration of Formaldehyde Fixation		
	1 Day	1 Week	6 Weeks
Squamous mucosa	60	180	180
Pancreas, ducts	30	30	120
Kidney, tubules	10	30	30

Figure 1.6. *Longer fixation times necessitate longer digestion of the tissue section in a protease (0.1% trypsin, in this case) to recover immunoreactivity to a monoclonal antikeratin antibody. The trypsin digestion time, which varies with the type of tissue, is given in minutes. (Modified from Battifora,* 1986.*)*

EFFECTS OF VARIOUS FIXATIVES

	Histology	Ultrastructure	Immunoreactivity	Recoverable?
Unfixed	0 – +	0	+ +	
Ethanol	+ +	0	+ +	
Acetone	+ +	0	+ +	
Formaldehyde	+ +	+	+ / + +	Yes
Glutaraldehyde	+	+ +	0	Sometimes
Bouin's	+ +	?	+ / + +	Yes
B-5	+ +	0	+ / + +	Yes
Osmium tetroxide	+ +	+ +	0	Sometimes

Figure 1.7. *The effect of a given fixative on each parameter is indicated on a scale from 0 (worst) to + + (best). "Recoverable?" indicates the degree to which antigenicity can be recovered by such maneuvers as digesting the tissue section with an enzyme. This is a chart of generalizations. In practice, the effect of each fixative on the immunoreactivity of a given antigen should be assessed.*

tentially affect antigenicity. In general, heating of already fixed tissues is not a significant cause of altered antigenicity.

The type of embedding medium may also have a differential affect on retention of molecules. For example, embedding in epoxy selectively removes luteinizing hormone from endoplasmic reticulum (Childs, *1983*). Common embedding methods (Fig. 1.8) are:

Freezing. Fixed tissue need not be embedded to obtain sufficient rigidity for sectioning. Freezing provides a sufficiently rigid structure for sectioning; its advantage over other media is that it alters antigenicity the least. One should use this approach to maximize the possibility of identifying poorly characterized antigens. The disadvantages of freezing as an embedding method are:

1. Storage needs to be done in freezers.
2. Sectioning is more labor intensive.
3. Light microscopic morphology is suboptimal.

Paraffins. The paraffins are complex hydrocarbons that are selected for their melting point, hardness, and ease of removal. The great advantage of paraffin is that it enables cutting of many sections with efficiency. Embedding in paraffin is not a significant cause of altered antigenicity.

Plastics. Of the currently used embedding media, methacrylates and epoxy resins have the most profound effects on antigenicity. For example, methacrylate and epoxy resins differ in their effect upon antigenicity of pituitary hormones (Nakane, *1975*). Glycol methacrylate has particular appeal because antigenicity is preserved and other histochemical reactions can be performed (Fig. 1.9). The most recent embedding media are LR White and Lowicryl; both retain antigenicity and both can be thin-sectioned for electron microscopy, although ultrastructural preservation is worse than that of epoxy-embedded tissue. Most of these plastics can be removed after sectioning to regain antigenicity. The advantage of plastic is that it possesses greater hardness, which facilitates thinner sectioning. As with frozen sections, their use is too labor intensive for routine use.

Nature of the Antigen

Because antigenicity depends upon molecular three-dimensional configuration, compounds that are chemically very similar but not identical may differ greatly in their antigenic nature. For example, antibodies to arginine-vasopressin only partially cross-react with levo-vasopressin, which differs by only a single amino acid substitution (Swaab, *1975*). Phosphorylation of proteins of identical amino acid composition is a perturbation that alters antigenicity. Pretreatment of tissue sections and of protein immunoblots of brain with phosphatase reveals different patterns of immunoreactivity by monoclonal antibodies to neurofilament protein (Sternberger, *1983*).

ANTIBODY

Definition

Antibodies, or immunoglobulins, are glycoproteins that bind with high affinity and specificity to antigens. They are classified according to electrophoretic mobility, which depends pri-

EFFECTS OF EMBEDDING MEDIA

	Histology	Ultrastructure	Immunoreactivity	Recoverable?
Freezing	+	0	+ +	
Paraffin	+ +	+	+/+ +	
Methacrylate	+ + +	0 – +	+/+ +	Yes
Lowicryl	+	+	+/+ +	
LR White	+	+	+/+ +	
Epoxy resin	+ + +	+ +	0	Sometimes

Figure 1.8. *The effect of each embedding medium on each parameter is indicated on a scale from 0 (worst) to + + + (best). "Recoverable?" indicates the degree to which antigenicity can be recovered by such maneuvers as removing the medium.*

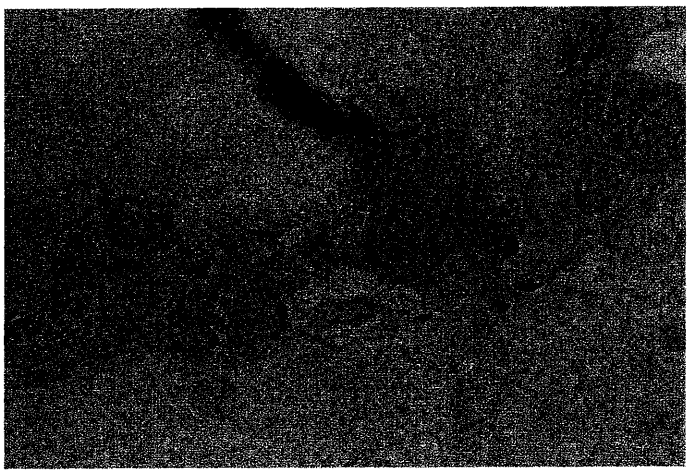
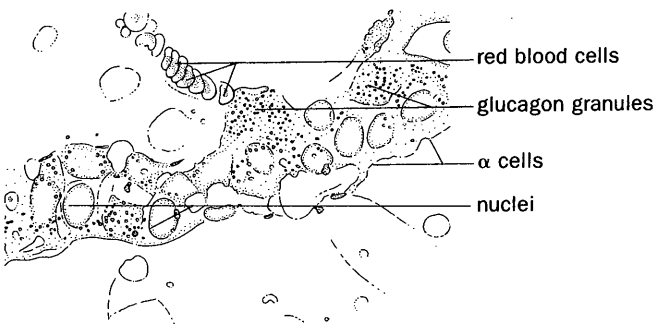


Figure 1.9. *Pancreas embedded in glycol methacrylate and reacted with antiglucagon antibodies. The thin (1 to 2 μm) sections of methacrylate-embedded tissue which can be obtained allow resolution of glucagon granules.* (DAB-PO)



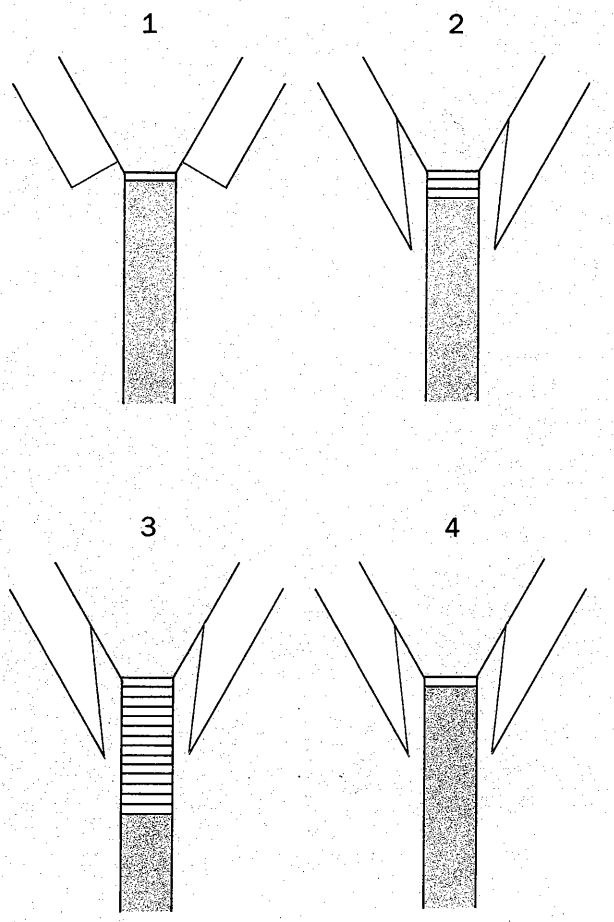
marily upon heavy chain composition and degree of multimerization. Accordingly, properties of the immunoglobulin classes are distinctive—molecular weight, percentage carbohydrate, and functional properties in the binding of other substances (Fig. 1.10).

Within the classes are different subclasses, which differ in interchain disulfide bonds and in functional properties (Roitt, *1985*) (Fig. 1.11). The functional properties of greatest relevance to immunohistochemistry are the ability to bind complement and the affinity for Staph protein A.

DISTINCTIVE PROPERTIES OF IMMUNOGLOBULIN CLASSES

	IgG	IgM	IgA	IgD	IgE
Molecular weight (kd)	146–170	970	160	184	188
Carbohydrate (%)	2–3	12	7–11	9–14	12
Function					
Complement fixation	Y	Y	—	—	—
Placental transfer	Y	—	—	—	—
Reactivity with Staph A	Y	—	—	—	—
Binding					
Neutrophils	Y	—	Y	—	—
Mast cells/basophils	—	—	—	—	Y
B and T lymphocytes	Y	Y	Y	—	Y
Platelets	Y	—	—	—	—

Figure 1.10. *For each class of immunoglobulin, this figure gives the range of molecular size (expressed in kilodaltons), the percentage of each immunoglobulin that consists of carbohydrate, and some functions and cell-specific binding properties. "Y" indicates that the given immunoglobulin has the indicated functional or binding capacity. (Adapted from Roitt, 1985.)*



IgG SUBCLASS

IgG subclass	1	2	3	4
Number of heavy chain bonds	2	4	15	2
Function				
Fixes complement	Y[a]	Y	Y	—
Staph A reactivity	Y	Y	—	Y
Binds neutrophils	Y	—	Y	Y

[a]"Y" indicates that the given IgG subclass has the indicated functional capacity. (Adapted from Roitt, *1985*.)

Figure 1.11. (Left) *Schematic representation of IgG subclasses by subclass number, with interchain disulfide bonds (blue).* (Right) *Description and functions of some subclasses.*

Basic Structure

The immunoglobulin molecules have been well characterized. This discussion focuses on IgG, which is the most common subclass used in diagnostic pathology. The basic structure of IgG features specific domains and bonds (Fig. 1.12).

Specific Domains. The Fab (antibody binding) end of the molecule contains multiple hypervariable domains. The variability of these domains allows the tremendous diversity of specificity and affinity of immunoglobulins (Kim, *1981*). The opposite end, the Fc portion of the molecule, contains domains for binding the following: complement; Fc receptor; Staph protein A; and inflammatory cells, ie, macrophages, mast cells, lymphocytes, and placental syncytiotrophoblasts.

Bonds. Interchain disulfide bonds covalently link the heavy and light chains. Cleavage of various combinations of these bonds and of the heavy chains yields immunoglobulin fragments (Fig. 1.13). The advantages of using fragments are in controlling some of the domain-specific functions (ie, Fab fragments lack complement and Fc receptor binding domains and will therefore not bind to such sites in tissue) and in producing a smaller antigen-reactive molecule whose size might allow it to penetrate tissue more easily (Fig. 1.14).

Polyclonal Antibodies

The successful immunization of animals produces a variety of antibodies with different specificities and binding affinities. Polyclonal antiserum theoretically maximizes the number of antibodies bound to a molecule that has little repetitiveness of epitopes. Molecule-specific antibodies of different subclasses may all bind to the desired molecule. Polyclonal antisera are also quicker and simpler to obtain than monoclonal antibodies.

A significant disadvantage is the heterogeneous nature of polyclonal antibodies. Because antisera comprise a mixture of antibodies to various epitopes of the antigen and to any other molecules in the immunizing preparation, as well as to immunoglobulins already present in the animal, and because these various antibodies have different affinities, both specificity and suitability for immunohistochemistry must be assessed. To minimize the development of antibodies to contaminants, the immunogen should be as pure as possible.

A further disadvantage of polyclonal sera is that the supply is limited to the serum of one animal. The antibody characteristics of serum from another animal immunized with the identical antigen may be quite different.

Monoclonal Antibodies

Monoclonal antibodies are the secreted immunoglobulins of the clonal progeny of a single hybrid plasma cell. Having molecular identity, they have identical antigen specificity and binding affinity.

An advance of great significance for diagnostic immunohistochemistry was the development of a method to generate immortalized clones that secrete monoclonal antibodies (Kohler, *1975*). Such monoclonal antibodies have the following advantages over polyclonal antibodies:

1. Monospecificity (to a single epitope).
2. Immortality. Because they are progeny of myeloma cells, they can proliferate indefinitely; polyclonal an-

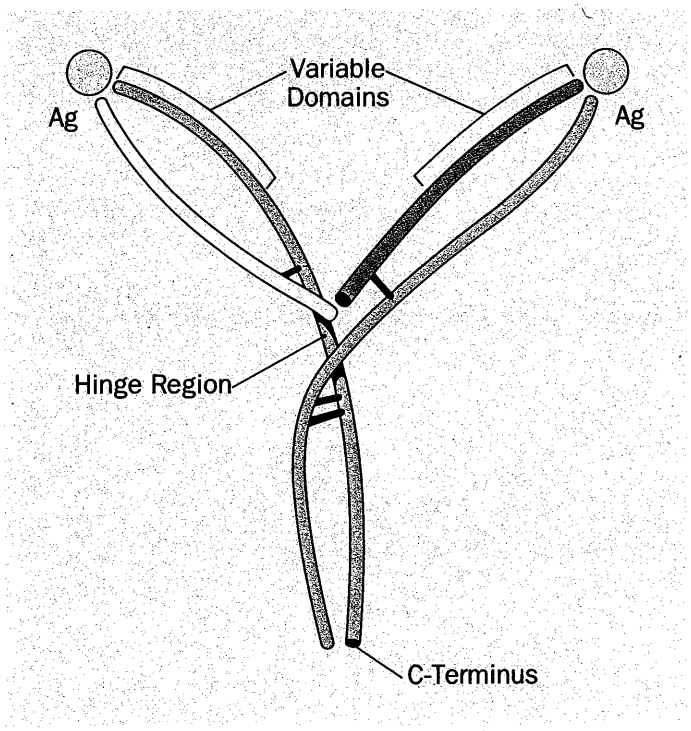


Figure 1.12. *Computerized three-dimensional model of an IgG molecule, showing antigen binding areas (Ag), variable domains, interchain bonds (black lines), and components—light chains (blue and red) and heavy chains (green). (Adapted from Roitt,* 1985.*).*

tibodies from animal serum are limited to the amount of available serum.

3. Because antibody specificity is generated during selection of clones to expand, the immunogen need not be pure.

A potential disadvantage of monoclonal antibodies is that they have a lower sensitivity than do polyclonals. Only single monoclonal antibodies will bind to a molecule containing only one copy of the epitope. Furthermore, the generation of monoclonal antibodies is more time-consuming and complex than is the production of polyclonal antisera. Finally, immortality of monoclonal antibody synthesis is not invariable. Monoclonal antibodies may spontaneously lose isotype or domains, at a rate of 1:100 to 1:1000 (Kohler, *1986*).

An option is the use of mixes of monoclonal antibodies. By the formation of circular antibody–antigen complexes with low antibody concentrations, such mixes have greater affinity than either antibody alone (Moyle, *1983*). Furthermore, mixes

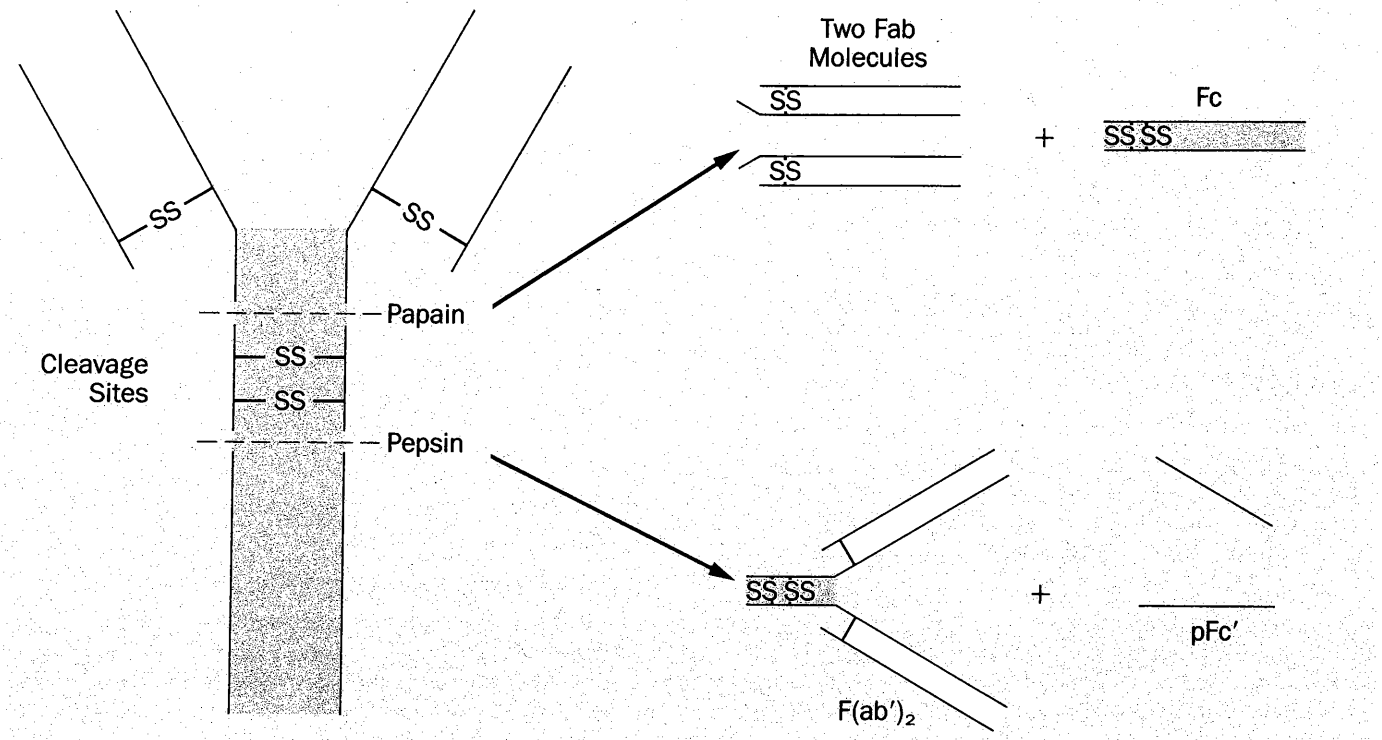


Figure 1.13. *Digestion pattern of IgG_1. Cleavage pattern of immunoglobulin by the proteases papain and pepsin.*

BINDING SITES FOR IMMUNOGLOBULIN FRAGMENTS

	IgG	$F(ab')_2$	Fab	Fc
Molecular weight (kd)	156	92	46	110
Binding sites				
Antigen	Y	Y	Y	—
C4	Y	Y	Y	—
C1q	Y	—	—	Y
Macrophage	Y	—	—	Y
Placenta	Y	—	—	Y
Neutrophil	Y	—	—	Y
Staph Protein A	Y	—	—	Y

Figure 1.14. *The presence of binding sites for specific molecules or cells on immunoglobulin fragments is signified with a "Y."*

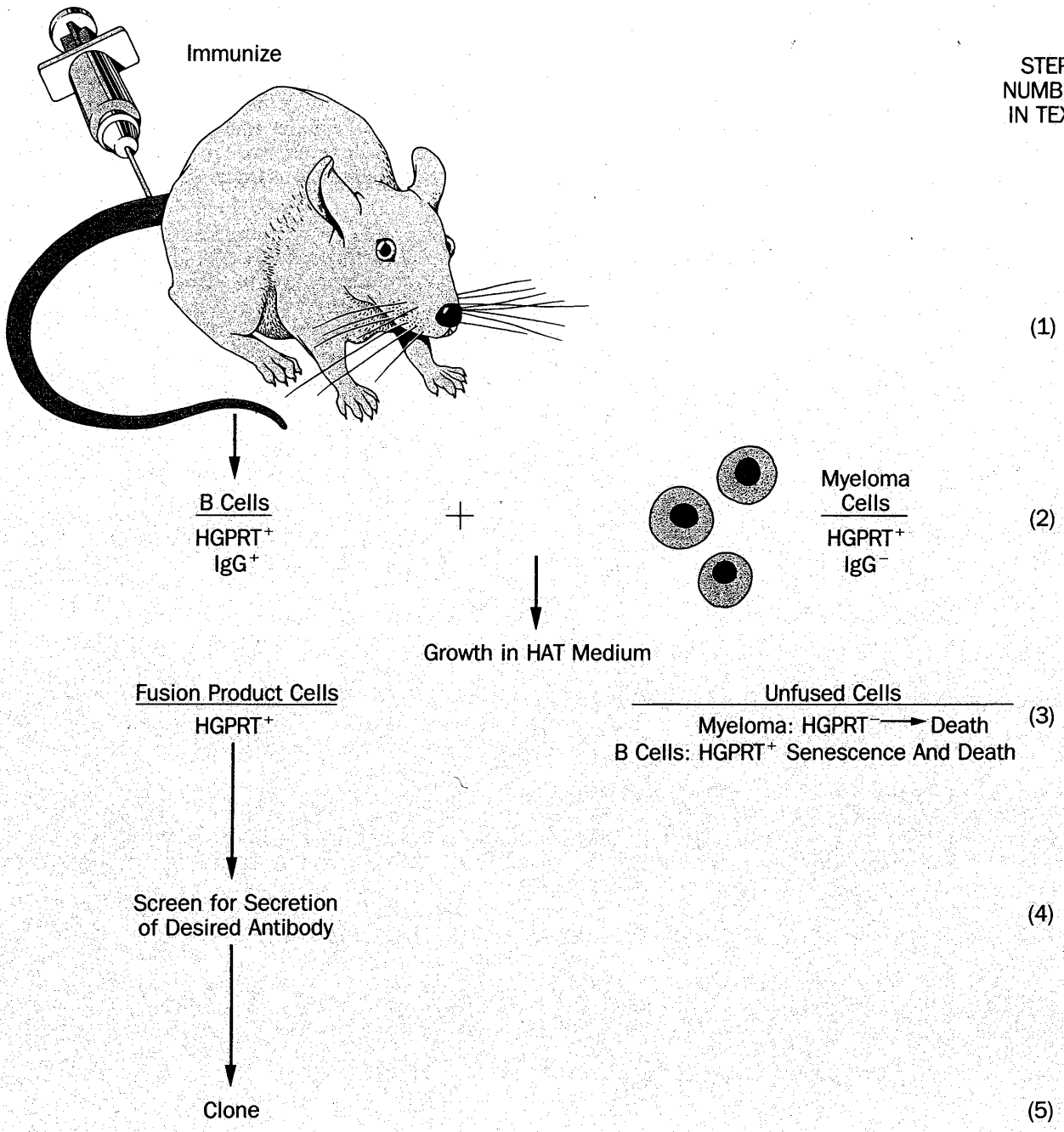


Figure 1.15. *Scheme of monoclonal antibody production. Normal cells contain the gene to synthesize the enzyme (HGPRT) necessary for survival in HAT medium. After several generations these cells senesce and die. In contrast, myeloma cells can propagate forever. However, lacking the enzyme HGPRT they cannot survive in HAT medium. Therefore, only recombinant cells can both survive in HAT medium and proliferate. The ability of cells to synthesize HGPRT, and thus to multiply in HAT medium, is designated "HGPRT$^+$." The ability of B lymphoid cells to synthesize and secrete IgG is designated "IgG$^+$".*

of monoclonal antibodies to different epitopes may be more sensitive. The monoclonals we use appear to have a sensitivity comparable to corresponding polyclonals.

Cells fulfilling the following conditions are required for the generation of monoclonal antibodies:

1. Immortality: One should use a myeloma cell line that will propagate indefinitely.
2. Nonsecretory status: The myeloma cell line should not secrete the immunoglobulins it synthesizes, because the specificity of this immunoglobulin is most likely irrelevant to that of the sought epitope.
3. Inability to proliferate in a controlled environment: To select against myeloma cells that lack recombinant genetic material, one should use myeloma cells that lack an enzyme in the recovery pathway of DNA synthesis, namely, hypoxanthine guanine phosphoribosyl transferase (HGPRT). When such myeloma cells are grown in an environment that necessitates the presence of HGPRT for survival, the cells die. Such an environment is created by use of aminopterin, which poisons the usual DNA synthesis pathway.
4. B cells from an animal syngeneic to the myeloma cell line: These B cells can be induced to synthesize and secrete antibody in response to immunization, and they constitutively synthesize HGPRT.

The multistep procedure for the generation of monoclonal antibodies (Fig. 1.15) involves the following:

1. Immunization of an animal, either mouse or rat, with antigen.
2. Harvesting of immunocompetent B cells, when specific antibody production has occurred, and fusion of these cells with myeloma cells. Fusion, or somatic cell hybridization, is induced by co-culture of cells in an environment that causes membrane fusion.
3. All cells are grown in an environment that selects for features of both the myeloma cells (immortality) and the immunocompetent B cells (HGPRT sufficiency). This environment contains the DNA poison, aminopterin, and substrates for DNA synthesis by the alternate pathway, namely, hypoxanthine and thymidine (HAT medium). Only successful fusions can proliferate in this medium.
4. Clones of these successful fusions are assayed for secretion of antibodies of selected specificity. The assay test should be able to efficiently screen the hundreds of resulting clones. Examples include solid-state imunoabsorbent assay (ELISA) and immunohistochemical assay. Additional characterization of antibody specificity should be done by immunoblotting or immunoprecipitation.
5. Clones producing and secreting antibodies of desired specificity and immunoreactivity are subcloned to ensure monoclonality and are then propagated either in vitro or by serial passages in animals.

Affinity

Affinity is the binding strength of an antibody to a specified antigen. Affinity is characterized as an affinity constant (Fig. 1.16). For immunohistochemistry, the higher the affinity constant, the better the antibody, because the antibody is more likely to remain bound to the antigen sought during the procedure. In contrast, the optimal antibodies for competition immunoassays, such as radioimmunoassays or ELISA, have an affinity constant that allows successful competition for a specific, labeled antigen. Nonetheless, antibodies used in competition assays have been successfully used in immunohistochemistry.

ANTIBODY–ANTIGEN REACTION

Binding of antibody and antigen is a noncovalent process highly dependent on the tertiary structures of binding sites on both antibody and antigen. The binding results from hydrogen bonding, electrostatic and van der Waals forces, and hydrophobicity from association of nonpolar groups (Roitt, *1985*). The bond strength determines antibody affinity for the antigen. Antibodies to an identical epitope may have different structures, hence different affinities.

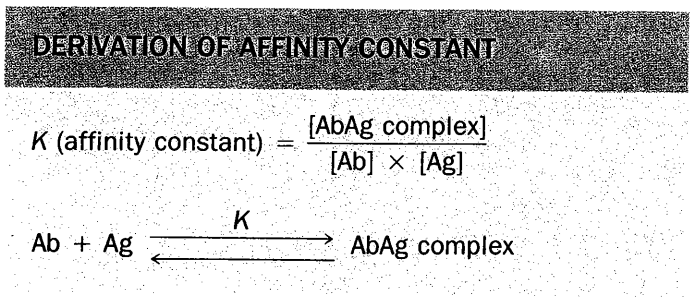


Figure 1.16. *Derivation of Affinity Constant*

Resolution of the antibody–antigen bond to 0.28 nm has enabled identification of factors important for the bond (Fig. 1.17). These factors include shape and complementarity of functional groups and the presence of clefts (Rebek, *1987*). Binding has a cascade effect; initial binding to amino acid residues promotes local side-chain displacements, which, in turn, allow participation of other previously buried residues in the antibody–antigen interaction, thereby enhancing binding affinity (Getzoff, *1987*). The most reactive antigenic sites are characterized by a negative electrostatic potential, a high local mobility, and the formation of a convex surface (Geysen, *1987*).

Using series of mammalian lysozymes and a specific antilysozyme, the interface of binding was defined by crystallography as being comprised of 16 lysozyme residues and 17 antibody residues, the majority in the hypervariable region of the heavy chain. Although amino acid differences outside the binding region do not inhibit binding, single differences in the region abolish binding (Amit, *1986*). Thus, successful antibody binding is sensitive to single amino acid differences (Fig. 1.18).

However, antigenicity is not restricted to continuous sequences of molecules. Unique epitopes can be formed from discontinuous portions of the molecule which form a continuous surface in the tertiary structure of the molecule (Fig. 1.17).

Model Systems

The following model systems can be used to more carefully characterize (1) the antibody–antigen reaction and (2) the influence of variables that affect the sensitivity and specificity of the reaction:

Competition Binding Assay

Such an assay characterizes specificity by assessing the ability of defined antigens to compete for binding to a known antigen (Berson, *1957*). The disadvantages of competition binding assays are that purified antigens must be used and that conditions to which tissue antigens are subjected cannot be precisely duplicated.

Immunoblot Assay

This assay assesses the binding of antibody to defined antigen, which is bound to a two-dimensional matrix such as nitrocellulose. Proteins in concentrations below 0.15 $\mu g/mm^2$ remain absorbed to such a matrix. The influence of some tissue variables (ie, heat, fixation, and buffers) on antigenicity and antibody–antigen binding can be assessed (Bosman, *1983*; Scopsi, *1986a*).

The best system creates a three-dimensional structure which

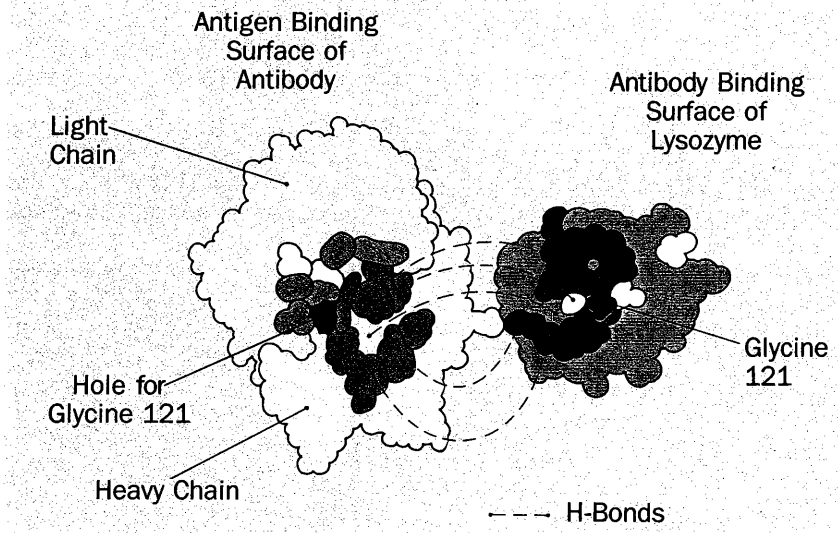


Figure 1.17 *Model of the binding surfaces of lysozyme and a specific antibody, determined by crystallography. All amino acid determinant points of contact are indicated (black circles); contiguous determinants are of the same pattern (blue or heavy chain, green on light chain, and black on lysozyme). Note that there are multiple discontinuous determinants. The contact surface is rather flat, occupying 748 Å² of lysozyme surface (11% of solvent accessible surface) and 690 Å² of antibody surface. Glycine residue 121 of lysozyme serves as a "key" that fits into the indicated "lock," or hole, on the antibody surface. Opposing groups bound by the hydrogen bonds* (H) *are indicated (From Amit,* 1986.)

exhibits tissue characteristics (ability to be fixed, sectioned, and immunostained) and in which antigen of known concentration can be uniformly distributed without loss of antigenicity. Embedment of antigen of known concentrations in a polyacrylamide gel matrix (Posthuma, *1987*) or in a gelatin gel matrix (Schipper, *1983*) satisfies these criteria. Only with such a system can the sensitivity and specificity of the antibodies be rigorously assessed.

The main constraints to using the latter system for evaluating antibodies are the unavailability of purified antigens and the novelty of initiating use of such a model. In current practice, the evaluation of the antibody–antigen reaction in immunohistochemistry is typically empirical. The staining pattern of antibodies in a range of tissue types is used to characterize the antibodies.

LABEL

The label is the visually identifiable material. The label may be conjugated to another substance (immunoglobulin or protein A), or it may be complexed with antilabel antibodies. The effect of the label on the binding properties of the protein to which it is conjugated must be assessed before the conjugate is used.

Antibody

Unconjugated antibody may be detectable (Wabl, *1974*). However, it can only be identified as an indistinct density at the edge of its organelle of localization, and only at the electron microscopic level.

Dyes

The first chromogenic materials conjugated to antibodies were dyes, ie, the red dye R-salt. However, these dye–antibody conjugates lacked sensitivity; conjugation of greater than 10 dye molecules per antibody altered immunoreactivity.

Metals

Substances such as mercuryl compounds, ferritin, and gold can be conjugated to antibodies to provide ultrastructural markers. This use will be discussed in more detail below (see section on immunoelectron microscopy). Of these, only gold can be readily seen by light microscopy.

Fluoresceinated Compounds

These compounds are excited by light to emit light (within 10^{-4} sec of excitation) at wavelengths that are characteristic

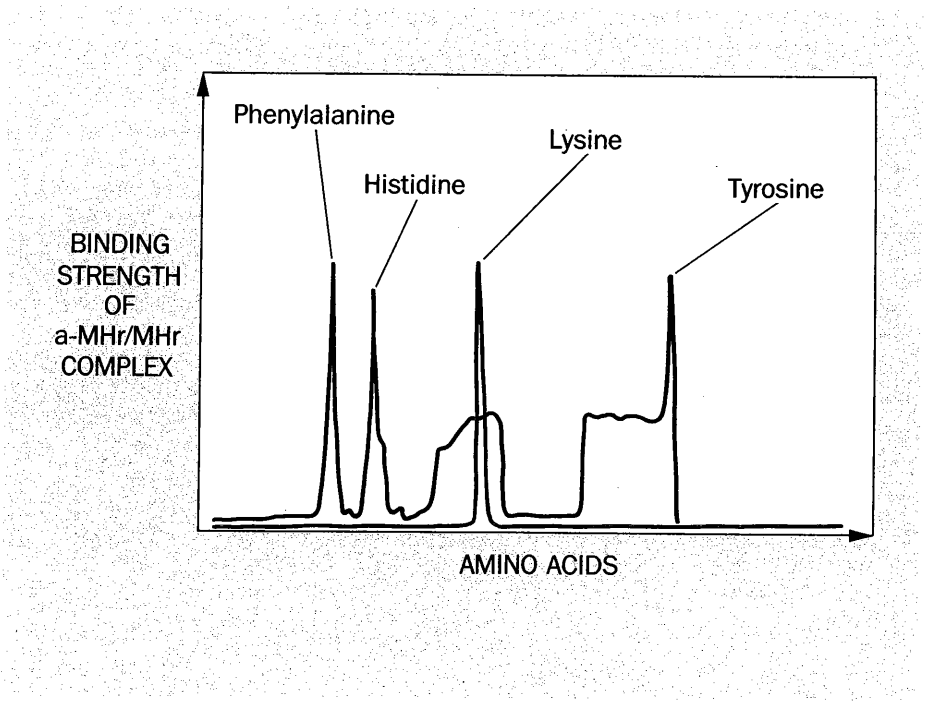


Figure 1.18. *Plot of strength of binding of antimyohemerythrin (a-MHr) to myohemerythryin (MHr), measured by ELISA. The effect on binding of substituting the 19 different amino acids for the native amino acid at two critical binding sites on MHr is illustrated. Lysine is essential for binding at MHr position 83 (blue line), whereas the native phenylalanine at position 80 is not essential because histidine or tyrosine can be substituted with similar binding affinity (red line). Substitution of other amino acids in position 80 variably affects binding. Although the binding strengths only of substitutions with the amino acids phenylalanine, histidine, lysine, and tyrosine are explicitly indicated, the graph reflects the binding strengths of all amino acid substitutions. (From Getzoff, 1987.)*

of the fluorescent compound and independent of the wavelength of the excitatory light. In accordance with Stokes' law, the emitted light will be at a lower energy level (ie, longer wavelength) than the excitatory light (Fig. 1.19).

Successful immunofluorescence requires, among other things, more complex hardware than that used for immunoenzymatic light microscopy (Fig. 1.20). Needed are: a light source that emits intense light at short wavelengths (the blue end of the spectrum); an optical path that optimizes the signal-to-background ratio of light intensity (currently an epifluorescence system is standard); and a filtration system that precisely separates the excitation light (the excitation filter) from the emitted light (barrier filter) (Rygaard, *1971*).

The advantage is general ease of use; ie, a direct fluorescein conjugate gives a higher signal-to-noise ratio than do direct enzyme conjugates and more precise localization of antigen (Wicker, *1971*).

The disadvantages of using fluorescent labels are:

1. Labels gradually self-quench, a process that is accelerated by incident light. Thus, fluorescent labels are not permanent, although this process of decay may be decelerated (Bock, *1985*).
2. The slides cannot be dehydrated and coverslipped without compromising the immunostain. Dehydra-

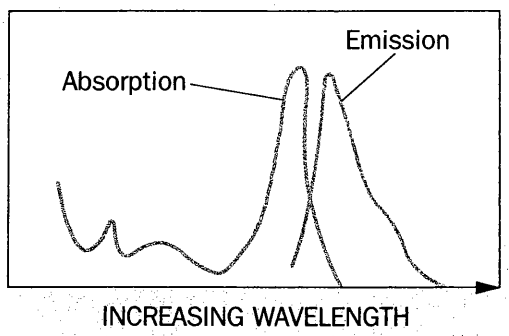


Figure 1.19. *This generalized spectrum of a fluorescent dye plots the degree of absorption (green) and the intensity of emitted fluorescence (yellow) against the wavelength of the excitation light. Emission wavelength is the same for a given dye and is always of longer wavelength than the absorption light.*

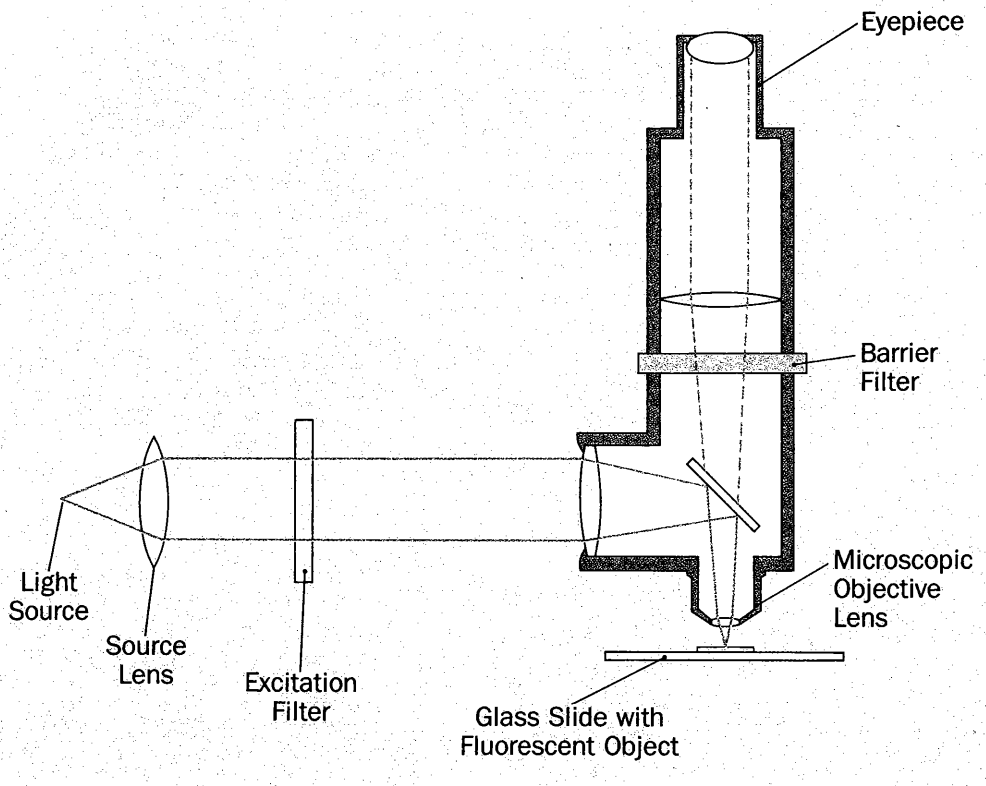
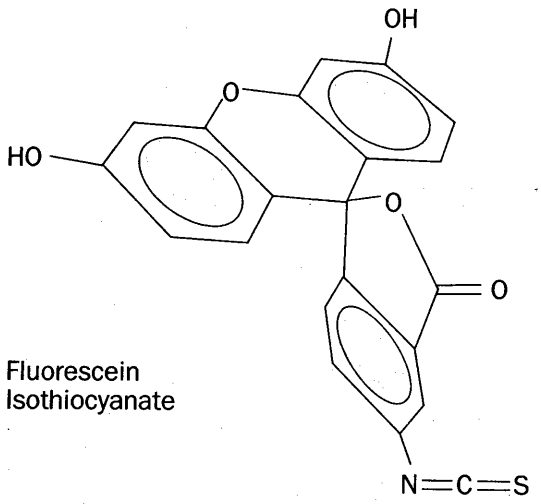


Figure 1.20. *The light path in an epifluorescence setup is schematicized. The incident light (green) excites the fluorescent label on the glass slide to emit light (yellow) that is filtered by the barrier filter to exclude the excitation light for final viewing.*

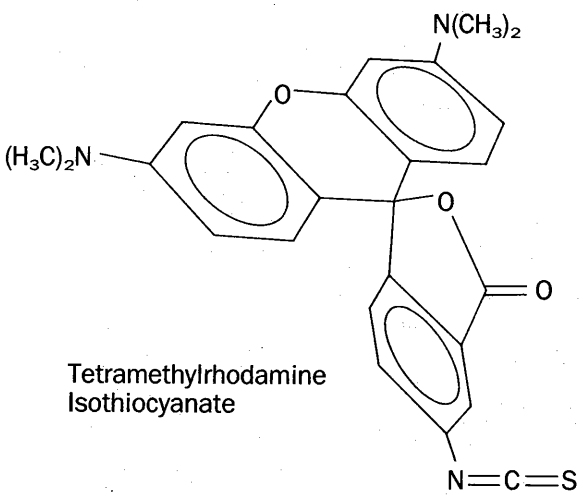
tion for coverslipping and optimal viewing resolution inactivates and solubilizes the fluorescent compound.

3. Light filtration requirements prevent simultaneous viewing by incandescent, broad spectrum wavelength light, because fluorescent signal cannot be visualized in the white light.
4. Complex filtration requirements. Because peak excitation frequency is typically within 30 nm of the emission peak (see Fig. 1.19), the wavelength of the excitation light must be restricted by filtration lest it overlap the frequency of the emission light and produce excess background illumination. For example, transmittance of light by the secondary barrier filter at 525 nm should be less than 0.1% for immunofluorescence (Ploem, *1971*).
5. Autofluorescence. The low-intensity autofluorescence of all tissue is enhanced by fixation and prolonged storage. When excessive, it may be indistinguishable from the specific signal.

Because the two common fluorescent dyes—fluorescein and rhodamine—have different emission wavelengths, they can be used as independent markers in double immunofluorescent staining (Figs. 1.21 and 1.22).



Fluorescein Isothiocyanate



Tetramethylrhodamine Isothiocyanate

Figure 1.21. *Structural formulas for fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate.*

EMISSION AND EXCITATION WAVELENGTHS FOR TWO COMMON FLUORESCENT DYES

	Excitation Wavelength (nm)	Emission Wavelength (nm)
Fluorescein isothiocyanate	490	525 (blue)
Tetramethylrhodamine isothiocyanate	530	580 (red)

Figure 1.22. *Emission and Excitation Wavelengths for Two Common Fluorescent Dyes.*

Enzymes

Peroxidase

Horseradish peroxidase (E.C. 1.11.1.7). a 40 kd protein consisting of an outer shell of eight neutral carbohydrate chains (comprising 18% of the molecular weight) and a heme–protein core, is the most widely used enzyme label (Welinder, *1979*). It consists of more than 20 isoenzymes that have different isoelectric points (Rennke, *1979*). The preferred peroxidase should have an isoelectric point near physiologic pH, which also optimizes antibody–antigen reaction, and an RZ > 3. RZ is the ratio of the optical density (OD) as expressed in nanometers. OD 403/OD 280 reflects the ratio of hemin to protein.

Before the development of the more sensitive PAP and ABC methods, peroxidase-conjugated antibodies were made by covalent bonding of peroxidase to antibodies by aldehyde groups of the carbohydrate shell. Peroxidase is demonstrable by its ability to reduce hydrogen peroxide to water in the presence of an electron donor. (Fig. 1.23). Multiple substrates for peroxidase exist. (Fig. 1.24). The following factors influence choice of substrate:

1. Color: The color should contrast with other colors. For example, a non-brown chromogen might be chosen to contrast with melanin, and a second chromogen of different color would then be selected for double immunohistochemistry.
2. Solubility in dehydrants and mounting media: Only DAB forms a precipitate; the other chromogens are soluble in organic solvents.
3. Sensitivity.
4. Suitability for immunoelectron microscopy.

Of the chromogens, only diaminobenzidine (DAB) (Graham, *1966*) forms an insoluble precipitate that survives long-term storage and dehydration for optimal resolution by light microscopy and permanent coverslipping.

Studies with an artificial system, using immunostains of dot-blots of pentagastrin and specific antisera, demonstrated that DAB provided maximum sensitivity of the chromogens (Scopsi, *1986a*). The reaction products of both DAB and 4-chloro-1-naphthol can be rendered electron dense for ultrastructural localization when osmicated. A final difference of apparent minimal significance is potential carcinogenicity. Of these chromogens, only benzidine is of proven human carcinogenicity. DAB and aminoethylcarbazole (AEC) have little apparent carcinogenicity (Weisburger, *1981*).

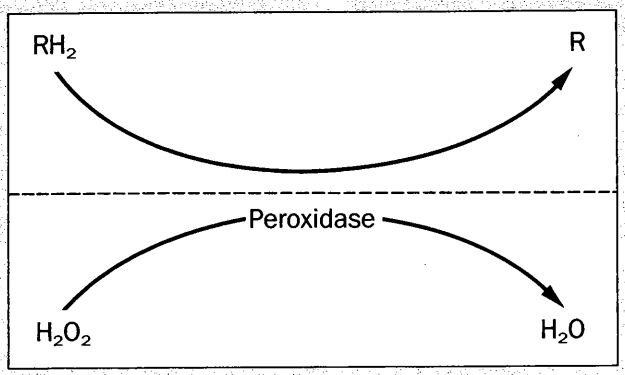
In direct immunostaining, the ability of both peroxidase and fluorescein conjugates to detect nuclear viral antigens is similar (Wicker, *1971*).

Alkaline Phosphatase

First used by Avrameas as an enzyme marker conjugated to the primary antibody (Avrameas, *1969*), alkaline phosphatase is commonly used only in double-immunostaining protocols. Substrates include naphthol salts as coupling agents (eg, naphthol AS-MX or AS-BI phosphate) and diazonium salts as chromogenic capture agents (eg, Fast Red or hexazotized new fuchsin) (Cordell, *1984*).

Glucose Oxidase

First used by Avrameas *(1969)*, glucose oxidase has been little used because traditional substrates were not stable. The current coupler molecule D-glucose and substrate *p*-nitro blue



RH_2 = Reduced Soluble Form Of Chromogen
R = Oxidized Colorized Form Of Reaction Product

Figure 1.23. *Ability of peroxidase to reduce hydrogen peroxide to water in the presence of an electron donor and to oxidize the chromogen to a colorized form, where RH_2 is the reduced and soluble form of the chromogen.*

SUBSTRATES FOR PEROXIDASE

Chromogen	Color	Solubility[a]
Diaminobenzidine	Brown	–
3-Amino-9-ethylcarbazole	Red	+
4-Chloro-1-naphthol	Blue	+
Alpha-naphthol pyronin	Pink	+
Benzidine	Blue	–
p-Phenylenediamine and pyrocatechol (Hanker–Yates reagent)	Black	–
Tetramethyl benzidine	Blue	–

[a]Solubility in organic solvents

Figure 1.24. *Substrates for Peroxidase.*

tetrazolium, with phenazine methosulfate, provide a stable, nondiffusible reaction product. As a marker enzyme, glucose oxidase has the advantages of being absent as an endogenous enzyme in tissue and of providing a chromogen of a different color for use in double immunostaining. A glucose oxidase–anti-glucose oxidase complex with enzyme-to-antibody ratio of 3:2 retains about 50% activity and has been used as a substitute for PAP (Clark, *1982*).

DETECTION SYSTEMS

Of numerous methodologies for localizing the primary antibody, the following are the most widely adopted (Fig. 1.25).

Direct

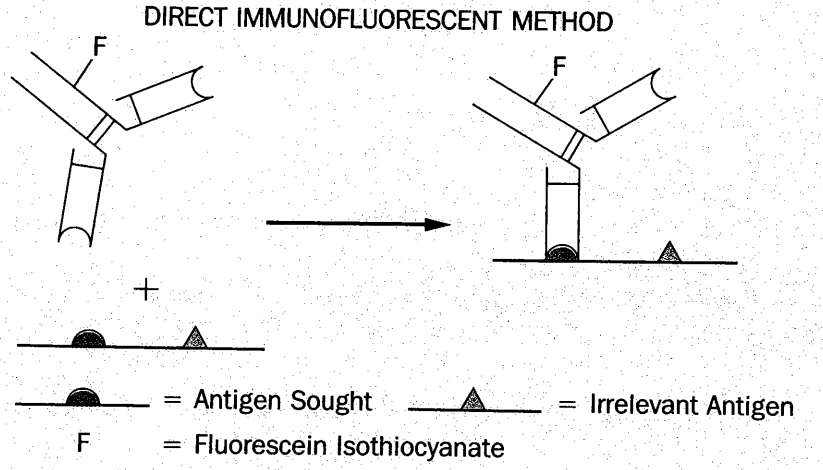
The primary antibody has the label. Introduced by Coons *(1941)*, this method is still employed in the localization of immune complexes in the kidney and skin. Any label may be used, although the common current label for immune complex localization is fluorescein.

Indirect

The primary antibody is localized by a labeled secondary antibody, which is specific for the primary immunoglobulin. There are up to eight immunoglobulin binding sites per IgG. Using fluorescein as the label, Coons found this technique 10 times more sensitive than the direct method, detecting as little as 10^{-15} g of antigen (Coons, *1956*). Although previously widely used in diagnostic pathology, this method is not used much at present because: it is less sensitive than three-step methods; it requires individually labeled secondary antibodies for each animal species of primary immunoglobulin; and residual unconjugated antibody may decrease sensitivity by competing with the conjugated antibody.

Peroxidase–Antiperoxidase (also termed "PAP" and "Bridging")

This widely used method (Sternberger, *1970*) localizes the primary antibody with a secondary antibody, termed the "bridging" antibody. When incubated with the section in excess, this secondary antibody "bridges" the primary to a tertiary complex, which contains the label. The tertiary peroxi-



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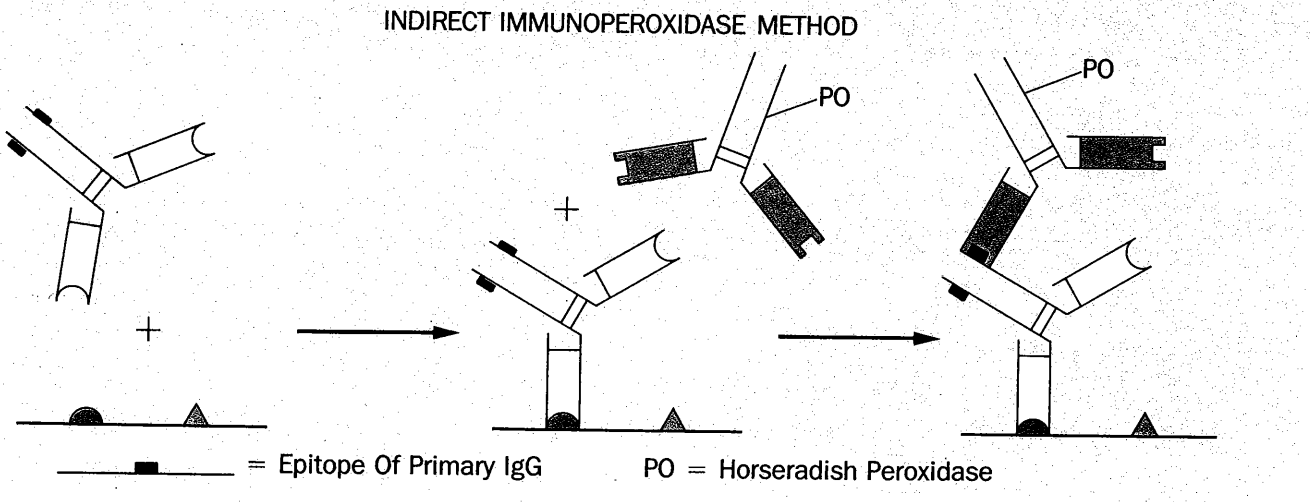


B

Figure 1.25. *Immunostaining methods.* (A) *Direct immunofluorescent method and* (B) *indirect immunoperoxidase method. The methods depicted in these diagrams are explained in the text. The final step, development of the chromogenic reaction product, is omitted (see Figure 1.23.).* *(Figure 1.25 is continued on the next page.)*

dase–antiperoxidase complex is developed in the same species of animal as is the primary antibody for successful "bridging." Analogous complexes with alkaline phosphatase (Cordell, *1984*) and with glucose oxidase (Clark, *1982*) can also be used.

Staph Protein A

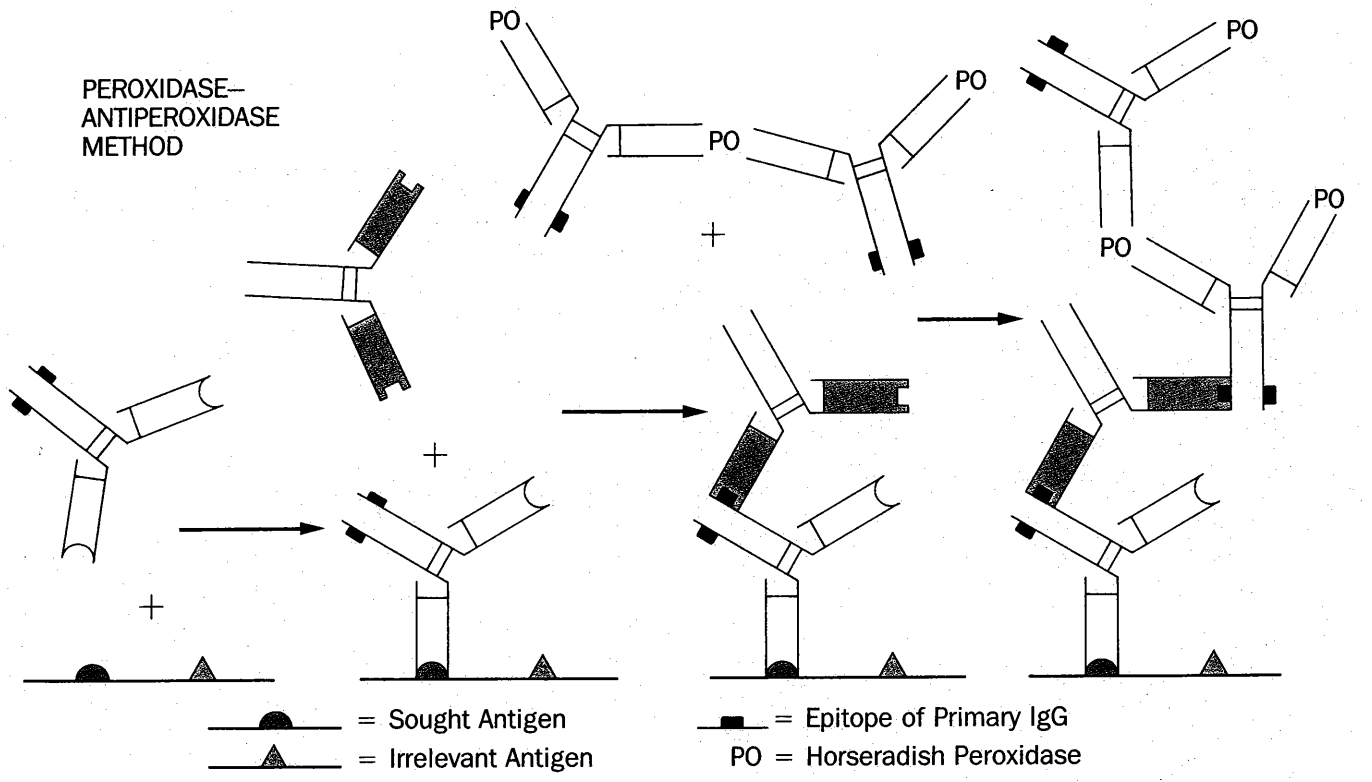
Protein A from the cell walls of many *Staphylococcus aureus* strains exhibits high-affinity (up to 10^8 liters/mole) (Goding, *1978*) binding with specific immunoglobulin subtypes. Each IgG contains two Protein A binding sites, which are in the Fc domain. Protein A is a single-chain 42 kd protein containing virtually no carbohydrate. It can be conjugated with various labels (ie, fluorescein, peroxidase, and gold) without impairing Fc binding. Staph Protein A as a secondary or tertiary reagent has the following advantages over antibodies:

1. Binding is more rapid.
2. Protein A reacts with many immunoglobulins of different species. However, Staph Protein A does not react with equal affinity with all immunoglobulin subclasses. Affinity varies with species and immunoglobulin subtypes; ie, human subclasses IgG_1, IgG_2, and IgG_4 and mouse subclasses IgG_2, IgG_3, and IgG_4 bind with high affinity (Goudswaard, *1978*).
3. Staph A–peroxidase conjugates are smaller (80 kd) than the 200 kd IgG–peroxidase conjugates.
4. Background staining is often at a reduced level because most tissues lack endogenous Protein A binding sites. Furthermore, any background binding may be inhibited by preincubation of sections with albumin or Protein A in buffer. Immunoglobulins should not be used for blocking Fc binding sites, because the Protein A may bind to the immunoglobulin.

The sensitivity of the two-step Protein A–peroxidase method is similar to that of the indirect immunoperoxidase method but has one-tenth the sensitivity of the PAP method (Falini, *1980*).

Avidin–Biotin–Peroxidase (ABC)

Avidins are glycoproteins having a binding affinity $K_D = 10^{-15}$ for the 244 kd vitamin biotin. This affinity is greater than any antibody–antigen binding. The most commonly used avidin is egg-white avidin, which is a basic glycoprotein (isoelectric pH 10) of 68 kd and has four high-affinity biotin-binding sites. This high binding affinity was first exploited by Avrameas' group using biotin-conjugated antibodies and avidin–peroxidase complexes (Guesdon, *1979*). Although several multistep methodologies have been developed, the most efficient and



C

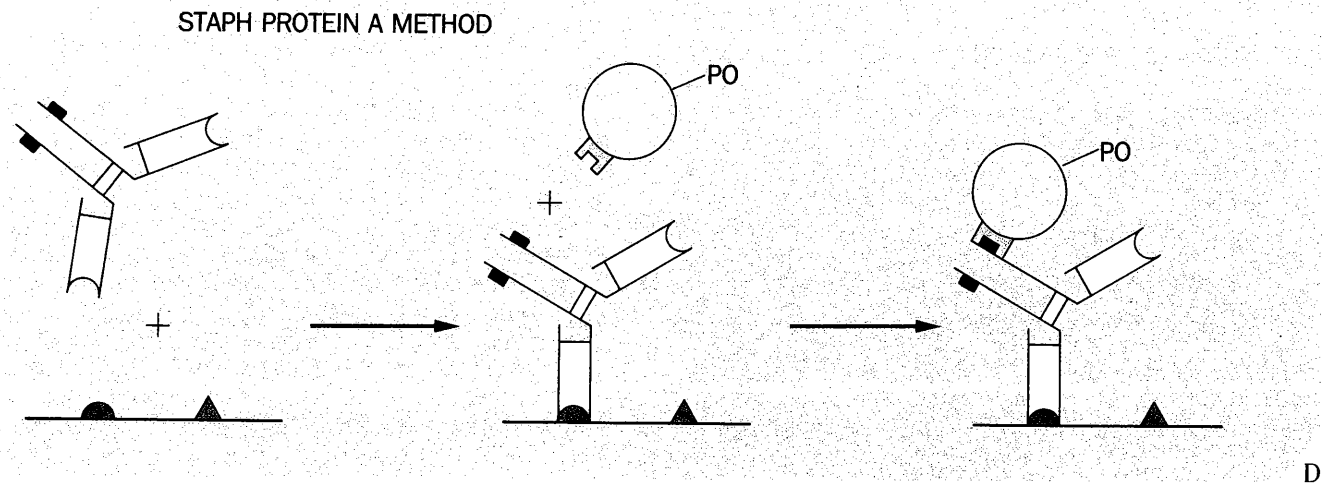
Figure 1.25. *Immunostaining methods (continued).* (C) *Peroxidase–antiperoxidase,* (D) *Staph Protein A, and* (E) *avidin–biotin–peroxidase complex methods. The methods depicted in these diagrams are explained in the text. The final step, development of the chromogenic product, is omitted (see Figure 1.23).*

most widely used employs preformed avidin–biotin–peroxidase complexes (Hsu, *1981*).

The primary antibody is detected by a secondary antibody to which biotin is conjugated (Jasiewicz, *1976*). The label is localized to the secondary by a complex of avidin, biotin, and peroxidase, which is prepared immediately before use. Theoretically, large complexes that include peroxidase might be likely to bind nonspecifically to tissue protein; in practice, such a source of background staining is not a problem.

Streptavidin is a 60 kd protein consisting of four identical subunits (each with a biotin-binding site) from the bacterium *Streptomyces avidinii.* Streptavidin has the advantage of reduced background staining, due in part to a near-neutral isoelectric point and absent carbohydrate side chains. In contrast, the carbohydrate side chains of egg-white avidin (which comprise 7% of the molecule), combined with the basic isoelectric pH of 10, theoretically predispose to greater nonspecific staining.

Whether the PAP or the ABC method has greater sensitivity is debatable. Assessed semiquantitatively, the ABC system can detect up to fivefold greater dilutions of primary antibody (Hsu, *1981*). In contrast, using densitometry, up to 10-fold greater



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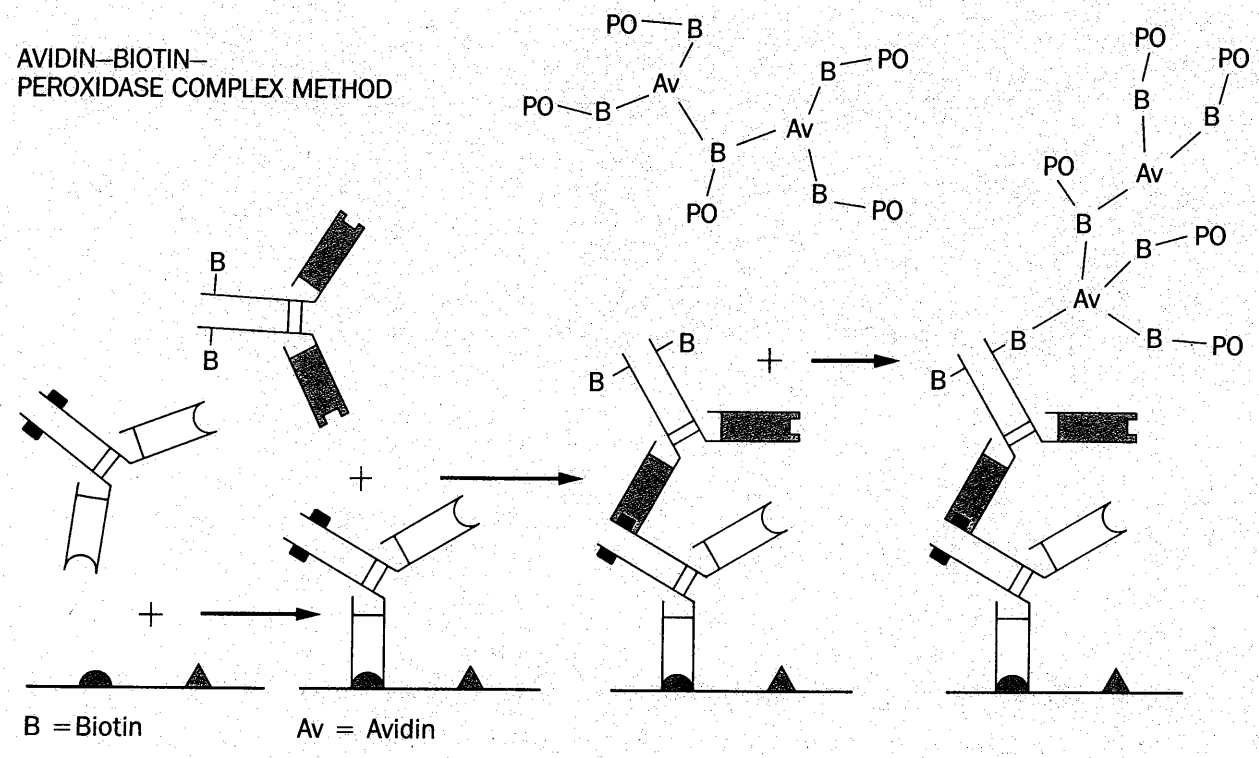


E

dilutions of primary antibody were detectable with the peroxidase–antiperoxidase technique (Sternberger, *1986*). Neither of these studies directly assessed sensitivity in detecting tissue antigen; rather, they assessed antibody efficiency, ie, the degree to which the primary antibody could be diluted (Petrusz, *1983*).

In fact, there is evidence that an indirect method may be as sensitive as the PAP method. As little as 8 ng of protein is detectable by both methods on immunoblots (Bosman, *1983*).

SENSITIVITY

The sensitivity of a detection system refers to the minimum concentration of antigen detectable by the assay system. This quality differs from "antibody efficiency," which refers to the minimal amount of antibody needed (Petrusz, *1983*). This definition contrasts with one applied to extracts of tissue; in the latter case, one is interested in the minimum concentration of antigen per unit volume of tissue. The distribution of antigen in a tissue dictates the choice of detection system; if an antigen is not uniformly distributed but is concentrated in a portion of the tissue, an immunohistochemical assay might be more sensitive, assuming that the portion of tissue containing the antigen is sampled. If the antigen is uniformly distributed, extraction assays may be more sensitive. In the extreme example, if one cell contains the antigen and that cell is sectioned, the antigen will be detected, whereas it might not with an extraction assay. Thus, immunohistochemistry detected sparsely granulated, luteinizing hormone cells, which comprise 10% of the pituitary cell population, whereas radioimmunoassay failed to detect these cells (Childs, *1983*).

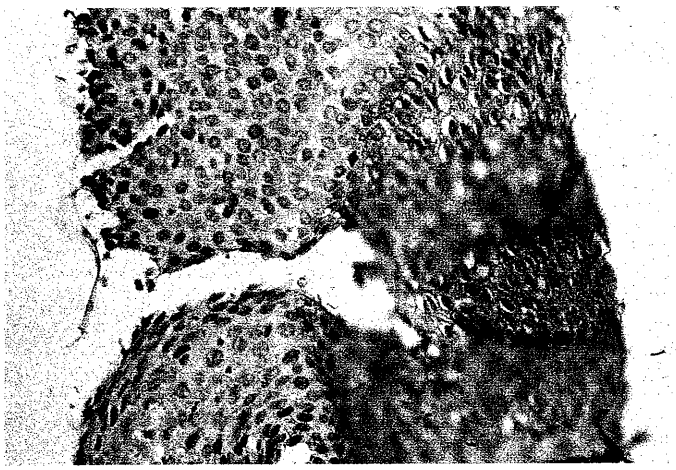


Figure 1.26. *Nuclei of squamous cells in cervical condyloma stain more intensely for human papilloma virus where the section has lifted from the slide* (DAB-PO; H-counterstained).

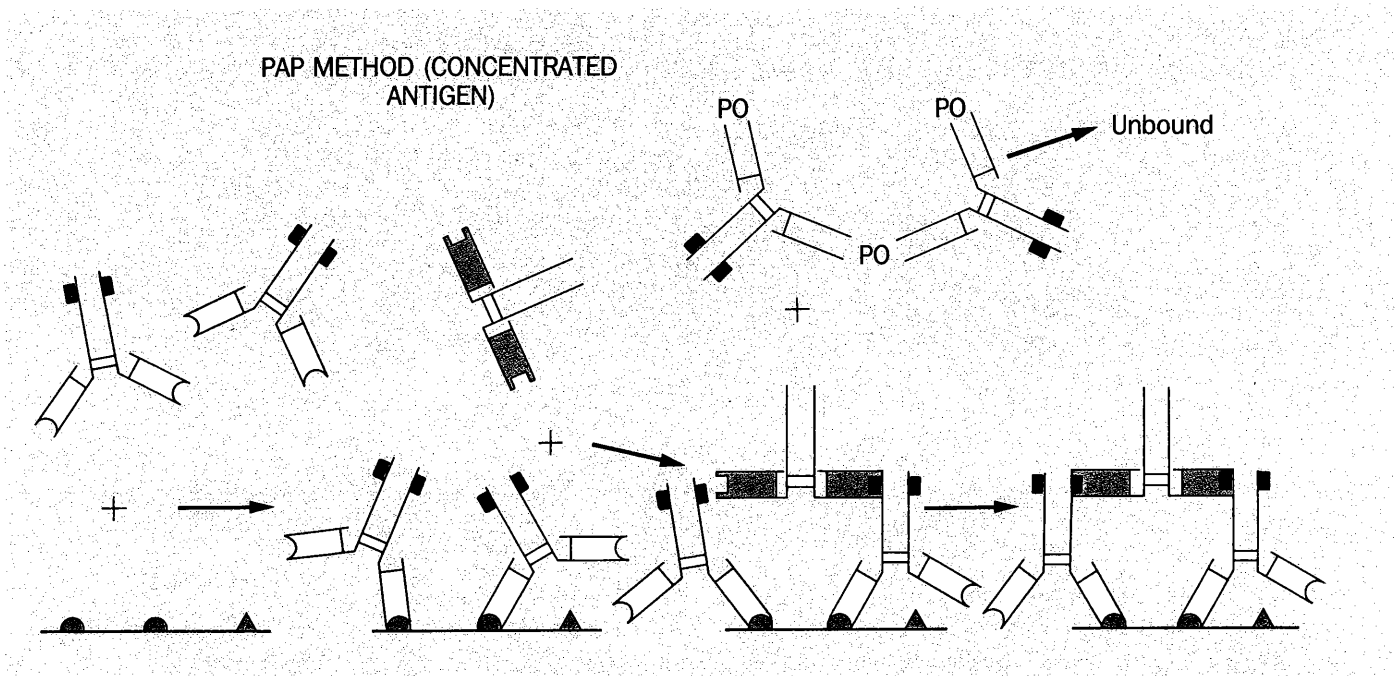


Figure 1.27. *Schematic of PAP method where the antigen is highly concentrated. In contrast to the ideal scheme in Figure 1.25, all of the antigen-binding regions of the secondary antibody are bound to the primary antibody in this figure. Hence, the secondary antibody cannot function as a "bridge" to bind the tertiary PAP complexes. Therefore, the antigen A is not detected.*

Several sets of factors influence sensitivity, as discussed in the following subsections.

Nature of Antigen

The factors discussed above which alter antigenicity can decrease sensitivity. And an antigenic sequence which is repeated multiple times (or multiple different antigens) is more easily detected than a single epitope. Sensitivity may be increased by exposing more antigenic sites (through protease digestion) and by removal of fixative and of embedding medium. Antibody affinity does not affect sensitivity, so long as affinity is greater than 10^6 (Petergy, *1983*).

Antigen Accessibility

Location of an antigen may affect sensitivity. In contrast to cell-surface antigens, which are immediately accessible to antibodies, intracellular antigens are shielded by membranes. Because cell membranes are naturally impermeable to immunoglobulins, holes must be created to allow antibodies access to intracellular antigens. Fixation and sectioning creates adequate access for antibodies. This access can be increased by immunoreacting both sides of the tissue (Ciocca, *1987*) (Fig. 1.26). However, other maneuvers often need to be performed on intact cells, ie, freeze-thawing, use of detergents, and use of enzymes (True, *1981*).

The size of the immunologic reagents may influence antibody penetration of tissues. Fab′ fragments, which are 46 kd in size, diffuse more readily through tissue than does whole IgG (156 kd) or PAP complexes (440 kd) (Nakane, *1975*). Presumably the size of holes in membranes, created by tissue handling, including fixation, is the determinant of antibody penetration.

Recently, a system using Fab fragments in a bridged, unlabeled method was shown to be very sensitive (Brandon, *1985*). The most important factor was, surprisingly, not the use of the 150 kd Fab–peroxidase complexes, but the use of an Fab′ as the primary antibody. Primary Fab′ fragments can better localize the tissue-fixed, sterically hindered antigen than can larger IgG's. The explanation is that Fab′ is better able to conform to the three-dimensional architecture of the antigen than can the larger and more rigid IgG's.

Detection System

Maximum sensitivity occurs with multistep detection systems that amplify the signal by increasing the label–primary-antibody (hence, label–antigen) ratio. Sometimes, particular detection systems create individual problems. A too concentrated antigen cannot be detected by the PAP system, presumably because of steric hindrance between antibodies (Bigbee, *1977*) (Fig. 1.27).

Studies assessing the most sensitive detection system are controversial. The PAP method is either more sensitive (Sternberger, *1986*) or less sensitive (Hsu, *1981*) than the ABC method in detecting the primary antibody.

The final element in the detection system is the eye. The wavelength of maximum visual acuity is 555 nm (green-yellow). DAB is near that frequency (Fig. 1.28). When a camera is used as an imaging device, particularly if applied to quantitative immunohistochemistry, the camera's response features

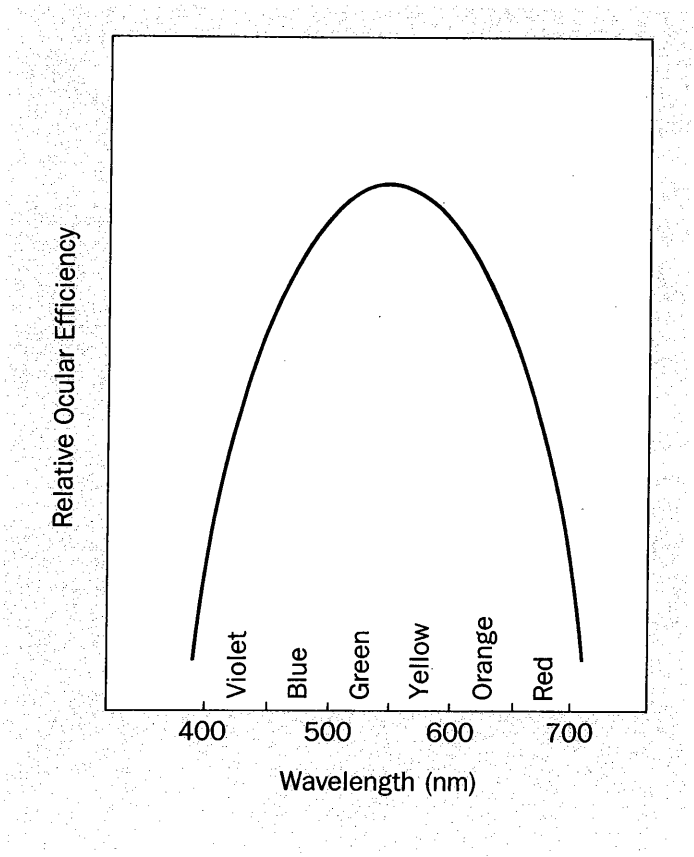


Figure 1.28. *The efficiency of the human eye in detecting light of constant intensity is plotted against the wavelength of the light. (Modified from Inoue,* 1986.*)*

to varying levels of illumination should be known. In contrast to the eye (and to photographic emulsions), which responds logarithmically to illumination, the response of digital cameras is closer to linearity (Inoue, *1986*).

Adequacy of the detection system for identifying a sought antigen is checked with a positive control. This is a section previously shown to contain the antigen, under identical conditions of immunohistochemistry. Selecting an adequate positive control can be difficult for antibodies to a new antigen; a negative immunostain of a tissue reported to contain the antigen cannot be fully interpreted and the distinction cannot be made between inactive antibody and inactive or absent antigen. In such a case, there is little choice but to immunostain other tissues reported to contain the antigen (at a range of antibody dilutions) or to return the antibody to the supplier.

Enhancement Methods

Diaminobenzidine reactivity can be enhanced by counterstaining with heavy metals such as osmium (Graham, *1966*), colloidal gold followed by silver, and nickel or cobalt (Figs. 1.29 and 1.30). Repeat bridges, ie, with subsequent peroxidase–antiperoxidase, also enhance detectability (Vacca, *1975*) (Fig. 1.31).

SPECIFICITY

Nonspecific immunoreactivity represents deposition of reaction product at a site other than the location of the desired antigen. Such false positivity can arise either from antibody binding or from nonantibody-specific binding.

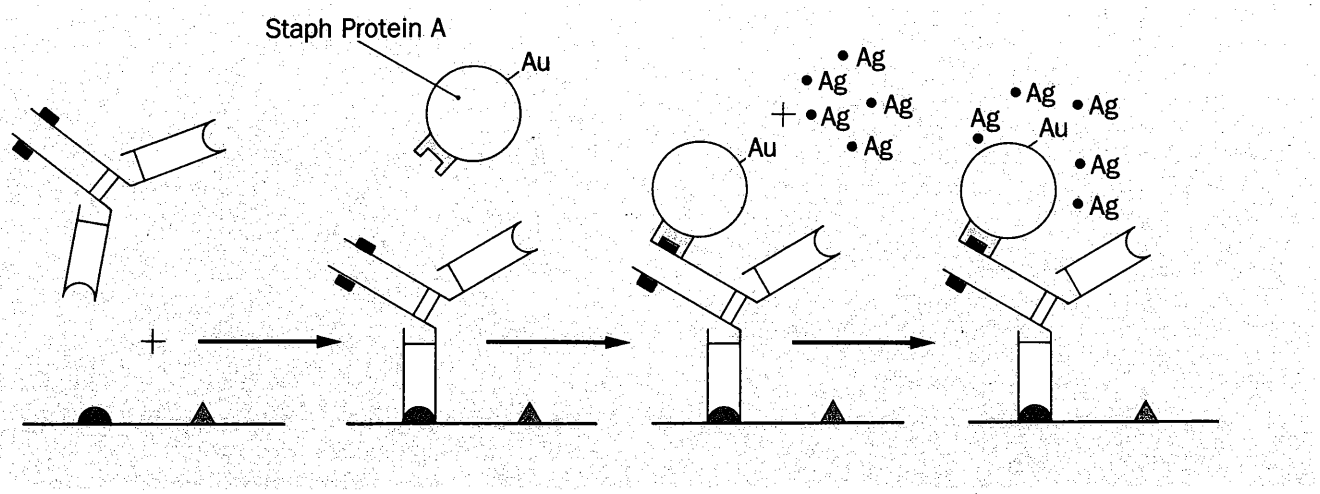


Figure 1.29. *Silver-enhanced, gold-labeled Staph Protein A immunohistochemistry. The primary antibody must contain a Staph Protein A-binding site, to which the Protein A–gold complex binds. Subsequent development of silver (Ag) deposits black reduced silver grains at the site of sought antigen A.*

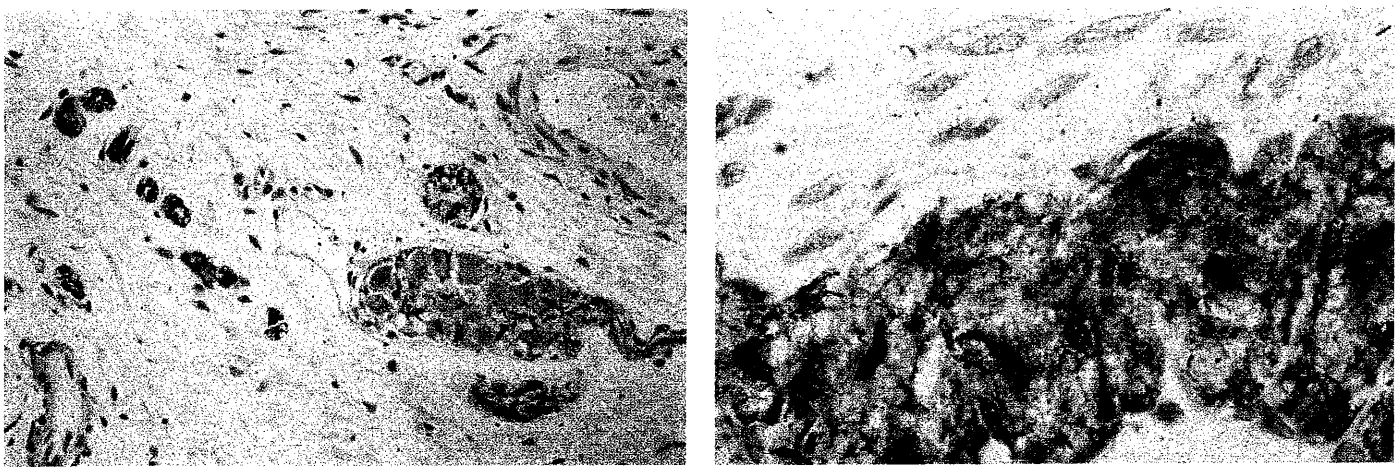


Figure 1.30. (Left) *Section of colon reacted with anti-S-100 protein, followed by Staph Protein A complex and silver. Granules of reduced silver stain nerve sheath cells in Auerbach's plexus black.* (Right) *Amplification of PAP staining. At higher magnification, the granular nature of the silver precipitate is seen. There is virtually no nonspecific binding.*

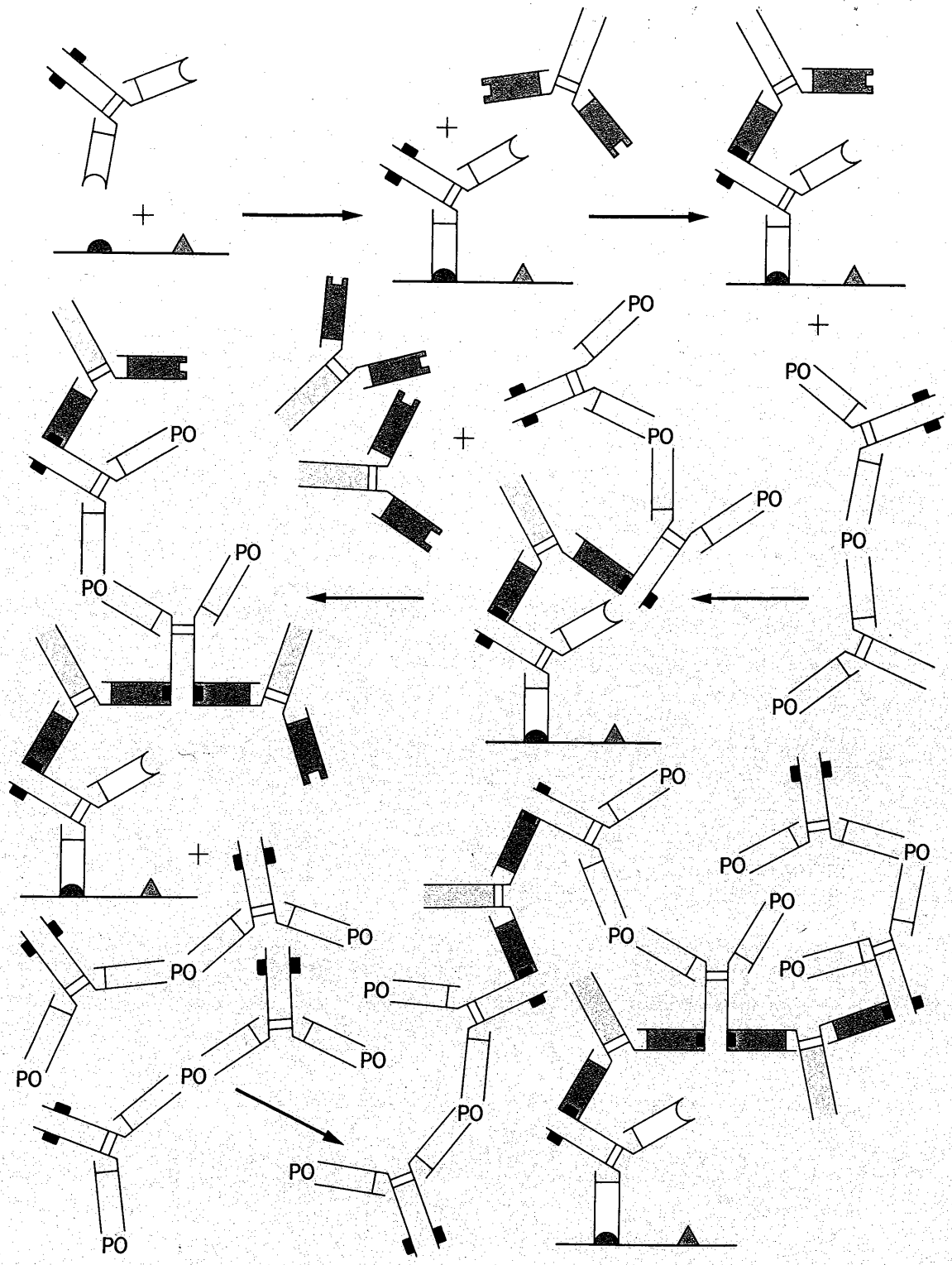


Figure 1.31. *By sequentially repeating the incubations with the secondary and tertiary (PAP complex) antibodies, greater amounts of peroxidase (PO) are localized to the antigen sought. Compare with Figure 1.25.*

Immunologic Nonspecificity

Here, immunologic nonspecific reaction refers to the successful immunohistochemical localization of an antigen which provides a false result. Sources of such false positivity include the following.

Sequence Homology

Many molecules share partial amino acid identity, ie, gastrin/cholecystokinin, the intermediate filaments, α_1-AT/α_1-ACT, and S-100/calmodulin. Antibodies to the homologous sequence may localize both molecules. Unless the investigator is aware of both the partial potential antigenic identity of these two molecules and the specificity of the antibody to the common antigen determinant, she/he may falsely conclude that one molecule has been specifically localized. For example, localization of antineurofilament activity to nuclei could represent cross-reactivity with nuclear lamin, which is partially homologous with the intermediate cytoplasmic filaments (see Chapter 4) (Figs. 1.32 and 1.33).

Similar Antigenicity

There need not be amino acid identity for there to be antigenic similarity. For example, Leu-7 monoclonal antibody binds any proteins containing a certain carbohydrate group of restricted configuration, and some lupus antibodies bind to a phosphodiester epitope regardless of whether the epitope is on DNA or cardiolipin (Lafer, *1981*).

Contaminating Antibodies

The primary antibody preparation may contain various antibodies in addition to the one expected. If directed towards endogenous molecules, these are termed "autoantibodies." They may be present congenitally. Six percent of hybridomas from plasma cells of newborn mice produce autoantibodies, most frequently directed to such cytoskeletal proteins as tubulin and actin (Dighiero, *1985*). Virtually all adult humans contain antibodies to the 200,000 kd neurofilament protein; these immunohistochemically localize to neurons at 1:50 dilutions (Stefansson, *1985*). Apparently normal people have a 4% to 8% incidence of antivimentin and/or antikeratin antibodies.

Rabbits are known to have endogenous antikeratin antibody activity. That a polyclonal antiserum may be localizing an antigen other than the desired antigen was probably the explanation for reported immunostaining of epidermal cells and of proven squamous-cell carcinomas using a polyclonal anti-factor VIII-related antibody (Wilson, *1984*) (Fig. 1.34).

False positivity owing to contaminating or autoantibodies can be corrected by purifying antibody preparations. When pure antigen is available, affinity chromatography can remove spe-

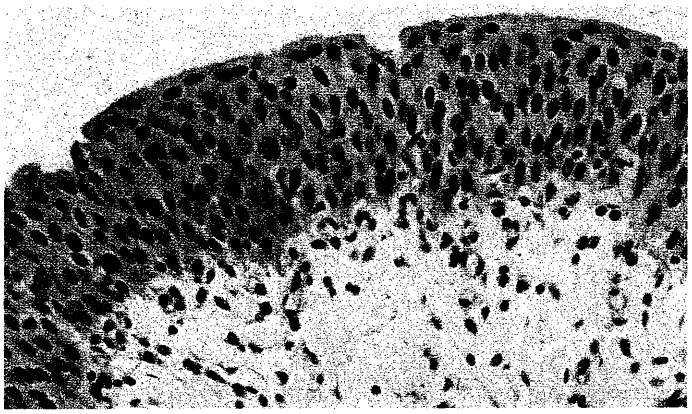


Figure 1.32. *Nuclei of urothelium localizing antineurofilament antibody. One explanation is that the nuclear lamin proteins of these urothelial nuclei share a specific epitope with neurofilament protein.* (DAB-PO; H-counterstained.)

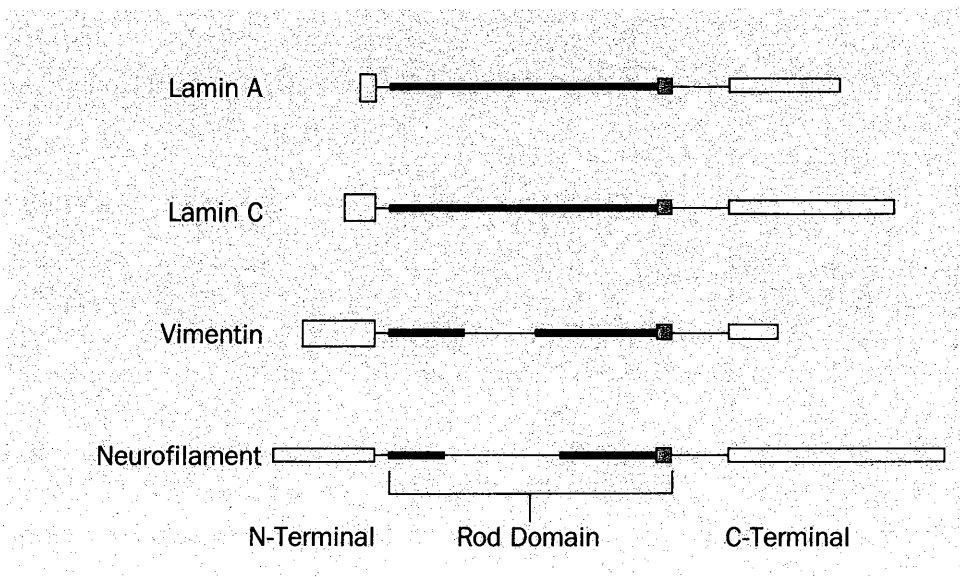


Figure 1.33. *Schematic of areas of extensive amino acid homology between the nuclear-membrane-associated proteins lamin A and lamin C. The cytoskeletal filaments vimentin and neurofilament protein are indicated in red. These four proteins differ at the amino and carboxy terminals. (McKeon, 1986).*

cific antibodies from a solution of multiple antibodies. When the specific antigen is unknown, the specific antibody cannot be isolated, but it can be concentrated using methods that isolate immunoglobulins, such as ammonium precipitation of immunoglobulins or separation with a Protein A column.

False positivity due to epitope identity cannot be corrected because successful antibody–antigen binding in these instances represents immunologic activity. Solutions of polyclonal antibodies containing multiple antigen-specific antibodies can be purified of antibodies that cross-react by affinity chromatography using the cross-reacting molecule as the immunoreactant. Only antibodies directed to unique epitopes will remain.

Nonimmunologic Nonspecificity

Many sources of non-antibody-binding false positivity exist. Fc portions of whole immunoglobulins may bind receptors of Fc phagocytes and mast cells (True, *1981*). Fc receptors are labile and readily inactivated by fixation. Complement-binding IgG molecules may localize to complement already present in tissue. (Buffa, *1979*). Complement is also labile. Certain antibodies and gut endocrine cells have an electrostatically mediated affinity that can mimic antibody–antigen reactivity (Grube, *1980*). Changes in the solution pH and salt concentration of reagents abolish this activity. Free aldehyde groups from incompletely reacted fixatives may nonspecifically bind antibodies to tissue (Farr, *1981*).

Neurohormonal peptides such as ACTH and vasoactive intestinal polypeptide have binding affinity for several reagents, including peroxidase-labeled immunoglobulin, Protein A, and streptavidin. Preincubation with excess ACTH(1-24) or use of poly-L-lysine in diluents will be corrective (Scopsi, *1986*b).

We have not suffered these sources of false positivity, which can be controlled for, in part, by using an irrelevant antibody as a positive control, and minimized with high dilutions of antibodies.

Endogenous Label Activity

Peroxidase. Hemoproteins with an iron porphyrin prosthetic group have peroxidase activity, which is highly variable and affected by factors that can be readily controlled in immunoperoxidase staining. The peroxidase activity of peroxidases in epithelial cells (mammary gland and secretory endometrium), megakaryocytes, and mast cells, catalase in liver, cytochrome C, myoglobin, and hemoglobin is suppressible by routine fixation and dehydration of tissues (Fahimi, *1979*; Escribano, *1987*).

The peroxidase activity in red cells, neutrophils, eosinophils, basophils, and histiocytes can be suppressed by multiple techniques that destroy or inactivate enzymatic activity (True, *1981*). (Fig. 1.35).

TUMORS IMMUNOSTAINED WITH ANTI-FACTOR VIII-RELATED ANTIGEN ANTIBODY

	Intensity of Immunostain		
Type of Tumor	Strong	Weak	Absent
Angiosarcoma	0	2	1
Squamous-cell carcinoma	3	3	0
Renal-cell carcinoma	2	0	1

Figure 1.34. *Number of cases of respective tumors immunostained with an anti-Factor VIII-related antigen antibody that also had anti-keratin activity. (From Wilson,* 1984.)

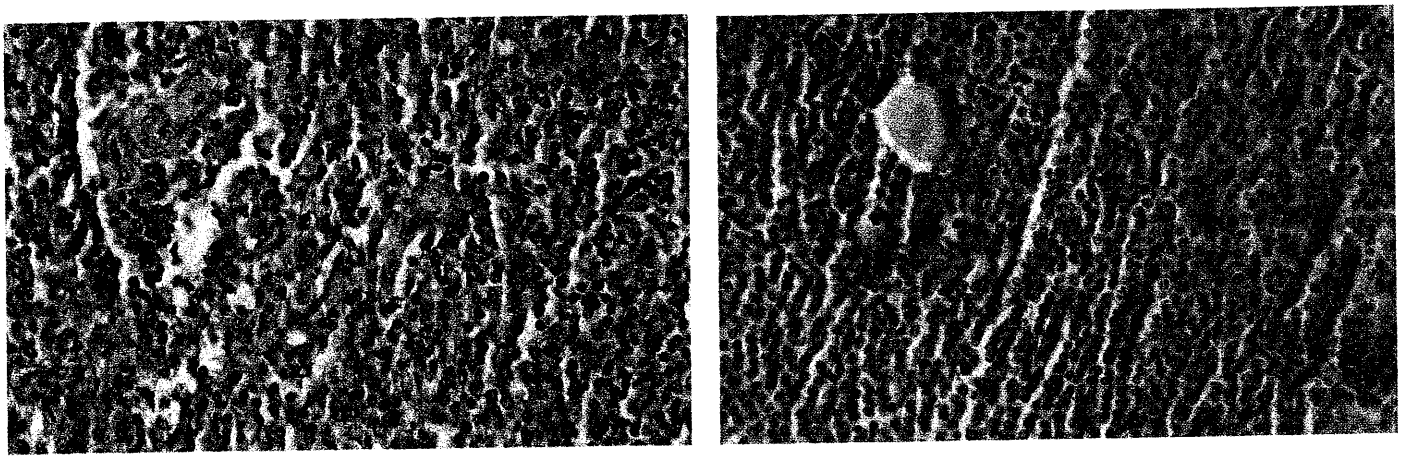


Figure 1.35. (Left) *Section of spleen incubated with antikeratin antibody. Reaction product is seen associated with red cells and neutrophils, because endogenous peroxidase activity was not suppressed.* (DAB-PO; H-counterstained.) (Right) *After endogenous peroxidase activity in an adjacent section of spleen is suppressed, red cells and neutrophils exhibit virtually no staining.* (DAB-PO; H-counterstained.)

Alkaline Phosphatase. The alkaline phosphatase activity present most intensely in epithelia of bladder, renal tubules, and small bowel, placental trophoblasts, neutrophils, and mast cells is suppressible with techniques that differ with the type of alkaline phosphatase. Because these methods may not suppress all activity, peroxidase may be preferred (Ponder, *1981*).

Biotin. Kidney, pancreas, and liver contain high concentrations of biotin, which may cause false localization of avidin–biotin complexes (Fig. 1.36). Preincubation with free avidin and biotin can block such binding (Wood, *1981*).

Label-Binding Activity

Peroxidase. Horseradish peroxidase has an affinity for cell membranes by two apparent mechanisms: a mannose-specific affinity and a calcium-dependent affinity for a cell-surface glycosyltransferase. These bindings are fixation-sensitive (Straus, *1987*).

Nonimmunologic binding of peroxidase to the hepatitis B virus may also depend on affinity between the carbohydrate components of both substances (Omata, *1980*). Furthermore, binding of cationic peroxidase conjugates to anionic sites in extracellular matrix is preventable by digestion of the negatively charged sites from the tissue or by neutralization of the positive charge of peroxidase (Pino, *1985*).

Avidin. Ionic binding of the basic residues of avidin to sulfate groups of heparin may give false localization of avidin–biotin complexes to mast cells (Fig. 11.37). Because this binding is dependent on a neutral environment, conducting the reaction in a high-pH environment will decrease or abolish such mast-cell granule binding.

Avidin also has an affinity for nuclei, which can be blocked with nonfat dry milk (Duhamel, *1985*). Avidin furthermore exhibits affinity for immunoblotted proteins, suppressible with high-salt buffers (Clark, *1986*).

Protein A. Protein A may bind to tissue immunoglobulins that retain Protein A-binding activity. Thus, Protein A is a poor label for immune complex localization.

Miscellaneous

Other localizations are poorly understood, such as localization to the edge of tissues, to necrotic tissue, and to stroma (Figs. 1.38 and 1.39).

Endogenous pigment may be confused with label, particularly lipochromes or melanin with DAB. A chromogen of contrasting color may be used instead of DAB (Figs. 1.40 and 1.41). Futhermore, the negative control section will demonstrate the distribution of such endogenous pigment.

The determination of specificity of antibody binding is a

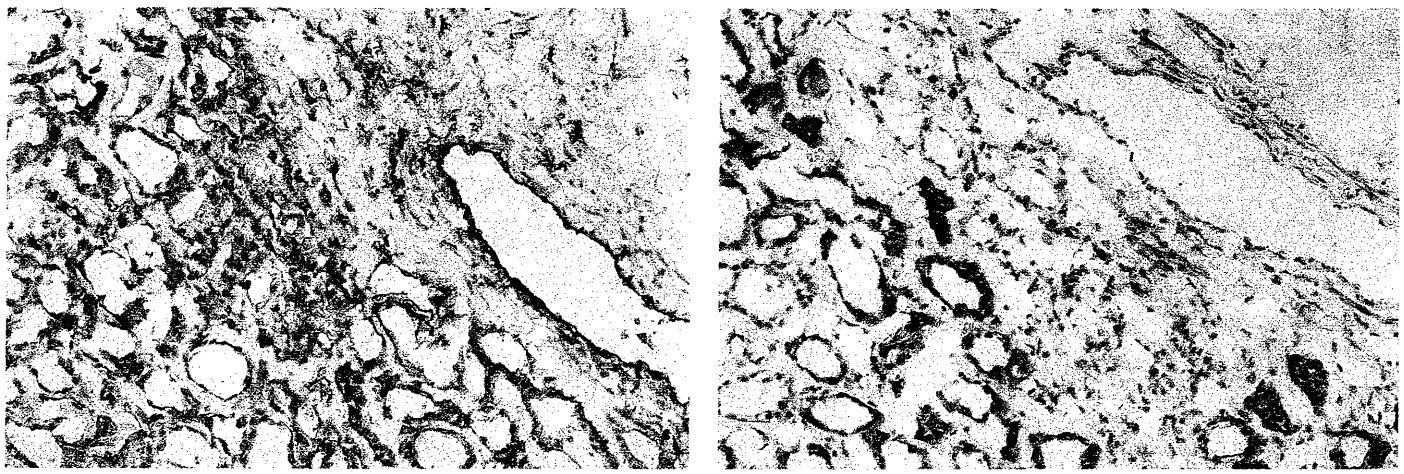
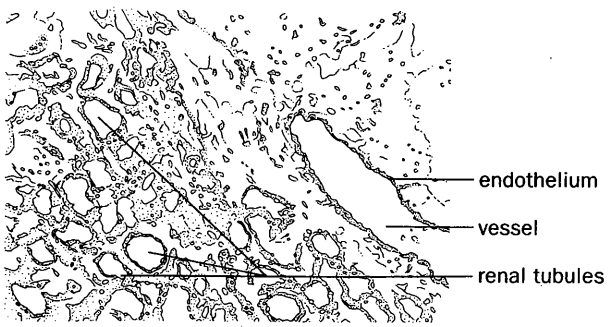


Figure 1.36. (Left) *Frozen section of kidney immunoreacted with anti-HLA-DR exhibits staining of both endothelial cells and tubules.* (DAB-PO; H-counterstained.) (Right) *An adjacent control frozen section in which the primary antibody has been omitted exhibits tubule staining. This negative control indicates that only the endothelial cells contained HLA-DR. The tubules stain because the endogenous biotin binds the avidin–biotin–peroxidase complex.*



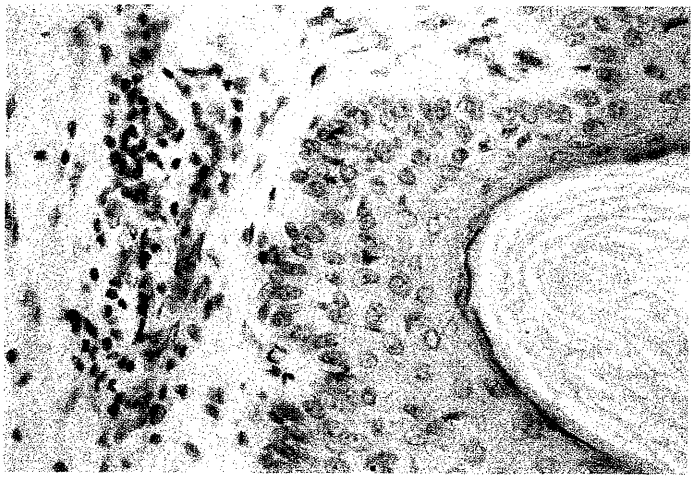


Figure 1.37. *Reaction product is localized to a single mast cell in this section of skin which has been incubated with avidin–biotin–peroxidase complex, followed by a solution of DAB and peroxide. Endogenous peroxidase activity was suppressed* (DAB-PO; H-counterstained.)

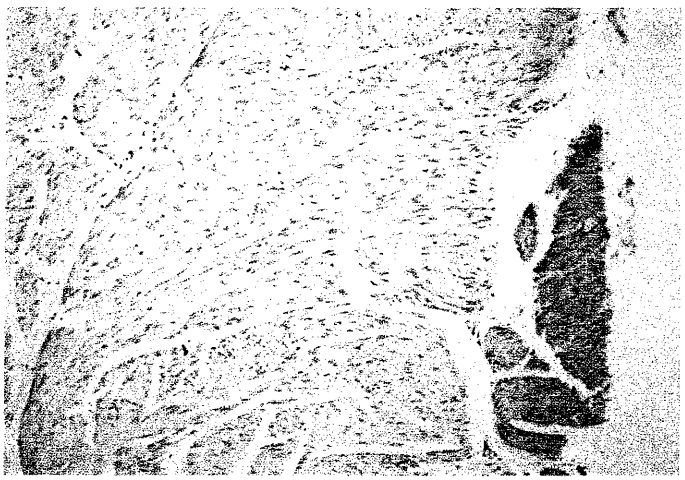


Figure 1.38. *Edge effect. Section of bowel immunostained for ACTH. Reaction product in this field is present only at one corner, where a portion of the muscularis presumably lifted from the slide and "trapped" the immunostain. As is typical, no other edges stained.* (DAB-PO; H-counterstained.)

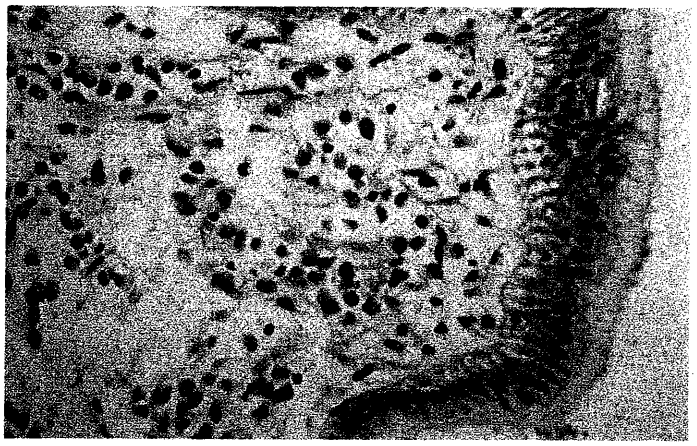


Figure 1.39. *The foveolar cells in this field of gastric mucosa exhibit Leu-7 immunoreactivity, which is apparently localized to the basal aspect of the cell cytoplasm. Because only this one area of stomach exhibited this staining pattern, which is atypical for the membrane protein identified by the Leu-7 antibody, the staining is interpreted as nonspecific.* (DAB-PO; H-counterstained.)

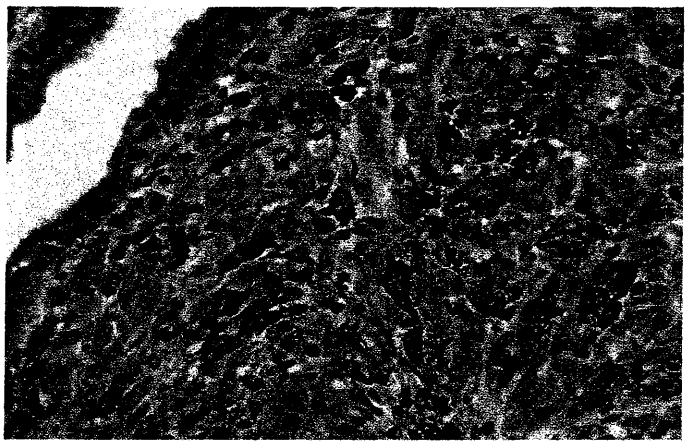


Figure 1.40. *Section of gastric mucosa containing large atypical cells and brown pigment. (H&E)*

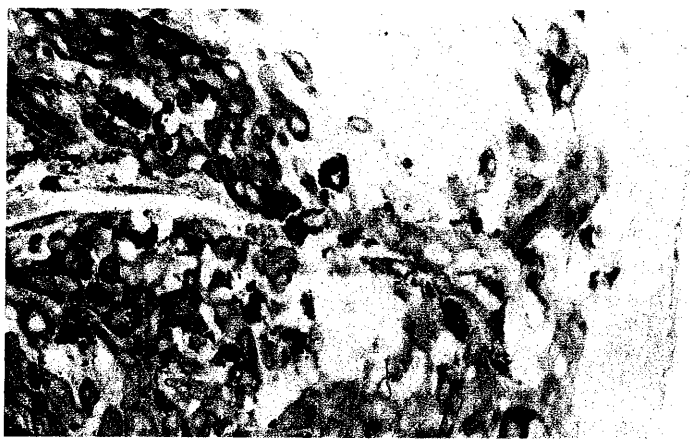


Figure 1.41. *Serial section immunostained for S-100 protein, using AEC as the chromogen. The atypical cells contain red AEC reaction product, which contrasts with the endogenous brown melanin pigment. S-100 positivity confirms that the atypical cells are metastases of a previously diagnosed acral melanoma.* (AEC-PO; no counterstain.)

very important aspect of assessing the results of an immune reaction. The following criteria of specificity should be satisfied (Swaab, *1975*; Petrusz, *1976*; Swaab, *1977*; Childs, *1983*; Petrusz, *1983*).

Preabsorption of the antibody with the antigen of interest should abolish immunoreactivity. This condition is insufficient because the immunogen may have contained impurities, the antigen may not fully neutralize antibody activity, or the antibody may cross-react with other molecules.

Substitution of non-immune-specific agents (ie, buffer or irrelevant antibody) for any of the immune reactions should abolish reactivity. This type of negative control will also identify nonimmunologic reactivity.

The immune reaction should not be sensitive to small changes in the physical–chemical environment of reaction (ie, changes in pH, salt concentration, temperature), duration of incubation steps, or type of embedding medium. This is a relative criterion, because antigens differ in their sensitivity to alterations in these conditions.

Antigenicity should be demonstrable by a different immunologic technique, such as an analysis of tissue homogenates by immunoassay.

Proof that a given molecule is present in a certain tissue ultimately rests on proof that the functional activity of that molecule is present.

One of the current limitations of immunohistochemistry as it is now practiced in diagnostic laboratories is the inability to satisfy all of these criteria of specificity. Therefore, labs must rely upon the suppliers of antibodies to supply only antibodies that are specific to the specified antigen or to supply data that detail nonspecific reactivity in the expected conditions.

The consequence of not controlling for nonspecificity has been exemplified by the reported nonspecific binding of anti-p21 antibodies (Samowitz, *1987*).

INTERPRETATION

Interpretation of an immunohistochemical stain takes into account the sources of false positivity and negativity discussed above. Good analysis of immunostains depends upon a final set of considerations.

Site of Synthesis

Localization of a substance to a given cell usually, but not always, indicates site of synthesis. For example, immunoreactive myoglobin can be localized to macrophages in the region of muscle necrosis (Eusebi, *1984*). Localization at the electron microscopic level to endoplasmic reticulum is strongly suggestive of site of synthesis, once the observer is satisfied there has not been artifactual displacement of reaction product (Novikoff, *1972*). Demonstration of uptake and incorporation of a radiolabeled amino acid into the substance by specific cell antigen, followed by release into the culture medium, is further important evidence.

Stable Expression

The assumption that cells of a certain histogenesis stably express certain antigens is not necessarily true. For example, the types of keratins synthesized depend upon the stage in the cell cycle (Franke, *1983*). Furthermore, mesothelial cells decrease their synthesis of certain keratins and increase their synthesis of vimentin and of other keratins under conditions of rapid growth, or when grown in suspension, either in ascites or in culture (Connell, *1983*; LaRocca, *1984*).

Homogeneity

The assumption that all cells of a tumor produce the same amount of a given antigen is inaccurate. For example, 1% to 100% of cells in squamous carcinomas of the lung contain identifiable immunoreactive keratin (Ramaekers, *1985*). Functional endocrine tumors are typically composed of a heterogeneous cell population, although usually only one hormone is produced in excess (Mukai, *1982*).

Nomenclature

"Positivity" for, say, keratin implies that all cells contain keratin. As pointed out above, keratin distribution may be heterogeneous. Furthermore, "keratin" is not a single protein or epitope but is, instead, a family of over 19 intermediate filament proteins. Thus, failure to detect keratin does not denote the absence of keratin but denotes, instead, the absence of the epitope detectable by the particular detection system. Proof of absence or nondetectability requires multiple methods, with consideration for the limitations of all methods.

The Set of Cells is Known

Assumptions that the specificity of a given substance for a given set of cells is known are not necessarily accurate. Lysozyme, α_1-antitrypsin, and α_1-antichymotrypsin are often referred to as markers of macrophages. Yet, many epithelial cells synthesize these proteins (see Chapter 7). And Leu-7, described as an anti-NK killer lymphocyte antibody, also reacts with prostate duct cells (Rusthoven, *1985*).

Even the significance of intermediate filament expression in histogenesis is uncertain. The assumptions that mesenchymal cells express vimentin and that epithelial cells express keratin are not always accurate. The spindle-cell component of some squamous carcinomas (Ellis, *1987*), and "epithelial" cells of some thyroid (Henzen-Logmans, *1987*), lung (Upton, *1986*), and renal-cell carcinomas (Herman, *1983*), may express vimentin. Furthermore, some subserosal stromal cells (Bolen, *1986*) and various sarcomas, eg, synovial sarcomas (Miettinen, *1984*), leiomyosarcomas (Miettenen, *1988*), and rhabdomyosarcomas (Colindre, *1988*), express keratin (Miettinen, *1984*).

Significance

A final question, yet unanswered, is whether tumor cells so morphologically undifferentiated as to be characterized only

by the presence of certain antigens behave and respond to therapy in a manner similar to that of better-differentiated tumor cells.

SPECIAL PROCEDURES

Quantitative Immunohistochemistry

Quantitative immunohistochemistry is the determination of the concentration of an antigen at the light-microscopic level within tissues or cells or at the electron-microscopic level within cell compartments, in either relative or absolute units.

The concentration of an antigen is most easily assessed by estimating relative visual intensity of a chromogenic label. To determine optimal antibody or reagent concentration in titration studies and to compare antibody efficiencies and sensitivities of different immunostaining protocols, such a semiquantitative method suffices. Results are typically expressed on a 3- or 4-point numerical scale, ie, 1+, 2+, etc. Although the use of numbers is potentially misleading, because parametricity is implied, in practice the use of such scales is an efficient means for communicating the variability and intensity of immunostaining (Fig. 1.42).

Only in the quantification of estrogen receptors (Sklarew, *1987*) and in the estimation of the variability of calcitonin concentration in thyroid C cells (Lippman, *1982*) has quantification seen a role in diagnostic pathology. Although the number of variables affecting immunostaining is so great as to suggest that sufficient control of conditions cannot be achieved, the notion that antigen density can be determined by immunostaining has been validated. Such validations have been achieved using model systems in which a variable produces a change in a known antigen. Validation has been achieved at both light-microscopic and ultrastructural levels, by correlating biochemical assays with immunostaining results. Castration produces a decrease in pituitary gonadotrophs. Image analysis has verified a decrease in total immunostain reaction product which parallels the decrease in extractable pituitary luteinizing hormone (Benno, *1982*; Gross, *1985*). Furthermore, the change in ratios of pancreatic enzymes in rats on a soybean trypsin inhibitor diet corresponds to the absolute numbers of gold-labeled antibodies to the respective two enzymes at the electron microscopic level (Posthuma, *1984*).

Successful quantitative immunohistochemistry should satisfy the following conditions (True, *1988*):

1. All antibody–antigen and enzyme–substrate reactions must be conducted under conditions where binding is saturated (Gross, *1985*).
2. The distribution of reagents must be homogeneous.
3. The matrix (tissue or embedding medium) and the antigen density should have a predictable effect on immunostaining intensity (Griffiths, *1986*).
4. Quantification should be done objectively. At the light-microscopic level, a densitometer should be used. At the electron-microscopic level, either the optical density of electron-microscopic negatives (Sternberger, *1975*) or the density of countable particles, ie, electron-dense gold particles (Kraehenbuhl, *1977*), can be determined (Posthuma, *1987*). Measuring devices should be standardized to optical density standards and should be periodically assessed for the effect of other influences, such as loss of sensitivity resulting from camera decay or low voltage.

Double Immunohistochemistry

Two or more antigens can be detected in the same tissue. Successful tissue immunostaining requires that the chromogens are distinctive and that there is no cross-reactivity of immunologic reagents. The choice of method is dictated by available reagents. If there is immunologic cross-reactivity, then staining needs to be done sequentially and the immunologic reagents from the first antigen localization must be

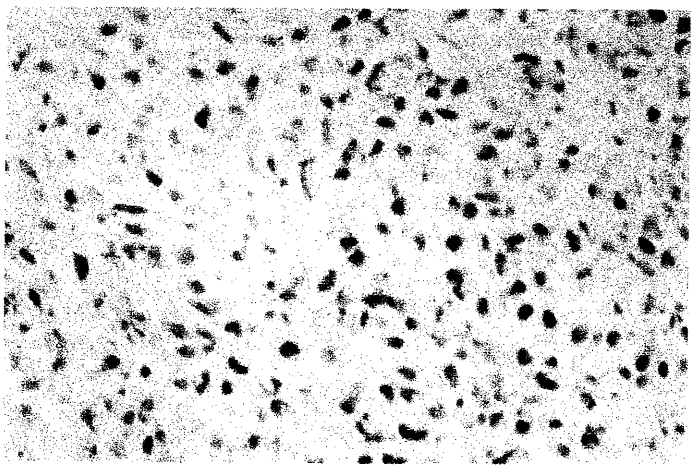


Figure 1.42. *An infiltrating ductal carcinoma of the breast, immunostained for estrogen receptor protein. The marked variability of staining intensity exhibited by tumor nuclei must be quantified, either by visual estimate of optical density on a "semiquantitative" scale or by a densitometer, to obtain a relative estrogen receptor content.* (DAB-PO; H-counterstained.)

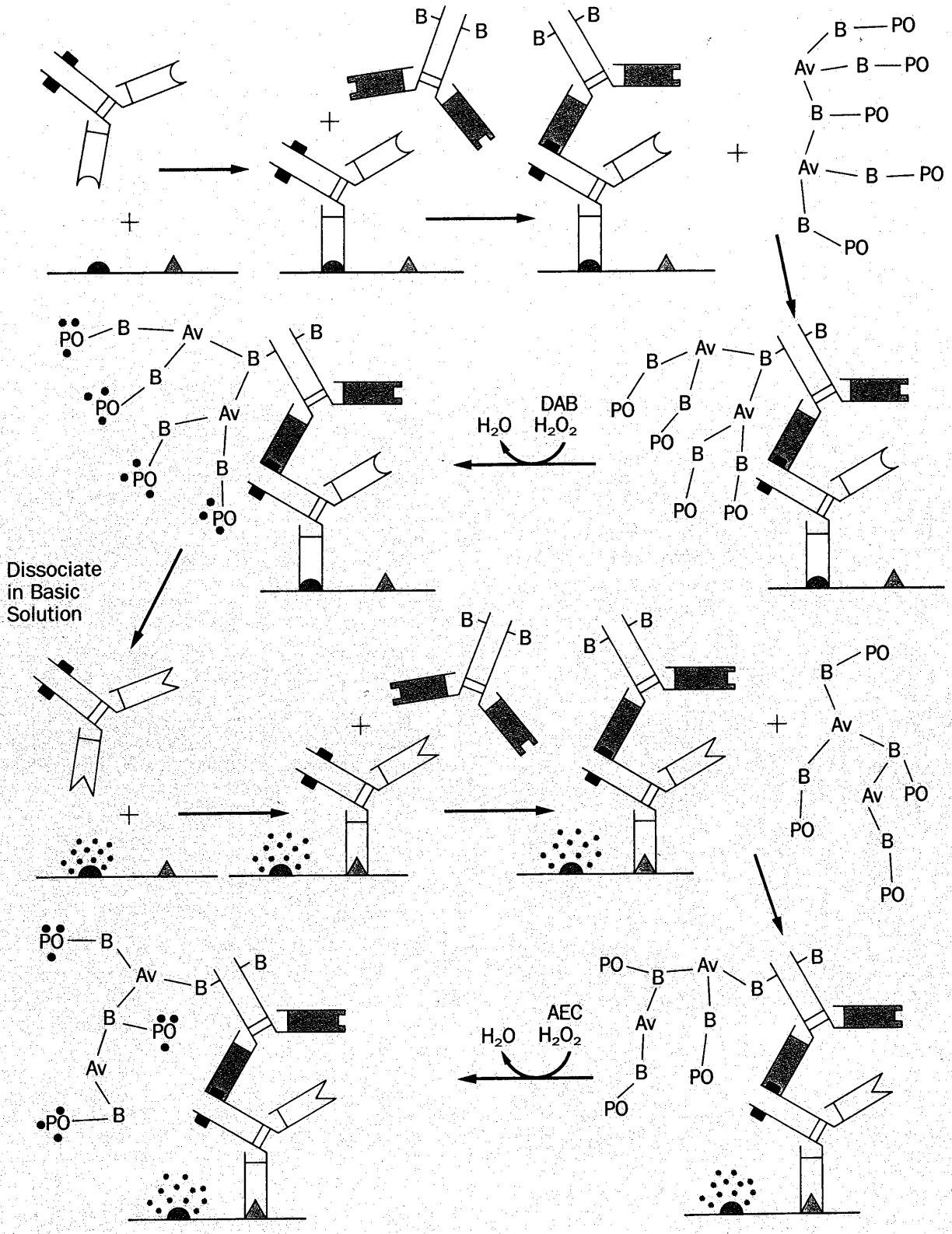


Figure 1.43. *A scheme for double immunostaining. If the secondary antibodies cross-react, the immune complexes must be dissociated after the first chromogen, which should be chosen to form a precipitate, is developed. In this scheme, the first chromogen, DAB, forms a brown precipitate (brown dots) that remains insoluble on the surface of the section throughout the procedure. The second chromogen, AEC, forms a contrasting red reaction product (red dots).*

dissociated (Nakane, *1968*). Simultaneous incubations with distinct reagents can be done more rapidly if there is no cross-reactivity (Mason, *1978*) (Figs. 1.43 and 1.44).

Immuno-Electron Microscopy (IEM)

The common goals of ultrastructural localization of antigens are the identification of the cell compartment(s) in which the antigen occurs and the quantification of the relative antigen concentrations in different cell compartments.

The essential, partially conflicting, requirements of IEM are preservation of ultrastructure and preservation of antigenicity (True, *1981*). A third requirement is use of an electron-dense tracer, which remains associated with the antigen during processing and which is stable under an electron beam. The following tracers are used currently: ferritin, a 650 kd molecule composed of four micelles of iron, which imparts the electron density; the substrates of peroxidase which are rendered electron-dense by chelation of osmium; and monodispersals of colloidal gold, which can be selected as uniformly sized dispersates ranging from 8 to 40 nm in diameter.

The choice of tracer and of method depends upon the location of the antigen, whether tissue orientation needs to be retained, whether the reaction product needs to be visualized at the light-microscopic level, and whether double IEM will be performed. There are two basic IEM methods—pre-embedding and post-embedding. These refer to the temporal relationship of immune reaction and embedding for electron microscopy (Fig. 1.45). The method we prefer is a pre-embedding method, where antigens are localized in cryoprotected frozen sections of fixed tissue which are mounted on slides and immunostained before embedding for electron microscopy (True, *1981*). For double IEM, various combinations of these tracers have been used.

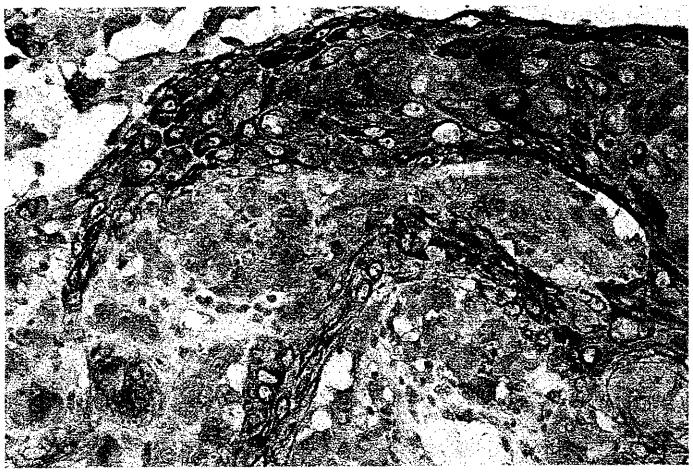


Figure 1.44. *Section of cutaneous melanoma, sequentially immunostained for keratin, using the brown chromogen DAB, and for S-100 protein, using the red chromogen AEC. Thus, keratinocytes are brown and the melanoma cells are red.* (DAB-PO; and AEC-PO; H-counterstained.)

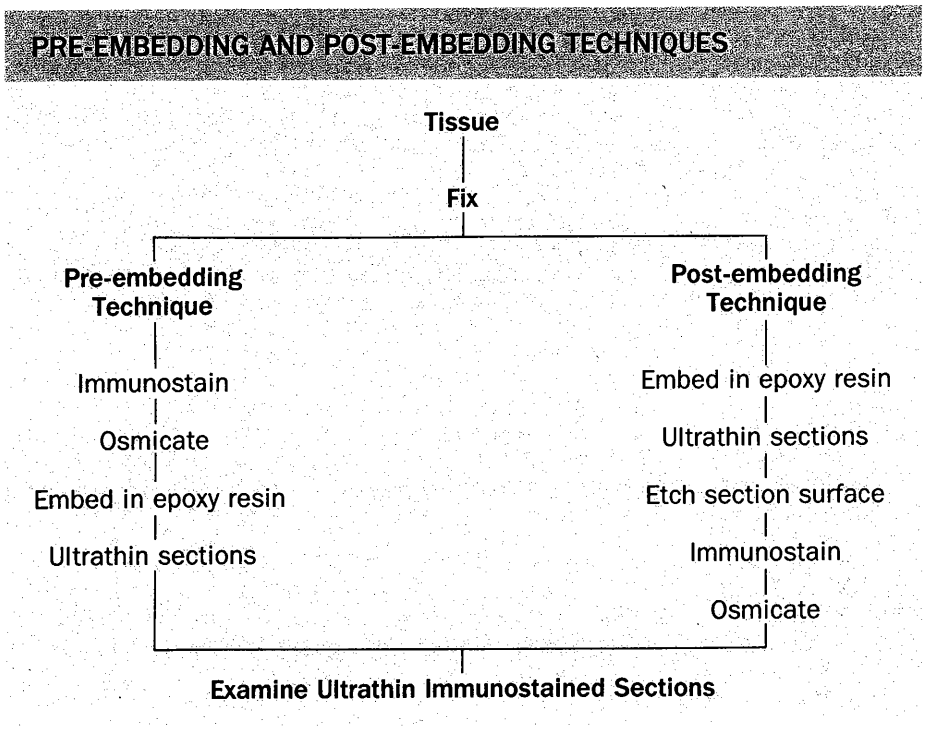


Figure 1.45. *Flow chart for pre-embedding and post-embedding techniques.*

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